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Histology and ultrastructure of canine hair follicle stem cells (cHFSCs)

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Abstract
The adult hair follicle contains stem cells that maintain and regulate the cyclical hair growth and differentiate into multiple cell types that collectively produce a pigmented hair. The present study was conducted on adult canine skin to identify the location of hair follicle stem cells and its surroundings. Canine skin samples were fixed and processed for normal routine histological and transmission electron microscopical analysis. The location of undifferentiated cells in the hair follicle was identified between the opening of the duct of sebaceous gland and the insertion point of arrector pili muscle and notified as bulge. The projected outer root sheath cells of the bulge are showing single nucleolus with euchromatic karyoplasm in their nucleus and the cytoplasm was devoid of keratin filaments and other organelles. These unspecialized cells are surrounded by spindle shaped telocytes and forming a niche in the bulge region. Thus, our results endorse the hypothesis that the canine Hair Follicle Stem Cells (cHFSCs) are located in the isthmus/bulge region of the canine hair follicle.

Keywords: Histology, cHFSCs, hair follicle, stem cells, telocytes

Introduction
The hair follicle (HF) had a wide range of functions including thermoregulation, physical and immunological protection against external insults, sensory perception, social interactions and camouflage [19]. The mammalian skin and its appendages were derived from ectoderm and mesoderm during embryogenesis. The epidermal-dermal interactions resulted in hair follicle morphogenesis [5]. Most omnivores and herbivores (horses, cattle, pigs, rats and mice) had simple follicle with single hair shaft. Carnivores (dogs, cats) and rabbits had compound hair follicle which contained thick primary hair shaft and thin secondary hair shafts [9]. The HF was composed of concentric rings of an external outer root sheath (ORS) attached to the basal lamina and contiguous with epidermis, a channel (inner root sheath, IRS) and hair shaft [11]. HF was divided into three major anatomic regions extending from epidermis to the base: infundibulum, isthmus and inferior segment of which the latter was composed of suprabulbar region and bulbar region. The infundibulum and isthmus were permanent portions whereas the inferior segment underwent regression during catagen phase and absent in telogen phase of hair cycle [19].

The skin was rich in easily-accessible stem cells. HF contained rapidly proliferating and differentiating cells similar to other self-renewing tissues such as bone marrow, gastrointestinal tract and epidermis [4]. The epidermis contained two subpopulations of progenitor/stem cells: basal keratinocytes and cells residing in the bulge region of the human hair follicles. The putative epithelial stem cells in the hair follicle bulge were thought to play pivotal roles in the homeostasis, ageing and carcinogenesis of the cutaneous epithelium [7]. Unna was the one who first described about bulge as when the new hair follicle emerged from skin and grew next to the old hair which persisted into next hair cycle. This created a protrusion or 'bulge' near the new hair follicle [17]. Cotsarelis *et al.* (1990), was the one who first localized putative epithelial stem cells as Label Retaining Cell (LRC) to the hair follicle bulge of mouse hair follicle and labeled them with [^{3}H] TdR in vivo [3]. Epidermal stem cells were located in the bulge area of the hair follicle as shown by label retaining assays [10]. The hair follicle stem cells (HFSCs) were located in the hair follicle outer root sheath (ORS) as a bulge region [14]. The neural crest cells from bulge explants were pluripotent in nature in *in vitro* clonal analysis studies [15]. The bulge was not just as out pouching of outer root sheath (ORS) cell. The bulge area was not only kinetically but also morphologically distinct from ORS cells [6]. Therefore considering the importance and uniqueness of these cells, the

following study was undertaken to identify the stem cells niche in canine hair follicles.

Materials and Methods

Ethical approval: The research work has been carried out as per the approval of the Institutional ethical committee for stem cell research and therapy and was processed in the Department of Veterinary Anatomy in collaboration with Centre for Stem Cell Research and Regenerative Medicine, Madras Veterinary College, Chennai-07.

Procedure

For light microscopy

Irrespective of sex, age and breed, canine hair follicle samples were collected from surgical cases brought to Madras Veterinary College Teaching Hospital with the consent of the owner (Sample No:12). Collected skin samples were rinsed in normal saline and fixed in 10 per cent neutral buffered formalin for 24 hours. The fixed tissues were dehydrated in ascending grades of alcohol (50%, 70%, 90%, 100%-I, 100%-II), cleared in xylene for three changes. Then tissues were infiltrated with paraffin wax (three times) and embedded in paraffin wax. Tissue sections were cut at 3-5µm thickness by rotatry microtome and stained with haematoxylin-eosin and Masson's trichorome method for histomorphological analysis.

For transmission electron microscopy

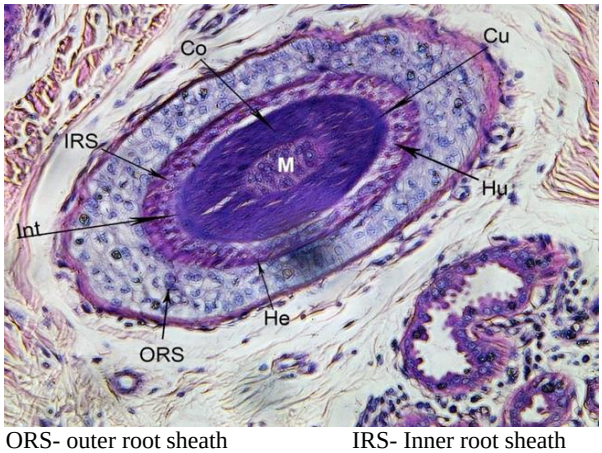
Skin samples were collected and prefixed at three per cent glutaraldehyde and stored at 4°C. Subsequently, the tissues were washed, three changes (each 30 minutes) in cold sodium cacodylate buffer solution (pH 7.4) and post fixed in one per cent osmium tetraoxide for two hours at 4°C. The tissues were then dehydrated in ascending grades of alcohol, propylene oxide: epoxy resin mixture and embedded in Epon-araldite mixture. Semi thin (one micron) sections were stained by toludine blue. Ultra thin sections (600Å to 900Å) were prepared on Leica ultracut microtome, mounted on uncoated copper grids and stained with saturated solution of uranyl acetate and lead citrate. The ultra thin sections were examined under Jeol JEM 1400 transmission electron microscope, computer augmented transmission electron microscope operated at 60-kilowatt ampere (KV).

Results and Discussion

Light microscopy

Hair papilla was observed beneath the hair bulb region. Four parts of hair follicle (bulb, supra bulb, isthmus and infundibulum) were observed. The hair follicle's bulb and the bulge region were well demarcated by the indentation of supra bulb region. In this study, the eight layers of canine hair follicle were observed in cross section of the follicular tissue [2]. The outer root sheath (ORS) was observed to be made up of large sized cells with dense nucleus. The inner root sheath of hair follicle (IRS) was divided into three layers such as Henle's layer (He), Huxley's layer (Hu) and inner most layer of ORS interlocked with cuticle (Cu). Henley's and Huxley's layer were made up of flat to cuboidal cells. The cuticle and the cortex (Co) were observed as keratinized layer and the central portion, medulla (M) appeared to be vacuolated (Plate-1). The location of bulge was identified between the opening of the sebaceous duct and the insertion point of arrector pili muscle. In longitudinal section, the isthmus area was densely packed with small cells than other area of outer root sheath. The cells of the bulge area had convoluted nuclei surrounded by a small area of cytoplasm (Plate-2).

In transverse section, the bulge area was noticed on the same section of arrector pili muscle [8]. In Masson's trichrome staining, diverse protrusions in the bulge region of HF were filled with more densely packed ORS cells exclusively at the insertion point of arrector pili muscle (APM) level [18] and the ORS protrusion was termed as Follicular Trochanter [16].

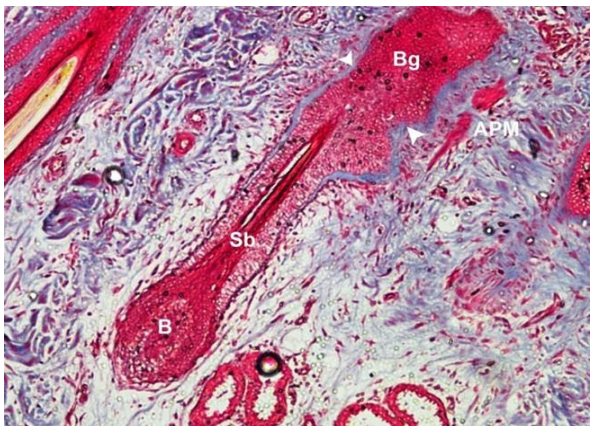


ORS- outer root sheath	IRS- Inner root sheath
He- Henle's layer	Hu- Huxley's layer
Int- Internal Root sheath	Cu- Cuticle
Co- Cortex	M- Medulla

Plate 1: Photomicrograph of cross section of canine hair follicle showing eight layers of hair follicle

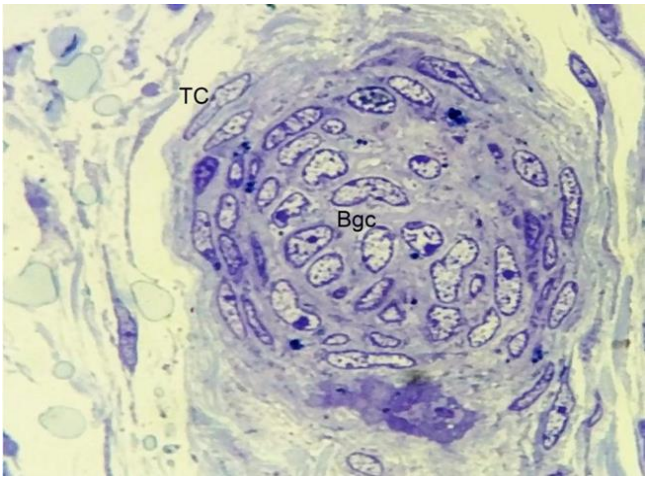
Semi thin sections

Semi thin sections of canine hair follicle stained with Toludine blue showed the stem cell niche, in the bulge region of the follicle as a cluster of cells with different morphological features. The cell sizes varied from small to large. Shape of the cells varied from spherical to oval and also few cells with irregular shape were noticed. Cell boundaries were indistinct except in certain areas. The cytoplasm of the bulge cells was homogenous and lightly stained. Most of the cells in the bulge region showed indentation in nuclear membrane. Electron dense, single round nucleolus was found in each cell nucleus with euchromatic karyoplasm (Plate-3). This bulge stem cell niche was surrounded by long spindle shaped telocytes with cytoplasmic processes on both ends. The nucleus of telocytes was uniformly elliptical in appearance. The cytoplasm of the telocytes was homogenous and darkly stained (Plate-3).



B- Bulb	Sb- supra bulb
Bg- Bulge	APM- Arrector pili muscle

Plate 2: Photomicrograph of longitudinal section of canine hair follicle showing bulge niche of cHFSCs (arrowheads)



Bgc- Bulge cells TC- Telocytes

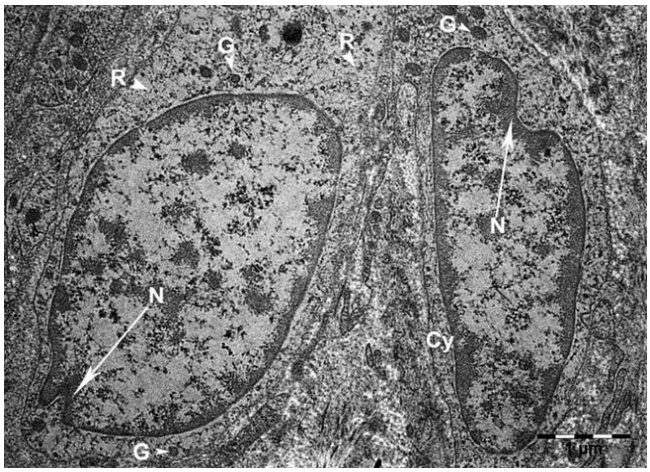
Plate 3: Photomicrograph of semi thin section of canine hair follicle showing the bulge niche with stem cells surrounded by telocytes

Transmission electron microscopy

The cytoplasm of the bulge cells was thin and filled with free ribosomes, glycogen particles and devoid of aggregated keratin filaments and cell organelles (Plate-4). But a larger amount of keratin filaments and cytoplasmic organelles were noticed in the other outer root sheath cells (Plate-5). Desmosomes were completely formed in between bulge cells. Bulge cells which were located on the periphery were larger when compared to inner cells. Nucleus of some bulge cells contained single nucleolus with euchromatic karyoplasm.

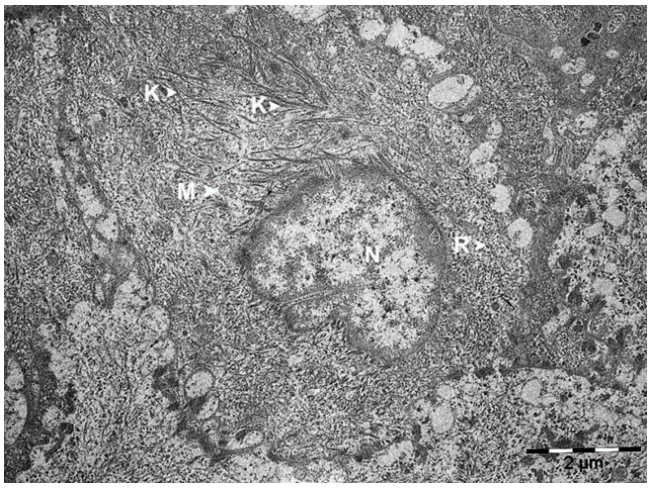
In line with previous findings, this present ultrastructural study also, the size of the bulge cell in the niche varied with each other and the nucleus was round to oval in shape with intendation [6] [13]. Nucleolus was noticed in few bulge cells. The cytoplasm of the bulge cells contained free ribosome and no keratin filaments. The cytoplasmic organelles were absent in bulge cells as noticed in human hair follicles HFSCs [1].

In the present study, the bulge niche of canine hair follicles was surrounded by telocytes with elongated telopodes as described in human hair follicle's bulge [12]. The nucleus of telocytes was elongated with absence of nucleolus and the cytoplasm consisted of very long prolongation or processes (telopodes). These telopodes contained fibrillar segments and dilated, cistern-like regions where cytoplasmic organelles were located.



N- Nucleus with intendation Cy- Cytoplasm
R- Free Ribosomes G- Glycogen particles

Plate 4: Transmission electron micrograph of the bulge cell showing undifferentiated morphological features.



N- Nucleus R- Free Ribosomes
K- Keratin filaments M- Mitochondria

Plate 5: Transmission electron micrograph of the outer root sheath cells (ORS) showing differentiated morphological features.

Conclusion and summary

The cHFSCs were located in the isthmus region of canine hair follicle between the opening of the sebaceous duct and the insertion point of arrector pili muscle. In light microscopic study, the cHFSC niche/ bulge was observed as a subtle projection from ORS to dermis near arrector pili muscle insertion in cross section of hair follicle. Ultrastructurally, the nucleus of bulge cells showed indentation on its nuclear membrane with granular karyoplasm and cytoplasm was filled with free ribosomes, glycogen particles and devoid of other cellular organelles and keratin filaments. The bulge niche was surrounded by telocytes with its elongated cytoplasmic processes. This study will be helpful to identify the stem cell niche of hair follicle for further translational studies.

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