World Journal of Stem Cells

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DOI: 10.4252/wjsc.v7.i5.866

*World J Stem Cells* 2015 June 26; 7(5): 866-872
ISSN 1948-0210 (online)


MINIREVIEWS

# Hair follicle stem cells: *In vitro* and *in vivo* neural differentiation

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Author contributions: Najafzadeh N wrote the paper; Esmaeilzade B and Dastan Imcheh M reviewed and edited the manuscript.

Conflict-of-interest: The authors declare that there are no conflicts of interest.



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Received: November 28, 2014
Peer-review started: December 2, 2014
First decision: January 20, 2015
Revised: February 22, 2015
Accepted: April 1, 2015
Article in press: April 7, 2015
Published online: June 26, 2015

## Abstract

Hair follicle stem cells (HFSCs) normally give rise to keratinocytes, sebocytes, and transient amplifying progenitor cells. Along with the capacity to proliferate rapidly, HFSCs provide the basis for establishing a putative source of stem cells for cell therapy. HFSCs are multipotent stem cells originating from the bulge area. The importance of these cells arises from two important characteristics, distinguishing them from all other adult stem cells. First, they are accessible and proliferate for long periods. Second, they are multipotent, possessing the ability to differentiate into mesodermal and ectodermal cell types. In addition to a developmental capacity *in vitro*, HFSCs display an ability to form differentiated cells *in vivo*. During the last two decades, numerous studies have led to the development of an appropriate culture condition for producing various cell lineages from HFSCs. Therefore, these stem cells are considered as a novel source for cell therapy of a broad spectrum of neurodegenerative disorders. This review presents the current status of human, rat, and mouse HFSCs from both the cellular and molecular biology and cell therapy perspectives. The first section of this review highlights the importance of HFSCs and *in vitro* differentiation, while the final section emphasizes the significance of cell differentiation *in vivo*.

**Key words:** Hair follicle; Stem cells; Bulge area; Neuron; Differentiation



**Core tip:** Hair follicle stem cells (HFSCs) can proliferate *in vitro* and retain the label for a long time. Various types of stem cells, including epidermal-neural crest stem cells, nestin-positive, keratin 15-negative cells, and CD34-positive cells have been demonstrated in

hair follicles. HFSCs normally give rise to keratinocytes, sebocytes, and transient amplifying cells *in vivo*. In addition, these cells differentiate into ectodermal lineages including oligodendrocytes, astrocytes, and neurons. Neural cells derived from HFSCs can replace lost cells in neurodegenerative diseases. Their easy accessibility along with their potential for neural differentiation makes HFSCs an ideal stem cell source for treatment of neurodegenerative disorders.

Najafzadeh N, Esmaeilzade B, Dastan Imcheh M. Hair follicle stem cells: *In vitro* and *in vivo* neural differentiation. *World J Stem Cells* 2015; 7(5): 866-872 Available from: URL: http://www.wjgnet.com/1948-0210/full/v7/i5/866.htm DOI: http://dx.doi.org/10.4252/wjsc.v7.i5.866

## INTRODUCTION

Skin stem cells reside in the stratum basale of the epidermis, sebaceous glands, and bulge area of the hair follicles. A promising start to derive hair follicle stem cells (HFSCs) was made in the early 1990s, when it was found that label-retaining cells were located in the upper portion of the hair follicle (bulge area). Subsequently, it was clarified that during the early anagen phase, the bulge cells grew downward in response to the stimulating factors of the dermal papilla cells and formed half of a hair follicle, which was degenerated during the catagen phase. Studies on hair follicles suggest that mouse HFSCs occupy a relatively fixed location in the hair follicle called the bulge area, which is important for hair follicle cycling[1]. HFSCs continuously supply new cells to the bulb during the anagen phase[2], and have the ability to differentiate into most of the ectodermal lineages[3]. These characteristics make them a promising cell source for grafting in various skin and nervous system diseases[4].

Kobayashi *et al*[5] has shown that the bulge area of rat whisker follicles contains colony-forming stem cells that can be expanded continuously on the feeder cell layer and form 95% of the total colonies. Moreover, the bulge cells form larger colonies than the non-bulge keratinocytes[6]. HFSCs have been seen to be able to differentiate either *in vitro* or *in vivo* upon grafting into a spinal cord injury (SCI)[7,8] and Alzheimer disease (AD)[9] animal models. The isolation of human HFSCs has generated tremendous interest, because the information gained from the study of human HFSCs, particularly those regarding cell differentiation, is of particular importance[10].

The neural potential of HFSCs was first reported by Sieber-Blum *et al*[11] and Amoh *et al*[12] who demonstrated that the population of HFSCs could be converted into Schwann cells after pretreatment with neuregulin-1 or upon transplantation into a severed sciatic nerve and SCI. Since then, many studies used inducers for neural differentiation of HFSCs, including neurotrophin-3 (NT-3)[13], a glial cell line-derived neurotrophic factor (GDNF), a brain-derived neurotrophic factor (BDNF)[14], a serum-free medium, *all trans* retinoic acid (RA), and other chemical neural inducers[15].

In this review, we have summarized the progress achieved in both the proliferation and neural lineage differentiation of HFSCs. Research on the application of stem cells for the treatment of neurodegenerative diseases such as peripheral nerve lesions, SCI, and AD has created a growing interest in the field of biology. Ultimately, a good knowledge of the wide variety of cell differentiation will provide the therapeutic application of HFSCs in degenerative nervous system diseases and skin pathologies.

## MAINTAINING HFSCS

HFSCs were initially identified in the bulge area of rat hair follicles[5]. In 1990, Cotsarelis *et al*[1] suggested that follicular stem cells retain the label and are slow cycling. Similarly, Morris *et al*[16] demonstrated that bulge cells are quiescent and maintain a label for a long time following induction of anagen. The label-retaining cells divide asymmetrically into both identical copies of themselves and transient amplifying cells[1,17]. The initial HFSCs culture is established and maintained by a co-culture, with a feeder layer of 3T3-J2 fibroblasts[5]. Subsequent studies have revealed that the epidermal growth factor (EGF) and a basic fibroblast growth factor (b-FGF) are the feeder molecules that play a significant role in the maintenance and proliferation of these cells[18,19]. HFSCs can also be grown in a medium supplemented with EGF, cholera toxin, insulin, and hydrocortisone[13]. Sieber-Blum *et al*[11] suggests that epidermal neural crest stem cells (Epi-NCSCs) are pluripotent stem cells residing in the bulge area and can also be expanded continuously in the presence of an alpha-modified MEM medium, 5% chick embryo extract, and 10% of fetal calf serum. With these new culture conditions, it is now possible to grow HFSCs with defined factors in the absence of a feeder cell layer.

## CELL SURFACE MARKERS

HFSCs can be routinely isolated from hair follicles and expanded *in vitro* to cell populations that are similar to the adult stem cells in respect to morphology and cell surface markers. In fact, numerous subsets of stem cells with varying differentiation potentials have been demonstrated in hair follicles, such as, Epi-NCSCs[9,11], nestin-positive, and keratin 15-negative cells[20], CD34-positive cells[15,21], and CD200 -positive cells[22].

Mouse bulges cells express specific markers including CD34 and K19, as well as CD200 and

K15[23]. Expression of CD200 protects the bulge area from inflammation and hair loss in alopecia areata[24] and these cell surface protein is the best positive marker for the human bulge stem cells[22]. A central problem is the lack of a specific and reliable marker for the identification of HFSCs. Among the different markers, CD34 is possibly the most promising marker for HFSCs[25]. Previously, bulge cells were isolated from mouse, rat, and human cells by manual dissection, but the purity of the isolated cells was unclear. Several years ago, Ohyama *et al*[22] used laser capture microdissection for precise bulge cell isolation. Now different isolation methods for bulge stem cells are accessible using CD34 as a hair follicle stem cell surface marker, by fluorescence-activated cell sorting (FACS)[26] and magnetic activated cell sorting (MACS)[8,27]. Therefore, the precise isolation of HFSCs having the ability to differentiate into many different cell types has generated a promising field for therapeutic application.

Some bulge stem cells are K15-positive and nestin-negative and differentiate into keratinocytes[28]. More recently, Snippert *et al*[29], reported Lgr6 expression in HFSCs, which differs from Lgr5/CD34$^+$ HFSCs. Lgr6$^+$ HFSCs particularly express Sca-1, α6-integrin, β1-integrin, sox9, and Lhx2, and contribute to wound repair. Sieber-Blum *et al*[7], showed that the other type of HFSCs express neural crest genes as well as neural stem cell markers, indicating a neural crest origin and neural differentiation potential. The other types of HFSCs in mouse hair follicles are called nestin-positive stem cells. These cells express some stem cell markers such as CD34, and can differentiate into non-follicular cell types, such as, neural cells[30], which are maintained in the presence of FGF2 and a low concentration of B27[20]. Nestin-positive cells exhibit a colony forming potential *in vitro* and are small oval or round shaped, with a dendrite-like structure. At the anagen stage, nestin-expressing cells form the lower part of the hair follicle[30].

## MULTIPOTENCY OR PLURIPOTENCY

This is an open question of whether bulge stem cells isolated from the hair follicle are pluripotent or multipotent cells and whether truly pluripotent cells can be isolated from the hair follicle by expansion, *in vitro*. HFSCs are considered to be multipotent cells because they differentiate into ectodermal derivatives such as neural cells, astrocytes, oligodendrocytes, and Schwann cells[14], as well as mesodermal lineages, including chondrocytes[11], myocytes[31], adipocytes, and osteocytes[17]. However, the expression of differentiation markers is often insufficient to conclude whether a cell has converted to a new state of differentiation[32]. Bulge cells can proliferate and differentiate into the bulb keratinocytes at the onset of the anagen phase[2,33]. In addition it is shown that these stem cells can also emigrate and continuously provide progenitors, regenerating the epidermis and sebaceous gland[34-36].

EP-NCSCs derived from the mouse vibrissae hair follicle express neural stem cell markers, such as Nanog and Oct4[37]. The neural crest transcription factors such as nestin, Slug, Snail, Twist, Sox9, Sox10, Wnt-1 and BMP4 play essential roles in maintaining the undifferentiated state of bulge cells[11,31]. The role of these transcription factors in neural crest stem cell renewal has been recently reviewed[38,39] and we are not going to discuss further. Together, HFSCs has an extensive developmental potential, however, it is necessary to evaluate the multipotency of HFSCs by differencing, *in vivo,* after cell grafting. For this purpose, HFSCs are transplanted into the skin[40], SCI[8], peripheral nerve injury and AD[9] models. The results suggest that HFSCs have extensive developmental potential. Further studies are required to determine whether the population of HFSCs can give rise to endodermal derivatives. Finally, the pluripotency of all subpopulations of HFSCs remains an unproven concept, but it seems that only Epi-NCSCs express two pluripotency factors, such as, Oct-4 and nanog[11].

## *IN VITRO* NEURAL DIFFERENTIATION OF HFSCS

HFSCs attract attention not only in dermatology, but also in the treatment of nervous system diseases. It has been shown that HFSCs retain the ability to generate differentiated progeny *in vitro*. Many differentiation approaches have been optimized using growth factors and other inducing factors. These protocols can also successfully generate certain lineages. HFSCs can be induced to produce an enriched population of glial and neuronal lineages following incubation in exogenous growth factors[14]. On account of the diversity of HFSCs, differentiation and the proliferative potential of HFSCs is strongly dependent on the exogenous growth factors. Neuroregulin-1 has been found to be required for differentiation of HFSCs to Schwann cell and β-Ⅲ tubulin positive neural cells. The Epi-NCSCs can also differentiate into smooth muscle cells in response to bone morphogenetic protein-2 (BMP-2)[11].

Of late, Ghoroghi *et al*[13], isolated rat HFSCs and cultured them on a poly-l-lactic acid (PLLA) scaffold to provide a suitable microenvironment for neural differentiation, and then used NT-3 to induce neural differentiation in the HFSCs.

New protocols have been developed to generate neural cells from HFSCs. Of late, we have isolated *CD34*$^+$ cells from the mouse hair follicle, using MACS, and then we applied RA, serum-free medium, and chemical treatments such as β-mercaptoethanol (BME), butylated hydroxyanisole (BHA), and dimethyl sulfoxide

(DMSO), for the neural differentiation in these cells. We have found that the serum-free condition and 1 μmol/L RA appropriately triggered neurogenesis in HFSCs[15]. In addition, it seems that prolonged serum deprivation induces formation of the ectodermal derivatives[41]. However, chemical treatments with DMSO, BME, BHA, and potassium chloride (KCL) induced rapid changes in cell morphology, which led to the $CD34^+$ cells dying within seven days of treatment[15]. It will be important to determine whether the neural cells derived from HFSCs possess the functional characteristics of neurons.

### *Therapeutic application of HFSCs*

HFSCs have advantages and disadvantages regarding the potential use of cell-based regenerative therapies. The main potential advantage of the HFSCs is that they are easily accessible adult stem cells, which have ectodermal differentiation potential[15]. Moreover, HFSCs are attractive tools for future burn and neurodegenerative disease treatments.

HFSCs are similar to embryonic stem cells and have a high proliferative potential *in vitro*. Similar to other types of adult stem cells, they do not form tumors. Without a doubt, autologous transplantation and avoiding immune rejection has been demonstrated in HFSC-treated animal studies[7,12,42].

HFSCs have the properties to differentiate into various neural cell types including neurons, astrocytes, oligodendrocytes, and Schwann cells. Among them some specific cell types can be produced in high purity, such as, Schwann cells, motor neurons, and oligodendrocytes, which are the main cells that degenerate in SCI, peripheral nerve lesions, and AD. One of the goals of the researchers in this field is to generate neural cells from stem cells, with the aim of replacing lost tissue in degenerative disorders. Therefore, we have used HFSCs as an alternative cell source for cell therapy in nervous system diseases. AD is one of the most common neurodegenerative disorders affecting about four million people throughout the world each year. AD brains have been characterized by amyloid β peptide plaque and neurofibrillary tangle formation, which leads to axonal transport defects and synaptic loss responsible for cholinergic neuron degeneration[43].

HFSCs have the potential to constitute an anatomical nervous system structure. Thus, in the case of an AD model, these cells are capable of replacing the degenerated neurons. In a recent study, researchers transplanted a subtype of HFSCs (Epi-NCSCs) to differentiate into cholinergic neurons in the AD rat model. These stem cell-derived cholinergic neurons improved learning and memory deficits in the AD model. In spite of rare studies on the therapeutic effects of Epi-NCSCs in an AD model[9], it will be possible to modify HFSCs to deliver neurotropic factors that modify the course of the disease, in the future.

It is well known that SCI induces paralysis in about 2.5 million people, affecting the patients and society. Unfortunately, no effective treatment has been discovered for SCI to date[44]. However, some studies are under way to promote regeneration and neural repair in patients with SCI[45]. Cell therapy plays a major role in axonal regeneration and neuronal replacement in AD[9], SCI, and other nervous system diseases. Potential strategies to repair neurodegenerative diseases include neuronal and glial replacement, axonal regeneration and remyelination, and increased production of neurotrophins by grafted cells[46,47]. Various cell types have been assessed to repair central and peripheral nervous system disorders, such as, fetal tissue, olfactory ensheathing glia, Schwann cells, skin derived precursors, mesenchymal stem cells, and HFSCs[48]. To this end, in this part of the review, we have focused on the transplantation of HFSCs in SCI.

Preclinical studies have shown a novel type of HFSCs known as Epi-NCSCs, which survive and differentiate into βⅢ-tubulin, glutamic acid decarboxylase (GAD67), RIP positive, and myelin basic protein-positive neural cells, but not Schwann cells, when transplanted into the contusion model of the SCI; the cells can be even integrated with host neurites in the contused spinal cord. However, the transplanted cells do not form tumors[7]. In another study, extensive Schwann cell differentiation of nestin-positive HFSCs has been reported after transplantation into a severed spinal cord. They have found that the differentiated cells facilitated the repair of SCI and promoted hind limb function recovery[49]. Likewise, when rat HFSCs are transplanted into a compression model of SCI, they generate oligodendrocytes and neuron-like cells expressing RIP and β-Ⅲ tubulin, respectively. Walking scale, limb coordination, and plantar stepping improved following HFSC transplantation[8]. Consistent with this study, Hu *et al*[50], also demonstrated that unilateral bulge-derived Epi-NCSC transplantation into a contused spinal cord promoted a 24% recovery in sensory connectivity and touch perception. Moreover, some grafted Epi-NCSCs differentiated into functional motor neurons. *In vivo* studies using Epi-NCSCs isolated from the mouse hair follicle showed similar results[7]. Briefly, these studies showed the safety of HFSC transplantation and enhanced recovery in animal models of SCI.

Damage to the peripheral nerve interrupts the axonal pathways and causes partial or total loss of motor, sensory, and autonomic functions. An important aim for the ongoing research is the development of therapeutic strategies that enhance axonal regeneration and replace lost neural cells, to bridge the gap of the lesion[51].

HFSCs possess a self-renewal ability and neural differentiation potential. Amoh *et al*[12,52] transplanted HFSCs in the severed sciatic nerves of mice. Eight weeks after grafting, many spindle cells grew in the severed sciatic nerve rather than in the control, differ-

entiated extensively into Schwann cells, and promoted motor function.

In another study, extensive neuronal differentiation of the nestin-expressing cells was reported after transplantation of RA-pretreated cells into the transected distal sciatic nerve. In this study, the nestin-positive bulge cells grew in the distal sciatic nerve stump and caused locomotor recovery owing to the presence of many Tuj1, Is1$_{1/2}$, and EN1-positive cells and nerve fibers. Muscle atrophy was also reduced after grafting, and re-innervation was promoted[53]. HFSCs transplanted to the subcutis and severed sciatic nerve also differentiated into blood vessels[40] and Schwann cells[12], respectively. It seemed that the differentiation of HFSCs supported neural regeneration and motor function of the lower extremity muscles, such as, the gastrocnemius[12].

There are still many problems that need to be resolved before HFSCs can be widely used clinically. The main problem is that approximately only a few neural cells derive from these stem cells *in vivo* and further studies are required to determine whether the differentiated neurons are functionally integrated into the AD brain, SCI, and peripheral nerve lesion tissues. Some other forms of cell differentiation, such as keratinocytes, myocytes, and melanocytes are the other disadvantages of HFSC transplantation in neurodegenerative disorders. The successful directed differentiation of HFSCs into specific neurons with the help of different neural induction methods may make neural transplantation widely available for neurodegenerative disorders at some point in the future.

Collectively, these results indicate that HFSCs can promote the recovery of peripheral nerve injury, SCI, and AD. Thus, these cells have the desirable properties of neural replacement and remyelination.

## CONCLUSION

In summary, we have explained that the hair follicle contains various multipotent stem cells. The ability of HFSCs to proliferate *in vitro* and the directed differentiation toward ectodermal and mesodermal lineage cell types render them potential for clinical application. These include the modeling of SCI, sciatic nerve injury, and AD, studies of cell therapy on progression, and amelioration of symptoms.

At present, HFSC transplantation provides the best chance of cure for many diseases. Future studies seek to characterize the signaling genes that regulate stem cell behavior.

## ACKNOWLEDGMENTS

We gratefully acknowledge to M Sagha, M Arzanlou and M Amani for their precious contribution in terms of language editing.

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**P- Reviewer**: Li ZJ, Liu L **S- Editor**: Ma YJ **L- Editor**: A
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