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TITLE:

Derivation and Differentiation of Canine Ovarian Mesenchymal Stem Cells

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KEYWORDS:

Ovary; dog; stem cells; isolation; differentiation; mesenchymal stem cell; plastic adherence; homogeneous population

SUMMARY:

Herein, we describe a method for the isolation, expansion, and differentiation of mesenchymal stem cells from canine ovarian tissue.

ABSTRACT:

Interest in mesenchymal stem cells (MSCs) has increased over the past decade due to their ease of isolation, expansion, and culture. Recently, studies have demonstrated the wide differentiation capacity that these cells possess. The ovary represents a promising candidate for cell-based therapies due to the fact that it is rich in MSCs and that it is frequently discarded after ovariectomy surgeries as biological waste. This article describes procedures for the isolation, expansion, and differentiation of MSCs derived from the canine ovary, without the necessity of cell-sorting techniques. This protocol represents an important tool for regenerative medicine because of the broad applicability of these highly differentiable cells in clinical trials and therapeutic uses.

INTRODUCTION:

The number of published studies that focus on stem cells has increased substantially over the past decade, a research effort that has been fueled by the collective goal of discovering powerful

regenerative medicine therapies. Stem cells have two primary defining markers: self- renovation and differentiation. Mesenchymal stem cells are responsible for regular tissue turnover and have a more restricted capacity of differentiation when compared to embryonic stem cells[1]. Recently, many studies have shown a wide range of differentiation of MSCs, and a topic under discussion is whether differences between embryonic and adult stem cells exist at all[2].

The ovarium surface epithelium is an uncommitted layer of cells, relatively less differentiated, which expresses both epithelial and mesenchymal markers[3], retaining the capacity to differentiate into different types of cells in response to environmental signals[4]. The exact location of stem cells in the ovary is not well known; however, it has been proposed that bipotential progenitors in the tunica albuginea give rise to germ cells[5]. Immunological studies have hypothesized that these cells have a stromal origin[6] or are located in or proximal to the ovary surface[7]. Since mesenchymal stem cells express numerous receptors that play an important role in cell adhesion[8], an experiment was designed to test the hypothesis that selecting a population of cells with rapid adhesion would isolate a population of cells clearly characterizable as mesenchymal in nature. Recently, our group reported the derivation of MSCs from ovarian tissue based on their capacity of adhesion to the plastic surface of the culture dish in the first 3 h of culture, in order to obtain a purified population of cells exhibiting rapid adhesion[9]. Here, we describe the developed method for mesenchymal stem cell isolation from the ovarian tissue.

PROTOCOL:

This experiment was performed with the ovaries of four mongrel female dogs donated after elective surgery at a canine sterilization program. This experiment was approved by the Ethics Committee on the use of animals of UNESP-FCAV (protocol no. 026991/13).

1. Experimental Preparation

1.1. Prepare or purchase 500 mL of sterile Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium.

1.2. Prepare a collagenase I stock solution by mixing 40 µg of the enzyme in 1 mL of DPBS. Filter using a 0.2 µm syringe filter, and store 2 mL aliquots at -20 °C.

1.3. Prepare the culture media from Dulbecco’s modified Eagle’s medium (DMEM) low glucose with 10% fetal serum and 1% of antibiotics.

1.4. Collect sterile materials: scissors and forceps, a tissue culture flask, dishes, tubes, and 10 mL and 5 mL pipettes.

1.5. Use standard cell culture laboratory equipment: a biological safety cabinet (BSC), a cell culture incubator set at 5% CO_2 and 38 °C, and a centrifuge.

1.6. Clean the BSC: with gloved hands, sterilize it with 70% EtOH, using UV lights.

2. Isolation of Mesenchymal Stem Cells from the Ovary

2.1. After surgery, keep the ovaries in PBS on ice in a conical tube, until their arrival at the lab.

2.2. Fill two 100 mm Petri dishes with 10 mL of PBS.

2.3. Remove the ovaries from the tube and transfer them to the first Petri dish with PBS. Wash them with mixing movements.

2.4. Gently retrieve the ovary and place it into another dish. With sterile tweezers and scissors, mince the tissue into very small pieces, approximately 1 mm in size.

2.5. Transfer the ovarian tissue to a 35 mm Petri dish and add 2 mL of collagenase. Mince the tissue a little more.

2.6. Place the Petri dish in the incubator at 38 °C for 3 h and gently shake the Petri dish with circular movements every 20 min.

2.7. After 3 h, remove the Petri dish from the incubator.

2.8. Transfer the contents of the dish to a 15 mL tube. Add 5 mL of expansion media.

2.9. Gently invert the tube before centrifugation. Centrifuge the tube at 2100 x *g* for 7 min. Remove the supernatant and resuspend the pellet with 3 mL of expansion media.

2.10. Transfer the pellet to a T25 bottle. Place the bottle in the incubator with 5% CO_2 at 38 °C for 3 h.

NOTE: The most important part of the procedure is to change the media after 3 h of incubation.

2.11. Remove the bottle from the incubator and remove the media with the remaining tissue. Add 3 mL of fresh expansion media to the bottle.

2.12. Change the media every 48 h and observe the bottle for cellular confluence.

NOTE: The main steps of the procedure can be observed in **Figure 1**.

3. Expansion of Mesenchymal Stem Cells from the Ovary

3.1. Cell passage

3.1.1. When the cells reach confluence, remove the media from the bottle.

3.1.2. Wash the bottle with 3 mL of PBS. Remove the PBS.

3.1.3. Add 1.5 mL of trypsin and put the bottle in the incubator at 38 °C for 3 min. Gently tap the bottle to help the cells to detach.

NOTE: Cells should reach confluence approximately 5 - 7 d after the initial plating.

3.1.4. Add 3 mL of expansion media and transfer the contents to a conical tube.

3.1.5. Centrifuge the tube at 2100 x *g* for 7 min. Remove the supernatant and add 1 mL of expansion media.

3.1.6. Gently homogenize the contents of the tube.

3.1.7. Take an aliquot of 10 µL of the sample to perform cell counting and put the tube with the cells back in the incubator.

3.1.8. Place 10 µL of the mix into the hemocytometer.

3.2. Counting the cells

3.2.1. Use the microscope's 10X objective and focus on the grid lines of the hemocytometer.

3.2.2. Count the cells in five small squares (**Figure 2**).

3.2.3. Multiply the counted number by 50,000 to estimate the number of cells per milliliter.

4. Differentiation of Mesenchymal Stem Cells from the Ovary

NOTE: Differentiation assays were performed according to the guidelines established in Hill *et al.*[9].

4.1. Seed 1.0 x 10^4 cells per well, in triplicate, using a 4-well culture dish.

4.2. Add 1 mL of expansion media.

4.3. Gently agitate the dish with circular movements.

4.4. After 24 h, replace the culture media with the desirable differentiation media.

4.5. For osteogenic, adipogenic, and chondrogenic differentiation, incubate the cells with the induction media for 30 d, with media replacement every 3 d. Specific differentiation kits were used for each of these assays.

4.6. For neurogenic lineage differentiation, incubate the cells for 10 d in the induction media, with media replacement every 3 d. The media used here contained DMEM low glucose, 2 mM valproic acid, 1 µM hydrocortisone, 10 µM forskolin, 5 mM potassium chloride, 5 µg/mL insulin, and 200 µM butylated hydroxyanisole.

4.7. For endoderm precursors, incubate for 5 d in endoderm media as per the reagent manufacturer's instructions.

4.8. For primordial germ cell lineage differentiation, incubate the cells in the induction media for 14 d, with media replacement every 3 d. The media used here contained DMEM, 10% FBS, 1 mM sodium pyruvate, 10 ng/mL LIF, 1 mM nonessential amino acids, 2 mM L-glutamine, 5 µg/mL insulin, 0.1 mM β-mercaptoethanol, 60 µM putrescine, 20 µg/mL transferrin, 10 ng/mL EGF (mouse epidermal growth factor), 1 ng/mL human bFGF (basic fibroblast growth factor), 40 ng/mL human GDNF (glial cell line-derived neurotrophic factor), and 15 mg/L penicillin.

REPRESENTATIVE RESULTS:

Mesenchymal Stem Cell Isolation from Canine Ovary:

The ovarian MSC isolation procedure is summarized in **Figure 1**. After surgery, tissue mincing, collagenase digestion, and a media change 3 h after the beginning of the culture, a putative MSC population with rapid plastic-adhesive properties was successfully isolated from canine ovarian tissue. The harvested cells rapidly adhered to the plastic surface of the culture dish and grew into a morphological homogeneous monolayer culture with typical MSC-like morphology (**Figure 3**).

Characterization of Ovarian MSC *In Vitro*:

The goal of MSC characterization is to ensure that isolated cells conform to standard MSC criteria. These include an adherence to plastic, a positive detection of mesenchymal surface markers, and an absence of hematopoietic surface markers, as well as the capacity to undergo mesodermal differentiation.

As shown in **Figure 3**, ovarian-derived cells were adherent to the plastic and exhibited a fibroblast-like morphology, fulfilling the first criterion that defines MSCs. Moreover, the isolated cells showed the expression of transcripts for the canine MSC markers CD44, CD90, and CD105. In addition, MSC-specific cell surface antigens CD90 and CD44 were detected by early passage FACS analysis and immunocytochemistry. The absence of the markers CD45 and CD34, which are hematopoietic-specific cell surface antigens, was also confirmed by the same techniques (**Figure 4**). This further confirmed that the ovary contains cells expressing an MSC-specific phenotype[9]. The antibodies that were used in this experiment for canine MSC characterization are listed in the **Table of Materials**.

The next step in the characterization of MSCs is to attain proof of their ability to differentiate into mesodermal lineages. After 30 days of exposure and culture in lineage-specific differentiation protocols, staining was performed to identify a commitment to different lineages (**Figure 5A**). A commitment to the osteogenic lineage was confirmed through Von Kossa staining, which

identified calcium deposits. Safranin O proteoglycan staining confirmed chondrogenic commitment, and Oil Red O lipid staining confirmed adipogenic commitment. Continuing the investigation of the differentiability of these cells, they were able to undergo differentiation into the ectodermic lineage, as shown by immunostaining for two neuroectodermal stem cell markers: β-tubulin and Nestin, following exposure of ovary-derived MSCs for to the neuronal lineage differentiation protocol for 10 days (**Figure 5C**). Transcripts for SOX17 and CD184, as well as cell morphologies specific to the endodermal lineage, were observed after exposure to specific differentiation protocols for 5 days (**Figure 5B**). Finally, after 14 days of exposure of ovarian-derived MSCs to germ cell induction media, OCT 4 and DDX4 were identified (**Figure 5D**). Together, these results support a robust and diverse capacity of the differentiation for MSCs derived from ovarian tissue, when exposed to specific differentiation protocols[9].

FIGURE LEGENDS:

Figure 1: Main steps of the isolation procedure. (1) After surgery, transfer the ovaries to sterile tubes with PBS, on ice. (2) In order to wash the ovaries, they should be transferred to a 100 mm plate with PBS. (3) Mince the tissue into small pieces, approximately 1 mm in size. (4) In order to liberate the cells from the tissue, transfer the pieces of tissue to 35 mm plates and add collagenase for a 3 h digestion in the incubator. (5) Transfer the contents of the plate to a conical tube for centrifugation. (6) Plate the pellet in a T25 bottle and incubate for 3 h. (7) After the first 3 h of culture, change the culture media.

Figure 2: Hemocytometer. The five small squares to be counted are marked in red.

Figure 3: Cell morphology. When the media was changed 3 h after the beginning of the culture it resulted in a homogeneous population of mesenchymal cells, which exhibited a fibroblast-like morphology. On the contrary, when the first media change is done after 48 h other cell types besides the fibroblast-like cells can be observed. The scale bar = 1,000 µm. This figure has been modified from Hill *et al.*[9].

Figure 4: Molecular profile of ovarian MSC. (**A**) The isolated cells exhibited a classic MSC marker profile, being positive for three MSC markers by real-time PCR (CD90, CD105, and CD44). (**B**) By protein analysis, CD44 was positive in those cells, and they lacked the expression of two hematopoietic markers (CD34 and CD45). The scale bar = 70 µm. This figure has been modified from Hill *et al.*[9].

Figure 5: Differentiation of ovarian MSC. The isolated cells were able to differentiate into (**A**) mesodermal lineages (chondrogenic, osteogenic, and adipogenic; the scale bar = 70 µm). Moreover, the cells were able to differentiate into both (**B**) endodermal precursors (the scale bar = 70 µm) and (**C**) neurogenic lineages (the scale bar = 50 µm). Interestingly, the cells underwent (**D**) primordial germ cell differentiation, demonstrating a wide capacity of differentiation for ovarian mesenchymal stem cells. This figure has been modified from Hill *et al.*[9].

DISCUSSION:

Herein we provide evidence that MSCs can be isolated from canine ovarian tissue, which is considered biological waste after ovariectomy. Due to the fact that many cell types can be found in the ovary, we proposed a protocol to select MSCs based on their rapid adherence to plastic, which successfully selected cells that grew in a monolayer with a fibroblast-like morphology.

The first report of the derivation of MSCs from bone marrow was based on the plastic adhesion capacity of the MSCs during the first 48 h of culture[11]; however, it has been reported that this method has the potential to isolate a heterogeneous population, phenotypically and functionally, at least for bone marrow[12]. The proposed culture method also selects cells by plastic adhesion capacity and isolates mesenchymal cells from a tissue that has many cell types, without the necessity of sorting the desirable cells. Due to the fact that mesenchymal stem cells express numerous receptors that play an important role in cell adhesion[8], it was hypothesized that if a population of cells with rapid adhesion were selected, it would result in a more homogeneous population at the first passage, with a reliable MSC profile. The representative results provide evidence that a shorter duration plastic adhesion assay can be used to isolate a morphologically homogeneous population of MSCs at the first passage, without the necessity of waiting for more advanced passages or cell sorting.

A critical step in this protocol is to change the media 3 h after the beginning of the culture. As shown in the representative results section, the stem cells obtained from canine ovaries met the three criteria established for the characterization of MSCs: they exhibited a fibroblast-like morphology, were positive for the presence of MSC markers, and were able to differentiate into such mesenchymal lineages as osteogenic, adipogenic and chondrogenic lineages. Cells isolated in this experiment were also successfully differentiated into endodermal precursors, ectodermal lineages, and primordial germ cell lineages. The cells should reach confluence approximately 5 - 7 days after the initial plating. The cells from animals with an advanced age may grow more slowly than those of young animals, and of those, fewer cells may adhere to the plastic. In these cases, the concentration of FBS in the media can be increased to 15%.

Therefore, the selection for MSCs based on the cells with more rapid adherence capacity represents an inexpensive, streamlined, and effective procedure. From a therapeutic perspective, it is most important to highlight that these cells represent a promising tool for regenerative medicine, especially because of their wide range of differentiation.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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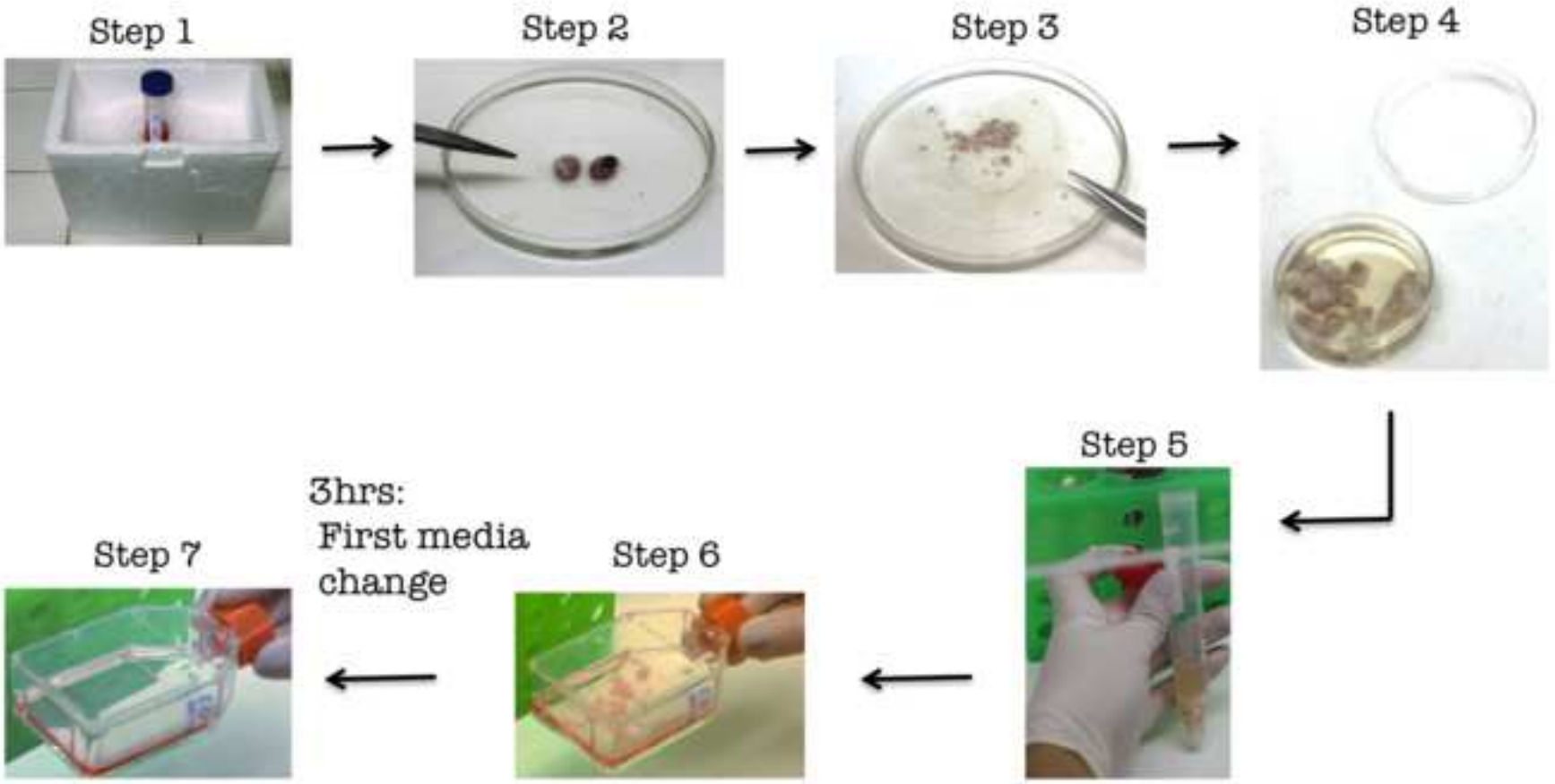

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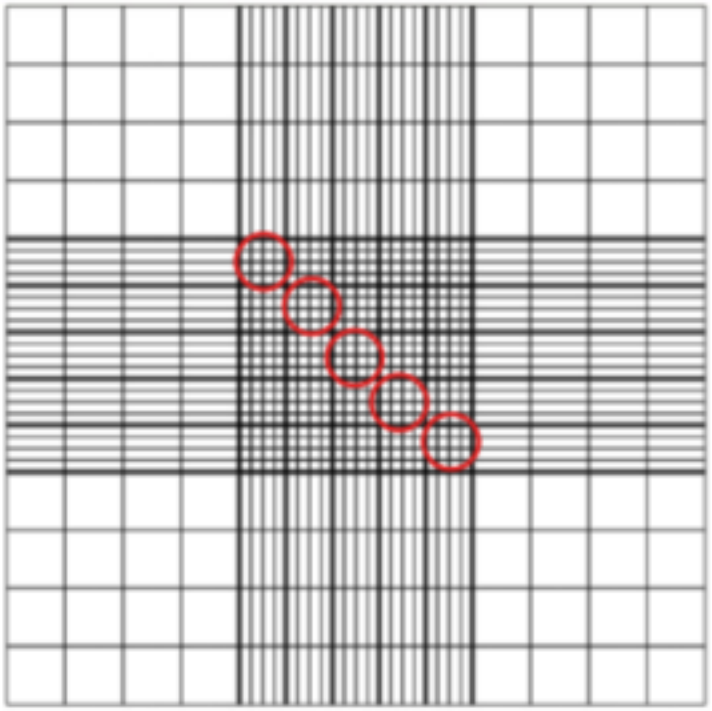

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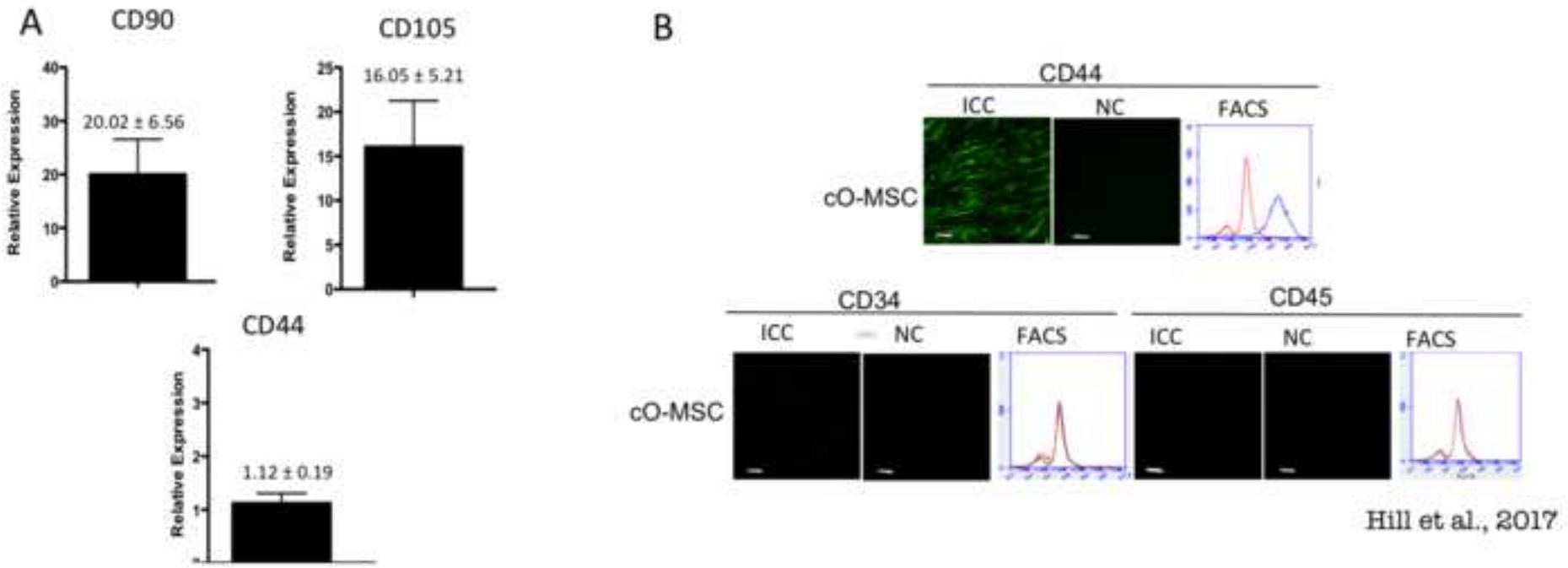

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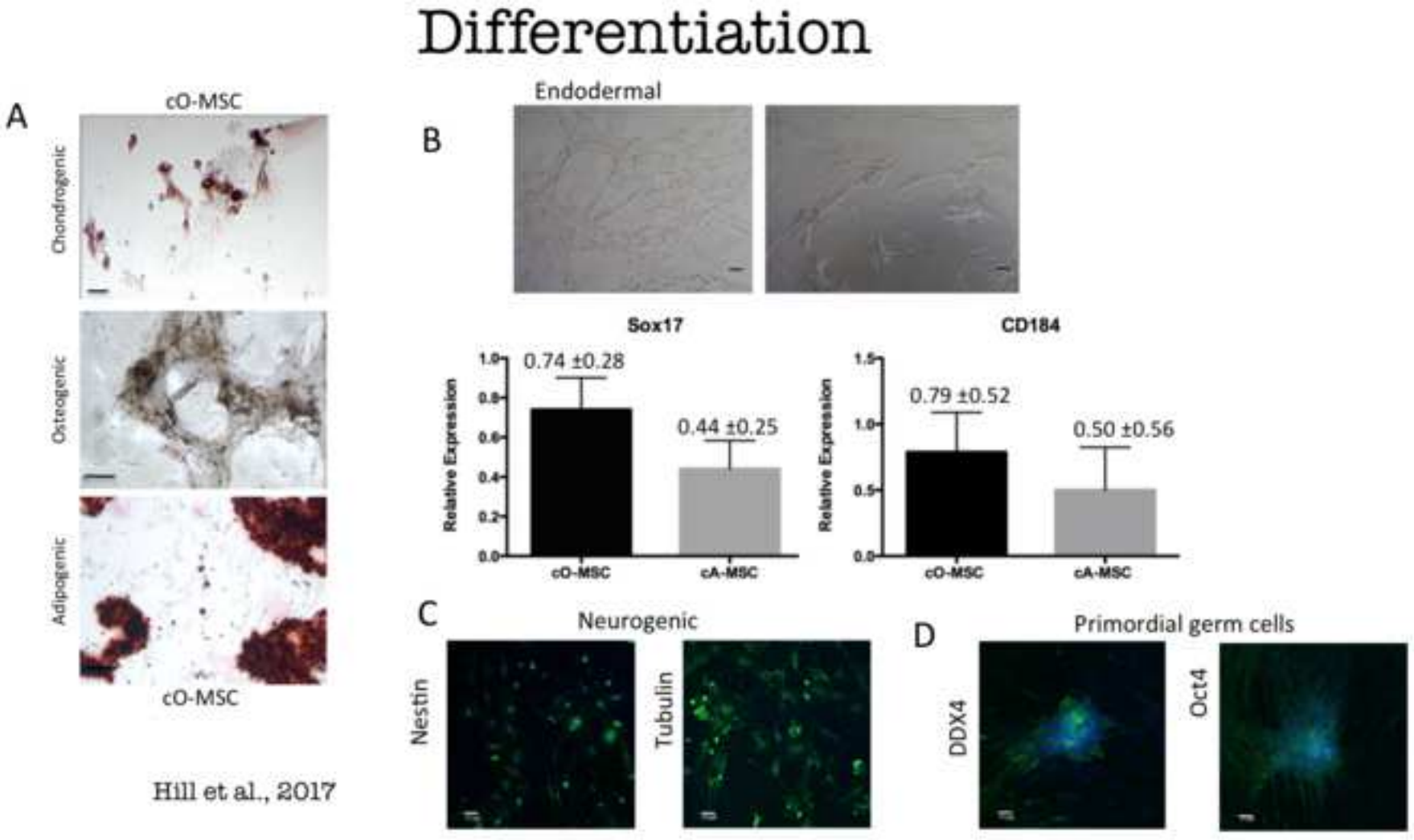

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
DPBS	Thermo Fisher	14190144	
Collagenase I	Thermo Fisher	17100017	
Tissue flask	Corning	CLS3056	
DMEM low glucose	Thermo Fisher	11054020	
FBS	Thermo Fisher	12484-010	
TrypLE express	Thermo Fisher	12604021	
StemPro Adipogenesis Differentiation Kit	Thermo Fisher	A1007001	
StemPro Chondrogenesis Differentiation Kit	Thermo Fisher	A1007101	
StemPro Osteogenesis Differentiation Kit	Thermo Fisher	A1007201	
STEMdiff Definitive Endoderm Kit	StemCell	5110	
Penicillin-Streptomycin	Thermo Fisher	15070063	
CD45	AbD Serotec	MCA 2035S	
CD34	AbD Serotec	MCA 2411GA	
CD90	AbD Serotec	MCA 1036G	
CD44	AbD Serotec	MCA 1041	
Nestin	Milipore	MAB353	
β-Tubulin	Milipore	MAB1637	
DDX4	Invitrogen	PA5 -23378	
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Dear Editor,

Thank you for the comments, we have revised the manuscript and video to make sure that we formatted everything according to the recommendations. Importantly, the authors note that written permission has not yet been obtained from Cell Proliferation to use reproductions of published images. The authors will send updates regarding this situation.

Reviewer #1:

Thank you for attention. We said castration because the dogs had ovariohysterectomy procedures, however we want only the ovaries so we did not include the uterus in the video. Anyhow, we changed castration for ovariectomy.

Reviewer #2:
Thank your for your comments.
We used this technique because many cell types can be found in the ovary and we wanted to select MSC. When we use the three-hour protocol, the population that grows is morphologically homogenous and fibroblastic-like when compared to the population for which media was changed after 48 hours, as we can see in the figure 3. No existing MSC differentiation protocol that the authors are aware of achieves differentiation of all cells; there are always some cells that do not respond to the treatment, and it was not our intention to suggest that the differentiation product was completely homogenous.
Regarding the opportunity to define gene products unique to the tissue MSC, the authors thank the reviewer for this thoughtful contribution. Though gene profiling was not conducted, the authors will take this insight into consideration in future experiments.

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1.1. Gently invert the tube before centrifugation. Centrifuge the tube at 2100 g for 7 min. Remove the supernatant and re-suspend the pellet with 3 mL of expansion media.

1.1.1. Centrifuge the tube at 2100 g for 7 min. Remove the supernatant and add 1 mL of expansion media.

Characterization of ovarian MSC *in vitro*

The goal of MSC characterization is to ensure that isolated cells conform to standard MSC criteria. These include adherence to plastic, positive detection of of mesenchymal surface markers and absence of hematopoietic surface markers, as well as the capacity to undergo mesodermal differentiation.

As shown in **Figure 3**, ovarian derived cells were adherent to the plastic and exhibited fibroblastic-like morphology, fulfilling the first criterion that defines MSC. Moreover, the isolated cells showed the expression of transcripts for the canine MSC markers CD44, CD90, and CD105. In addition, MSC-specific cell surface antigens CD90 and CD44 were detected by early passage FACS analysis and immunocytochemistry. Absence of the markers CD45 and CD34, which are hematopoietic-specific cell surface antigens, was also confirmed by the same techniques(**Figure 4**). This further confirmed that the ovary contains cells expressing an MSC-specific phenotype[9]. The antibodies that were used in this experiment for canine MSC characterization are listed in the **Table of Materials**.

The next step in the characterization of MSC is to attain proof of their ability to differentiate into mesodermal lineages. After 30 days of exposure and culture in lineage-specific differentiation protocols, staining was performed to identify commitment to different lineages (**Figure 5A**). Commitment to the osteogenic lineage was confirmed through Von Kossa staining, which identified calcium deposits. Safranin O proteoglycan staining confirmed chondrogenic commitment, and Oil Red O lipid staining confirmed adipogenic commitment Continuing the investigation of the differentiability of these cells, they were able to undergo differentiation into the ectodermic lineage, as shown by immunostaning for two neuroectodermal stem cell markers: β-Tubulin and Nestin, following exposure of ovary-derived MSCs for to the neuronal lineage differentiation protocol for 10 days (**Figure 5C**). Transcripts for SOX17 and CD184, as well as cell morphologies specific to the endodermal lineage, were observed after exposure to specific differentiation protocols for 5 days **Figure 5B**). Finally, after 14 days of exposure of ovarian derived MSC to germ cell induction media, OCT 4 and DDX4 were identified (**Figure 5D**). Together, these results support a robust and diverse capacity of differentiation for MSC derived from ovarian tissue, when exposed to specific differentiation protocols[9].