Original Article

Bulge Cells of Rat Hair Follicles: Isolation, Cultivation, Morphological and Biological Features

Maliheh Nobakht, Ph.D.[1, 2, 3]*, Nowruz Najafzadeh, M.Sc.[1, 4], , Manouchehr Safari, Ph.D.[5], Nahid Rahbar Roshandel, Ph.D.[6], Hamdollah Delaviz, Ph.D.[7], Mohammad Taghi Joghataie, Ph.D.[1], Mehrdad Bakhtiyari, Ph.D.[1], Sara Asalgoo, M.Sc.[1], Farid Safar, M.D.[8]

1. Anatomy Department, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
2. Antimicrobial Resistance Research Center, Iran University of Medical Science, Tehran, Iran
3. Cellular and Molecular Research Center, Iran University of Medical Science, Tehran, Iran
4. Anatomy Department, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran
5. Anatomy Department, School of Medicine, Semnan University of Medical Sciences, Semnan,Iran
6. Pharmacology Department, School of Medicine, Iran University of Medical Sciences, Tehran,Iran
7. Anatomy Department, School of Medicine, Yasuj University of Medical Sciences,Yasuj, Iran
8. Dermatology Department, Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran

** Corresponding Address: P.O. Box: 14155-6183, Anatomy Department, Cellular and Molecular Research Center, School of Medicine, Iran University of Medical Sciences, Tehran, Iran*
Email: manob@iums.ac.ir

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Abstract

Objective: Transplants of multipotent stem cells have been shown to have a neuroprotective effect after central nervous system injury. The bulge region of the hair follicle has been reported as a putative source of hair follicle stem cells (HFSC) for many years; however, few studies have documented the properties of bulge derived cells in vitro until now. This study was conducted to isolate and culture bulge cells from rat hair follicles and to determine the morphological and biological features of the cultured cells.

Materials and Methods: The bulge region of the rat whisker was isolated and cultured in Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12) supplemented with epidermal growth factor (EGF), cholera toxin. Dissociated bulge stem cells were differentiated on coated substrates together with NT-3. The morphological and biological features of cultured bulge cells were observed by light microscopy and immunocytochemistry methods.

Results: Our results showed that newly proliferated cells could be observed on the 4th day after explantation. The expression of a neural progenitor marker, nestin, was seen before differentiation of the bulge cells. The differentiated cells expressed βIII-Tubulin and RIP, which are the markers of neural and glial lineages.

Conclusion: The results indicated that the bulge cells cultured from the rat hair follicle had the characteristics of stem cells and could differentiate into neural and glial lineages.

Keywords: Nestin, Beta III Tubulin, RIP, Hair follicle

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Introduction

Stem cells were first identified as pluripotent cells in embryos. Named embryonic stem cells they reside in the inner cell mass of the blastocysts (1). Throughout life, stem cells in the body have the ability to produce new cells, either as a result of phisiological function. It has been reported that their regeneration capacity is dependent on adult stem cell sources (2). Adult stem cells are located in a compartment called a niche. In this area stem cells are surrounded by a variety of differentiated cell types (3).

Skin is the largest organ and has fundamental functions in the human body, including the regulation of body temperature, preservation of the fluid balance, and keeping the body safe from injuries. The skin has three well defined histological appendages; the epidermis, hair follicles and sebaceous glands, (2, 4, 5) which form through complex epithelio-mesenchymal interactions during embryogenesis(6).

Hair follicles undergo a cyclical process from an active growing stage (anagen), through a regression stage (catagen) to a rest stage (telogen) (2, 7). The hair follicle is made up of an outer root sheath, inner root sheath and a shaft. These adult

stem cells are located in a particular area of the hair follicle called the bulge. The bulge region contains a population of stem cells capable of forming the follicle, epidermis and sebaceous gland (4, 6). The bulge region of the hair follicle has different stem cells for hair and skin. Some of them are nestin expressing cells (5, 6). Such stem cells have been derived from the bulge region of the adult mouse and human (6, 8-10). However, a major obstacle in this field has been the lack of operative isolation methods and reliable molecular markers. Bulge cells have properties characteristic of epithelial stem cells in that they are slow cycling and live a long time within the hair follicle (6, 11, 12).

Although it is more than 15 years since Cotsarelis et al demonstrated the location of infrequently-cycling, label-retaining cells in the bulge region of the outer root sheath (13) , the characteristics and the behavior of hair follicle stem cells have not yet been fully understood. Extensive knowledge of the bulge region is crucial to answer this question. A recent observation in the field of skin biology was that epidermal keratinocytes could be grown in culture and the properties of the cells were also discussed (14).

In recent years, many researchers have been devoted to establishing cultures of hair follicle cells of the mouse, rat and human (11, 15, 16). Growth of the rat hair follicle in primary culture has been reported using vibrissa follicles (16), but the exact characteristic of cells from the bulge region has not been investigated in detail. In this report, we describe a modified method to remove rat hair follicles. The rat bulge, dissected on the basis of morphologic features, was cultured and passaged successfully. Subsequently, the biological nature and the expression of nestin in undifferentiated cells, and βIII-Tubulin and RIP in differentiated cells were also studied. We believe that work on the characteristics of hair follicle stem cells is essential for understanding follicle regeneration and cell therapy for the treatment of alopecia and central nervous system (CNS) disorders.

Materials and Methods

Animals

All animal experiments were carried out according to the guidelines of the Iranian Council for Use and Care of Animals and were approved by the Animal Research Ethical Committee of Iran University of Medical Sciences. Female Wistar rats (n=20, 3-5 weeks) were purchased from the Razi Institute animal facility. The rats were permitted free access to water at all times and were maintained under light dark cycles (17).

Media

Media used were Dulbecco's modified Eagle's medium and Ham's F12 medium (3:1) (Sigma), supplemented with 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 µg /ml streptomycin, 0.5 µg/ml amphotericin B), 10ng/ml epidermal growth factor (EGF, Sigma-Aldrich), 10-9 M cholera toxin (Sigma-Aldrich), 0.5mg/ml hydrocortisone, 3.4 mM L-glutamine, 5µg/ml insulin and 0.135 mM adenine.

Hair follicle Isolation and Cultivation

The rats were sacrificed with ether and the whisker follicles were dissected as described by Sieber-Blum (18, 19). Briefly, the animal head was rinsed in a mixture of 1:1 betadine and hydrogen peroxidase for 3 minutes. After rinsing with 70% alcohol, the tissues were trimmed into small pieces (4×8 mm^2) and the samples were incubated in 2mg/ml collagenaseI/dispaseII solution (Sigma-Aldrich) at room temperature for 30 minutes. Most of the connective tissue and dermis around the follicles was removed and the whisker follicles lifted out (Fig 1A). After two rinses, the follicles were transferred into a 35-mm dish. The bulge region was then amputated from the upper follicle by making two transversal cuts respectively at the site of the enlargement spots of ORS with a fine needle, and the collagen capsule was incised longitudinally (Fig 1B).

The culture procedure used was that previously described by Yang and colleagues (17) with a slight modification. After an additional two rinses the bulges were transferred into a new dish at a density of 20 per dish. Briefly, 20 isolated bulges were cut into small pieces, plated into 6-well culture plates precoated with collagen type I (Sigma) prior to cultivation (the plates were pre incubated for 2 hours with medium then the medium was removed) and immersed in a 3:1 supplemented mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) containing 10% fetal bovine serum as described. The bulges were allowed to attach to the collagen for 1 hour. All dissection and cultivation procedures were performed under sterile conditions and incubation was at 37°C and 5% CO_2.

Subculture and proliferation

Within approximately 4 days initiation of the outgrowth of bulge cells from the bulges was observed. One week after onset of this outgrowth the bulges were removed from the culture plates and the cells collected by incubation with a 1:1 mixture of 0.125% trypsin (Sigma) and 0.02% EDTA

(Sigma) for 20 minutes at 37°C. The dispersed cells were centrifuged for 10 minute at 2000 rpm and placed in other collagen coated plates and incubated for another week with a medium change every 3-4 days. Cells were routinely passaged every 7 days.

Colonal Culture

To assess proliferation, isolated bulge cells were cultured at colonal density. A colonal density cell suspension consisting of 20-50 cells was plated into fresh collagen coated plates. The cells adhered within 45-60 minutes. Three hours after plating single cells were marked by circling the underside of the plate with a permanent marker (5mm diameter).

Immunocytochemical Staining

Cells plated on collagen coated coverslips were washed 3 times for 5 minutes with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 minutes. The fixed cells were then washed for 3×5 minutes with PBS and incubated in blocking buffer (10% Goat serum, Invitrogen/ 0.3% Triton X-100, Fluka) for 30 minutes at room temperature. They were then incubated overnight at 4°C with the following primary antibodies: mouse monoclonal βIII-Tubulin (1:200; Chemicon), mouse monoclonal RIP antibody (1:50,000; MAB1580, Chemicon), and mouse anti-nestin monoclonal antibody (1:200; MAB353, Chemicon). The next day the cells were rinsed for 3×5 minutes to remove unbound primary antibodies. Subsequently they were incubated for 2 hours at room temperature with the following secondary antibodies: sheep anti-mouse fluorescein isothiocyanate (FITC) conjugate IgG (1:200 F2266; Sigma), Alexa Fluor 546-conjugated goat anti-mouse (1:400; Invitrogen). After washing, coverslips were removed from the 6 wells and mounted on a slide with mounting media for visualization using a fluorescence microscope.

To examine the specificity of the nestin antibody, 3T3 fibroblast-like cells were used as negative control cells (Pasteur Institute, Tehran); PC12 cells were used as positive controls for βIII-Tubulin (Pasteur Institute, Tehran). Labeled cells were identified using fluorescent microscopy (Olympus Ax70) and cell colonies were observed using inverted microscopy.

Neural and glial differentiation

Bulge cells that had already been sub-cultured 3 times were trypsinized and plated afresh at a cell density of 50000 cells/well. Neuronal differentiation was induced by plating the bulge cells on glass coverslips coated with collagen and medium supplemented with 10ng NT-3 (Invitrogen) (20-23). For glial cell differentiation, bulge cells were plated on glass coverslips coated with collagen and differentiation induced for 10 days by increasing the cellular density to 70-90% confluence for about two months (21, 24, 25).

Results

Cell Culture

A modified method was successfully used to segregate and culture the bulge cells from dissected rat whisker hair follicles in this experiment. One isolated follicle is shown in (Fig 1A, B).

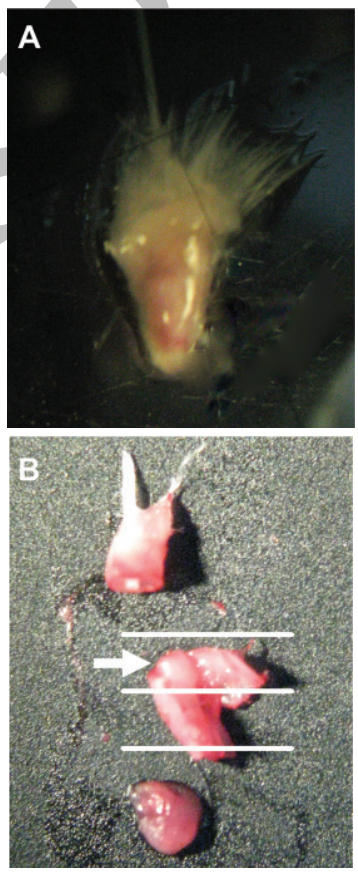


Fig 1: Dissection of the Bulge from adult rat whisker follicle. (A) Photograph of adult rat whisker follicle surrounded by connective tissue and capsule. (B) Bulge region shown after longitudinal incision.

The earliest outgrowth of cells from the bulge was observed within 4 days of explantation of the bulges (Fig 2A, B). In the beginning the cells grew slowly, gathered around the bulge fragment and became stratified. Then the cells proliferated quickly. On reaching a larger size, the bulge cells started to accumulate around the bulge fragment which led to the formation of dome-like cell layers (Fig 2C).

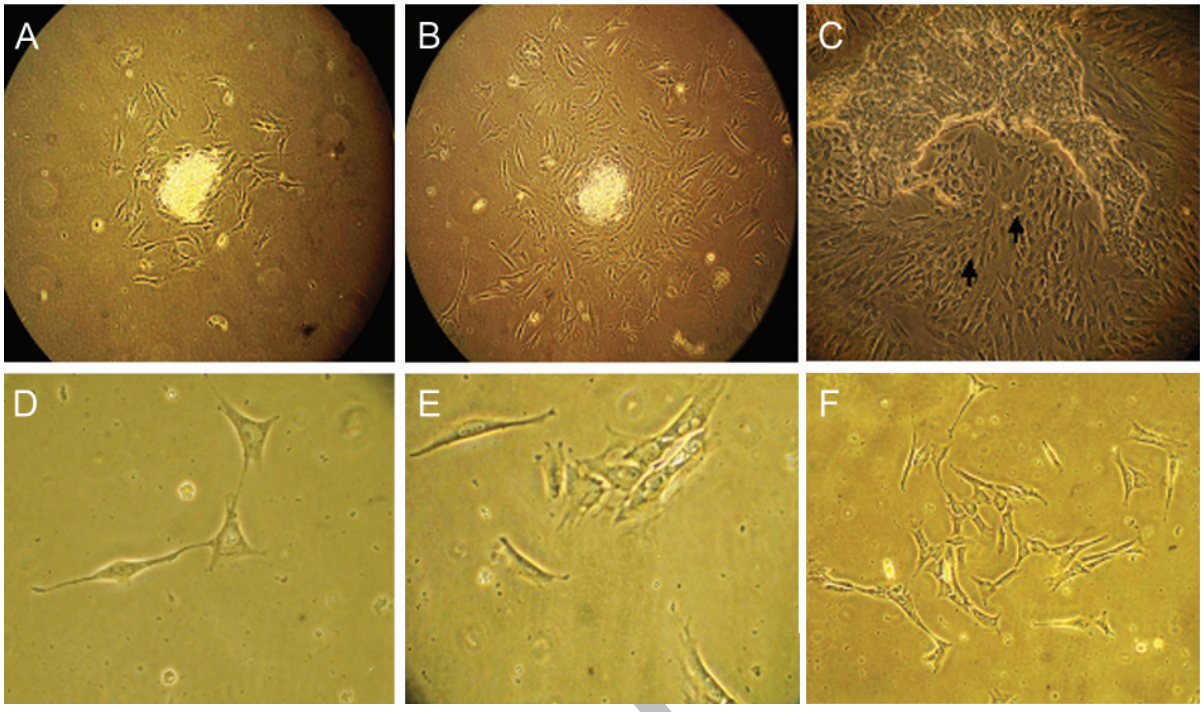


Fig 2: The primary culture of bulge cells from rat hair follicles. (A) The growth of bulge cells after 4 days and (B) 8 days (×100). (C) The bulge cells formed of domes (arrows) (×200). (D) The attached cell 24 hours after subculture (×400). (E) The cells produced daughter cells 48 hours after subculture (×400). (F) The cells three days after subculture (×200).

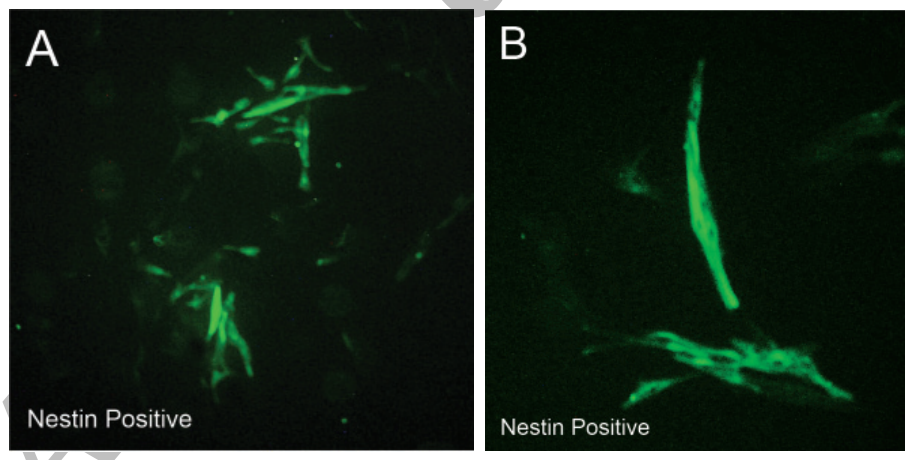


Fig 3: (A) Immunostaining with nestin antibody: Bulge cells expressed nestin 7 days after first subculture (×100) (B) (×200).

Subsequently, outgrowth could be seen along the edges of the domes, a result which showed that bulge cells retain strong proliferative capacity *in vitro* (Fig 2A, B). Bulge stem cells can be trypsinized and subcultured at low density. Within primary culture, the single cells have a sparse arrangement (Fig 2D) but the formation of colonies was observed in the sub-cultured plates (Fig 2E, F).

Immunocytochemical staining
Expression of nestin marker in bulge cells

To evaluate the biochemical nature of the bulge cells nestin was used as a hair follicle stem cell marker. Our results showed that the nestin antibody stained the bulge cells in culture and the positive cells clonally arranged. The proportion of positive cells decreased gradually with long-time culture (Fig3 A, B).

Expression of nestin marker in differentiated cells

After two months' differentiation most of cells were negative for nestin marker (Fig 4).

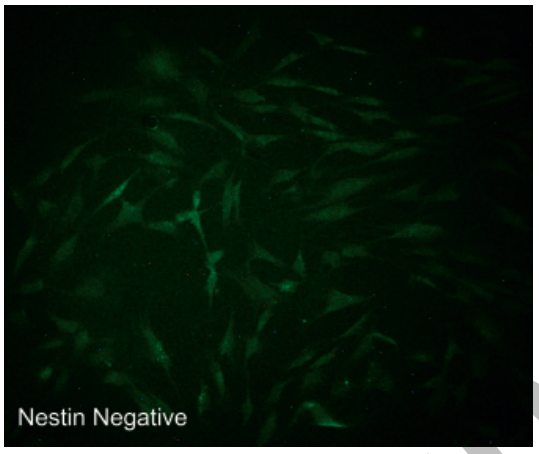
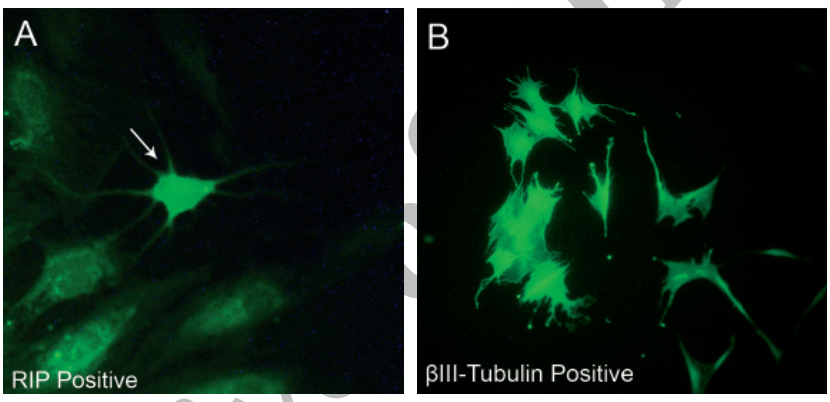


Fig 4: Immunostaining with nestin antibody: Differentiated bulge cells did not stain with nestin (×100).



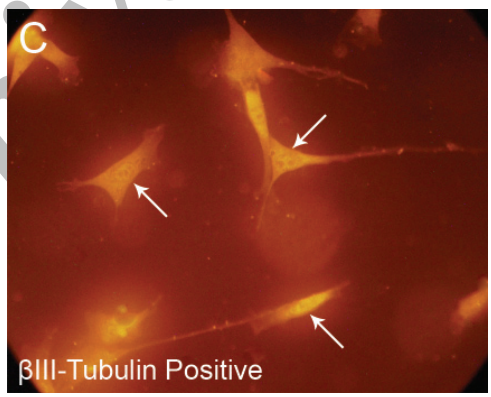


Fig 5: Immunostaining with RIP and βIII-Tubulin antibody: Differentiated cells expressed RIP and βIII-Tubulin. (A) White arrow shows RIP positive cell that has oligodentrocyte morphology (×200). (B, C) Dark arrows show βIII-Tubulin positive cells that have neural morphology (×400).

Expression of neural and glial markers in differentiated cells

Two months after differentiation in high cellular density plates, some cells showed glial morphological characteristics. In the immunocytochemical staining these cells expressed RIP, an oligodendrocyte marker (Fig 5A). In the plates that were nourished with NT-3, a lot of cells were positive for βIII-Tubulin, a neural marker (Fig 5B, C).

The bulge cells expression of neural and glial markers

Immunocytochemical staining of the colonies demonstrated that neural and glial markers were not observed and the undifferentiated cells were negative for RIP and βIII-Tubulin (Fig 6A, B).

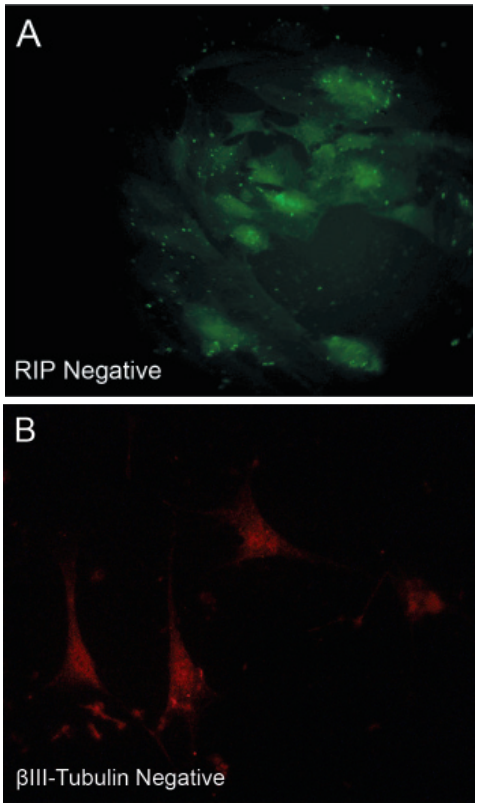


Fig 6: Immunostaining with RIP and βIII-Tubulin antibodies: Bulge cells before differentiation did not express RIP and βIII-Tubulin (A) (×100) and (B) (×200).

Discussion

Recent research has demonstrated that stem cells exist in most organs of the body, including the musculoskeletal, cardiac, hepatic, adipose, nervous, epidermal and hematopoietic systems (26). Situated near the insertion of the arector pili muscle and below the sebaceous gland, the bulge region has been shown for many years to be a candidate location for hair stem cells. However, despite their essential role in skin homeostasis and wound repair, bulge stem cells remain poorly characterized (6, 13, 27).

Cotsarelis et al, found that label-retaining cells were observed in the bulge region of the upper outer root sheath of mouse hair follicles (13). Rat hair follicle cell culture was established by Kobayashi et al. who report that in the rat vibrissa, keratinocyte colony-forming cells are highly clustered in the bulge containing region (16). The present study explored the growth potential and properties of cells derived from the bulge, a source of adult stem cells. Using, bulge cells from rat hair follicle, cultured and differentiated for observation of their biological and morphological properties, our results demonstrated that newly proliferated cells could be observed on the fourth day after explantation. Consistent with previous reports, these results confirm that cells with a high proliferative capacity and potential reside in the bulge region (17, 28).

Furthermore, our findings showed that newly proliferated cells (after 4 days), derived from the bulge region of the rat can be nestin positive. Again these results are consistent with previous reports. Li et al. found nestin driven - green fluorescent (ND-GFP) positive stem cells localicalized in the bulge region of mice (29). Mignone et al demonstrated that nestin expressing cells in the bulge region of the human hair follicle have stem cell like properties, are multipotent, and can effectively generate cells of neural lineage *in vitro* and *in vivo* (30).

Neural stem cells generate a wide range of cell types in both the developing and adult nervous system. These cells are marked by the expression of an intermediate filament of nestin (31). The expression of the unique protein, nestin, in both neural stem cells and hair follicle stem cells suggests their possible relation (32).

However, according to our results, bulge cells, gradually lose their nestin positivity and two months after differentiation most of the cells were nestin negative and could express RIP and βIII-Tubulin. In other words, these cells differentiated *in vitro*, lost their primitive biological characteristics and obtained new properties. Based on these morphological and biological results, it appears that bulge cells have the ability to differentiate and express RIP, an oligodentrocyte marker, and βIII-Tubulin, a neural marker.

Other work has demonstrated the potential of stem cells in the bulge region of the mouse hair follicle to differentiate into βIII-Tubulin positive neurons, GFAP positive astrocytes and 2', 3'-cyclic nucleotide 3'-phosphodiesterase-positive oligodendrocytes (28, 30, 32). It may be that nestin positive cells are self renewing and capable of expressing neuron, oligodendrocyte, Schwann cell and smooth muscle markers in culture (30, 33).

In vivo studies in lesioned murine spinal cord have also shown hair follicle grafts to express βIII-Tubulin, glutamic acid decarboxylase and RIP (19, 34).

Many factors, including collagen matrix, high cellular density and medium supplemented with neural growth factors, such as NT-3, play substantial roles in the development and survival of neurons (22, 24, 35, 36). To stimulate the differentiation of bulge cells to glial lineage (oligodentrocyte) we used the same medium as used by Rask-Andersen, under high-density conditions (24, 25). Yong et al. demonstrated that collagen could be suboptimal as

a neural substratum because it limits the extensive arborization that favors bipolar neuron development (23, 36), and that nestin positive cells from hair follicle stem cells were able to differentiate into neural-like cells in plates coated with polyornithine and laminin (30). For this reason, we also used medium supplemented with NT-3 and collagen coated plates for the culture and differentiation of bulge cells.

Conclusion

According our results, rat bulge stem cells have the ability to express nestin and differentiate into neural and glial lineages

Not only can these results provide useful cell culture information for the basic study of hair biology, but also in the field of CNS degenerative disorder repair.

Acknowledgments

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