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In vitro identification of a stem cell population from canine hair follicle bulge region

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ABSTRACT

Skin is an extensive and easily accessible organ possessing various cell types that are constantly renewed. Previous studies have suggested the presence of a stem cell niche at the bulge region of the hair follicle, which contains cells positive for CD200 and CD34. Thus, this study sought to identify these cell populations in canine skin cells using the following methods 1- collecting samples of adult and fetal skin and isolating and culturing these cells using a method of simple enzymatic digestion and 2- testing the cell cultures for CD200 and CD34 *in vitro* and comparing them with skin tissue samples (*in situ*). Immunofluorescence results were negative for both CD200 and CD34 in frozen and paraffin embedded tissue, whereas the analysis showed that cultured cells positive for CD34, CD200 and double positive cells could be visualized in different percentages. Additionally, the pluripotency marker OCT4 was positive in the isolated cells. Analysis of CD34, CD200 and OCT4 by RT-qPCR showed that there is expression in fetal and adult cells, although no difference was observed between groups. Our results suggest that bulge stem cells from both fetuses and adult dogs were reported with the use of CD34 and CD200 markers in this study, and further techniques for cell isolation and *in vitro* cultivation are needed in order to obtain enriched populations of skin stem cells in dogs.

1. Introduction

The skin is a complex and extensive organ comprising multiple organized cell arrangements derived from the embryonic layers. The skin's two primary layers are the epidermis, which originates from the ectodermal layer, and the dermis, which originates from the mesenchymal layer (Blanpain and Fuchs, 2006). Epidermis and its appendages contain specialized epithelial cells and keratinocytes, whereas the dermis mostly consists of mesenchymal cells (Lanza et al., 2012).

Skin lesions, such as extensive burns and large wounds, are common occurrences in routine veterinary practice. Thus, finding a graft that functions as a skin substitute in skin lesion cases is a subject of many studies. Green et al. described that once a considerable number of cells were available through laboratory cell culture, a patient's own keratinocyte mesh was employed successfully for the regeneration of human skin lesions (Green, 2008). However, according to Tan et al. (2014), a disadvantage of using the patient's own cells is the long period of time required before a significant sample of cells could be obtained for application (Tan et al., 2014). These authors, therefore, suggest the use of fetal skin cells as a possible substitute for autologous cells. Thus, the evaluation of cell cultures derived from fetuses and adults is important for the establishment of a more suitable skin substitute.

Previous studies have proven the existence of hair follicle bulge stem cell niches in mice, humans and dogs (Cotsarelis et al., 1990; Lyle et al., 1998; Pascucci et al., 2006). Analyzing mice follicle cells, Cotsarelis et al. (1990) identified a slow-cycling cell population capable of retaining a tritiated thymidine stain during its cell cycle at the outer root sheath of the hair. Later, Morris and Potten (1999) associated this population of cells with keratinocyte stem cells (Morris and Potten, 1999). These cells, which are self-renewable and responsible for

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maintaining the homeostasis of the tissue, are slow-cycling *in vivo* and can be activated whenever wounds emerge. Additionally, conditions provided by the cell culture medium *in vitro* indicate a high proliferative potential (Lavker and Sun, 2000). These cells are capable of generating epidermal cells and hair follicles, and therefore, this region is responsible for providing cells not just for the growth of new hair but also for the regeneration of the skin (Taylor et al., 2000).

CD200 has already been found in the hair follicle bulge of human skin (Inoue et al., 2009; Kloepper et al., 2008; Ohyama et al., 2006). In dogs, mRNA for K15, CD200 and follistatin in addition to sebaceous gland lineage markers were found in the bulge within reconstituted pilosebaceous structures, suggesting the canine bulge stem cells have contributed to the reorganization not just of hair follicles but also of sebaceous glands (Kobayashi et al., 2010). This makes the multipotency of cells evident in this region. Similarly, CD200 is a molecule associated with the diminishment of graft rejection and immune system regulation (Rosenblum et al., 2004; Yu et al., 2013).

CD34 is a well-known hematopoietic stem cell marker that has been found to be positive for cells at the follicular bulge region in mice and canines (Pascucci et al., 2006; Trempus et al., 2003), although interestingly, it is not found in the human bulge (Inoue et al., 2009; Poblet et al., 2006). Previous studies found that it is possible to isolate living cells from this region through the use of fluorescence-activated cell sorting (FACS) using a CD34 marker (Tumbar et al., 2004). Similarly, a positive CD34 population was obtained with magnetic separation, and these cells were differentiated into neural cells (Najafzadeh et al., 2015).

Some proteins are known for their presence in pluripotent cells. The OCT4 expression occurs in germinal lineages during the preimplantation of embryos and post-implantation epiblasts, making it ideal for the identification of pluripotent stem cells (Shi and Jin, 2010). For this reason, the presence of these stem cells was also monitored in our research.

Thus, the objective of this experiment was to prove the existence of cell populations of CD34 and CD200 within the hair follicle bulge, which could be obtained through simple enzymatic digestion methods. These cells, once identified, could be utilized in basic or applied research protocols, such as in regenerative medicine. Therefore, the isolation and *in vitro* culture of cells derived from fetal and adult canine skin, the labeling of membrane proteins and the quantification of transcripts known to be present in the stem cells of the hair follicle were conducted.

2. Materials and methods

2.1. Sample collection

The procedures from this work were approved by the ethical committee for the use of animals (CEUA) from the "Faculdade de Ciências Agrárias e Veterinárias/Faculdade de Ciências Agrárias e Veterinárias da UNESP – Jaboticabal – FCAV, UNESP" under protocol number 011904/14.

Fragments of skin from adult animals utilized in this study were collected from routine surgeries performed at the "Hospital Veterinário Governador Laudo Natel" at FCAV/UNESP with the consent of the animals' owners. For samples, a healthy skin fragment with a surface area of approximately 1 cm^2 was obtained from each animal (n = 3). Preference was given to extractions during orthopedic surgery, as incisions are generally made at skin regions rich in hair follicles, such as the limbs or the dorsum of an animal.

Fetal skin fragments were obtained from routine surgeries performed at the Obstetrics Department of the same institution. For fetal cell isolation, fragments from three fetuses from different females, aged between 40 and 45 days, were collected from the lateral thoracic region. Fetal age was determined by measuring the distance between the atlanto-occipital joint and the sacrum, as described earlier (Evans and Sack, 1973; Pieri et al., 2015).

2.2. Histological analysis: hematoxylin and eosin staining (HE)

Skin samples collected from the adults and fetuses were frozen in Tissue-Tek O.C.T. Compound Medium (SAKURA N° Cat. SAKU-4583) and stored in a freezer at −80 °C. For sample analysis, frozen sections were infused in cold acetone and stored for 10 min at −20 °C. They were then air dried and placed in 100% alcohol for five minutes, then 95% alcohol for 5 min, 70% alcohol for 5 min, and then they were rinsed in running water for 5 min. Samples were then placed briefly in hematoxylin and rinsed in running water. The slides were then briefly placed in eosin and rinsed in running water. Another alcohol passage at 70° was performed, followed by another at 95°, two more at alcohol 100°, one using a mixture of alcohol and xylene (1:1), and then three passages using xylene only. A drop of Permount mounting medium (Fisher Scientific) was added to the section, and a cover slide was added. The analysis was performed using Olympus IX70 fluorescence microscopy.

2.3. Immunofluorescence of frozen and paraffin embedded tissues

Collected skin samples were frozen in Tissue-Tek O.C.T. Compound Medium (SAKURA N° Cat. SAKU-4583) and stored in a freezer at −80 °C. For analysis of the frozen tissue, 5 µm histological sections were made in a cryostat (SLEE Mainz MEV) and placed on Star Frost® slides, which were then air dried for 30 min and fixed in cold acetone for 10 min in a freezer. For analysis of paraffin embedded tissues, the samples were fixed for 24 h in 4% in PFA, dehydrated in ethanol, and embedded in paraffin. Next, 5 µm thick sections were cut with a microtome.

For immunofluorescence staining, three 5-min rinses in tris-buffered saline (TBS) were performed, then the sample was blocked with 10% goat serum for one hour in a humidified chamber. After this period, the primary antibodies (Table 1) were added to the sections. For the control section, only TBS with 1% goat serum was added. The sections were maintained in a humidified chamber at 4 °C overnight. The following morning, another set of three 5-min rinses with TBS with 1% goat serum was performed, and the secondary antibody was added at a 1:500 dilution rate for one hour. After rinsing, PROLONG® (Gold Antifade Reagent with DAPI – N° Cat. P36935 ThermoFisher Scientific) was used before slide covers were added. The analysis was performed using Olympus IX70 fluorescence microscopy.

2.4. Cell isolation and in vitro culture

For cell isolation, healthy skin fragments with a surface area of approximately 1 cm^2 of both canine fetuses and adults were cleaned, and the subcutaneous tissue was removed. Then, the skin samples were submitted to the process of simple enzymatic digestion, which consists

Table 1
Antibodies used for dog skin stem cell characterization.

Antibodies	Brand	Catalog number	Type	Species	Dilution
CD34	Santa Cruz	Sc7045	Polyclonal	Goat	1:50
CD200	Santa Cruz	Sc323725	Polyclonal	Goat	1:50
OCT4	Santa Cruz	Sc8629	Monoclonal	Goat	1:100
Alexa Fluor 488 Anti-goat IgG	ThermoFisher	A11078	Polyclonal	Rabbit	1:500
Alexa Fluor 680 Anti-goat IgG	ThermoFisher	A21084	Polyclonal	Donkey	1:500

Antibodies used for immunofluorescence, immunocytochemistry and flow cytometry.