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ORIGINAL ARTICLE

Differentiation and multipotential characteristics of mesenchymal stem cells derived from adipose tissue of an endangered wild cat (*Leopardus guigna*)

Diana M. Echeverry, Daniela M. Rojas, Constanza J. Aguilera, Daniel M. Veraguas, Joel G. Cabezas, Llеretny Rodríguez-Álvarez, Fidel O. Castro*

ABSTRACT. Adipose tissue derived mesenchymal stem cells (AMSCs) had been isolated and used for cell therapy in domestic cats. For wild cats, the isolation of AMSCs has only been reported in the black-footed cat (*Felis nigripes*). AMSCs obtained from wild cats may be useful to treat injuries of endangered cat species that remain in captivity or arrive at wildlife rehabilitation centers. Additionally, AMSCs might allow improvement of cloning techniques or assist in derivation of induced pluripotent stem cells. Endangered wild cats such as the guigna (*Leopardus guigna*), an endemic and endangered species from Chile and Argentina, might benefit greatly from the development of novel treatments or techniques that can be applied for its conservation. The objective of this study was to characterise putative AMSCs from guigna in terms of their main biological attributes, particularly, growth kinetics, differentiation ability and surface marker expression. Results obtained from this characterisation were compared with AMSCs isolated from domestic cats. AMSCs were isolated from peritoneal adipose tissue of female cats and subcutaneous tissue from a female guigna. Migration potential, colony-forming unit assay, mesodermal differentiation and surface marker expression (CD45, CD44, CD90, MHCI and MHCII) were evaluated. Domestic cat and guigna AMSCs displayed similar growth properties in culture. Both AMSC types showed mesodermal differentiation potential, *in vitro* homing potential and similar surface marker expression. These results indicate that AMSCs from subcutaneous tissue of guigna could have potential use as regenerative treatment for this species and might be considered for use in other biotechnological applications.

Key words: cell therapy, guigna, stem cell, wild cat.

INTRODUCTION

Cell therapy is a promising alternative for the treatment of several pathologies in animal species. For instance, adipose tissue derived from mesenchymal stem cells (AMSCs) has been successfully applied to experimentally treat domestic cats affected by chronic kidney disease, asthma and gingivostomatitis (Quimby *et al* 2015, Trzil *et al* 2015, Arzi *et al* 2016). This treatment was also used to restore elbow functionality in the cougar (*Puma concolor*) (Gómez *et al* 2015). Adipose tissue is considered to be the best source of mesenchymal stem cells (MSCs) as it can be accessed with relative ease, a large number of cells can be isolated per gram of tissue and this approach is less invasive than other methods (Martin *et al* 2002, Kono *et al* 2014). The collection of tissue samples from wild cats is a difficult task because most species suffer from conservation issues and specimens are rarely available to perform this procedure. Among wild cats, AMSCs have only been isolated from the endangered black-footed cat (*Felis nigripes*), with cells displaying multilineage differentiation potential and a proliferation rate similar to AMSCs obtained from domestic cats (Gómez *et al* 2015). The potential of these MSCs is of great relevance for the treatment of endangered species as well as an approach to improve reproductive techniques, such as cloning and gamete derivation. The guigna (*Leopardus guigna*) is a threatened felid species with a distribution restricted only to Chile and Argentina (Napolitano *et al* 2014). This species is endangered due to its interaction with humans. Many of these individuals get run over on roads or arrive at rehabilitation centers with gunshot wounds or blow trauma. Guigna is a threat to rural poultry producers due to the predation of chickens and eggs (Galvez *et al* 2013). Recently, this species was affected by large forest fires, arriving at wildlife rehabilitation centers with burns[1], leading to a significant reduction in the population of guigna. Currently, there are very limited alternatives available for the treatment of wild cat species. The Wildlife Rehabilitation Center at Universidad de Concepción occasionally receives injured guignas that cannot receive proper treatment to improve their health condition. The isolation of AMSCs from guignas could be beneficial for the development of novel techniques for the treatment of wild cats maintained in captivity or rehabilitation centers. The purpose of this study was to isolate and characterise guigna AMSCs and to perform a comparison between AMSCs isolated from guignas and domestic cats.

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Departmento de Ciencia Animal, Facultad de Ciencias Veterinarias, Universidad de Concepción, Chillán, Chile.

*Corresponding author: F Castro; Avenida Vicente Mendez 595, Chillán, Chile; fidcastro@udec.cl

1 Facultad de Ciencias Veterinarias y Pecuarias Universidad de Chile. 2017. Fauna silvestre de zonas afectadas huye de los incendios hacia sectores poblados. http://www.veterinaria.uchile.cl/noticias/130676/fauna-silvestre-de-zonas-afectadas-huye-hacia-sectores-poblados.

MATERIAL AND METHODS

SAMPLES

This study was conducted following standard veterinary practices and with approval of the Ethical Committee for Animal Experimentation of the Universidad de Concepción, permit number: CBE-29-16. Tissue donors were female domestic crossbreed cats (6 to 36 months-old) during routine ovariohysterectomy (n = 3). Informed consent was obtained from all cat owners. Subcutaneous adipose tissue from a guigna (n = 1) was obtained immediately post-mortem from a 6 month-old female who arrived at the wild animal rescue center. The researchers were not granted permission from regulatory authorities for sampling other tissues of the deceased animal.

ISOLATION OF ADIPOSE MESENCHYMAL STEM CELLS (AMSCS) FROM CAT AND GUIGNA

Preparation of cultured AMSCs was performed as previously described (Gómez *et al* 2015) with minor modifications. Adipose tissue was minced and digested in 0.1% (w/v) collagenase I (Sigma-Aldrich) at 37 °C for 20 min. After filtration and centrifugation at 1200 rpm for 5 min, the pellet containing the stromal vascular fraction (SVF) was isolated. Cell number and cell viability were measured by the Luna Automated Cell Counter from Logos Biosystems. The cells (~2000 cells/cm^2) were then plated in 35 mm culture dishes with HyClone Dulbecco's Modified Eagles Medium (DMEM) supplemented with 50% fetal bovine serum - FBS (HyClone Fetal Bovine Serum Standard). After 2 days, the medium was changed to 10% FBS/DMEM/F12. When the cells were confluent (80%), they were trypsinised and subcultured. Cells were used for differentiation experiments at passages two to three (P2-3). Guigna and domestic cat AMSCs were cultured until P5 to evaluate growth kinetics.

ISOLATION OF SKIN FIBROBLAST FROM CAT AND GUIGNA

For the establishment of fibroblasts cells from domestic cat and guigna skin samples were taken from the abdominal region of cat females subjected to ovariohysterectomy and from a female guigna post-mortem. The skin samples obtained were cut in small pieces (approximately 1 mm^2). Once cut, they were placed in 35mm plates and allowed to dry for 15 minutes to promote its adherence to the culture dish. Once this was achieved, special media for fibroblasts was added (DMEM/F12 without HEPES, 10%FBS, 2,4 mM L-Glutamine, 2,4 mM sodium piruvate, 10 U/ml ampicilin/amphotericin, 10ng/ml EGF, 1% essential and non-essential aminoacids) and the explant was further cultured at 38°C with 5% de CO_2 for 7 days. Culture plates were examined microscopically every two days until fibroblasts were seen emerging from the explant. After reaching 80% confluence the explant was removed with a sterile forceps and the obtained fibroblasts were expanded in 60 and 100 mm plates.

GROWTH KINETICS

The assay was performed as previously described with brief modifications (Kono *et al* 2014). P1 Cat and guigna AMSCs were plated at a concentration of ~1000/cm^2 into 35mm dishes and cells were cultured until P5. Cells were counted when they reached confluency in each passage; likewise, the days in which they reached each passage were calculated and growth curves were plotted.

IN VITRO MULTILINEAGE DIFFERENTIATION OF DOMESTIC CAT AND GUIGNA AMSCS

For differentiation assays, domestic cat and guigna AMSCs at P2-P3 were plated at ~8000cells/cm^2 in six-well culture dishes containing 3mL of DMEM/F12 medium supplemented with 10% FBS and cultured at 38.5 °C under humidified air supplemented with 5% CO_2. When cells reached 70-80% confluency, the culture medium was replaced with the respective differentiation medium. Adipogenic differentiation was performed as previously described with minor modifications (Gómez *et al* 2015). The culture medium was replaced with adipogenic induction medium (AIM) consisting of DMEM/F12 medium supplemented with 20% FBS, 5% insulin-transferrin-selenium-X (ITS-Gibco), 100 nM dexamethasone, 0.5 mM 3-isobutyl-l-methylxanthine (IBMX) and 100 mM indometacine and cultured for 3 additional days. At day 3, the AIM was switched to adipogenic maintenance medium (AMM), which consisted of the same components as AIM excluding dexamethasone and IBMX. At day 14, cells were fixed and stained with oil red. Osteogenic differentiation was performed as previously described with minor modifications (Castro *et al* 2014). The tissue culture medium was replaced with DMEM/F12 supplemented with 10% FBS, 0.25 mM ascorbic acid, 100 nM dexamethasone and 10 mM β-glycerophosphate and cultured for 21 days. Cells were fixed and stained with Alizarin red. Chondrogenic differentiation was performed as previously described with minor modifications (Castro *et al* 2014). For chondrogenic induction cells were cultured in DMEM/F12 supplemented with 10% FBS, 4.5 g/L D-glucose, 10 µL/mL insulin-transferrin-selenium-X (ITS; Gibco), 100 nM dexamethasone, 1 µM ascorbic acid 2-phosphate and 2.5% equine platelet rich plasma (ePRP) for 30 days. Cells were fixed and stained with Alcian Blue. Cells of control groups were cultured in DMEM/F12 supplemented with 10% FBS without inducers or other supplements for exactly the same time periods as experimental groups. All stained cells were visualised with phase-contrast optics using an inverted microscope (Olympus CKX-41).

MIGRATION ASSAYS

Transwell and scratch assays were used for the migration experiments. Domestic cat and guigna AMSCs at P4 were placed in 6.5 mm diameter transwell dishes with 8 µm cut-off pore filters (Corning Costar, Cambridge, MA). AMSC were placed on the upper layer of a cell-permeable membrane, and a medium with 1% ePRP was placed below the cell-permeable membrane as a chemoattractant agent. Medium without FBS was used as control. Incubation period was 3 hours at 38°C, 5% CO_2; the cells that migrated and remained trapped in the membrane were stained and counted. Scratch migration assays were performed with the same mediums. Domestic cat and guigna AMSCs were plated in six-well tissue culture plates and were grown to 80% confluence and then scratched with a sterile pipette tip to leave a gap of approximately 0.4-0.5 mm in width. The culture medium was then removed and replaced with fresh culture medium without FBS (control) and a medium with 1% ePRP. Migration was observed at 0, 2, 4, 8, 24, 48 and 72 hours, and the area was measured with Motic Images 2.0 software. The scratch was drawn freehand in the software to obtain the measurement in cm^2 of the area during each of the measurement times.

COLONY-FORMING UNIT ASSAY

Colony-forming unit (CFU) assays were performed at P1 on freshly isolated cells at different densities (250, 500, 1000, 2000, 4000 and 8000 cells/4 cm^2) as described by Gomez *et al* (2015) with minor modifications. Cells were plated in 12-well plates in duplicate serial dilutions and cultured in 5% CO_2 and 90% humidity at 38.5°C for two weeks in High glucose DMEM-supplemented medium with 20% FBS. Then, colonies were fixed with 4% formalin and stained with 0.5% crystal violet in methanol for 5 min, washed twice in distilled water and subsequently photographed. Cell clusters of 50 or more cells were considered to be a colony. The rate of colony-forming units-fibroblast (CFU-F) was calculated by dividing the average number of colonies/well by the total number of cells plated/well (Gómez *et al* 2015).

FLOW CYTOMETRY

Surface marker expression on domestic cat and guigna AMSCs and domestic cat fibroblasts was assessed by flow cytometry (FCM). Cells at P3-P4 from domestic cat and guigna were incubated with primary mouse anti-CD90 (1:100; cat. no. 14-0909, eBioScience, San Diego, CA, USA), anti-CD45 (1:50; cat. no. MCA2727T, AbD Serotec, Raleigh, NC, USA), anti-CD44 (1:200; SantaCruz Biotechnology), anti-MHCI (1:50; Kingfisher Biotech) and feline anti-MHCII antibodies (1:50; Kingfisher Biotech). Antibody-binding reactions were carried out in a blocking solution of 1% bovine serum albumin (BSA) and 5% sheep serum in Dulbecco's Phosphate-Buffered Saline (DPBS) at 4 °C for one hour. Then, cells were washed in DPBS twice and incubated with secondary sheep anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (1:50; cat. no. S3772, Aldrich, St. Louis, MO, USA) for 2 hours at room temperature. Finally, cells were washed with 5 mL of DPBS to remove excess/unbound antibodies before FCM analysis. Control for nonspecific binding was performed by incubating cells with sheep immunoglobulin G (IgG) and the secondary conjugated antibody, but not the primary antibody. Unstained cells were used as control for autofluorescence.

RNA EXTRACTION AND QUANTITATIVE RT-PCR ANALYSIS

The expression of *Oct4*, *Nanog*, *Cd44, Cd90, Aggrecan, PPARγ* and *Runx2* mRNAs were detected by quantitative RT-PCR (qRT-PCR). Feline *Sdha* (succinate-dehydrogenase-complex-flavoprotein subunit A) housekeeping was used as internal standard. Total RNA was isolated from guigna and domestic cat AMSCs and also from dermal fibroblasts to compare expression. RNA was extracted from each sample using an EZNA RNA extraction kit (Omega, Georgia, USA). The first-strand cDNA was synthesised from 500 ng of DNase-treated total RNA using 50 ng random hexamers (Invitrogen, Waltham, Massachusetts, USA) and 200U of MMLV reverse transcriptase (New England Biolabs Ipswich, Massachusetts, USA) according to the manufacturer's instructions. The primer sequences used for amplification are described in table 1. qRT-PCR amplification was performed in a 10 µL reaction mixture for 40 cycles under the following conditions: 94°C for 30 s, 58°C for 30 s and 72 °C for 40 s, with additional 7 min incubation at 72 °C after cycle completion. The data of target genes were plotted as fold changes in relation to the expression level of the housekeeping gene, *Sdha*. The transcript level of *Sdha* was used as normalisation control for all analysed samples.

STATISTICAL ANALYSIS

Data were presented as the mean value ± standard error mean (SEM) of each cell line for CFU assays. To assess statistical significance of mRNA expression, the Kruskal-Wallis and t-student test were performed to obtain *P*-values using Infostat Software (free version). Additional replicate experiments were also performed when necessary. Differences were considered as significant at $P<0.05$.

RESULTS

ISOLATION AND GROWTH KINETICS OF AMSCS FROM DOMESTIC CAT AND GUIGNA

AMSCs isolated from domestic cat and guigna adhered to plastic and displayed initial morphological heterogeneity

Table 1. Primer sequences and qRT-PCR conditions of housekeeping and studied genes used for gene expression analysis in AMSCs and fibroblasts from domestic cat and guigna.

Gene name	Primer sequences (5'-3')	Annealing temperature (°C)	Product lenght (bp)	Accession number (NCBI)
Sdha	F: GCAGCAGAAGAAGCCATTTG R: GTCATTGACGGGTCTGTACTC	58	103	XM_003981595.1
Oct4	F: CCGAAAGAGAAAGCGAACAAG R: GACCACATCCTTCTCCAG	55	136	NM_001173441.1
Nanog	F: CAGCCCCAGATACAGTTACAG R: GCTGGGCACTAAAATACTTGG	55	115	NM_001009340.1
Cd44	F: TCGAGGCACCCCATTTCATAGACA R: ATCAGCTGGCTACTCTGTTGGACT	59	128	XM_011286810.2
Cd90	F: AGCACGTGATCTTTGGCACTATGG R: ACATGTGTACATCCCCTCGTCCTT	59	134	XM_006936729.3
PPARγ	F: TCGGTTTCAGAAGTGCCTTGCT R: TGGAGATCTCCGCCAACAGCTTTT	59	101	NM_001113176.1
Sox9	F: CGTCAACGAATTCGACCAGTACCT R: TGCTGTTGATGCCGTAGCTC	60	101	XM_023243815.1
Aggrecan	F: GCACTGTGGATGTAAGTGGCGAAT R: ACCCTCCACGAACTCAGAAGTGAT	59	104	XM_023254934.1

with fibroblastic-like appearance. For domestic cat AMSCs, a primary culture with an average of 9.2 x 10^4 (± 2.5 x 10^4) cells/cm^2 was established. Guigna AMSCs were obtained from an initial cell population of 8.9 x 10^4 cells extracted from 400 mg of subcutaneous adipose tissue. Cell viability of at least 80% for both species was demonstrated. Clusters of rapidly expanding cells were observed in guigna AMSCs. The growth curves of domestic cat and guigna AMSCs were similar (figure 1).

COLONY-FORMING UNIT ASSAY

Domestic cat and guigna AMSCs demonstrated ability to form colonies of 250 to 1000 cells/well. The percentage of CFU decreased when the seeding density was higher than 1000 cells/well for both domestic cat and guigna AMSCs (table 2). However, no significant statistical differences were observed in colony counts for cat AMSCs (P=0.16). No statistical analysis was performed for guigna AMSCs.

Table 2. CFU of domestic cat and guigna AMSCs. Cells were seeded at different densities and CFU was calculated in duplicate for each cell line.

Plating cell density/well (4 cm^2)	CFU guigna AMSC	% CFU guigna AMSC	CFU cat AMSC	% CFU cat AMSC
250	45.5	18.2	7.6 ± 0.6	3.1
500	22.5	4.5	13.3 ± 2.5	2.7
1000	23.5	2.35	22.3 ± 1.5	2.2
2000	4.5	0.22	11 ± 11.5	0.55
4000	21.5	0.53	21 ± 10.5	0.52
8000	23.5	0.29	19 ± 13.1	0.23

FLOW CYTOMETRY

Flow cytometry analysis (figure 2) showed that both domestic cat and guigna AMSC and domestic cat fibroblast samples were barely positive for stromal marker CD90. Cat fibroblasts were slightly positive for CD45 (2.33%), MHCI (5.99%) and MHCII (1.25%). Domestic cat AMSCs were slightly positive for CD45 (1.82%) and negative for markers CD44, MHCI and MHCII. Guigna AMSCs were slightly positive for CD45 (4.25%) and CD44 (1.49%) and were negative for MHCI and MHCII. CD90 expression for domestic cat and guigna AMSCs and fibroblasts was similar. Other markers assayed, CD73, 105 and 271, were not detected, most likely due to the species-specific binding of the antibodies (anti-mouse or humans).

IN VITRO MULTILINEAGE DIFFERENTIATION OF DOMESTIC CAT AND GUIGNA AMSCS

Adipogenic differentiation of domestic cat and guigna AMSCs was indicated by the presence of intracytoplasmic lipid droplets after two week (figures 3 and 4) using oil red staining; whereas, control undifferentiated cells showed no lipid deposits. When compared to control, non-differentiated cells, PPAγ expression was significantly higher in the guigna (P=0.0079) and cat (P=0.0039) AMSCs differentiated towards the adipogenic lineage (figures 3 and 4). Domestic cat and guigna AMSCs (figures 3 and 4)

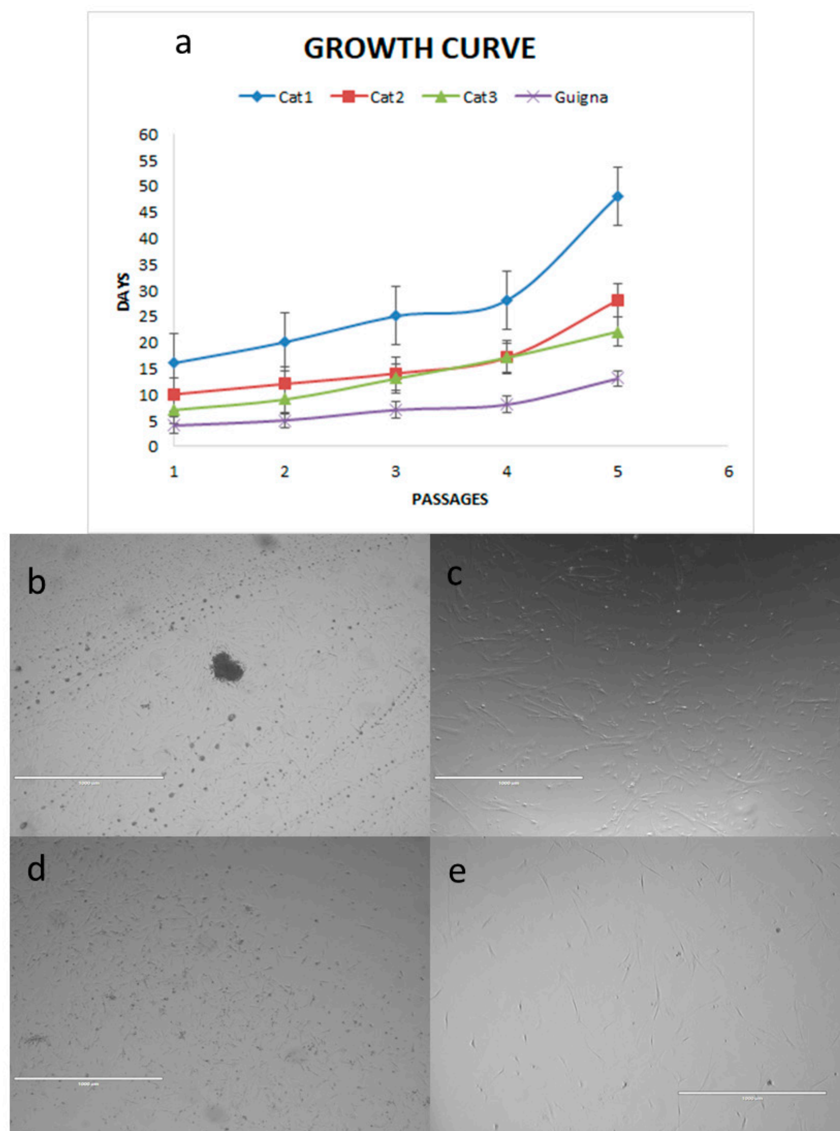

Figure 1. a) Growth curve of cultured domestic cat and guigna AMSCs. Three lines of cat AMSCs and one of guigna AMSCs were cultured until passage 5 (P5) to evaluate growth kinetics; b) AMSCs from guigna at P1 and c) AMSCs from guigna at P5; d) AMSCs from cat at P1 and e) AMSCs from cat at P5. Scale bars represent 1000 μm.

induced towards osteogenic differentiation for 21 days revealed calcium mineralization after Alizarin red staining and *Sox9* expression significantly higher in cat (*P*=0.028) and guigna (*P*=0.0079) AMSC. Osteogenic control of undifferentiated cells were negative for Alizarin red stain (figures 3 and 4). Proteoglycan and glycosaminoglycan depositions following chondrogenic differentiation were shown by Alcian blue staining in domestic cat and guigna AMSCs at day 30 (figures 3 and 4). Chondrogenic control of undifferentiated cells were negative for Alcian blue stain after being cultured with standard medium. The expression of *Aggrecan* showed a tendency to a higher expression in cat (*P*=0.1) and guigna (*P*=0.16) AMSCs with respect to the control group, however there was no significant statistical difference (figures 3 and 4).

MIGRATION ASSAYS

Transwell and scratch assays proved to be similar for assessing cellular migration. There was a significant increase in migration in the presence of attractant versus non-treated controls in transwell assays for domestic cat AMSCs (*P*=0.03) (figure 5d). A greater number of guigna AMSCs migrated through the transwell; however, it was not statistically significant (figure 5d). To complement the results obtained in the transwell assay, it was decided to perform the scratch migration test at different times. AMSCs from domestic cat and guigna migrated and filled the scratch gap efficiently after 48 hours of exposure to 1% ePRP (figure 5a-c).

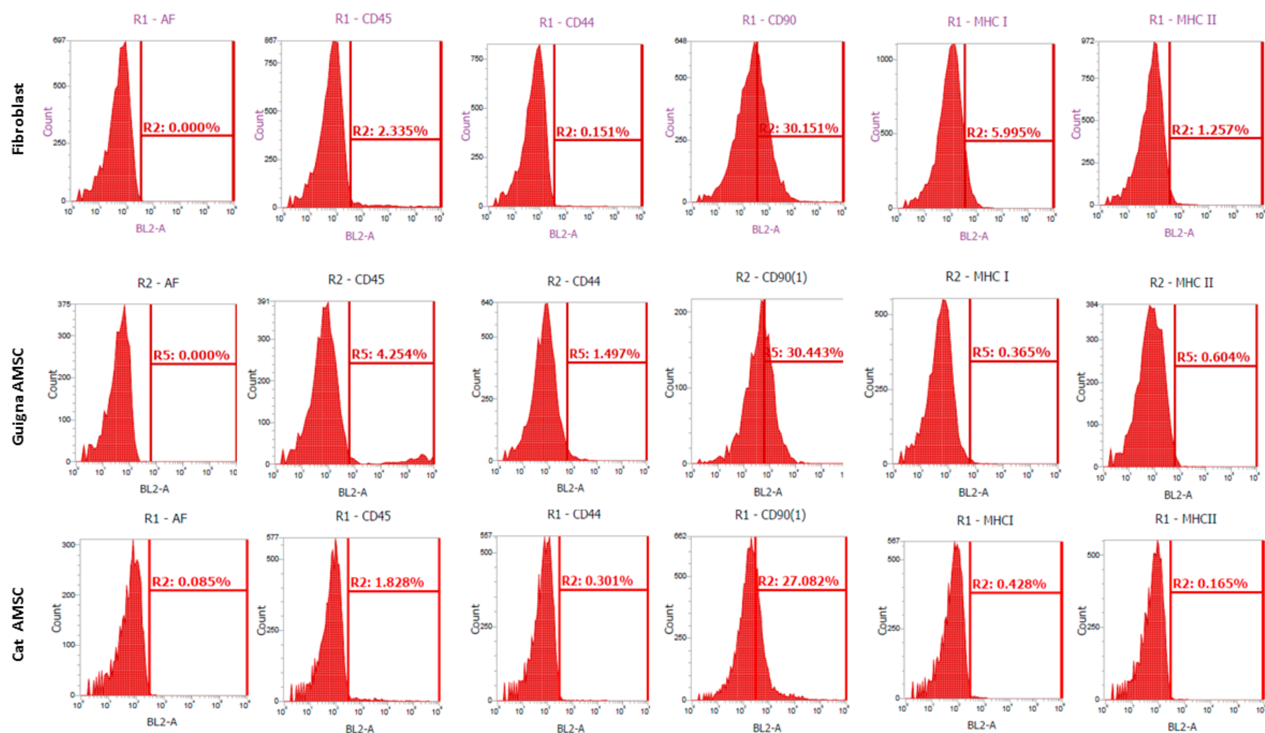

Figure 2. Surface marker expression for domestic cat and guigna AMSCs and for domestic cat fibroblasts. There was a positive expression of CD90 surface marker for fibroblast and AMSCs from domestic cat and guigna. A low percentage of expression was detected in domestic cat fibroblasts and domestic cat and guigna AMSCs for CD45. Guigna AMSCs had a low percentage of positivity for CD44. For domestic cat fibroblasts, MHCI and MHCII expressions were low. For both domestic cat and guigna AMSCs, MHCI and MHCII expressions were 0.16 and 0.6% respectively. AF = Autofluorescence, BL2A = Fluorescence channel associated with the blue laser, R1-R2 = Regions of a specific group of cells in the histogram that were analysed.

QUANTITATIVE RT-PCR ANALYSIS

The qRT-PCR analysis revealed that cat cell lines expressed OCT4, NANOG, CD44, and CD90 (figure 6). There was a significant difference in the expression of NANOG (P=0.007) and CD44 (P=0.02) between cat AMSCs and fibroblast (figure 6a). Testicular tissue containing primordial germ cells was used as internal control (figure 6a). AMSC and dermal fibroblasts from guigna were analysed by RT-PCR to determinate if some of the genes of interest were expressed in this specie. Expression of OCT4, NANOG, CD44 and CD90 was detected in guigna AMSCs. NANOG, CD44 and CD90 were expressed as well in guigna fibroblast (figure 6b).

DISCUSSION

In the present study, AMSCs were successfully obtained from guigna for the first time. The isolation of AMSCs from subcutaneous and abdominal tissue of cats had been previously reported (Kono *et al* 2014, Gómez *et al* 2015). Results for the growth kinetics of domestic cat and guigna AMSCs were consistent with other reports in human and cat (Kern *et al* 2006, Kim *et al* 2015, Gómez *et al* 2015). Colony-forming unit-fibroblast (CFU-F) was similar for domestic cat and guigna AMSCs and 250 cells seeded in 4 cm^2 were enough to form colonies. When initial plating densities were above 2000 cells/4 cm^2, the efficiency of CFU formation was diminished. Similar results were published before for domestic cat AMSCs (Kono *et al* 2014, Gómez *et al* 2015). Authors found that the cell growth was not affected by cell contact inhibition in small cell clusters and they continued to proliferate until covering 100% of the dish surface. Moreover, it has been described that cell proliferation of cat AMSCs plated at a density of 1000 cells/cm^2 formed colonies with the highest frequency rate per number of cells plated compared to those plated at cell densities $>$ 4000 cells/cm^2, and CFU was constant and similar at plating densities $<$ 4000 cells/cm^2 (Gómez *et al* 2015).

CD90 was detected in flow cytometry analysis of both domestic cat and guigna AMSCs and fibroblasts; this is consistent with another report (Mumaw *et al* 2015). Previous descriptions have mentioned that fibroblasts express certain surface markers of MSCs (Halfon *et al* 2011). Interestingly in humans, it has been shown that the canonical markers of adult MSCs such as CD105, CD166, CD90, CD44, CD29, CD73 and CD9 are also expressed

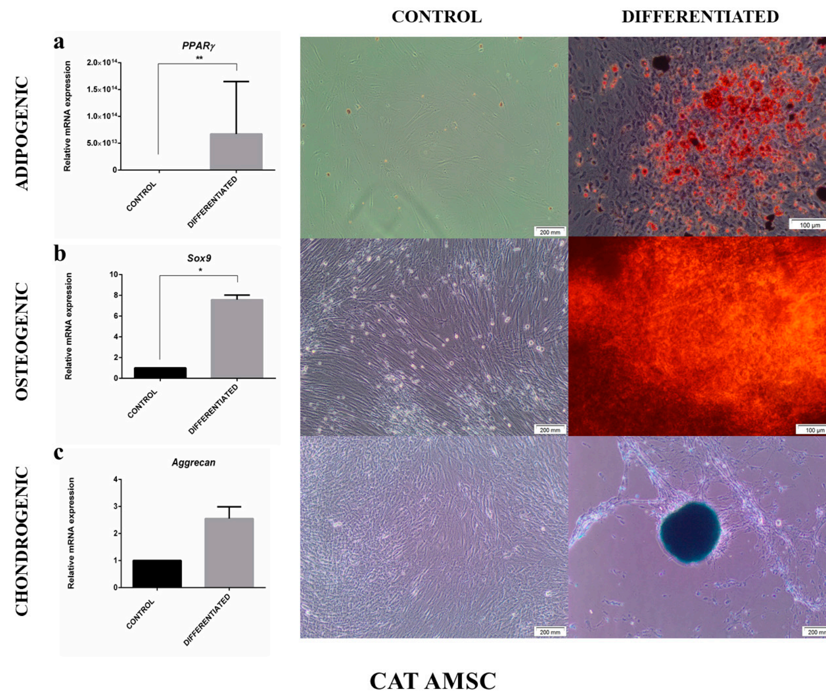

Figure 3. *In vitro* multilineage differentiation of domestic cat AMSCs. a) Undifferentiated and differentiated domestic cat AMSCs at day 14 of adipogenic differentiation and PPARγ expression. After oil red staining at day 14 intracellular lipids were visualized; b) undifferentiated and differentiated domestic cat AMSCs at day 21 of osteogenic differentiation and *Sox9* expression, the presence of calcium deposits was detected by Alizarin red; c) undifferentiated and differentiated domestic cat AMSCs at day 30 of chondrogenic differentiation and *Aggrecan* expression. Glycosaminoglycans and proteoglycans were observed in induced domestic cat AMSC at day 30 after Alcian blue staining.

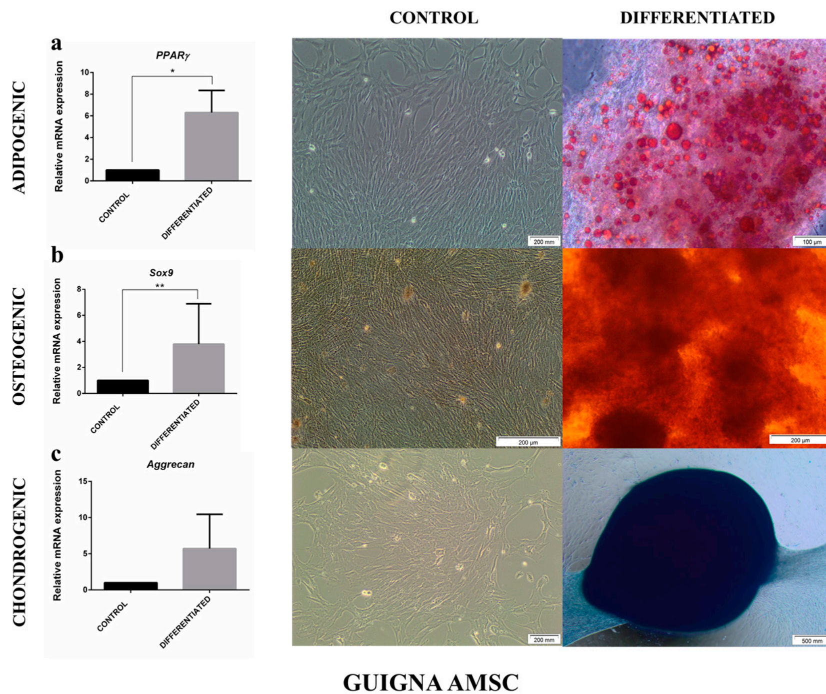

Figure 4. *In vitro* multilineage differentiation of guigna AMSCs. a) Undifferentiated and differentiated guigna AMSCs at day 14 of adipogenic differentiation and PPARγ expression. After oil red staining at day 14 intracellular lipids were visualized; b) undifferentiated and differentiated guigna AMSCs at day 21 of osteogenic differentiation and *Sox9* expression, the presence of calcium deposits was detected by Alizarin red; c) undifferentiated and differentiated guigna AMSCs at day 30 of chondrogenic differentiation and *Aggrecan* expression. Glycosaminoglycans and proteoglycans were observed in induced AMSC at day 30 after Alcian blue staining.

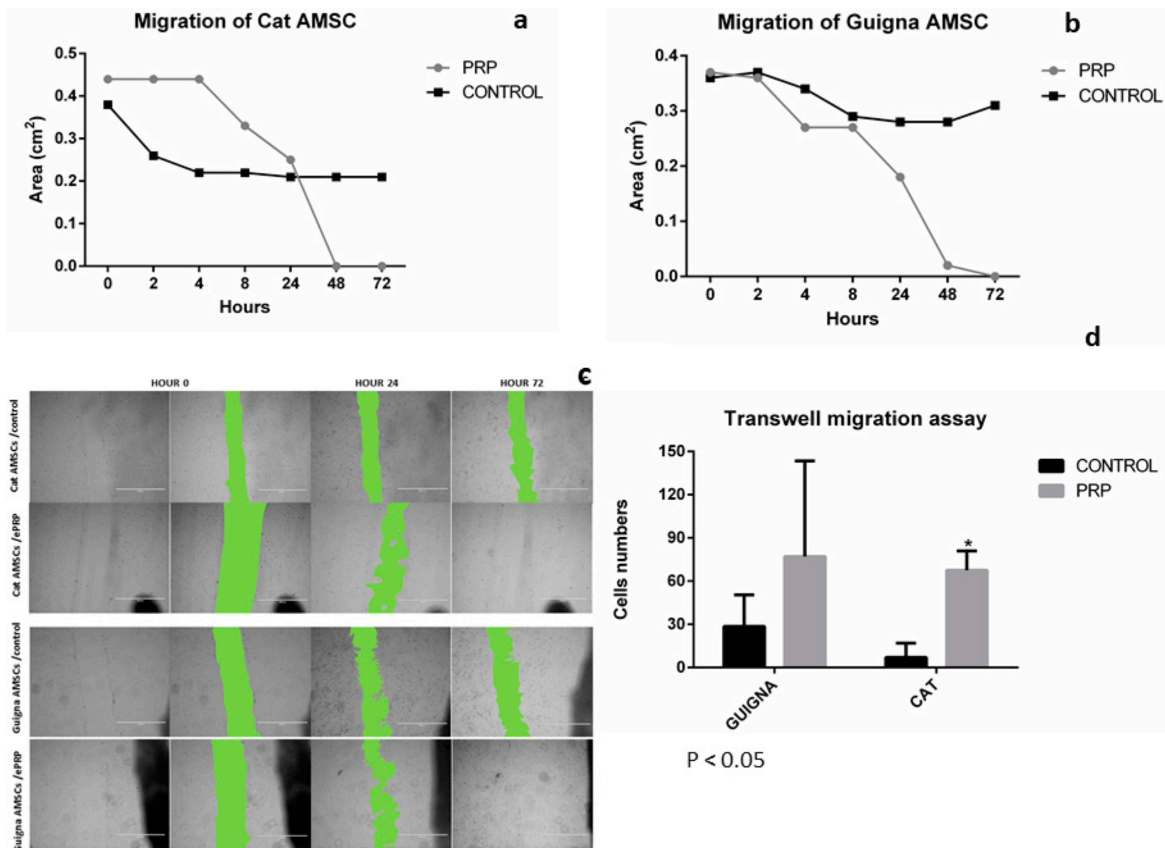

Figure 5. Migration assays. Figures a-c correspond to scratch migration assays. Guigna and domestic cat AMSCs with 1% ePRP or control medium were photographed at 0, 2, 4, 8, 12, 24, 48 and 72 hours (c). The area of migration was measured (a and b). Transwell migration assay was evaluated with 1% ePRP and control medium. The test was carried out in triplicate (technical replicates). Migrated cells were counted after 2 hour (d). Scale bars represent 1000 μm. (*) There was a significant statistical difference between the control and treatment with ePRP in the AMSC of domestic cat.

in skin and lung fibroblasts, but this expression decreases with passages (Halfon *et al* 2011). For *Felidae* in general, there are no surface markers indicative of stemness. The results of this study confirm that the commonly used CD antigens for human MSCs are not useful to qualify feline stem cells. Other markers should be pursued if accurate selection is required based on surface proteins. Here, the lack of expression of some surface markers, such as CD44, may be related to the passage number of the MSCs used or to the low specificity of antibodies to feline species. It cannot be ruled out that the cell cultures were actually contaminated with fibroblasts from the surrounding tissues. This has been previously reported in literature; fibroblast contaminating cultures could create a potential obstacle for long-term expansion of MSC cultures due to their senescence and may also result in decreased or complete loss of differentiation potential (Prockop and Olson 2007, Rosland *et al* 2009). Lack of expression of MHCII in cat AMSCs was found, while cat fibroblasts expressed this marker. A very low expression of MHCII was found in guigna AMSCs. The lack of MHCII expression may grant AMSCs a beneficial effect in allogenic cell therapies due to their low immunogenicity (Rutigliano *et al* 2013). Although the percentage of expression of this marker was close to zero, more studies are required to determine if the guigna AMSCs are suitable for allogenic treatments.

Although inconclusive, our cytometry studies led us to consider complementary characterisation of the putative guigna MSCs with qRT-PCR to evaluate expression of pluripotency genes and surface markers and to compare them with cat cell lines. These results were similar to those reported by Gómez *et al* (2015), in which *Nanog* had a higher expression in cat AMSCs when compared to cat fibroblasts. Regarding surface markers, there was significant expression of *Cd44* in domestic cat fibroblasts despite its lack of detection by flow cytometry. This result reinforces the hypothesis of the low specificity of the antibodies used against feline cells. On the other hand, expression of *Oct4*, *Nanog*, *Cd44* and *Cd90* was observed in guigna AMSCs and expression of *Nanog*, *Cd44* and *Cd90* in guigna fibroblasts; however, it is necessary to refine the methods to evaluate the expression of these genes in this species.

Guigna and domestic cat AMSCs showed multilineage differentiation potential by stain and gene expression, as

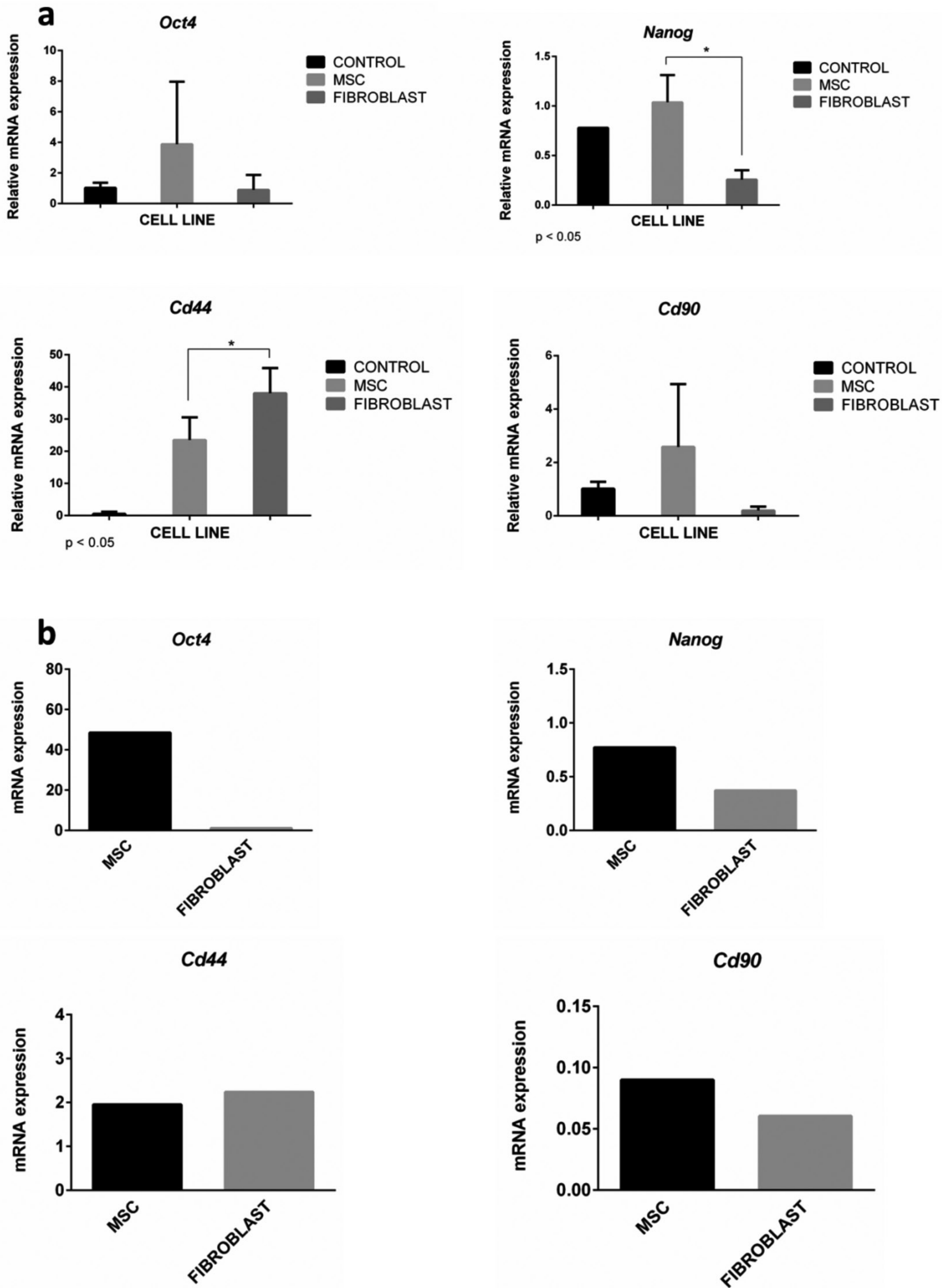

Figure 6. Relative mRNA expression of *Oct4*, *Nanog*, *Cd44* and *Cd90* in domestic cat AMSCs and fibroblasts (a). Significant differences (P<0.05). Cat testicular tissue was used as comparative control for mRNA expression of *Oct4*, *Nanog*, *Cd44* and *Cd90* domestic cat fibroblasts and AMSCs. Qualitative expression of *Oct4*, *Nanog*, *Cd44* and *Cd90* mRNA for guigna AMSC and fibroblasts (b).

has been previously reported for domestic cats (Kono *et al* 2014, Gómez *et al* 2015, Sato *et al* 2016). For chondrogenic differentiation, in this study ePRP was used instead of TGF-beta with similar results to those previously reported (Castro *et al* 2014). ePRP is a good source of TGF-beta when used with vitamin C it as a synergistic action in the up-regulation of *Runx2*, allowing differentiation towards the chondrogenic lineage (Castro *et al* 2014).

Homing of cells to the site of the injury is a big issue in cell therapies. Migration assays are used *in vitro* to

assess the potential of MSCs to move towards a gradient of chemo attractants, which in turn could be indicative of future homing *in vivo*. To evaluate the migration potential of domestic cat and guigna AMSCs, we used transwell and scratch assays. In both approaches, domestic cat and guigna AMSCs demonstrated migration potential in medium conditioned with ePRP. Previous reports on the migration ability of domestic cat or guigna MSCs were not found, which makes it difficult to perform a comparison. The choice of PRP as attractant was based on its use in combination with MSCs in some cell therapies. PRP contains important growth factors that act as chemo attractants and mitogens, such as TGF-β1, VEGF, PDGF and IGF-1 (Bartold and Raben 1996, Vater *et al* 2011, Marx *et al* 2015). In general, the present migration studies yielded similar results to canine AMSCs (Al Delfi *et al* 2016). Results suggest that guigna AMSCs have migration potential, an important characteristic of multipotent cells. New studies on the potential use of wild-type MSCs are required because of their applicability in cell therapy and potential biotechnological use. Furthermore, other molecular tools are required to allow better analysis of the characteristics of stem cells in non-traditional species.

In this study, guigna AMSCs displayed features of MSCs such as colony formation, differentiation to trilineage and migration towards chemoattractant molecules present in the ePRP. This leads us to propose these cells are MSCs and potential candidates for possible autologous treatments in individuals of this species or to be used in other biotechnological applications, such as cloning. Although more samples of guigna tissue are required in order to confer statistical power to the present study it was showed that, at least in a single isolation of cells from adipose tissue of guigna, there were cells with similar properties as MSC.

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