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Adipogenic Differentiation of Canine Hair Follicle Stem Cells (cHFSCs)

N. Hemavathi[1], Sabiha Hayath Basha[1], S. Usha Kumary[1], R. Sivashankar[1]

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ABSTRACT

Background: Homeostasis in adult body is maintained by stem cells which is a powerful reservoir harbored in the body which regenerate tissue 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. Hair follicle protects the body from cold and potential injuries. To know the pluripotency nature of hair follicle stem cells,a study was conducted on canine HFSCs (cHFSCs).

Methods: *In-vitro* culture of cHFSCs was done by William's E medium with several growth factors and passaged. Stemness of the cultured cells was assessed by immunostaining of cells with Anti-cytokeratin 15 (CK15) marker. Viability of cells was checked by acridine orange staining and passage 2 cells were induced for adipogenic differentiation using adipogenic induction media for a period of seven days.

Result: A spindle shaped cell with accumulation of fat droplets was observed in differentiated cHFSCs. Differentiated cHFSCs were positively stained for Oil Red 'O' staining. Thus, this research proves that cHFSCs were multipotent cells which can be differentiated into many other type of cells such as adipocytes, chondrocytes, *etc.*

Key words: cHFSCs, CK15, Differentiation, Hair follicle, Stem cells.

INTRODUCTION

Stem cells are undifferentiated cells, defined by their ability at the single cell level to both self-renew and differentiate to produce mature progeny cells, including both non-renewing progenitors and terminally differentiated effector cells (Wagers and Weissman, 2004). 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). Hair follicle stem cells (HFSC) were located at the bulge/ isthmus region of the human hair follicles (Unna, 1876). The keratinocytes of the bulge region of mice hair follicles were quiescent and long-lived, multipotent (could give rise to all cell lineages of the hair follicle) and had high proliferative potential, thus fulfilling many of the traditional criteria for true stem cells (Lin and Andersen, 2008). Hair follicle stem cells were also characterized by expression of gene markers such as β1 integrin,cytokeratin 15, cytokeratin 19, α6 integrin, CD71, S100A, Caveolin1, CD200, PHLDA1, follistatin, frizzled homolog 1, nestin (neuron progenitor cell marker) and CD34 glycoprotein in human (Ohyama *et al.*, 2006).

The hair follicle bulge cells were thought to differentiate into hair matrix keratinocytes, medulla, cortex, cuticle and basal sebocytes of the sebaceous glands and epidermis which could be used for wound healing (Lavker *et al.*, 1993). Murine HFSCs differentiated into epidermis and called them as bipotent cells.They can also differentiate into neurons, glial cells, keratinocytes and smooth muscle cells *in vitro* in mice (Amoh *et al.*, 2004). The HFSCs were implanted into gap region of a severed sciatic nerve. The HFSCs greatly enhanced the rate of nerve regeneration and the restoration of nerve function. The follicle stem cells transdifferentiated largely into Schwann cells which were known to support neuron regrowth (Hoffman, 2006). Goat hair follicle stem cells (gHFSCs) were differentiated into osteocytes, chondrocytes and myocytes and differentiated osteocytes were confirmed for Von Kossa staining and expressed Osteocalcin. Differentiated chondrocytes were positive for Alcian blue staining and COL2A1 expression and myogenic induction confirmed by positive expression of gHFSCs for MyoG (He *et al.*, 2016). Hence the present study was carried out to find the multipotent ability of canine hair follicle stem cells (cHFSCs) and differentiated as adipocytes.

[1]Department of Veterinary Anatomy, Madras Veterinary College, Chennai-600 007, Tamil Nadu, India.

Corresponding Author: Sabiha Hayath Basha, Department of Veterinary Anatomy, Madras Veterinary College, Chennai-600 007, Tamil Nadu, India. Email: sabihahb@yahoo.com

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MATERIALS AND METHODS

Ethical approval

The research work has been carried out at the Department of Veterinary Anatomy, Madras Veterinary College, TANUVAS, Chennai during 2018-19 as per the approval of the Institutional ethical committee for stem cell research and therapy, Approval No.01/ICSCRT/2018 dated 23.11.2018 of the Chairman, IC-SCRT, Madras Veterinary College, Chennai-07.

Isolation and culture of cHFSCs

Canine skin samples were collected and washed with 2% chlorhexidine for 3 minutes and washed with 70% isopropyl alcohol for a minute (Hibbard *et al.*, 2002). Canine hair follicles were separated from skin by digesting them in Dispase-II enzyme (neutral protease, grade II; Roche; Cat. No. 04942078001; 2.4 U/ml in DMEM) at 4°C for overnight. In this method, to prepare single cell suspension, plucked canine hair follicles were placed into 0.25% trypsin-EDTA solution (SIGMA®; Cat. No. T4049) for digestion at 37°C in CO_2 incubator for 15-20 minutes.Trypsin-EDTA was neutralized by adding serum and the digested bulge follicular cells were dissociated into single cell by gentle pipetting repeatedly and cultured in growth medium containing William's E medium (Sigma- Aldrich; W0397) or DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco®; Cat. No. 10082-147) and 10 ng/ml of epidermal growth factor (EGF) (Thermofisher;PHG0311), 5 ng/ml of insulin growth factor (IGF-I; Cat. No. I8779) two per cent ABAM (ABAM) (Gibco®; Cat.No.15240-062) and one per cent amphotericin B (Sigma- Aldrich;Cat. No. A2942) in a humidified atmosphere at 37°C with 5 per cent CO_2. Culture plates were coated with collagen coating solution (CELL; Cat. No. 125-50) prior to seeding the cells. Cultures of cHFSCs were maintained upto passage 2 (P2) level for differentiation.

Cell proliferation(CD) and population doubling time (PDT) were calculated by following CD=ln (Nf/Ni)/ln 2(Nf-final cell number; Ni- initial cell number; ln- natural logarithm) (Spencer *et al.*, 2012); PDT= 1/r (r = log N_2- Log N_1/ t_2-t_1; N_2- total cells harvested at the hour of t_2. N_2- total cells harvested at the hour of t_1) (Hilmi *et al.*, 2013).

Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 5 minutes and incubated with 8% blocking buffer for 25 minutes. Fixed cells were incubated with 250 µl of primary antibody Anti-Cytokeratin 15 (CK15) (Cat. No. ab80522) (5 µg/ml) at 4°C for overnight and washed further 250 µl of secondary antibody (Goat Anti-mouse IgG FITC conjugated) of 1:1000 dilutions was added and incubated for one hour and counterstained with DAPI. Washing with DPBS was followed after every step of incubation.

Viability assessment

Growth media was removed from cHFSCs culture plates and washed with DPBS. Cells were fixed with methanol for 5 minutes in room temperature. Fixative was removed and cells were washed with distilled water. Acridine orange stock solution was prepared by dissolving 1 g in 1000 ml of Dulbecco's phosphate buffered saline (DPBS). For working solution, Acridine orange stock solution (5 ml) was mixed with 20 ml of 0.1 M Citric acid and 1.3 ml of 0.3 M Sodium heptahydrate. Working solution was poured over the cell colonies and incubated for five minutes in room temperature. Culture plates were washed and observed under 530 nm filter for green fluorescence and 600 nm for red fluorescence.

Adipogenic differentiation and staining

cHFSCs of passage 2 (P2) with 70% confluency were incubated with adipogenic differentiation medium (Stem Pro™ Adipogenesis Differentiation Kit; Gibco™; A1007001) for 7 days in CO_2 in cubator. Adipogenic differentiation medium was changed for every two days. At the end of 7 days of induction, cells were fixed with 4% paraformaldehyde for 10 minutes in room temperature and washed with DPBS twice. Cells were stained with 0.3 per cent of Oil Red 'O' (Sigma®; O1391) for 50 minutes in room temperature to detect the lipid droplets in the cell cytoplasm.

RESULTS AND DISCUSSION

In primary culture of canine hair follicles bulge, round cells with clear cytoplasm was observed. On second day most of the cells were shown plastic adherence. The adhered cHFSCs showed small cytoplasmic processes surrounding round to oval nucleus with two or more nucleoli. The colonies became larger and united together forming a monolayer of cHFSC in the culture plate. Most of the colonies looked like cobblestone on appearance in the seventh day (Fig 1). Then cHFSCs were subjected to passage 1 (P1) (Fig 2) and passage 2 (P2) (Fig 3). The CD at primary culture was found



Fig 1: Photomicrograph of cHFSCs showing cobble stone morphology with 70-80 per cent confluency on seventh day of culture (×200).



Fig 2: Photomicrograph of cHFSCs in P1 (×200).

to be $0.11\pm0.21\times10^6$ which remained the same to $0.11\pm0.24\times10^6$ at P1. The CD at P2 was higher than P1 and measured about $0.49\pm0.10\times10^6$. The PDT of cHFSCs in primary culture was observed as 4.36±0.02 hours in collagen coated culture plates. The PDT slightly increased in P1 as 4.38±0.02 hours than primary culture. The PDT of P2 was found to be 4.29±0.06 hours which was lesser than P1 culture.

Immunocytochemistry

The cultured cells of the hair follicle were immunostained with Anti-Cytokeratin 15 and DAPI on day seven of post incubation. The positive stem cells were confirmed by observing intra cytoplasmic green fluorescence and intra nuclear blue fluorescence (Fig 4).

Acridine orange staining

The viability of cells during culture was assessed by staining the primary cultured cells and P1 cells with Acridine orange. Viable cells were observed with emission of green fluorescence. The cells which were not viable appeared to emit light orange to red fluorescence in nucleus and yellow fluorescence cytoplasm. In primary culture, about 70 per cent of cHFSCs emitted green fluorescence (Fig 5).

Adipogenic differentiated cHFSCs

After incubation of cHFSCs in adipogenic media, on the second day of induction, the cell morphology changed from cobblestone appearance to long spindle shape cells with long cytoplasmic process (Fig 6). On day three, fat droplets were observed in the cytoplasm of differentiated cells and became rounded in appearance. On day seven of post induction with adipogenic media, cytoplasm of cells contained clusters of fat droplets (Fig 7) which were positive to Oil red 'O' staining (Fig 8).

In this study, cultured cHFSCs gave rise to macroscopic colonies within six days after initial seeding and colonies were homogenously made up of small flat cells near the periphery and the interior of the colony was stratified and consisted of large flat cells as described by Barrandon and Greens in human in 1987. Cells were closely aligned and gave cobblestone morphology which was similar to the results of goat and human hair follicle stem cell cultures respectively (Zhang *et al*., 2006; Hilmi *et al*., 2013).

In the present study, only flat keratinocyte type of cells were isolated from canine hair follicles whereas de Castro reported that mixed population of cells, mostly round and

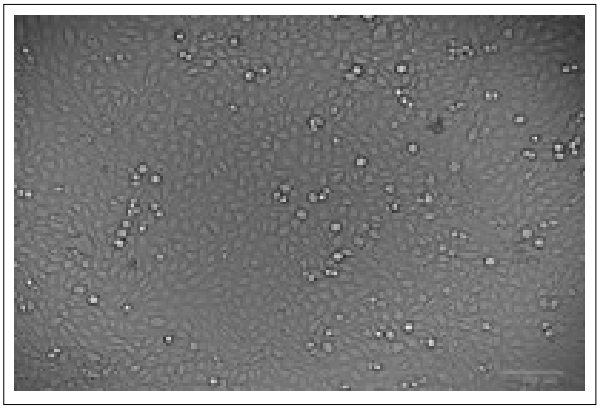

Fig 3: Photomicrograph of cHFSCs in P2 (100 µm).

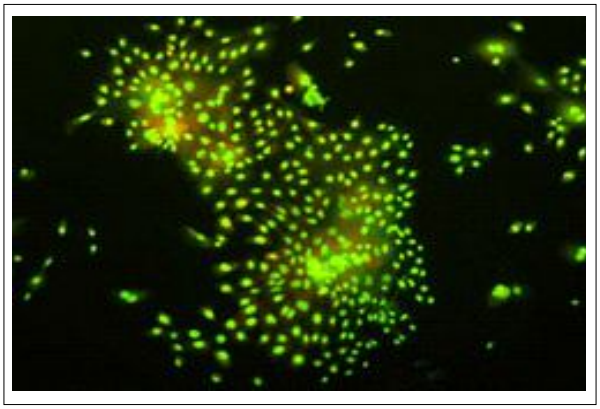

Fig 5: Photomicrograph of cHFSCs showing 90 per cent viable cells emitting green fluorescence-Acridine orange staining (×100).

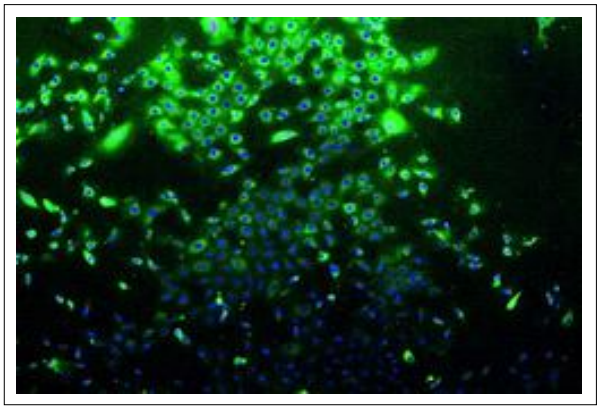

Fig 4: Photomicrograph of cHFSCs culture showing positive expression for cytokeratin 15 (CK15) surface marker with DAPI (×200).

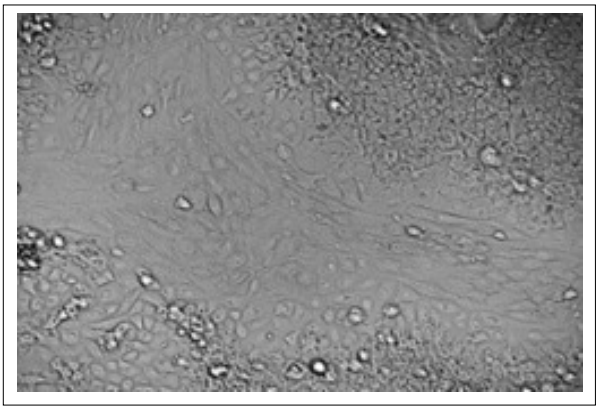

Fig 6: Photomicrograph of cHFSCs showing *in vitro* adipogenic differentiation: day-2 morphology of cHFSCs changed from flat cells into spindle shaped cells (25 µm).

fusiform shaped cells were isolated from canine fetal and adult skin (de Castro *et al*., 2018). In the cell viability assay, the dead cells were observed as yellow to red in fluorescence and viable cells fluoresced green in colour as reported by Bank (1988). Sieber-Blum *et al.* (2004) reported that the initial doubling time of mouse HFSCs was approximately six hours and the PDT for human HFSCs cultured in uncoated plate with CnT07 medium was 21.48±0.44 hours and in KSFM coated plates, it was 30.73±0.75 hours (Hilmi *et al*., 2013) which was reduced to 4.36±0.02 hours in primary culture of cHFSCs cultured in collagen coated plates and PDT was decreased in P2 level and calculated as 4.29±0.06 hours in the present study.

The expression of CK15 transcription factor and DAPI was demonstrated as intra cytoplasmic green fluorescence and intranuclear blue fluorescence respectively in cultured cHFSCs from 7-14 days of culture as in gHFSC (He *et al*., 2016) and in human HFSCs, positive expression was noticed along with CD200, CD34, K14, K15, CD271 and K1 markers (Hilmi *et al*., 2013; Inoue *et al.,* 2009). Shen also performed immunofluorescence detection for K19 and β1-integrin in human HFSCs (Shen *et al*., 2017). The hair follicle pluripotent nestin-driven GFP stem cells were positive for the stem cell marker CD34 but negative for CK15 and β-III tubulinin mice (Amoh *et al*., 2005).

Stem cells from dermal papilla of human hair follicles exhibited mesenchymal stem cell (MSC) immunophenotype and differentiated into all mesenchymal lineages and they were termed as human hair follicle derived mesenchymal stem cells (hHF-MSCs) (Liu *et al*., 2010). Adipogenesis property had decreased significantly in over time (Bajpai *et al.,* 2012) Interestingly, loss of adipogenic differentiation potential was prevented by the adhesion of human Bone marrow mesenchymal stem cells to denatured type I collagen, suggesting a critical role of extracellular matrix in the maintenance of stem cell multipotency (Mauney *et al*., 2005). Adipogenic differentiation of canine hair follicle bulge stem cells has not been reported yet. *In-vitro* adipogenesis from cHFSCs was evident by aggregation of lipid rich droplets with cluster of differentiated cells stained positive by Oil red 'O' cytochemical staining after seven days of induction period. Review of earlier reports have evinced that the adipogenic differentiation of HFSCs from canine bulge cells was not proved in any species so far. Further validation of adipogenic differentiation of cHFSCs through qPCR is needed to confirm the differentiation property which was not performed in this study.

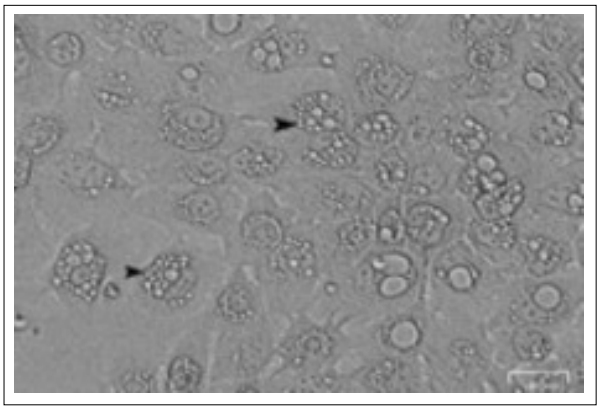

Fig 7: Photomicrograph of cHFSCs showing *in vitro* adipogenic differentiation (arrow heads) on seventh day of post induction (25 µm).

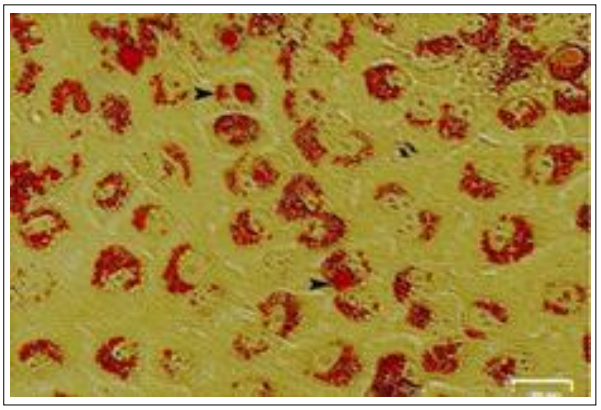

Fig 8: Photomicrograph of differentiated cHFSCs showing positive reaction to Oil red 'O' (arrow heads) after seven days of adipogenic induction (25 µm).

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Conflict of interest: None.

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