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The Hair Follicle: An Underutilized Source of Cells and Materials for Regenerative Medicine

Mehrdad T Kiani[1,2], **Claire A Higgins**[1,*], and **Benjamin D Almquist**[1,*]

[1]Department of Bioengineering, Royal School of Mines, Imperial College London, London SW7 2AZ UK

[2]Department of Materials Science, 496 Lomita Mall, Stanford University, Stanford CA 94305 USA

Abstract

The hair follicle is one of only two structures within the adult body that selectively degenerates and regenerates, making it an intriguing organ to study and use for regenerative medicine. Hair follicles have been shown to influence wound healing, angiogenesis, neurogenesis, and harbor distinct populations of stem cells; this has led to cells from the follicle being used in clinical trials for tendinosis and chronic ulcers. In addition, keratin produced by the follicle in the form of a hair fiber provides an abundant source of biomaterials for regenerative medicine. In this review, we provide an overview of the structure of a hair follicle, explain the role of the follicle in regulating the microenvironment of skin and the impact on wound healing, explore individual cell types of interest for regenerative medicine, and cover several applications of keratin-based biomaterials.

Keywords

hair follicle; keratin; wound healing; biomaterials; regenerative medicine; stem cells

Introduction

Outside of a few locational exceptions, such as the palms of the hands or the soles of the feet, human beings are covered in hair. Surprisingly, we have as many hair follicles on our bodies as our closest relative, the chimpanzee 1–2. However, we do not appear hairy like chimpanzees since our hair follicles often contain small unpigmented fibers termed vellus hairs, rather than large pigmented hair fibers which are known as terminal hairs 3. On humans, these terminal hairs are limited to restricted body sites such as pubic areas, the face, and the scalp. Their presence in these areas, in particular on the eyebrow region on the face, enable us to effectively communicate and interact with other humans 4. This feature imbues hair with important sociological functions, highlighted by the common use of hair based monikers that people use to describe one another. In addition to a sociological function, there are many cultural references alluding to the strength of long hair. Perhaps the most well-known story relating to the power of the hair follicle is the biblical story of Samson and

*Corresponding authors. c.higgins@imperial.ac.uk and b.almquist@imperial.ac.uk.

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Delilah. Samson's forte lay in his locks, and upon cutting his hair while he slept, Delilah depleted Samson of his strength.

Despite the well-established sociological and cultural importance of hair, humans appear to have 'lost' hair on their bodies; we are regarded as the naked ape. However, as is the case with all evolution, the loss of body hair must have conferred a competitive advantage at the time it occurred. A popular hypothesis explaining the loss of human body hair, or rather the miniaturization of terminal hairs to vellus hairs, is entwined with the savannah hypothesis of human evolution 5–6. This suggests that movement from the jungle, where early humans were relatively inactive, to the savannah, required humans to become increasingly active 7. As a result of this intensified activity humans needed to efficiently sweat to cool down quickly, thus we needed more sweat glands that are hypothesized to have arisen at the expense of terminal hair follicles. While the sweat duct, or eccrine duct, has its pore at the skin surface separate from the pore of the follicle, the eccrine gland itself is intertwined and directly connected with the hair follicle 8.

Compared to other mammals, humans are incredibly efficient at sweating. In support of the hypothesis above is a recent publication that explored the natural variation in the number of sweat glands and hair follicles in several strains of mice. The authors found that as the number of sweat glands in mouse skin increases, the number of hair follicles concomitantly decreases, with activity of the transcription factor engrailed 1 shown to direct the relative numbers of each ectodermal appendage (the ectoderm is the outermost germ layer in the early embryo that gives rise to the nervous system and skin epithelium) 9.

From a regenerative medicine perspective, humans are intriguing since our 'loss' of hair is unique amongst mammals, but at the same time the hair follicles we have on our scalp actively produce hair fibers for several years at a time 10. This is an anomaly since hair follicles in other mammalian species are often in a resting state, not actively producing a hair fiber 11. Thus, hair follicles from the human scalp provide an intriguing case study. In this review, we will cover how various cell types within these growing scalp hair follicles, and materials produced by these follicles, can be used for regenerative medicine.

Orientation around the hair follicle

Before discussing the hair follicle as a source of cells and materials for regenerative medicine, we need to introduce this mini organ in more detail. All hair follicles on the body are formed prior to birth, and arise as a result of tissue interactions wherein one tissue influences the differentiation and development of the other tissue. This process of morphogenesis is known as secondary induction 12. Once these developing follicles have matured, they continue to progress through cycles of regression, rest and growth throughout their lifetime. During the growth stage, which is known as anagen, the hair follicle actively produces a hair fiber. As mentioned above, hair follicles from the human scalp can remain in anagen for several years at a time, enabling the growth of long hair on this body site. In comparison, sites such as the eyebrows and eyelashes contain follicles that have a relatively short anagen phase of only a couple months, leading to the growth of short hair on these sites 13. Upon the termination of anagen a new cycle starts that is characterized by entry into

a destructive regression phase termed catagen. This destruction phase is thought to last approximately two weeks in human scalp hair follicles 10. During catagen the growth of the hair fiber stops, becoming known as a club fiber due to the ‘club’ shaped morphology at its root. Following catagen, follicles progress into a resting phase termed telogen, wherein the club fiber is retained until the following anagen stage when a new growing fiber is produced. The club fiber is eventually shed in a process known as exogen, which occurs independently of the other cycle stages 14–15.

In terms of spatial structure, the follicle can be subdivided into an upper permanent portion and a lower cycling portion. While both are present during the anagen stage, the lower cycling portion degenerates in catagen and telogen, before regenerating again in the next anagen stage. This makes the hair follicle the only structure, other than the mammary gland, that selectively degenerates and regenerates in the adult 16, making it an intriguing organ for studying the processes of regeneration.

On the human scalp approximately 90% of follicles are in anagen at any given time 17–18. Since we are discussing human hair follicles and their application in regenerative medicine, we will therefore cover the morphology of the anagen follicle rather than the morphology of the telogen or catagen follicle. Despite the base of anagen follicles residing deep within the adipose tissue of the skin (which is derived from the mesoderm germ layer of the early embryo), the majority of the follicle structure is derived from the ectoderm. Within the epithelial (ectodermal) compartment of the follicle, there are distinct stem cell populations that enable follicle growth, the first of which was identified in the early 1990’s.

The location of this original population of stem cells is termed the ‘bulge’, and is found in the ‘permanent’ compartment of the follicle (Figure 1). It is a small cluster of cells located in the basal layer of the follicle, beneath the sebaceous gland. It was first identified in murine hair follicles as a slow cycling and thus label retaining population 19. In human hair follicles, bulge cells are Krt15+ 20, and possess the abilities to self-renew and differentiate into multiple cell types, fulfilling the criteria for designation as multipotent stem cells. Cell tracing experiments in murine skin have demonstrated that in homeostatic conditions, bulge stem cells give rise to all the lower epithelial cell lineages of the hair follicle including outer root sheath cells, matrix cells, the companion layer, three layers of inner root sheath cells, the hair cuticle, the cortex and medulla 21. It should be noted that this capacity for bulge cells to contribute to the anagen follicle is established earlier during the hair cycle; in telogen there is a subset of activated stem cells that are both derived from and are located just beneath the bulge in a germ structure 22. In the telogen to anagen transition these germ cells contribute to the hair matrix 22, which then differentiates into the concentric layers of the follicle during anagen 23. Thus, bulge cells indirectly give rise to all the lower epithelial cell lineages of the follicle via the hair germ.

Once in anagen, stem cells exit the bulge and migrate down the basal layer of the follicle where they proliferate and contribute to the outer epithelial layer known as the outer root sheath. The outer root sheath contains hair keratinocytes that express the cytoskeletal protein Krt14. In the bulb of the follicle is the hair matrix, which contains transit amplifying cells derived from the hair germ in the previous telogen to anagen transition 22. In response to

signaling from the dermal papilla mesenchyme, these matrix cells differentiate, and moving upwards they form concentric layers of the follicle including the hair shaft, the inner root sheath and companion layer 24. The inner root sheath is comprised of three layers; Henle's layer, Huxley's layer, and an innermost cuticle layer, while the hair fiber is comprised of the hair cuticle, hair cortex, and hair medulla. The inner root sheath and hair fiber move towards the skin surface together as a unit, with the fiber in the center surrounded by the inner root sheath. At the level of the sebaceous gland the inner root sheath is sloughed off by proteolytic enzymes, which leaves the hair fiber clean to exit the skin surface 25. The hair fiber itself is rich in both type I and type II keratins, including keratin 33a, 33b, 34, 39, 40 (Type I's), and keratin 81, 83, and 86 (Type II's)26.

In the preceding discussion of the bulge, we are referring to the epithelial stem cells within the bulge that give rise to the epithelial lineages of the follicle. However, there is a second stem cell population that is also found anatomically within the bulge compartment, known as melanocyte stem cells 27. Melanocyte stem cell progeny reside in the hair bulb during anagen, where they produce melanin pigment granules that are transferred to epithelial cells, in turn giving the hair fiber its color 27.

Following the discovery of the bulge, other markers such as CD200 (human) and CD34 have been shown to mark the epithelial cell population that resides there 28–29. Markers such as Lgr5 and Gli1 mark sub-populations of epithelial cells that also reside within the bulge 28, 30. More recently, an additional epithelial stem cell population, anatomically close to the bulge cells but functionally distinct, was identified 31. This Lrig1+ population that is juxtaposed to the bulge cells contributes to the formation of the sebaceous gland, isthmus, and infundibular regions of the hair follicle 31. Specifically, Lrig1+ stem cells give rise to Krt79+ cells that line the lumen of the infundibulum; the canal opening at the skin surface where the hair fiber exits. These Krt79+ cells express high levels of matrix metalloprotease 9 (Mmp9), leading to the suggestion that the lumen is established as a result of proteolysis 32.

Recently, single cell RNA sequencing was performed on all epithelial populations in murine skin. The unparalleled resolution afforded by this method enabled identification of 16 populations of epithelial cells within the hair follicle, several of which were previously undefined 33. Going forward, understanding the transcriptional regulators of cell hierarchy within the hair follicle will help resolve how stem cells with different transcriptional identities give rise to the different cell types within the follicle. Stem cells are a powerful source of cells for rebuilding tissues, and using the right cell at the right time will advance the field of tissue engineering. Their increased potency compared to differentiated cells means multicellular tissue structures can be engineered using a single starting cell that is at the top of the hierarchical tree, and has the capacity to differentiate into all the cells within the end tissue 34.

While the epithelial portion of the follicle comprises the majority of the tissue structure, there are key mesenchymal components of the follicle that cannot be overlooked 35. These are predominantly the dermal papilla and the dermal sheath, which is also known as the connective tissue sheath. The dermal papilla is engulfed by the epithelial matrix and lies at the base of the follicle. Despite this proximity, the dermal papilla and hair matrix are

separated by a glassy membrane, which is a thickened and thus specialized basement membrane 36. The dermal papilla is connected via a small neck at its base to the dermal sheath that wraps around the exterior of the follicle, and is separated from the outer root sheath by a basement membrane that runs continuous with the basement membrane between the skin dermis and overlying epidermis. The dermal papilla and dermal sheath are derived from the same cellular progenitors as the fibroblasts that reside in the interfollicular skin dermis 37; however, they are distinctly different in their gene expression profiles and biological function. While the interfollicular fibroblasts support growth and differentiation of the overlying epithelial cells (i.e. keratinocytes), the dermal papilla and sheath have key roles in directing hair growth 38–39.

In human skin, individual hair follicles are organized into follicular units that each contain 2-5 hair follicles. Despite there being multiple follicles per unit, all the hair fibers produced in these units exit through a single pore at the skin surface. Each of these follicular units is also connected to just one arrector pili muscle, which connects the follicles at the level of their bulge to the basement membrane of the skin epidermis 40. Work in mouse skin has demonstrated that the fibroblast progenitors that contribute to the arrector pili muscle come from the upper skin dermis during skin development 37, while we also know that the loss of a specific extracellular matrix protein in the basement membrane of the follicle next to the bulge perturbs attachment of the muscle at this site 41. It seems there is an interesting relationship between the follicle and the arrector pili muscle, as observational studies in human skin have found follicles that miniaturize in male pattern baldness do so after losing attachment to the arrector pili muscle 42–43.

Impact of hair follicles on skin regeneration

Now that we have an understanding of how an anagen hair follicle is structured, it is possible to explore the role of this complex structure on skin regeneration. There are numerous studies in murine skin that have led to the proposal that hair follicles modulate skin regeneration. Many of these studies have focused on the skin macroenvironment, and changes that occur here in response to changes in the hair cycle. In human skin hair follicles grow in a mosaic pattern, and each follicle grows independently from hair follicles surrounding it. This makes studies of the macroenvironment in relation to hair follicle cycle stage relatively difficult. However, in murine skin hair follicles grow in a wave along the back of the mouse, from anterior to posterior 44. This means that murine skin can be isolated when all follicles are in either anagen, catagen, or telogen, and the surrounding macroenvironment can be assessed with regard to the cycle stage of the follicle.

Some of the first studies into the patterns of hair cycling in murine models looked at skin thickness. It is now well accepted that the skin thickens during anagen, and decreases in thickness during catagen and telogen 43. The majority of this increase in thickness in anagen is due to increases in the adipose layer, however, the skin dermis is 1.2-1.5 fold thicker in anagen then it is in telogen 45–46. There is little evidence for proliferation of fibroblasts in the skin dermis of adult mice 47 to explain this increase in thickness. An alternative suggestion is that dermal extracellular matrix is redistributed to accommodate the growth of follicles during anagen, resulting in an increase in dermal thickness but not total dermal

volume 46. Researchers have also looked at the relationship between other dermal constituents such as vasculature, finding that the number of capillaries in the skin decreases during catagen and telogen, but then increases during anagen 48–49. This leads to the question as to which comes first; the anagen hair follicle or skin vascularisation. Skin biologists will argue that the anagen hair follicle is a source of pro-angiogenic factors, and expression of these factors within the follicle during anagen promotes angiogenesis in the surrounding skin dermis 48. One such factor is Vascular Endothelial Growth Factor (VEGF), a well-known pro-angiogenic factor that is highly expressed by cells within the anagen hair follicle 50.

Macroenvironment fluctuations are not limited to the vascular networks around the follicle; neural networks, immune cell populations, and dermal white adipose tissue all have cyclic activity analogous with the follicular cycle 51. Neuronal networks display a high degree of remodeling in coordination with the follicle cycle; degeneration occurs during telogen, followed by reinnervation of the skin during anagen, particular around the bulge area of the follicle 52. Studies into the immune populations have mainly focused on macrophages that intriguingly decrease concomitantly with the onset of anagen 53. Dermal white adipose tissue follows a similar oscillation trend as the skin dermis, with adipose precursor cells differentiating into mature lipid filled adipocytes during anagen, resulting in a 2-3 fold increase in intradermal tissue during this stage of the hair cycle 45, 54–55. Upon completion of anagen, mature adipocytes apoptose resulting in a decrease in tissue bulk size, while proliferation of adipose precursor cells ensures there are enough in reserve for when the follicle re-enters the next anagen stage 54.

This dermal remodelling that is associated with the hair cycle most likely has an impact on the ability of skin to heal. Research has shown that when wounds are created in the dorsal skin of mice with hair follicles in anagen, they close significantly quicker than wounds established in dorsal skin containing telogen hair follicles 56. However, the specific role of the hair follicle in wound healing cannot be deconvoluted from the other morphological changes in the skin dermis such as the altered numbers of blood vessels, neural networks and immune cell populations. Care should be taken by researchers when studying wound healing in skin, since the growth state of the hair follicle can influence and alter results. In addition, caution should be exercised when translating findings from murine skin to human. For example, dermal $\gamma\delta$ T cells are critical modulators of skin and hair regeneration in mice, however, humans lack appreciable numbers of these cells in their skin dermis 57.

Perhaps alluding to a role for human hair follicles in wound healing are early self-experimentation studies performed by George Holman Bishop, who created wounds on his own skin and followed their closure 58. His observations tell us that re-epithelialization of wounds first occurs in pockets around the hair follicle, suggesting that bulge stem cells can form skin epidermis in a wound situation. In the 1940’s when Bishop performed these experiments, researchers did not know the location of the stem cells within the follicle. However, more recent lineage tracing experiments in murine models has confirmed his early observations, and we now know that hair follicle stem cells within the bulge can migrate upwards to replenish the skin epithelium after wounding (Figure 2) 59–60.

Finally, perhaps the most convincing evidence that hair follicles have a role in skin regeneration comes from recent work arising in the field of hair transplantation. Fueled by the above observations that hair follicles promote both dermal and epidermal remodeling, a number of groups have transplanted plugs of skin containing terminal anagen hair follicles from the scalp into chronic venous ulcers. These are predominantly observation studies conducted in human skin, but the early results indicate that the skin plugs containing hair follicles promote wound closure significantly faster than skin plugs devoid of terminal hair follicles 61. Wound closure is defined as re-epithelialization, although the working hypothesis is that the follicle promotes both dermal remodeling and provides a source of cells for re-epithelialization 62. When focus is moved to specifically assess the regenerated epithelium, its clinical appearance is significantly improved when skin plugs containing hair are used compared to skin plugs devoid of hair 63.

Hair follicles as a source of cells for regenerative medicine

In the section above we focused on whole follicles and their role in dermal and epidermal remodeling, both during the hair cycle and after wound healing. We will now switch tack, focusing specifically on various tissues within the follicle, notably the mesenchymal dermal papilla and sheath, and the epithelial bulge.

One of the more intriguing aspects of the dermal papilla and dermal sheath, as introduced above, is that they direct the growth of hair in adult skin. Hair follicles go through cycles of anagen, catagen and telogen throughout their lifetime, and the presence of, and signals from the dermal papilla and dermal sheath are fundamental for progression through these cycle stages 64. Perhaps even more intriguing is the observation that both dermal papilla and dermal sheath can have similar effects ex vivo. When these mesenchymal tissues are removed from both rodent and human hair follicles and grafted into non-hairy skin or inactivated follicles, they can induce new hair follicle and fiber growth in the recipient epithelium 65–66. Thus, specific mesenchymal cell types from the follicle can induce formation of new hair growth after transplantation into non-hairy skin. While this can potentially serve as an autologous cell therapy, the hair follicle is immune privileged and dermal sheath cells are known to avoid rejection and elicit hair growth in an allograft 67. Their immune privilege is thought to arise due to their virtual lack of MHC Class I and II antigens 68, which makes the dermal papilla and sheath potentially advantageous to other cell types for allogeneic cell therapies.

As components of the hair follicle, it has also been postulated that dermal papilla and dermal sheath cells can contribute to the skin dermis where they have roles in skin formation and wound remodeling 69–70. In this context, both dermal papilla and sheath cells are able replace dermal fibroblasts to form the dermal component of engineered skins 71. Engineered skins are one of the first tissue engineered products to be used clinically, and are usually comprised of a simple collagen-based dermis containing dermal fibroblasts, and an epithelial component. In response to signaling from the underlying dermis, the epithelial keratinocytes proliferate, then differentiate and stratify to form the multiple layers of the skin epidermis. A key component of these skins are the stem cells in the basal layer of the epidermis. These

stem cells are able to self-renew, maintaining continuous turnover of the epithelium and ensuring successful skin replacement 72.

In engineered skin containing either dermal papilla or sheath cells substituted for dermal fibroblasts, normal differentiation and stratification of the overlying epithelial cells occurs, while basal layer cells continue to proliferate. In addition, the hair follicle dermal sheath cells appear superior to dermal fibroblasts in one aspect; they are able to induce the formation of a thickened basement membrane 71. In a regenerative medicine context, this may strengthen the replacement skin when engineered skins are used in grafting.

Regardless of their role in hair follicle cycling and skin development, dermal papilla and sheath cells have other understudied properties that are of interest in the field of regenerative medicine. Dermal sheath cells are prolific producers of collagen I, and because of this RepliCel Life Sciences are currently conducting a Phase I/II autologous cell therapy trial in 28 patients (Clinical trial # NCT02330146) using dermal sheath cells isolated from the hair follicle to treat chronic Achilles tendinosis. At the time of writing this review there are no results informing us of dermal sheath efficacy in tendon repair, however previous trials with skin-derived fibroblasts have been shown to be safe.

When isolated from the follicle and grown in cell culture, both dermal papilla and sheath populations are multipotent. It is known that in vitro, these hair follicle-derived cells are able to differentiate into cells from several other mesenchymal lineages including osteoblasts, adipocytes, contractile smooth muscle cells, and chondrocytes (Figure 2) 73–75. Given the immunogenic properties of dermal papilla and dermal sheath cells, along with their multipotency and accessibility, they represent a source of cells that may be employed for applications such as cardiac regeneration or the replacement of bone and cartilage in joint repair 76.

However, when thinking of clinical applications a single dermal papilla from one hair follicle will not go very far; a dermal papilla from a scalp hair follicle contains on average 1300 cells 77. To be suitable for clinical use the cells have be first micro-dissected from the follicle, then their numbers expanded in culture 78. Dermal papilla cells grow according to a Gompertz function, which means they grow very slowly at the initiation of the culture 79. The perturbing event of growth in culture also elicits dramatic changes in the signature and behavioral traits of the cells, such as the ability to induce de novo hair growth 65. Once cultures are established, dermal papilla cells have an average doubling time of 93 hours 79, and to increase in number to 5 million+ cells required to repair a critical size bone defect 80 would take approximately 50 days. While obtaining a culture of 5 million cells is achievable with dermal papilla culture, in normal culture conditions they will not double enough times prior to exhaustion to produce a sufficient number of cells to repair larger defects. Many times these large defects require hundreds of millions to billions of cells 81. Alternative cell culture approaches, where bioreactors are used to maintain cells in a stirred suspension are starting to be employed for the culture of skin cells 82. In addition, materials scientists are currently devising ways to alter the growth kinetics of cells in order to promote proliferation while maintaining key biological properties such as stemness 83–84.

In addition to the exciting applications in regenerative medicine for the mesenchymal populations of the follicle, cells from the epithelial stem cell region of the follicle (the bulge) also have intriguing properties. As mentioned above, we know that in response to wounding in the skin epidermis, bulge stem cells differentiate into interfollicular epithelial cells and contribute to healing the epithelium 59. A similar response of bulge cells occurs when homeostasis of the sebaceous gland is perturbed; bulge cells migrate to the sebaceous gland and differentiate into sebocytes 85–86. In addition, hair follicle bulge cells have been incorporated into bioengineered skin constructs, where they are capable of forming a stratified epithelium 87. Epithelial sheets established using hair follicle bulge cells have also been used as an autologous therapy to treat chronic ulcers 88–89, drawing attention back to their wound healing capacity.

We also know from recent work assessing the epigenetic status of cells in the follicle that hair follicle bulge stem cell identity is governed by transcription factor binding at super-enhancers associated with hair follicle stem cells 90. In the past few years, there has been increasing interest in super-enhancers as governors of cell identity. Super-enhancers are enhancers (>28kb) that have elevated H3K27ac occupancy, and are bound by multiple transcription factors at a time. In turn, the genes transcribed as a result of super-enhancer binding are usually unique to specific cell types, and therefore associated with cell identity 91. In response to wounding in the skin or growth of bulge cells in culture, the super-enhancers associated with bulge stem cells lose their H3K27ac occupancy and become actively repressed. Instead, super-enhancers associated with a wound healing identity acquire H3K27ac marks, enabling transcription of genes associated with an interfollicular fate 90. This highlights the dramatic epigenetic changes, in addition to genetic ones, which occur when cells are removed from their in vivo surroundings and grown in cell culture.

In recent years the interest in bulge stem cells has increased as they can be isolated from a single plucked hair fiber; a skin biopsy is not required. Specifically, bulge stem cells gained attention when cells isolated from plucked hair fibers were used to generate bonafide populations of induced pluripotent stem cells (iPSCs) 92. This may have large implications for the field of regenerative medicine, where iPSCs have had a significant impact since their derivation in 2006 93. This is because iPSCs are pluripotent stem cells, which share many similarities with embryonic stem cells and can differentiate into cells from all germ layers: the endoderm, mesoderm and ectoderm. However, unlike embryonic stem cells, iPSCs can be generated with patient specific cells, enabling the study of specific disease mechanisms and methods of gene therapy in addition to regenerative cell therapies. Being able to generate iPSCs from cells from plucked hair fibers makes the hair follicle bulge extremely accessible as a source of cells for regenerative medicine 92, and enables the generation of iPSC from patient groups where skin biopsies cannot be easily taken, such as children.

While accessibility is a large benefit when it comes to optimal cell sources for iPSC, the slightly less accessible hair follicle dermal papilla may actually be an even better source for iPSC than bulge stem cells. Dermal papilla cells are a somatic cell population that express Sox2, Klf4, and cMyc 94, which readers with a knowledge of iPSC will realize are 3 of the 4 suggested factors used to reprogram somatic cells into a pluripotent state 93. Thus, murine dermal papilla cells can be isolated from hair follicles and with the addition of Oct4 alone,

can be reprogrammed into iPSCs 95. The reprogramming efficiency of dermal papilla cells with only Oct4 is 0.088%, similar to the efficacy observed in other cell types when all 4 factors are used 95. When all 4 transcription factors (Oct4, Sox2, Klf4, cMyc) are used to reprogram murine dermal papilla cells, reprogramming efficiency is as high as 1.38% 96. Hence, dermal papilla cells currently share similarities with neural stem cells 97, as these are the only two types of cells that have been reprogrammed using a single factor. However, it is easy to appreciate that while dermal papilla cells are not as accessible as bulge stem cells, they are much easier to obtain compared to neural stem cells, and a simple scalp biopsy is all that is needed for their isolation.

Taken together, the diverse collection of cell populations within the hair follicle provide an exciting source of cells for applications in regenerative medicine. Their low immunogenicity, ease of access, and efficient differentiation all support continued exploration of their translational potential. With that being said, while the individual cell types are indeed interesting for regenerative medicine, the materials that they produce are equally interesting for use as biocompatible materials for regeneration.

Hair follicles as a source of biomaterials for regenerative medicine

In ectodermal appendages like the hair follicle, keratin is the major constituent that provides strength and resilience to the end product, the hair fiber. In this final section of this review, we will focus on the importance of keratin from hair follicles as a biomaterial for regenerative medicine. Despite being studied for over 100 years, keratin remains relatively underexplored as a biomaterial when compared to collagen and fibronectin. However, keratin provides unique properties and benefits that make it an intriguing material to use.

Keratin represents a class of important intermediate filament proteins that are ubiquitous across numerous animal species and possess unique mechanical and structural properties 98. Their classification is based on these properties, and the first major classification of keratin aims to separate these proteins by their predominant secondary protein structure. β-keratin, characterized by β-sheet structure, is much stronger than α-keratin, characterized by α-helices 99. β-keratin constitutes the major component of scales, claws, beaks, and shells, providing the strength and rigidity seen in these appendages. Compared to β-keratin, α-keratin is not nearly as rigid 100, however it is the only type found in mammals and is present in hair, nails, wool, and horns. In humans there are 54 known α-keratin monomers, further separated into Type I (acidic) and Type II (basic/neutral) keratins. Of these 54 keratins, approximately half are specifically expressed in the hair follicle 26. A heterodimer unit is formed between one Type I and one Type II keratin that form a “coiled-coil” tertiary structure. This constitutes the basis of a functional keratin unit 99.

While the secondary and tertiary structure provide ways to classify keratins, the strength and insolubility of keratin arises from their amino acid constituents and the covalent interactions between amino acids 100. In particular, keratin from human hair possesses a high amount of cysteine, ranging from 8.7-17 mol% depending on gender, race, and hair color 101. Cysteine contains a thiol group that is easily oxidized, enabling the formation of disulfide bonds

between keratin units. This common formation of disulfide bonds provides the robust mechanical properties and insolubility of keratin 102.

In ectodermal appendages such as the hair follicle, the disulfide characteristic of keratin can lead to difficulty in breaking down the tertiary structure. While human cells produce proteolytic enzymes such as caspases 103, these enzymes are not typically secreted outside cells and do not target disulfide bonds (these enzymes, termed keratinases, can be found in bacteria and fungi 104). Thus, hair is stable for most time scales of interest and does not undergo significant degradation. This stability is ideal in the case of nails and hair, but it can also give rise to medical problems. Highlighting keratin stability is the clinical presentation of a condition known as Rapunzel Syndrome. This is a rare condition where eating a large quantity of hair leads to potentially fatal intestinal blockage 105. Since trypsin and pepsin are unable to digest keratin, surgery is the only option for such cases. The issue of degradability also poses a problem in many analytical techniques such as mass spectrometry, HPLC, and SDS-PAGE 106, and researchers must that care that their own skin or hair doesn’t lead to erroneous experimental results due to contamination.

Although keratin is relatively resistant to degradation, the process for extracting it from hair, feathers, and wool is straightforward and inexpensive (Figure 3). The first step involves washing the raw material with soap or surfactant to remove any oil residues on the surface that may interfere with subsequent processing. After drying, it is necessary to break down the α-helical structure by chemically reducing or oxidizing the disulfide bonds between cysteine groups in the keratin. Without breaking the disulfide bonds, keratin is insoluble in nearly all solvents and cannot be easily modified. To reduce the raw keratin, the washed source material is placed in a solution with reducing or denaturing reagents such as 2-mercaptoethanol, thioglycolic acid, urea, dithiothreitol, or ionic liquids 107–108. The solution is then mixed at an elevated temperature and left for several hours to days depending on the reducing agent, followed by filtering to remove any undissolved raw material. This is necessary to remove components of source material that do not readily dissolve, such as the cuticle of hair fibers. At this point, the solution contains the reduced keratin, which is called kerateine109. This solution can be dialyzed and extracted as kerateine, or modifications such as carboxymethyl groups added to the reduced thiols to prevent the formation of disulfide bonds; iodoacetic acid is commonly used for this modification.

Oxidation of keratin is commonly done using peracetic acid to promote fission and subsequent conversion of the disulfide crosslinks in keratin to sulfonic acid groups. This oxidized form of keratin is known as keratose, and consists primarily of keratins from the cortex of the hair fiber110. As materials keratose is more susceptible degradation (degrades over days to weeks) due to presence of the acid groups and inability to reform disulfide bonds, while kerateine retains the ability to form disulfides and has a backbone that is less susceptible to hydrolytic degradation (degrades over weeks to months)109. One unique benefit of these differing degradation profiles is that it allows for tunable rates of degradation via simple mixing of kerateine and keratose111. In the end, kerateine (via reduction) and keratose (via oxidation) are the basic building blocks for most applications that use keratin.

Due to the availability and easy extraction process, in recent years keratin has become more popular as a regenerative biomaterial (Figure 3), complementing, and sometimes replacing more traditional biomaterials such as collagen and fibronectin. While there is a wide body of literature exploring how cells interact with these more traditional biomaterials, in the case of keratin biomaterials there is much less is known regarding the specific interactions between cells and the molecular features of keratin. Despite numerous studies demonstrating that many cells can attach to keratin 112–116, the underlying mechanisms of attachment have remained elusive. Binding regions such as RGD and LDV are thought to be the dominant integrin binding sites117, however, studies have not been decisive as to whether integrin-dependent binding is the dominant mode 112, 118–119. For instance, inhibition of the β1 & β2 subunits in hepatocytes does not affect cell binding to a keratin film, whereas inhibition of the asialoglycoprotein receptor inhibits binding 114. However, inhibition of the β1 & β3 subunits, talin staining, and inhibition of focal adhesion kinase phosphorylation in platelets all indicate integrin-mediated binding to keratin hydrogels 120. These divergent examples highlight the need for a better understanding of how specific cells interact with keratin, in order to facilitate tailoring the properties to fit the needs of different applications.

Even though the interactions of keratin with specific cell types remains to be elucidated, there is still a large body of literature that uses keratin as a biomaterial in wound healing, tissue regeneration, ocular surface reconstruction 121–123, and in models to study fungal infections of nails 124–125. Below, we will highlight some examples that have used keratin extracted from human hair, however, a large body of work also exists for keratins that are extracted from other ectodermal appendages such as feathers and wool. To begin, we will explore how keratin is a unique material for modulating multiple phases of wound healing, followed by exploring how keratin's resistance to degradation can be utilized for applications addressing long-term tissue regeneration.

Wound healing is an attractive area for applying new innovations in biomaterials. Due to the highly dynamic and complex process of wound repair, biomaterials have the potential to work as exogenous orchestrators of the repair process 126. Keratin biomaterials are interesting in this regard, as they have unique properties that can be harnessed for promoting hemostasis, delivering antimicrobials, modulating the immune response, delivering biologics such as growth factors, and modulating scar formation.

We will begin with keratin's role in promoting hemostasis, since stopping blood loss and promoting coagulation is the first step down the path of wound repair 127. For small wounds this step occurs without the need for intervention, however in cases such as battlefield injuries or surgery, significant blood loss can be fatal. To address this problem, the van Dyke Group has pioneered the development of numerous keratin biomaterials for hemostasis. In vivo studies, assessing liver injury in both rabbits 128 and porcine models 120, 129, have demonstrated either comparable or better survival rates and decreased blood loss using keratin hydrogels instead of commercially available hemostats. Post-sacrifice, histological analysis of keratin hydrogels revealed cell infiltration and the beginning of granulation tissue formation, which was not observed in commercially available hemostat samples. More recently, a keratin nanoparticle-based approach demonstrated decreased coagulation time both in vitro and in vivo using a rat laceration model, providing an alternate "powder" based

approach that may have a better shelf life and require less preparation time than a keratin hydrogel 130.

The underlying mechanism that promotes coagulation in kerateine gels is thought to depend on thrombin-mediated fibrin polymerization 115. Platelets have been shown to bind to kerateine hydrogels in an integrin-dependent manner 120, which concentrates platelets in the wound region and enables coagulation via fibrin polymerization. An in vitro study of kerateine in solution with plasma found increased fibrin polymerization in clots 115, and while the coagulation cascade is unclear, this phenomenon sheds light on why a keratin hydrogel can perform better than commercially available hemostats that work by predominantly absorbing fluid, concentrating clotting compounds, and providing a seal around the wound 129. Interestingly, keratose shows good blood compatibility 131, indicating that the thiol groups in kerateine likely play an important role in coagulation through disulfide-mediated interactions 132.

One bleeding has stopped the process of tissue repair can begin. In situations where it is difficult to ensure a clean wound environment, or where infection has been established (e.g. chronic wounds), it is advantageous to have a method for localized, controlled delivery of antibiotics to keep infections from inhibiting successful healing. Keratin-based biomaterials may offer a promising delivery strategy to achieve sustained release of antibiotics at the wound site due to slow degradation profiles, resistance to numerous enzymes, and flexible fabrication processes that can produce antimicrobial electrospun dressings 133, films 134, sponges 135, and hydrogels.

Several studies have investigated using keratin-based biomaterials to deliver ciprofloxacin, a broad-spectrum antibiotic commonly used against Gram-negative bacteria. When ciprofloxacin is incorporated into unmodified 20% w/v keratose hydrogels, 40% release is observed in the first 24 hours, and full release occurs after 10 days. This is in comparison to agarose gels (the authors were unable to synthesize collagen hydrogels with the drug), which show a plateau in ciprofloxacin release within 24 hours 136. Ciprofloxacin release from keratose hydrogels correlates with hydrogel breakdown, and is effective in preventing *Staphylococcus aureus* colony formation for at least 3 weeks in vitro, and at least two weeks in vivo 136. While this first in vivo study was conducted in mice, ciprofloxacin-loaded keratose hydrogels are similarly effective at preventing bacterial infection in porcine excisional wounds 137 and thermal burns 138. It is important to note that some bacterial strains produce keratinases, which have the potential to significantly alter the rate of hydrogel degradation, and therefore impact release profiles. However, if properly designed this feature may provide a method for tuning the release of specific drugs that target different bacterial populations. This can then be coupled with the ability to tune the rate of release using a variety of methods including kerateine-keratose mixtures111 or by controlling the levels of disulfide formation in kerateine hydrogels 118.

Whether or not antibiotics are delivered, there is a localized immune response that begins almost immediately following wounding 127, 139. Mast cells, neutrophils, and macrophages take on numerous roles including killing bacteria, removing apoptotic cells, and releasing cytokines that promote proliferation, angiogenesis, and ECM remodeling. This response

begins as an inflammatory response that is important for successful wound healing, but transitions to an anti-inflammatory response as damaged tissue is removed and the process of rebuilding and repair begins. However, biomaterials present in a wound have the potential to prolong the inflammatory phase and inhibit efficient healing.

Limited studies have been performed assessing the immune response of cells to keratin biomaterials. Studies on mast cells 140 and macrophages 141 showed no significant response to keratin compared to other commonly used materials. However, in the case of macrophages, keratose coatings can promote monocyte differentiation towards anti-inflammatory ‘M2-type’ macrophages over pro-inflammatory ‘M1-type’ macrophages 142. This is in contrast to collagen coatings, which promote equal monocyte differentiation into both M2 and M1 macrophages.

This biased differentiation can potentially be used as a means of mitigating glial scarring by reactive astrocytes following spinal cord injury. After injury, chondroitin sulphate proteoglycans (CSPGs) are produced by astrocytes in a process known as astrogliosis, and act to inhibit further cell damage. Inflammatory cytokines released by ‘M1-type’ macrophages can lead to excessive CSPG production by reactive astrocytes, hampering effective recovery. In an in vitro model for astrogliosis, media from monocytes grown on keratin films inhibited the process of astrogliosis 142. Thus, it opens up the possibility of controlling glial scarring by targeting and enhancing M2 macrophages, rather than astrocytes. The idea of exploiting biased induction of differentiation is not new, and other studies have used biomaterials to create pro-regenerative microenviroments by biasing the recruitment of alternatively activated macrophages 143–144.

The final step in wound healing aims to develop new tissue to replace the damaged tissue 127. Ideally, this new tissue recreates the form and function of uninjured tissue, and therefore minimal to no scar tissue will be produced. Scar tissue is aesthetically unpleasing, mechanically distinct, and impairs the function of the host tissue. In the case of skin, scar tissue is a biological bandage that lacks sweat glands and hair follicles, while for many internal traumas, scar tissue can lead to serious issues such as arrhythmia 145 or surgical adhesions 146.

In addition to glial scarring, keratin biomaterials have been shown to reduce scarring following myocardial infarction, which results in production of scar fibroblasts that replace cardiomyocytes and impair cardiac function 147. One theory as to how keratin biomaterials reduce scar formation arises from our understanding of the process of keratin extraction. A number of residual cytokines, including TGF-β, NGF, and BMP4 are found in human hair, and keratose hydrogels have been found to release approximately 40pg of growth factors per 100μg of hydrogel. It is postulated that these residual cytokines found in hair may have a role in stimulating cell proliferation, and be one of the reasons why keratin biomaterials elicit beneficial scar reducing effects in vivo 147.

In terms of skin, scar formation is not only disfiguring, but scar contracture can result in severely impaired movement 148. Several studies have looked at the ability of keratin or keratin-blend hydrogels and sponges to regenerate functional skin 149–154. The positive

effects of keratin are generally attributed to its ability to promote cell infiltration, proliferation, collagen production, and angiogenesis. However, specifically with regard to scar contraction, kerateine hydrogels can direct increased fibroblast clustering compared to collagen hydrogels with similar shear and storage modulus. This in turn results in considerably less fibroblast contraction in kerateine hydrogels 113, 155, and may be a feature that makes them beneficial for mitigating scar formation. At this point it should be noted that compared to more traditional scaffolds such as collagen and fibronectin, there is still much that is unknown about how keratin-based materials are manipulating the process of wound repair. Future studies that elucidate more detailed insight into the molecular mechanisms and interactions between keratin-based materials and cells involved with repair will be key to optimizing the design and use of these materials as scaffolds for wound repair and scar reduction.

While skin provides an attractive area for using keratin biomaterials to promote healing, the timescales of interest are relatively short. In many ways, this doesn't take advantage of one of the unique features of these materials, which is the ability to design them to be stable in the body for extended periods of time. This makes keratin biomaterials ideal for applications that require longer-term regeneration, such as the healing of bone, nerves, muscle, and cartilage 156. Most work looking at bone regeneration involves delivering bone morphogenic protein-2 (BMP-2), a growth factor that promotes osteoinduction and osteoblast differentiation at the site of injury. Currently, the FDA has approved the use of rhBMP-2 in an resorbable crosslinked collagen sponge for anterior lumbar spinal fusion (INFUSE®) 157. While clinical trials have demonstrated successful ossification and fusion with INFUSE®, collagen sponges have limited use for longer delivery due to their low binding affinity for rhBMP-2. In addition, INFUSE® has not been as effective in other orthopedic surgeries due to a variety of issues including heterotopic ossification (bone tissue formation in soft tissue) 158. It has long been postulated that the presence of BMP-2 exacerbates heterotopic ossification, however, less than half the amount of ectopic bone is produced when BMP-2 kerateine sponges are used compared to BMP-2 collagen sponges; thus, the biomaterial carrier has a large effect on the clinical outcome 159. These findings likely arise from kerateine, alkylated kerateine, and keratose hydrogels all demonstrating increased binding affinity for rhBMP-2 compared to collagen hydrogels, and thin films of all keratin derivatives have better adhesion of mice pre-osteoblast cells compared to collagen 118. Kerateine sponges can also minimize leakage of BMP-2 compared to amine-terminated keratin sponges 160, highlighting the advantages of this relatively unused biomaterial for longer term growth factor delivery and tissue regeneration.

To directly compare keratose scaffolds with INFUSE® collagen sponges, the van Dyke group assessed both scaffold degradation and BMP-2 release profiles 161. While BMP-2 release from keratose scaffolds can be correlated to the degradation of the scaffolds over 1 month, with INFUSE® the release of BMP2 was independent of the collagen degradation rate. INFUSE® sponges showed slower initial degradation and slower initial release of BMP-2, although a faster subsequent rate of release resulted in similar amounts released after 1 month. Despite BMP-2 release being correlated with scaffold degradation, the structural integrity of keratose scaffolds was found still to be intact after 5 months, whereas at this point in time collagen scaffolds were fully degraded. Comparing femoral gap repair in

a rats with both biomaterials, healing times, bone mechanics, bone volume, and density were similar between INFUSE® and keratose scaffolds, however there was significantly less adipose tissue found in the healed gap of keratose scaffolds 161. Taken together, these studies demonstrate several key advantages of keratin over collagen-based materials for healing bone.

Keratin-based materials have also shown promise as guides for peripheral nerve repair. They have the potential to replace autografts, which pose complications when harvesting 162, and they can possibly replace FDA approved conduits, some of which are made of Type I collagen 163. However, in vivo studies have been limited and their full potential is still unknown. In the studies that have been performed, keratin-based nerve guides have been found to promote Schwann cell proliferation and migration into the wound site 116. It is not fully known how these materials promote Schwann cell proliferation and migration; it may be that the minute amounts of various growth factors that remain in keratin biomaterials following the extraction process 147 provide some latent bioactivity, while the keratin itself provides adhesion sites that facilitate the migration of Schwann cells 116. Keratose hydrogels (15% w/v) in silicone, polycaprolactone, or collagen conduits can also improve nerve recovery after injury in mice 116, 164, rats 165–166, rabbits 167, and monkeys 168. While there is some variation between model systems, keratose hydrogels can generally decrease conduction delay (time of impulse transmission) and improve conduction motor action potential (amplitude of impulse) compared to saline filled conduits. It seems that the effect of keratose hydrogels is to accelerate the initial repair process, since while differences are observed between keratin conduits, saline conduits, and autografts early on, at 6 months these differences even out. Overall, these animal studies seem to indicate that keratin improves initial healing by promoting early Schwann cell migration but long term recovery is similar to autografts.

Conclusion

Hair follicles are a truly unique source of cells and materials for regenerative medicine. In theory, this accessible mini organ has the potential to provide all the cells and scaffolds necessary for a wide variety of applications in tissue engineering and regenerative medicine, something that is not possible with other tissue and organ sources. The ease of harvesting stem cell populations from donor sites, facile ability to perform iPSC transformation, and abundant supply of keratin make the hair follicle a very underappreciated and underexplored player in the field. To date, there has been some promising work using keratin-based materials to augment tissue repair, while follicular cells have been used in clinical trials to promote tissue repair. However, there is still much that we have to learn from the follicle. By having a natural role in the process of wound repair, along with the ability to promote key processes such as angiogenesis and neurogenesis, this fascinating appendage is a treasure-trove of information regarding key endogenous mechanisms of tissue repair. If we take the time to learn from the follicle, understand how it coordinates such complex processes of repair and regeneration, and elucidate how keratin interacts with our tissues, there is surely a wealth of potential to uncover. We are excited by this potential, and are confident that new and exciting insights and applications are just around the corner.

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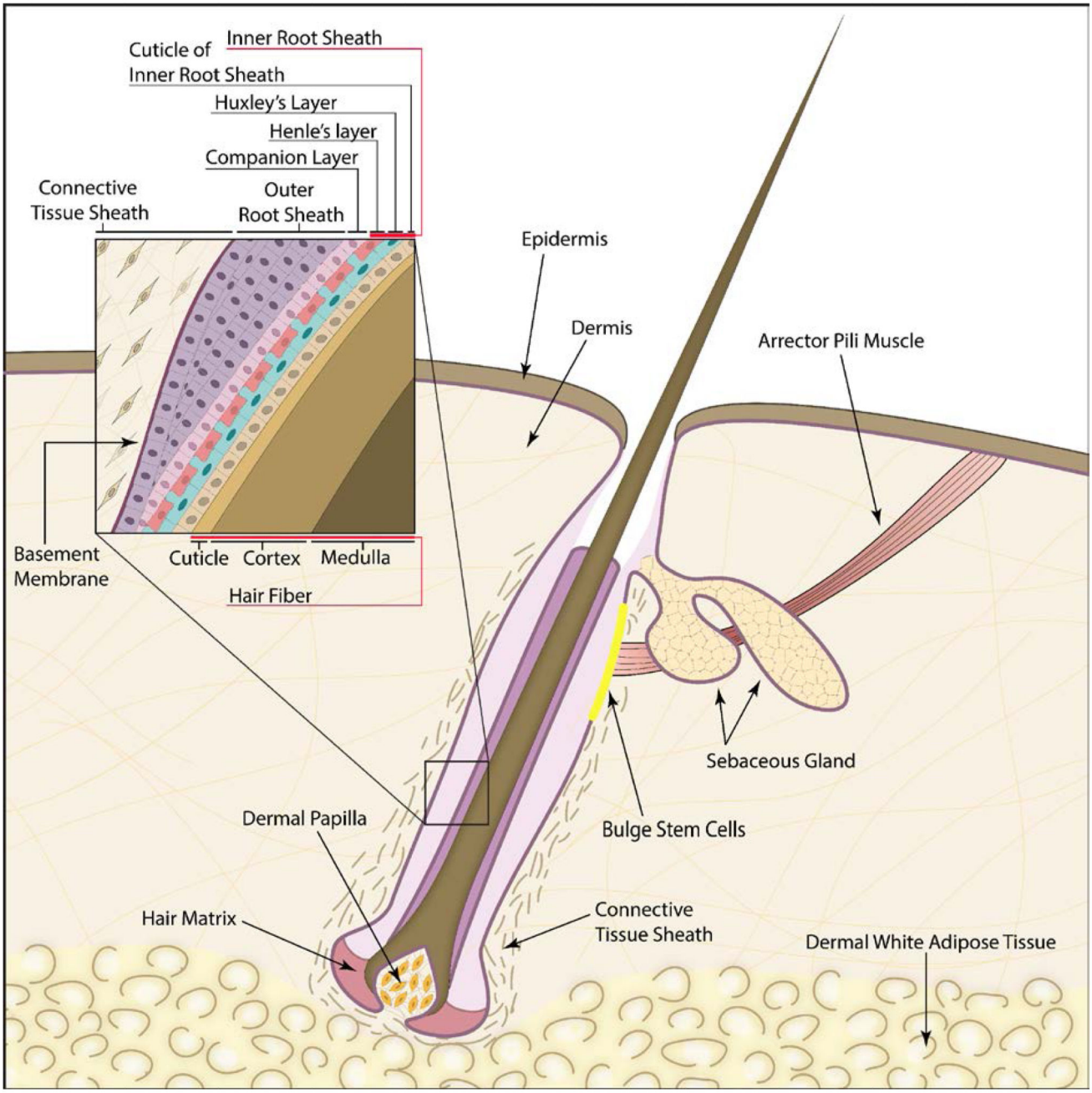

Figure 1. Schematic of the human hair follicle.

The hair follicle contains both mesenchymal and epithelial components, separated by a basement membrane. The mesenchymal dermal papilla and connective tissue sheath (dermal sheath) direct growth and differentiation of subjacent epithelial cells. In the epithelial compartment, stem cells exit the bulge, proliferate, and contribute to the outer root sheath. Cells in the matrix, which forms from the hair germ in the telogen to anagen transition, proliferate, differentiate, and migrate upwards towards the skin surface giving rise to the

three concentric layers of the hair fiber (medulla, cortex and cuticle), the three layers of the inner root sheath (cuticle, Huxleys, and Henles), and the companion layer.

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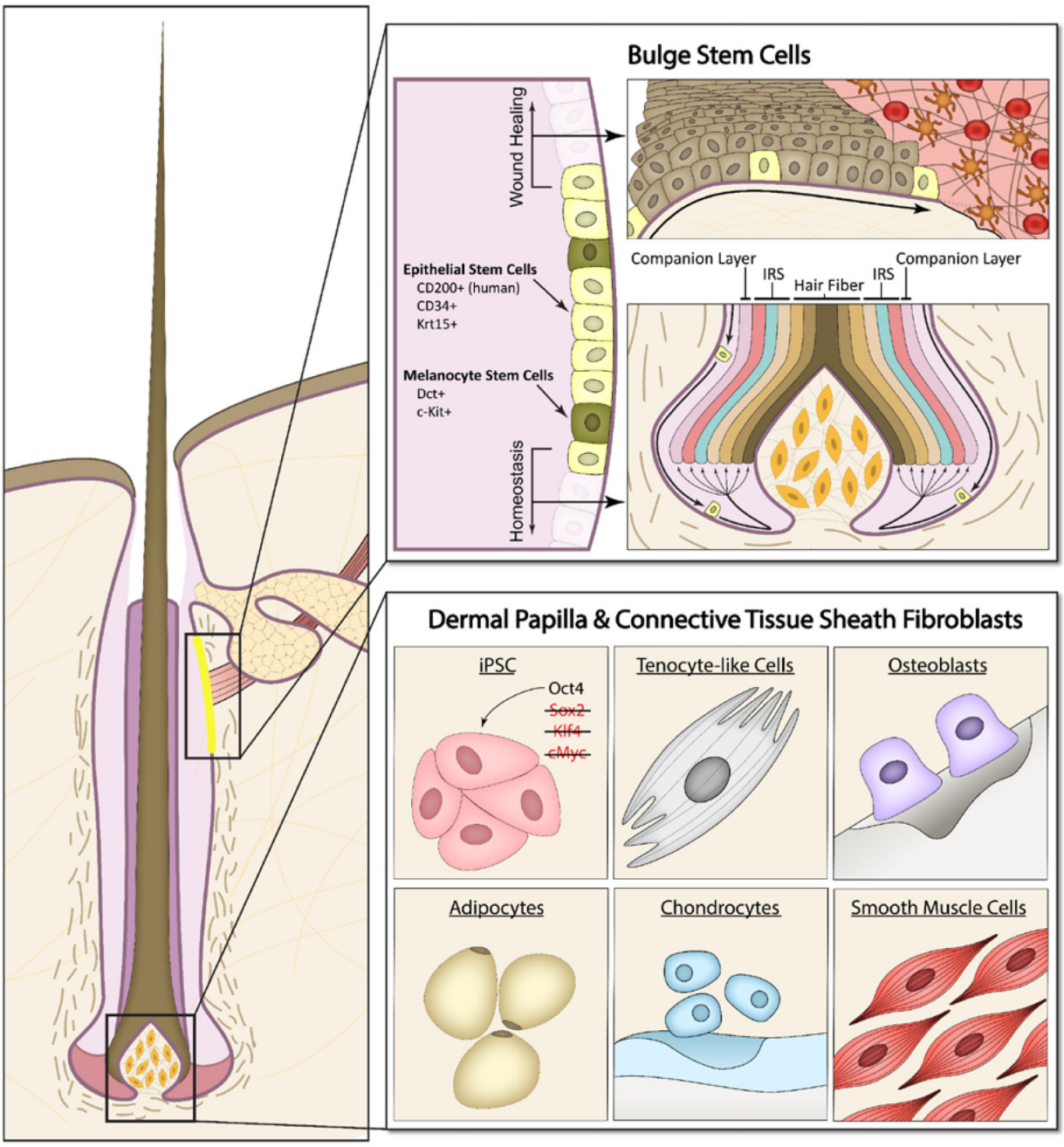

Figure 2. Regenerative medicine application of cells from the hair follicle.

Epithelial stem cells located in the bulge can give rise to the different epithelial lineages of the hair follicle. In response to wounding in the skin, bulge stem cells contribute to re-epithelialization of the epidermis. Comparatively, hair follicle dermal papilla and connective tissue sheath (dermal sheath) cells can differentiate into many cell types including osteoblasts and adipocytes. In a clinical trial for tendinosis, sheath cells are believed to differentiate into tenocyte-like cells.

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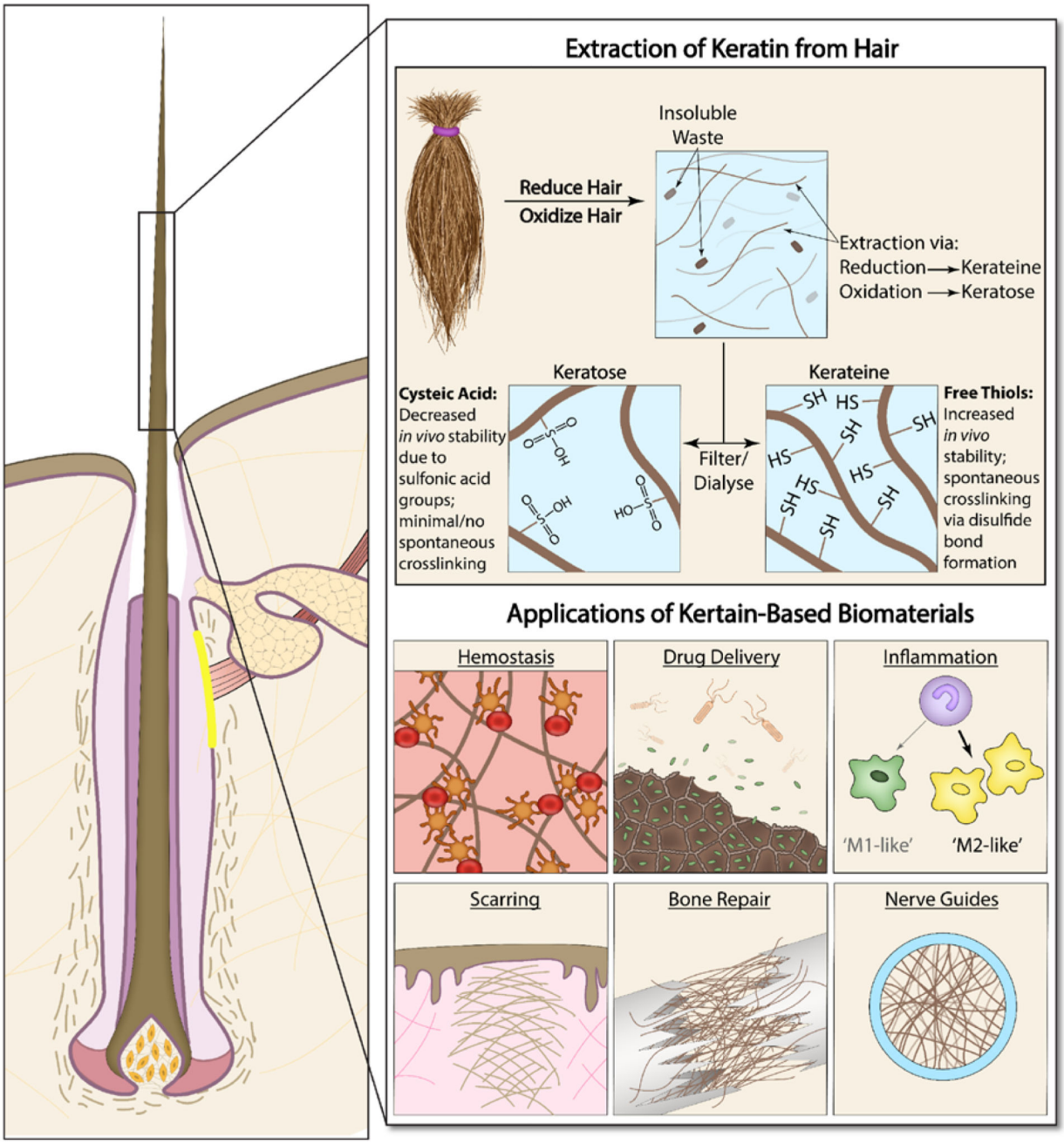

Figure 3. Regenerative medicine applications of biomaterials from the hair follicle.

The use of keratin as a biomaterial. (Top) Extraction of keratin from source materials such as hair is a straightforward process. Following reduction or oxidation of disufide bonds, the solution is filtered and extracted as either kerateine or keratose. (Bottom) Keratin-derived materials have been developed for a range of medical applications. These include promoting hemostasis, controlling drug delivery, modulating inflammation, reducing scarring, promoting bone repair, and acting as neural guides.