Chapter 5
Hair Follicle: A Novel Source of Stem Cells for Cell and Gene Therapy

Panagiotis Mistriotis and Stelios T. Andreadis

Abstract The adult body harbors powerful reservoirs of stem cells that enable tissue regeneration under homeostatic conditions or in response to disease or injury. The hair follicle is a readily accessible mini organ within the skin and contains stem cells from diverse developmental origins that are shown to have surprisingly broad differentiation potential. In this chapter, we discuss the biology of the hair follicle with particular emphasis on the various stem cell populations residing within the tissue. We summarize the existing knowledge on putative hair follicle stem cell markers, the differentiation potential, and technologies to isolate and expand distinct stem cell populations. We also discuss the potential of hair follicle stem cells for drug and gene delivery, tissue engineering, and regenerative medicine. We propose that the abundance of stem cells with broad differentiation potential and the ease of accessibility make the hair follicle an ideal source of stem cells for gene and cell therapies.

Keywords Hair follicle • Stem cells • Tissue engineering • Regenerative medicine Gene therapy • Drug delivery • Reprogramming

P. Mistriotis
Bioengineering Laboratory, Department of Chemical and Biological Engineering,
University at Buffalo, The State University of New York,
908 Furnas Hall, Amherst, NY 14260-4200, USA

S.T. Andreadis, PhD (✉)
Department of Chemical and Biological Engineering,
Department of Biomedical Engineering, and Center of Excellence in Bioinformatics
and Life Sciences, University at Buffalo, The State University of New York,
908 Furnas Hall, Amherst, NY 14260-4200, USA
e-mail: sandread@buffalo.edu

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5.1 Introduction

The hair follicle (HF) is a dynamic "mini" organ supporting important biological functions of the body. HFs protect against cold and potential injuries; they also have an important sensory and immunologic functions in addition to affecting the social behavior of a person [1, 2].

HFs are easily accessible and contain stem cells from diverse developmental origins that continuously self-renew, differentiate, regulate hair growth, and contribute to skin homeostasis. Hair follicle stem cells have been shown to be highly proliferative in vitro and multipotent [3–5] that allows engineering a variety of different tissues for organ replacement and regenerative medicine. In addition, genetic engineering of the hair follicle stem cells in vivo has shown promising results, suggesting that treatment of genetic diseases of skin and hair via the hair follicle may be feasible. This chapter summarizes the existing literature regarding the differentiation potential of hair follicle stem cells, their putative markers, the common isolation methods, and their application in cell and gene therapies.

5.2 Hair Follicle Biology

HF is part of the pilosebaceous unit that contains the sebaceous gland, the apocrine gland, and the arrector pili muscle. The HF is composed of two main compartments: the upper part includes the infundibulum and the isthmus, whereas the bulb, matrix, and dermal papilla comprise the lower part. The exact position of the lower part varies during hair cycling. Adjacent to the lower portion of the infundibulum lies the sebaceous gland that waterproofs the skin by secreting sebum. The bulge is a part of the isthmus that is believed to be the stem cell reservoir, which regenerates the HF during hair growth. Cells migrate from the bulge toward the bulb, where they proliferate and differentiate in order to produce the hair shaft and all the epithelial cells that constitute the HF [2]. Finally, the inner and outer root sheaths are composed mainly of keratinocytes surrounding the hair shaft (Fig. 5.1).

HF undergo numerous cycles of growth and retraction throughout life. This dynamic process in adult life has three distinct phases, that is, anagen, catagen, and telogen, each regulated by different signals. Anagen is the growing phase. Stem cells that are located in the bulge region differentiate to all hair lineages, resulting to hair elongation. The duration of anagen in human body varies depending on the anatomic location of the follicle. On the scalp, anagen may last as long as 8 years resulting in long hair, but in other places such as the eyebrow, anagen maybe as short as 3 months. Catagen is the regression phase. At this stage the majority of the HF cells undergo apoptosis, causing reduction of the lower compartment that brings the dermal papilla cells close to the bulge cells. Exchange of signals between the papilla and the bulge regulates the duration of catagen. The cells that escape apoptosis during this phase comprise the reservoir that leads to the next anagen. Telogen is the last phase of the HF cycle, also known as resting phase. In telogen, cells enter

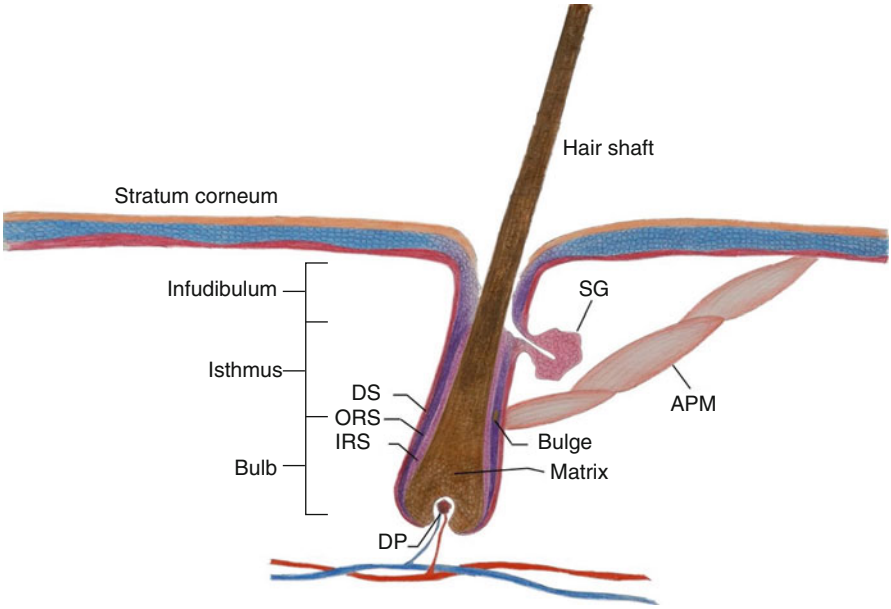

Fig. 5.1 Schematic drawing of hair follicle. *DS* dermal sheath, *ORS* outer root sheath, *IRS* inner root sheath, *DP* dermal papilla, *SG* sebaceous gland, *APM* arrector pili muscle. The illustration is not drawn in scale

a quiescent state waiting for the necessary signals to restart the cycle via the anagen. It is argued that 5–15 % of HF in the scalp remain in telogen [1, 2].

5.3 Location and Differentiation Potential of Hair Follicle Stem Cells

Stem cells can be characterized by three unique properties: self-renewal, capacity to differentiate into one or multiple cell types, and the ability to form tissues in vivo. Based on their differentiation potential, they can be categorized as totipotent, pluripotent, multipotent, and unipotent. Totipotent cells can reproduce all the cells of a living organism including the extraembryonic tissues. Pluripotent cells differ from the totipotent cells in their inability to form the extraembryonic tissues necessary for proper growth of the embryo. Multipotent stem cells have even more restricted differentiation potential, but they can still differentiate into more than one cell type. The lowest in the hierarchy are the unipotent stem cells or progenitor cells, which can generate only one cell type. Stem cells can also be classified into embryonic or adult according to their origin [6]. Although embryonic stem cells have broader differentiation potential, adult stem cells can be isolated from the patient, directly overcoming possible immune rejection after transplantation.

Adult stem cells in vivo reside in multiple tissues usually in a well-protected microenvironment called niche. Examples of stem cell populations that are surrounded by niche include the intestinal stem cells, the neural stem cells, and the HF stem cells. This way, the body holds a powerful reservoir of cells that can readily respond in case of emergency, such as an injury. Some adult stem cells like mesenchymal stem cells (MSC) have multilineage differentiation potential so that a single cell can contribute to the regeneration of multiple tissues such as fat, bone, cartilage, and muscle [7].

Although in vitro stem cells proliferate markedly in response to appropriate signals in the culture media, in vivo they remain quiescent until they are coaxed to proliferate and/or differentiate only when needed, for example, in case of injury. Under homeostatic conditions, the stem cell pool is maintained through asymmetric division, where the parent stem cell divides into two cells with varying differentiation potential: one retaining the stem cell characteristics (self-renewal) and the other assuming a more differentiated phenotype (differentiation). However, expansion of the stem cell pool would require symmetric division, where two stem cells are generated from a parental stem cell [8].

Similar to other organs, the HF contains a rich stem cell pool that resides in different anatomic locations within the HF. As a result, some scientists call the HF as a stem cell "zoo" [9]. In the next chapter, we will present the different stem cell populations, and we will elaborate on their broad differentiation potential.

5.3.1 Bulge and Hair Germ

Due to the complex architecture of the HF, the location of the stem cell reservoir remained elusive for many years. Initial studies reasoned that stem cells resided in the bulb [10], but this hypothesis was abandoned, as removal of the bulb did not inhibit the generation of new hair follicles [11]. In the early 1990s, Cotsarelis and colleagues were the first to propose that stem cells reside in the bulge area of HF. They took advantage of the fact that in vivo stem cells cycle very slowly so that long time after administration of tritiated thymidine, only the cells that retain the label (label-retaining cells) are the slow-cycling stem cells [12, 13]. Several years later, this finding was verified using transgenic mice that were engineered to express the fusion protein histone H2B-GFP under the keratin-5 promoter in a tetracycline-regulatable manner. As a result skin cells expressed GFP except when the mice were fed doxycycline, which suppressed GFP expression. The fast-cycling cells lost the GFP, whereas the slow-cycling stem cells retained it. These label-retaining cells were localized in the bulge region of hair follicles [14]. Furthermore, tracing studies with transgenic mice expressing the LacZ transgene under the control of either keratin-15 or Lgr5 promoter further supported the bulge activation hypothesis, which states that during anagen, stem cells from the bulge migrate in the bulb region where they are induced to proliferate and differentiate to all epithelial cell types of the HF [15, 16].

Notably, transplantation of keratin-15+ or Lgr5+ cells along with dermal fibroblasts in the dermis of nude mice generated new HF with high efficiency [15, 16]. Interestingly, damage of the bulge from autoimmune disease lichen planopilaris resulted in permanent hair loss [17], further highlighting the importance of bulge-derived stem cells for hair regeneration. In addition to hair regeneration, bulge stem cells were found to contribute to wound healing following skin injury by migrating and differentiating to epidermal keratinocytes [18, 19]. However, they are not necessary for the maintenance of the epidermis (ablation of the cells does not affect the homeostasis of the epidermis), and in the long run, they fail to stay at the sites of injury [19]. Additional studies have shown robust multipotency of bulge stem cells in vivo, where they were found to participate in angiogenesis, and in vitro where they were coaxed to differentiate into neurons, glial cells, melanocytes, keratinocytes, and mesenchymal cells [20–24].

Although it is widely accepted that the bulge harbors stem cells, the exact stem cell population is still under debate. Jaks and colleagues challenged the notion of label-retaining cells as the true stem cell population in HF, as $Lgr5^{+}$ cells can regenerate the whole follicle but do not coincide with the label-retaining cells of the bulge. The same study reported that $Lgr5^{+}$ cells were found in the hair germ, a region between the dermal papilla and the bulge, which remains discrete during telogen but overlaps with the matrix during anagen [16]. Others believe that the hair germ originates from the bulge and contributes to the generation of the new HF in the beginning of anagen [25]. In agreement, Greco and colleagues showed that the transcriptional profile of hair germ cells resembles that of bulge cells. They also found that hair germ cells proliferate faster than bulge cells and respond first to the dermal papilla signals at the late telogen. However, they also lose their proliferative capacity faster than bulge cells during long-term expansion in vitro [26].

5.3.2 Isthmus/Infundibulum

Cells located above the bulge are believed to retain multipotent properties. Studies have reported that they can differentiate not only into the epithelial lineages of the HF but also into the sebaceous gland and the epidermis. However, it is yet not known whether these cells represent a unique stem cell population, or a subset of bulge stem cells, or even progenitors with limited differentiation capacity.

Isolated cells from the area between the bulge and the sebaceous gland were found to be distinct from the bulge-derived stem cells since they did not express bulge-specific markers such as keratin-15 and CD34. Although they maintained their high clonogenic potential in vitro, they were also actively proliferating in vivo – in contrast to the notion that in vivo stem cells are the slow-cycling, label-retaining cells [27]. Similarly Jensen and colleagues reported that cells isolated from the upper bulge region and were not quiescent in vivo could generate new follicles after implantation, suggesting that stem cells need not be slow-cycling cells in vivo in order to be multipotent [28].

Although, during homeostasis, bulge-derived cells do not contribute to the generation of epidermis [15, 16, 19], several studies showed that cells derived from a region above the bulge can give rise to epidermis and persist there for a long time following injury [28–30].

5.3.3 Sebaceous Gland

There are two theories with regard to the origins of the sebaceous gland. The first asserts that stem cells residing in the bulge region migrate and give rise to resident gland cells. This theory is supported by transplantation studies showing that bulge cells generated functional sebaceous gland in vivo [15, 16]. The second theory suggests that stem cells located above the bulge differentiate into sebocytes [29–31]. Horsley and colleagues identified a unique cell population in the region of sebaceous gland that expresses the transcription factor Blimp1 and has unipotent differentiation potential into sebocytes. Loss of Blimp1 in HF resulted in activation of bulge cells, which may suggest a possible connection between bulge and sebaceous gland. The same study also showed that implanted bulge stem cells could give rise into Blimp1$^+$ cells [31].

5.3.4 Dermal Papilla and Dermal Sheath

Dermal papilla (DP) and dermal sheath (DS) are cell populations within the HF that are believed to contain stem cells. Whereas bulge cells originate from ectoderm, DP and DS cells are derived from mesoderm, and they are known to regulate hair cycling by exchanging signals with the bulge [2]. Multiple studies showed that DP and DS cells have broad differentiation potential. In a pioneering study, Lako and colleagues demonstrated that DP and DS cells could reconstitute multiple lineages of the hematopoietic system in lethally irradiated mice [32]. Rat and human HF-derived DP and DS cells could also be induced to differentiate toward the myogenic, osteogenic, chondrogenic, and adipogenic lineage resembling bone marrow mesenchymal stem cells [3–5, 33, 34]. A recent study showed that DP/DS stem cells are the precursors of dermal stem cells and contribute to dermal maintenance and wound healing [35].

5.4 Putative Hair Follicle Stem Cell Markers

The majority of the studies in HF have been conducted in murine models. However, there are several differences that have to be taken into account between human and murine models, and conclusions derived from experiments with mice models do not

Table 5.1 Common stem cell markers and their location within hair follicle

Species	Marker	Location	References
Mice	CD34	Bulge	[36]
	TCF3	Bulge	[37, 38]
	NFATC1	Bulge	[39]
	Nestin	Bulge	[20]
	Label-retaining cells	Bulge	[12]
	K15	Bulge, hair germ	[15]
	Lgr5	Bulge, hair germ	[16]
	Lhx2	Bulge, hair germ, early hair progenitors	[40]
	Sox-9	Bulge, early hair progenitors	[41]
	MTS24	Upper bulge	[27]
	a6LowCD34–Sca-1–	Upper bulge	[28]
	Lrig1	Upper bulge	[42]
	Lgr6	Upper bulge	[30]
	Blimp 1	Upper bulge	[31]
	Sox-2	Dermal papilla, dermal sheath	[35, 43]
	Versican	Dermal papilla	[44]
	Alkalinephosphatase	Dermal papilla, hair germ	[45]
	Nexin	Dermal papilla	[46]
	CD133	Dermal papilla	[47]
Human	Mesenchymal stem cell markers	Dermal sheath	[4]
	CK15	Bulge/isthmus	[48–50]
	CD 200	Bulge/isthmus	[48–50]
	CK19	Bulge/isthmus	[48–50]

necessarily apply in human HF cells. Whereas humans have only two types of hair (vellus and heavily pigmented hairs), mice are endowed with several distinct hair types (pelage, vibrissae, cilia, hairs on the tail, ear, genital, perianal area, nipples, and around the feet). In addition, the biological cycles of human and mouse HF are different; while human HF cycle independently after birth, mouse HF cycle in synchrony [2]. Finally, the biological markers characterizing the stem cell populations in human and mouse are strikingly different. Table 5.1 summarizes the most common markers of HF stem cells based on the species they are derived from and the location where they are expressed.

5.4.1 Murine Hair Follicles

5.4.1.1 Bulge

Several markers have been proposed to characterize murine bulge stem cells. In addition to keratin-15 and Lgr5, CD34 is co-expressed with keratin-15 and has also been proposed as a potential stem cell marker of the bulge. CD34+ cells are relatively

quiescent and have higher clonogenic potential in vitro as compared to $CD34^-$ cells [36]. Several transcription factors have been identified in the bulge region including Tcf3, Sox-9, Lhx2, and NFATc1. Tcf3 was shown to maintain the undifferentiated cell state by repressing numerous genes that induce sebaceous gland and HF differentiation [38]. Another key transcription factor that is expressed in the bulge area is Sox-9. $Sox\text{-}9^+$ cells are first detected during the formation of hair placode, the precursor of HF during prenatal life. The cells co-localize with early label-retaining cells, which subsequently give rise to bulge stem cells. Notably, deletion of Sox-9 decreased the proliferation of bulge stem cells, impaired the generation of proliferative matrix cells, and resulted in inhibition of HF morphogenesis [41, 51].

Similar to Sox-9, Lim-homeodomain transcription factor, Lhx2 is also expressed during hair placode formation as was seen by microarray analysis in the $P\text{-cadherin}^+$ cells that mark early hair progenitors. In postnatal life, Lhx2 is expressed in the bulge and suppresses differentiation, prompting some investigators to hypothesize that it may be required for stem cell maintenance [40]. However, a recent study challenged this notion and reported that Lhx2 is required for the induction of anagen and not for the maintenance of stem cells [52]. The fourth bulge-specific transcription factor is NFATc1, which is regulated by the intracellular levels of calcium. Under high calcium conditions, NFATc1 is dephosphorylated and translocates to the nucleus, where it downregulates cyclin-dependent kinase 4 and suppresses proliferation in bulge region. As a result, downregulation of NFATc1 leads to activation of bulge-derived stem cells [39]. Interestingly, NFATc1-expressing cells coincide only partially with $CD34^+$, $Tcf3^+$, $Lhx2^+$, and $Sox\text{-}9^+$ cells in the bulge region, suggesting there is no unique marker of bulge stem cells but rather a group of transcription factors that regulate stem cell maintenance and activation through a series of complex and dynamic interactions.

Finally, other studies provided evidence that nestin is expressed in the mouse bulge stem cells. Transgenic mice expressing GFP under the nestin promoter showed that nestin-positive cells are located in the bulge region during telogen but in the upper two thirds of the outer root sheath during anagen. In vivo these cells participated in the formation of new blood vessels, and in vitro they could be coaxed to differentiate into neurons, glial cells, smooth muscle cells, melanocytes, and keratinocytes, demonstrating the multipotency of hair follicle stem cells [20, 21, 53].

5.4.1.2 Upper Bulge

Several markers have been identified over the years that target putative murine stem cells in the upper bulge region. Lgr6, an orphan G protein-coupled receptor, is expressed in the region immediately above the bulge. $Lgr6^+$ cells were shown to play a critical role in the formation of HF, sebaceous gland, and epidermis during development [30]. MTS24, a cell surface glycoprotein, also marked potential stem cells in a region above the bulge. $MTS24^+$ cells exhibited increased colony-forming capacity as compared to $MTS24^-$ cells and showed similar gene expression profile with $CD34^+$ bulge cells [27]. However, the differentiation potential of these cells

was not examined. In addition, cells residing in the upper isthmus were shown to be multipotent as they could form HF, sebaceous gland, and epidermis after implantation. These cells expressed low levels of integrin α6, were negative for the hematopoietic markers CD34 and Sca-1, and exhibited distinct gene expression profile as compared to bulge cells [28]. Finally, another putative stem cell marker characterizing the region right above the bulge is transmembrane protein leucine-rich repeats and immunoglobulin-like domain protein 1 or Lrig1. Lrig1 was shown to regulate epidermal growth factor signaling by promoting the degradation of epidermal growth factor receptor [54] and to keep cells in this region in a quiescent state [42]. Indeed, in vivo Lrig1^{+} cells appeared to be quiescent and multipotent, two of the main attributes of stem cells [29].

5.4.1.3 Dermal Papilla and Dermal Sheath

The DP and DS are known to induce HF generation by interacting with epidermal stem cells [55]. In 1999, Kishimoto et al. reported that cells in DP express the proteoglycan versican, which is usually present in the condensed mesenchyme. The same group employed the versican promoter to express either LacZ or GFP and found that when implanted on the back of nude mice along with keratinocytes, the versican^{+} cells could reconstitute the HF but versican^{-} cells could not [44]. Others observed that nexin-1, a protease inhibitor, was highly expressed in DP during anagen and that the nexin-1 expression level correlated with the rate of hair growth [46]. Similarly, the expression of alkaline phosphatase – an enzyme expressed in bone cells and embryonic stem cells – correlated with hair growth and was also highly expressed in DP during anagen, suggesting a positive correlation between hair induction and alkaline phosphatase activity [45]. Finally, CD133 was expressed in DP cells during HF development, but its expression was greatly diminished after birth. Nevertheless, when co-implanted with embryonic epithelial cells, CD133+ cells enabled generation of HF in vivo [47]. Interestingly, a subpopulation of CD133+ Sox2+ cells within the DP was shown to be essential for the formation of particular types of hair such as awl/auchene follicles [43]. Rendl and colleagues compared the transcriptional profile of five distinct cell populations within the HF, namely, melanocytes, dermal papilla, matrix, outer root sheath, and dermal fibroblasts. This approach successfully identified several genes and signaling pathways that were unique to each population and need to be further explored in the future [56].

5.4.2 Human Hair Follicles

Murine HFs have been largely explored with respect to stem cell markers; however, human HF have remained unexplored. In contrast to murine bulge, the human bulge cannot be identified as a distinct anatomic projection, rendering isolation of

bulge cells very challenging. Screening a number of markers in vivo, Kloepper and colleagues identified CD200 and keratin-15 and keratin-19 as putative bulge stem cell markers, although their location is not restricted to the bulge but extends to a wider area of isthmus as well. In contrast to the mouse, human bulge does not express CD34, nestin, or Lhx2 [48]. In a more recent study keratin-15^{high}/CD200^{+}/CD34^{-}/CD271^{-} bulge-derived cells showed increased clonogenic potential as compared to keratin-15^{low}/CD200^{+}/CD34^{-}/CD271^{-} cells [50]. In agreement, CD200-expressing cells that were isolated from a population of label-retaining cells using laser capture microdissection showed increased clonogenic potential in vitro [49]. However, multipotency of CD200+ cells has not been examined. More recently our laboratory reported that DP/DS cells display a cell surface profile characteristic of mesenchymal stem cells being positive for CD90, CD44, CD49b, CD105, and CD73 [4, 5]. In addition, these cells are clonally multipotent as they can differentiate in fat, bone, cartilage, and smooth muscle with high efficiency [5].

5.5 Methods for Isolating Hair Follicle Stem Cells

Three techniques have been routinely used for the isolation of putative stem cells from the HF: microdissection, enzymatic digestion, and fluorescence-activated cell sorting (FACS). In the following, we describe each technique and elaborate on their advantages and disadvantages.

5.5.1 Microdissection

Microdissection is a technique that has been commonly applied for the isolation of cells from DP [11, 32, 33, 57, 58] as well as the bulge [49, 59]. This technique requires the use of fine forceps and blades for the isolation of the area of interest. Subsequently the isolated areas are transferred into tissue culture plates, where the cells migrate out of the tissue and proliferate in the presence of appropriate culture medium.

For DP cell isolation, application of pressure on the suprabulbar region by forceps was shown to compress the bulb and facilitate removal of the connective tissue sheath surrounding the DP, which is subsequently detached from the epithelium using a scalpel blade [60]. Finally, a highly reliable technique that has been used for isolating human bulge cells is laser capture microdissection [49]. A thermolabile membrane is placed on top of the sample, and the area of interest is targeted by laser, which melts the membrane locally marking the cells that are subsequently separated [61]. The major advantage of microdissection is that this approach preserves the whole tissue, thereby increasing the efficiency of cell isolation. However, this technique is quite laborious and requires experienced technicians.

5.5.2 *Enzymatic Digestion*

Another approach that has been employed for isolation of HF stem cells involves enzymatic digestion of the follicle from the surrounding dermis, usually with dispase or collagenase. The incubation time and concentration of enzymes used vary depending on the amount of extracellular matrix present around the follicle. Generally collagenase treatment requires few hours of incubation at 37 °C whereas dispase needs overnight treatment [3–5, 23, 49]. Others use a combination of enzymes to isolate DP cells. Specifically, dispase was employed initially to remove the follicle from the cutaneous fat, followed by collagenase D to digest the dermal sheath and isolate the DP. The remaining dermal sheath fibroblasts could be removed by low-speed centrifugation of the DP [62]. Enzyme digestion is a simple method of HF stem cells isolation but with little control over the type of cells that are obtained leading to possible variations between different isolations.

5.5.3 *Fluorescence-Activated Cell Sorting*

FACS is a common method for isolating stem cells, especially from murine HF [15, 16, 20, 28, 30, 43, 44, 47]. Fluorescently labeled antibodies are used to tag the cell surface, and cells are sorted based on fluorescence intensity, which is proportional to the expression level of the particular target receptor. FACS can also be applied for isolating cells based on markers that are not expressed on the cell surface. Our group made use of the smooth muscle alpha-actin (αSMA) promoter-driven GFP to isolate a homogeneous population of smooth muscle cells (SMC) from ovine and human HF-MSC [33, 34]. FACS yields highly purified cell populations that can be further expanded or directly analyzed for mRNA or protein expression. Regrettably, lack of reliable stem cell markers hampers use of this method in sorting human HF stem cells.

5.6 Hair Follicle Stem Cells for Tissue Engineering and Cell Therapy

5.6.1 *Tissue-Engineered Vascular Grafts*

Cardiovascular disease is the leading cause of death in USA as being reported by American Heart Association. In 2006 heart diseases accounted for more than 600,000 deaths. Almost half of the deaths were caused by coronary heart diseases, and 400,000 surgical bypass operations were performed highlighting the importance of an artificial arterial substitute (www.americanheart.org). A functional

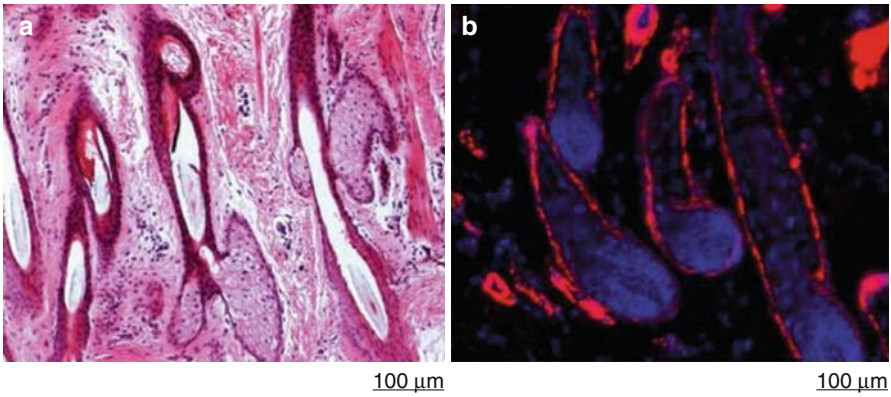

Fig. 5.2 Cells comprising the dermal sheath of hair follicle are positive for αSMA. (**a**) H&E staining from neonatal ovine dermis. (**b**) Immunohistochemistry showing αSMA$^+$ cells in the dermal sheath of hair follicles (Image taken from Peng et al. [63])

arterial graft should contain both endothelial cells (ECs) and SMCs. ECs line the lumen of a vessel, endow it with thromboresistant properties, and are selectively permeable to substances circulating in the blood. SMCs form the medial layer of an artery and are mainly responsible for the dilatation and constriction of the vascular wall in response to vasoactive agonists.

Our laboratory showed that DS cells of ovine and human HF stained positive for αSMA, a marker of SMC (Fig. 5.2). This finding prompted us to hypothesize that functional SMC can be derived from HF. To this end, HF were transduced with a lentivirus encoding for GFP under the control of the αSMA promoter, and GFP+ cells were sorted out using flow cytometry. We found that both ovine and human HF-derived SMC exhibited significantly higher proliferation and clonogenic potential compared to vascular SMC. In addition, tissue-engineered vascular grafts prepared from HF-derived SMCs displayed high reactivity in response to vasoactive agonists and generated significant mechanical force as shown by compaction of fibrin hydrogels [3–5, 63]. More recent studies in our laboratory showed that these vascular grafts could be implanted into the arterial circulation of an ovine animal model where they remained patent for at least 3 months [64] (Row S. et al., 2013, manuscript in preparation), suggesting that the HF may be a readily accessible source of stem cells for cardiovascular tissue regeneration and cell therapies.

5.6.2 Tissue Engineering of Cartilage, Bone, and Fat

In addition to myogenic differentiation, rodent DP/DS cells have the capacity to differentiate into the osteogenic, chondrogenic, and adipogenic lineage, similar to bone marrow-derived MSCs [33, 34]. Extending these studies, we demonstrated that human HF cells also possess multilineage differentiation potential [4]. We also showed that

single clones give rise to all four lineages, strongly indicating that human HF-MSC represent a true stem cell population and not a mixed population of progenitors with uni-lineage differentiation potential [5]. These results suggest that human HF can be an easily accessible source of true MSC that could be employed for regeneration of bone and cartilage for the replacement of joints or for meniscus repair.

5.6.3 Skin Regeneration

Several studies suggested that HF cells migrate to the epidermis during homeostasis and to a larger extent following skin injury [18, 19, 30, 35, 65], suggesting that HF cells could be used to generate the epidermis and enhance wound healing. Indeed, Hoeller and colleagues reported generation of bioengineered skin by introducing fibroblasts and HF tissue into the dermis. Interestingly, epidermal keratinocytes migrated out of the hair follicle and developed multiple layers of epidermis and stratum corneum [66]. In addition, HF-derived melanocytes have been used to develop a pigmented skin equivalent [67]. Most importantly, transplantation of tissue-engineered skin from HF-derived stem cells was shown to enhance healing of ulcers and burns significantly [68–70]. Notably, when hair buds were introduced into bioengineered skin before implantation, they sped up and guided nerve regeneration, suggesting that HF may recover the lost sense of touch [71].

5.6.4 Nerve Regeneration

Mouse HF-derived nestin^{+}/K15^{-} stem cells have the capacity to differentiate into neurons in vitro, suggesting a possible application to nerve regeneration in a variety of central and peripheral nervous system diseases [22]. Indeed, Amoh and colleagues transplanted mouse HF nestin + stem cells into a severed sciatic nerve or spinal cord, where they differentiated into Schwann cells and promoted nerve regeneration [53, 72]. The same group also reported that human HF stem cells have the capacity to restore the function of injured nerves [73, 74]. HF-derived neuronal and Schwann cells have also been introduced into acellular sciatic nerve conduit, where they exhibited long-term survival and significant electrophysiological properties in vitro but failed to induce repeated potentials [75].

5.6.5 Engineering Functional Hair Follicle

An important application of HF stem cells is bioengineering of HF to restore abnormal hair loss (alopecia). Common forms of alopecias include (a) the androgenetic alopecia which results from the miniaturization of the hair; (b) the alopecia areata,

which results from an autoimmune response that damages the hair follicle; and (c) permanent alopecia which can be caused, for example, from severe trauma [1].

Bioengineering a HF has been a topic of intense scientific research over many years. To date two strategies have been developed to achieve this goal. The first approach includes the transplantation of intact HF from a HF-rich area into the bald area. This technique requires initially the surgical excision of a thin strip of scalp that contains dense HF and subsequently the isolation of the individual follicles and implantation back to the bald scalp [76]. Although transplantation of whole follicles is considered as the gold standard for hair restoration, studies demonstrated that segments of the HF can also induce hair growth after transplantation [57, 77–82]. Transplantation of a truncated human HF after amputating the bulb has shown hair renewal suggesting bulb reformation possibly from the DS compartment [77, 78, 82]. Interestingly, transplantation of intact DP and/or DS into murine models demonstrated mesenchymal interaction with the host epithelium and subsequent hair induction as shown with the transplantation of both murine [57, 79] and human dermal compartments [81]. However, in contrast to human DS when human DP was transplanted into human skin, it failed to induce hair regeneration [80].

In severe cases of alopecias, the number of available HF is not sufficient to restore the bald site. On the other hand, HF stem cells can be expanded in culture into large numbers that may be sufficient to cover the whole area and result in hair restoration. Jahoda and colleagues were the first to report that implantation of DP cells resulted in the hair growth in mice [55]. Although the hair-inductive properties of DP cells were lost after long-term expansion in vitro, coculture with keratinocytes or in keratinocyte-conditioned medium could maintain the inductive properties of DP cells for almost 70 passages [83]. Similar to DP, DS cells were also found to induce HF growth [84]. Finally, HF restoration was enhanced by the mixture of bulge/hair germ stem cells from adult HF with neonatal dermal cells [15, 16, 29, 30, 85]. Notably, when mixed with embryonic mouse dermal and epidermal cells, mouse bone marrow-derived cells differentiated into HF cells, suggesting hair-inductive properties of bone marrow cells [86]. Although the results with mouse models are very encouraging, the significance of these findings in large animal models or humans has yet to be demonstrated.

5.6.6 Drug Delivery Through the Hair Follicle

Skin is an easily accessible organ that has been widely considered as a unique target for drug delivery. In contrast to the conventional delivery methods (oral, injections), the transdermal route allows drug administration to the circulation through the dermal vasculature and may increase drug bioavailability while avoiding painful injections. However, the presence of stratum corneum, the outermost layer of the skin, severely limits the penetration of hydrophilic and high molecular weight substances [87]. To bypass this drawback, microscale devices have been developed to enable transdermal delivery including liquid jet injectors, microneedles, and thermal ablation devices [87].

Alternatively scientists have focused on drug administration via the follicular route. The HF disrupts the stratum corneum and provides an opening to the epidermis. In certain areas such as the scalp or the face, the total area of openings can reach up to 10 % of the skin area, contributing significantly to solute permeation [88–90]. In addition the dense network of blood vessels that are associated with the HF suggests that drug release to the circulation may be feasible [91]. The heterogeneity of the harboring cell population in the HF (stem cells, gland cells, immune cells, etc.) may enable cell-specific drug targeting for treatment of skin diseases or vaccination [92–94]. Last but not least, the relatively large volume of infundibulum renders the HF a reservoir for sustained drug release to the circulation, further highlighting the importance of follicular delivery [91].

Several studies highlighted the contribution of follicular penetration during drug delivery through the skin. Mitragori and colleagues modeled the permeability of hydrophilic and hydrophobic compounds in skin, assuming that the solutes can transport through one or more of the following mechanisms: free-volume diffusion, lateral diffusion of the lipids, diffusion through pores, or diffusion through shunts (hair follicles and glands). The model predicted that high molecular weight and highly hydrophilic molecules penetrate the skin preferentially through the shunts [95]. Others suggested that there is a critical value of octanol/water partition coefficient beyond which the flux through the follicle is greatly diminished [96]. However, most studies omit the significance of sebum (a lipophilic product of sebaceous gland) during drug delivery due to lack of representative experimental models. The presence of sebum in the HF and its upward flow may hinder the delivery of hydrophilic compounds and may favor the delivery of hydrophobic compounds. Indeed, apart from molecular weight and molecular orientation, diffusion through the sebum was found to be affected by compound lipophilicity [97].

To further improve tissue targeting and drug delivery via the HF, studies have incorporated particle-based formulations. Lademann et al. demonstrated that nanoparticle-containing dye could penetrate up to 1,400 μm into the follicle of porcine skin whereas the non-particle formulation reached only 500 μm. Interestingly, the nanoparticles prolonged the storage of the dye into the follicle [98]. Nanoparticle size was shown to play critical role in follicular penetration, which was optimal for particles between 750 and 1,500 nm and decreased for larger particles [99]. In addition to this, Vogt et al. demonstrated that the size of the particles affects its uptake by the cells. They reported that only the 40 nm size nanoparticles could enter Langerhans cells that are localized around the HF. This suggests that size-specific particle formulation can be engineered to target antigen-presenting cells via the follicular route and deliver vaccines [93].

Finally, systemic delivery of a chemical through the HF has also been examined in vivo [91, 100]. Caffeine was introduced into a shampoo formulation, and its delivery into the circulation via the skin was examined in human subjects. Interestingly, the follicular route not only accelerated the delivery, but it also prolonged detection of caffeine in the blood indicating that HF may act as reservoir of chemical compounds.

5.6.7 Cell and Gene Therapy Using Hair Follicle Stem Cells

The goal of gene therapy is to restore the lost tissue function by introducing the correct gene copy at the sites where the gene is missing or is mutated [101]. Application of gene therapy for hair restoration has been attempted and showed promising results. Transduction of rat bulge-derived hair follicle stem cells with *LacZ*-encoding retrovirus showed stable expression of the transgene in the HF epithelial compartments for at least 6 months after implantation of transduced cells in an immunodeficient mouse model [102]. Retroviral gene transfer of the streptomyces tyrosinase gene was used to treat albinism. Specifically, transduction of ex vivo cultured skin from albino mice restored melanin production from the skin HF [103]. Direct gene transfer into the skin in vivo has also been reported to restore hair growth. Intradermal administration of the Sonic Hedgehog gene into C57BL/6 mice using an adenovirus resulted into anagen induction and subsequently enhanced hair growth [104]. More recently, in vivo transfection of the human telomerase reverse transcriptase DNA complexed with polyethylenimine induced telogen to anagen transition in the rat dorsal skin [105]. In addition to the treatment of hair- or skin-related disorders, gene transfer to HF could be used for delivery of proteins into the systemic circulation through the vascular plexus surrounding the follicles. To this end, it may be feasible to engineer HF that produce insulin and reverse diabetes as we have previously shown with epidermal cells using a diabetic mouse model [106].

5.6.8 Reprogramming of Hair Follicle Stem Cells

In a breakthrough study in 2006, Yamanaka and colleagues demonstrated that introduction of four transcription factors (OCT4, SOX2, KLF4, and c-Myc) into mouse embryonic fibroblasts or adult fibroblasts endowed them with enhanced proliferation capacity and potential for differentiation into all three germ layers, similar to embryonic stem cells (ESC) [107–110]. The Thomson group demonstrated that two of the transcription factors (KLF4 and c-Myc) could be replaced by NANOG and LIN28 with similar outcome [111]. The resulting cells were designated as induced pluripotent cells (iPSCs). An explosion of studies that followed demonstrated that iPSC could be generated from many human cells including blood cells [112, 113], MSC [114], fetal [114] and neonatal fibroblasts [111, 114], adipose-derived stem cells [115], adult testis [116], β-pancreatic cells [117], and T lymphocytes [118]. Interestingly, HF-derived primary keratinocytes could be reprogrammed with 100-fold higher efficiency than fibroblasts [119]. HF-derived MSC were also reprogrammed and used to understand the feedback loops that sustain self-renewal using global genomic and proteomic strategies [120]. DP cells were shown to reprogram using only two factors (Oct4, Klf4) [121], possibly suggesting the presence of endogenous factors that facilitated reprogramming. Reprogramming with fewer

transcription factors or higher efficiency suggests that HF cell-derived iPSC may be useful for regenerative medicine applications as well as for development of models to study the genetics and pathophysiology of human disease.

5.7 Conclusions: Future Directions

In summary, HF stem cells have great potential for tissue engineering and regenerative medicine applications. The ease of accessibility along with the broad differentiation capacity of HF stem cells makes the HF an ideal stem cell source. However, human HF stem cells remain relatively unexplored as compared to their mouse counterparts or other human adult stem cells. As a result more studies are required to address a number of challenges that hinder application of these cells in regenerative medicine. To this end, identification of reliable HF stem cell markers is urgently needed to facilitate HF stem cell isolation. More studies are also needed to evaluate the differentiation potential of human HF stem cells and establish culture conditions for efficient differentiation. The ease of reprogramming should be further explored to identify potential small molecules that may induce reprogramming even in the absence of genetic modification [122]. Finally, more studies are necessary to establish the HF as a site for drug and gene/protein delivery, for treatment of skin diseases and wound healing, or to the blood circulation for treatment of systemic disorders.

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