



Immunohistochemistry of Canine Hair Follicle Stem Cells (cHFSCs) by using CK15 and CK19

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ABSTRACT

Adult body harbors powerful reservoir of stem cells that maintains homeostasis by tissue regeneration and in response to disease and injury. Hair follicle is a dynamic mini organ supporting important biological functions of the body in maintaining homeostasis and skin tissue self-renewal. This study was carried out with the objective of finding the adult stem cells in canine hair follicular tissue. To conduct this study, adult canine skin samples (n=12) irrespective of breed and sex were collected. To characterize the hair follicle stem cells, paraffin sections of canine hair follicles were immunostained with positive hair follicle stem cell markers like Anti-cytokeratin 15 (CK15) and Anti-cytokeratin 19 (CK19) and FITC conjugated and HRP conjugated secondary antibodies were used. Immunoreactivities for CK15 and CK19 were observed in the bulge/isthmus region of hair follicles in between the infundibulum and suprabulbar regions and occupied most part of the peripheral layer of outer root sheath cell. Immunophenotyping of canine Hair Follicle Stem Cells (cHFSCs) in the bulge region of hair follicle helps in confirmation of *in vitro* culture of cHFSCs from the bulge region which will be further used for translational research.

Keywords: Cytokeratin15 (CK15), Cytokeratin 19 (CK19), Hair follicle stem cells, Stem cells

Adult Stem Cells (ASC) are undifferentiated cells located throughout the body and characterized by self-renewing capacity and multipotency; these cells participated in tissue regeneration of damaged tissues and replenishment of dying cells (Maleki *et al.*, 2014). Hair follicles contained rapidly proliferating and differentiating cells similar to other self-renewing tissues such as bone marrow, gastrointestinal tract and epidermis. The hair follicle was functioning as the “bone marrow of the skin” and continuously remodeled its cutaneous microenvironment including skin innervation and vasculature (Schmidt-Ullrich and Paus, 2005). According to Kobayashi *et al.* (1993) over 95 per cent of the keratinocyte colony forming cells isolated from rat vibrissa were located mainly in the bulge and remainder were present in the bulb region of the hair follicle. These bulge cells were quiescent throughout

the hair follicle cycle but they transiently proliferated at the onset of anagen to generate rapidly proliferating progeny that regenerate a new follicle (Lyle *et al.*, 1999).

Hair follicle stem cells (HFSCs) were characterized by expression of gene markers such as β 1 integrin, cytokeratin 15 (CK15), cytokeratin 19, α 6 integrin, CD71, S100A, Caveolin1, CD200, PHLDA1, follistatin, frizzled homolog 1, nestin (neuron progenitor cell marker) and CD34 glycoprotein in human (Ohshima *et al.*, 2006). The coexpression of cytokeratin 19 (CK19) (HFSCs marker) and Bcl-2 (apoptosis suppressing protein) but not Bax (apoptosis promoting protein) was observed in two distinct areas localized in upper and lower third of human hair

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follicles from both skin biopsies and plucked hairs (Gho *et al.*, 2004). The bulge region of hair follicles of canine, positively stained with CK15, CD200 and follistatin was observed in the outermost layer of the ORS between the insert point of the arrector pili muscle and the sebaceous gland, similar to the distribution of K15 immuno-reactivity in human hair follicles (Kobayashi *et al.*, 2010).

MATERIALS AND METHODS

Canine hair follicle samples (n=12) were collected from surgical cases brought to Madras Veterinary College Teaching Hospital. Collected skin samples were rinsed in normal saline and fixed in 10 per cent neutral buffered formalin. The fixed tissues were dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax (Bancroft and Stevens, 1990). Tissue sections were cut at 3-5µm thickness and used for the routine haematoxylin-eosin staining method.

Immunostaining with FITC conjugated secondary antibody

Deparaffinized skin sections were permeabilized by 500µl of 0.025% triton-100X in Tris Buffered Saline (TBS) for five minutes with gentle agitation in room temperature for two times. 10% goat serum was used as blocking agent for 2 hours. Sections were incubated with primary antibody Anti-Cytokeratin 15 (Cat. No. ab80522, Abcam) at 1.25µg/ml concentration in 1% BSA for overnight at 4°C. Then sections were permeabilized with triton-100 for 10 minutes and then incubated with 1:1000 dilutions of FITC conjugated secondary antibody in 1% BSA for one hour at room temperature. Sections were counterstained by 4'-6-diamino-2-phenylindole (DAPI) with fluoroshield and mounted.

Immunostaining with HRP conjugated secondary antibody

Heat mediated antigen retrieval was done in antigen retrieval device (MERE) with antigen retrieval buffer of Tris/EDTA, with a pH between 8.5-9.0 for 15-20 minutes. Blocking of endogenous peroxidase was done with three per cent hydrogen peroxide for 10 minutes and washed with washing buffer. Sections were incubated with primary antibody, Anti-Cytokeratin 15 (1µg/ml), Anti-Cytokeratin

19 in a moist chamber for one hour and polyexcel HRP conjugated secondary antibody was added and incubated for 12 minutes and sections were washed with three times in wash buffer. Diaminobenzidine (DAB) chromogen solution (1ml DAB buffer + one drop DAB chromogen) added and kept for two to five minutes and washed in distilled water. The sections were counterstained with Gill's haematoxylin.

Hair follicle stem cells were isolated and cultured from canine hair explants by using William's E medium as a growth medium supplemented with 10% fetal bovine serum, and 10 ng/ml of epidermal growth factor, 5ng/ml of insulin growth factor, 2% ABAM and 1% amphotericin B in a humidified atmosphere at 37°C with 5 per cent CO₂. Growth medium was changed every three to four days. Primary culture was maintained upto 14 days to obtain the colonies (Hemavathi *et al.*, 2019). Colonies were immunostained with hair follicle stem cells primary antibody Anti-Cytokeratin 15 (CK15) and FITC conjugated secondary antibody.

RESULT AND DISCUSSION

Canine hair follicles were divided into four regions as bulb, supra bulb, isthmus and infundibulum from the base to outward. The hair follicle's bulb and bulge regions were well demarcated by the indentation of supra bulb region. Similar to murine hair follicles, canine hair follicle also showed eight layers in their cross sections: outer root sheath (ORS), inner root sheath (IRS), henley's layer, huxley's layer, internal layer, cuticle, cortex, medulla from outward to inwards in their cross section (Alcolea and Jones, 2014).

Paraffin sections of the canine skin samples were immunostained with CK15 and CK19 antibodies and location of HFSCs in the hair follicle was detected. In longitudinal sections of hair follicles, the positive CK15 (Fig. 1) and CK19 cells (Fig. 2) were observed to be located above the supra bulb region of the hair follicles and defined as bulge. In human anagen hair follicle, CK19 was expressed within the bulge and suprabulbar ORS and also intermittently between these two region in the bulge region (Purba *et al.*, 2014).

In cross sections of hair follicles, the positively stained cells for CK15 (Fig. 3) and CK19 (Fig. 4) respectively

were found to be on the periphery of the outer root sheath of the hair follicle. The cHFSCs were located on the peripheral layer of the ORS and near the insertion point of APM, as described by Ohyama *et al.* (2006) in human and Kobayashi *et al.* (2009) in canine hair follicle.

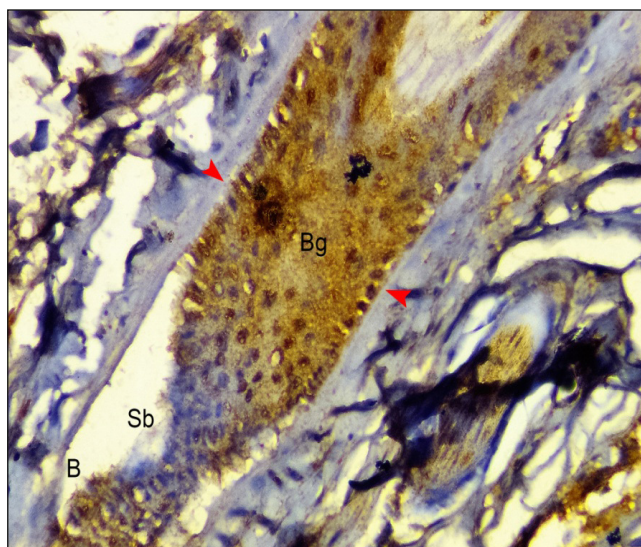


Fig. 1: Photomicrograph of longitudinal section of canine hair follicle showing Cytokeratin 15 (CK15) positively stained cells (arrowheads) at the bulge region. ×200 B- Bulb Sb- Suprabulb Bg- Bulge

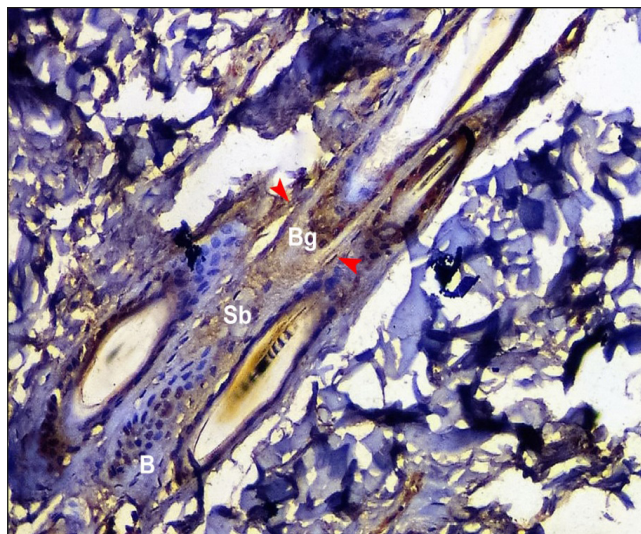


Fig. 2: Photomicrograph of longitudinal section of canine hair follicle showing Cytokeratin 19 (CK19) positively stained cells (arrowheads) at the bulge region. ×100 B- Bulb Sb- Suprabulb Bg- Bulge

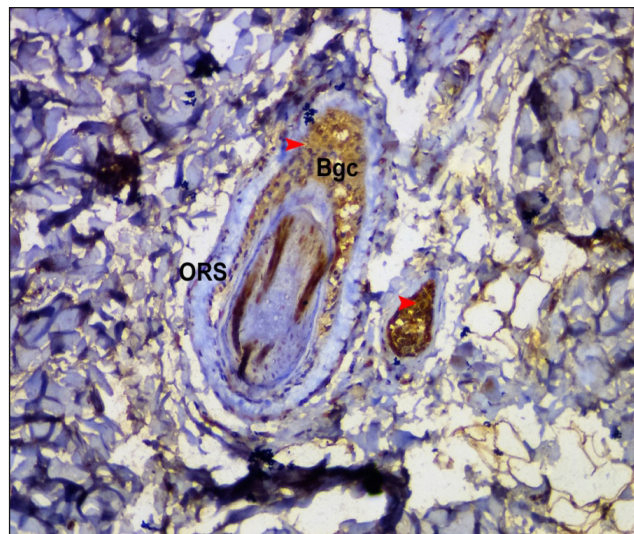


Fig. 3: Photomicrograph of cross section of canine hair follicle showing Cytokeratin 15 (CK15) positively stained cells (arrowheads) within the outer root sheath (ORS). ×200 Bgc- Bulge cells ORS- Outer root sheath

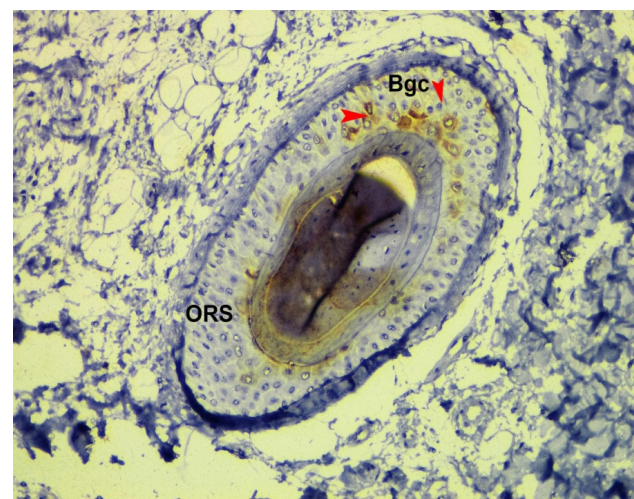


Fig. 4: Photomicrograph of cross section of canine hair follicle showing Cytokeratin 19 (CK19) positively stained cells (arrowheads) within the outer root sheath (ORS). ×200 Bgc- Bulge cells ORS- Outer root sheath

Arrector pili muscle was also immunostained with smooth muscle actin (SMA) antibody which showed the insertion point of the muscle near the hair follicle which served as a landmark to locate the cluster of bulge cells in cross section of hair follicles (Fig. 5). Diverse protrusions in the bulge region of HF were filled with more densely packed

ORS cells exclusively at the insertion point of arrectorpile muscle (APM) level and positively stained with CK15 surface marker (Fig. 6).

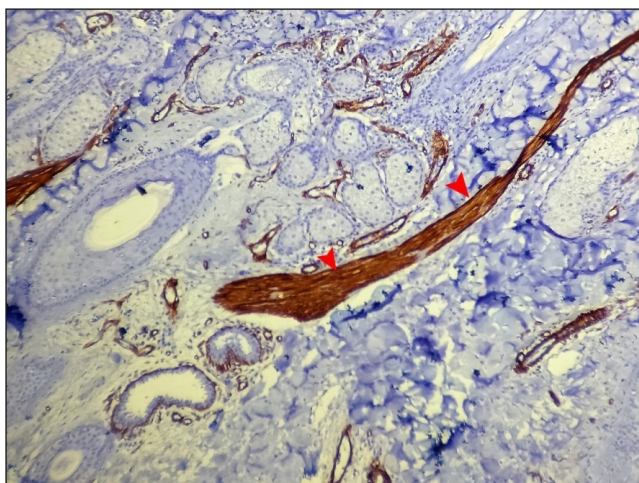


Fig. 5: Photomicrograph of arrectorpile muscle (APM) (arrowheads) immunostained with smooth muscle actin (SMA) antibody to earmark the location of the bulge region HF. $\times 100$

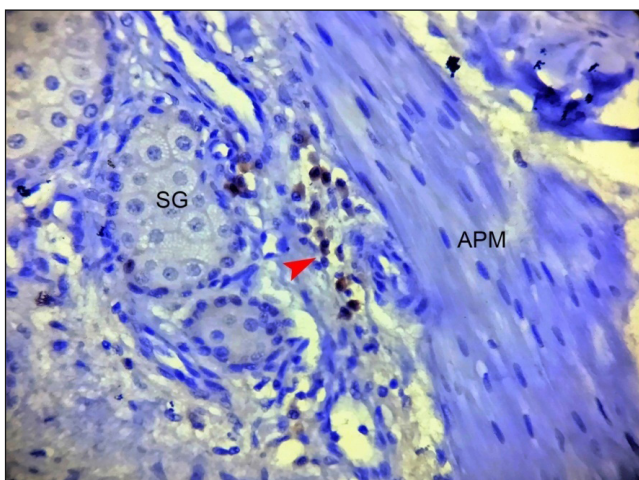


Fig. 6: Photomicrograph of canine hair follicle showing cytokeratin 15 (CK15) positively stained cHFSCs (arrowhead) near the insertion point of arrectorpile muscle (APM). $\times 400$ APM- Arrectorpile muscle SG- Sebaceous gland

ORS protrusions were reported in another study as follicular trochanter, observed at the point of APM insertion in humans (Tiede *et al.*, 2007). This result also corroborates well with findings of Lyle *et al.* (1998) and Wang *et al.* (2012) in human.

In FITC conjugated Antibody staining, the CK15 positively stained cells were observed on the peripheral layer of outer root sheath of the hair follicle. The positive cells showed green fluorescent cytoplasm and blue fluorescent nucleus (Fig. 7). The bulge region of canine hair follicles were positively stained with CK19 and CK15 marker in the outermost layer of the ORS as reported by Commo *et al.* (2000) and Gho *et al.* (2004) who observed CK19 positive reaction in human HF and Kobayashi *et al.* (2009) reported CK15 positively in canine HF.

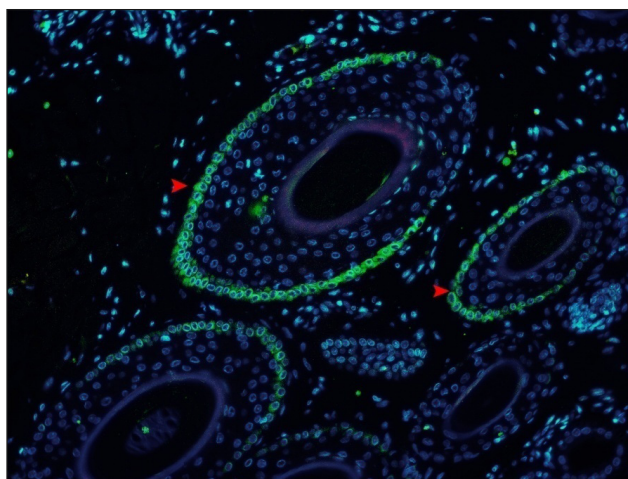


Fig. 7: Photomicrograph of cross section of canine hair follicle showing Cytokeratin 15 (CK15) positive stained cells with green fluorescence (arrowheads) located on the periphery of the outer root sheath (ORS) $\times 200$

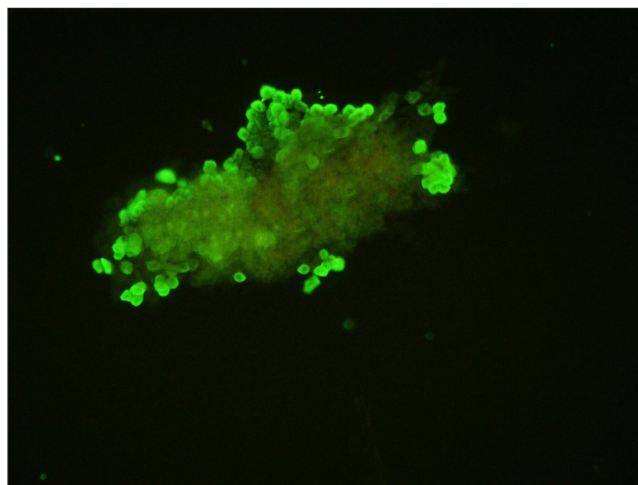


Fig. 8: Photomicrograph of cHFSCs derived from explant culture showing positive expression for Cytokeratin 15 (CK15) surface marker $\times 200$

Immunocytochemistry of isolated and cultured cells from canine hair follicle explants revealed positive expression of CK15 with green cytoplasmic fluorescence as per Kobayashi *et al.* (2009) (Fig. 8). According to Kloepper *et al.* (2008), CK15, CK19 and CD200 were the most useful positive surface markers for human bulge cells. Similar to the human scalp as demonstrated by Inoue *et al.* (2009), CK15 and CK19 expression was mostly found in peribulge and sub-bulge areas of the canine hair follicle in this study.

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