

## Epidermal stem cells and skin tissue engineering in hair follicle regeneration

María Eugenia Balañá, Hernán Eduardo Charreau, Gustavo José Leirós

María Eugenia Balañá, Gustavo José Leirós, Fundación Pablo Cassará -Instituto de Ciencia y Tecnología Dr. César Milstein, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Saladillo 2468 C1440FFX, Ciudad de Buenos Aires, Argentina

Hernán Eduardo Charreau, Technology Intelligence Unit, Clarke, Modet & C°, Lavalle 190, 3° Piso, C1047AAD Buenos Aires, Argentina

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**Correspondence to:** Dr. María Eugenia Balañá, Fundación Pablo Cassará, Instituto de Ciencia y Tecnología Dr. Cesar Milstein, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Saladillo 2468 C1440FFX, Ciudad de Buenos Aires, Argentina. [mbalana@fundacioncassara.org.ar](mailto:mbalana@fundacioncassara.org.ar)

Telephone: +54-11-41054127

Fax: +54-11-46874065

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pending in tissue engineering. The loss of hair follicles caused by injuries or pathologies such as alopecia not only affects the patients' psychological well-being, but also endangers certain inherent functions of the skin. It is then of great interest to find different strategies aiming to regenerate or neogenerate the hair follicle under conditions proper of an adult individual. Based upon current knowledge on the epithelial and dermal cells and their interactions during the embryonic hair generation and adult hair cycling, many researchers have tried to obtain mature hair follicles using different strategies and approaches depending on the causes of hair loss. This review summarizes current advances in the different experimental strategies to regenerate or neogenerate hair follicles, with emphasis on those involving neogenesis of hair follicles in adult individuals using isolated cells and tissue engineering. Most of these experiments were performed using rodent cells, particularly from embryonic or newborn origin. However, no successful strategy to generate human hair follicles from adult cells has yet been reported. This review identifies several issues that should be considered to achieve this objective. Perhaps the most important challenge is to provide three-dimensional culture conditions mimicking the structure of living tissue. Improving culture conditions that allow the expansion of specific cells while protecting their inductive properties, as well as methods for selecting populations of epithelial stem cells, should give us the necessary tools to overcome the difficulties that constrain human hair follicle neogenesis. An analysis of patent trends shows that the number of patent applications aimed at hair follicle regeneration and neogenesis has been increasing during the last decade. This field is attractive not only to academic researchers but also to the companies that own almost half of the patents in this field.

**Key words:** Adult stem cells; Skin grafts; Epidermis; Multipotential differentiation; Tissue regeneration; Dermal papilla; Epithelial-mesenchymal interactions

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### Abstract

The reconstitution of a fully organized and functional hair follicle from dissociated cells propagated under defined tissue culture conditions is a challenge still

**Core tip:** Loss of hair follicles caused by injuries or pathologies affects the patients' psychological well-being and endangers inherent functions of the skin. Different experimental strategies and approaches to obtain mature hair follicles have been designed based upon current knowledge of the epithelial and dermal cells involved in embryonic hair generation and adult hair cycling, and in the epithelial-mesenchymal interactions among them. This review summarizes the current advances in hair follicle neogenesis and regeneration, with emphasis on those involving neogenesis of hair follicles in adults from isolated cells and tissue engineering as well as an analysis on patent trends in this field.

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## INTRODUCTION

Regenerative medicine aims to create living, functional tissues that repair or replace lost or damaged organ function resulting from disease, injury, congenital defects or aging.

The main challenge of tissue engineering is the reconstitution of fully organized and functional organ systems from dissociated cells that have been propagated under defined tissue culture conditions.

Skin is the largest organ in the human body, acting as a barrier with protective, immunologic and sensorial functions. Deep skin injuries produce a complete destruction of skin regenerative elements. These wounds heal by contraction, with epithelization from the edges only and extensive scarring, resulting in reduced joint movements and cosmetic defects<sup>[1]</sup>. Moreover, if these lesions are too extensive, the healing process is unsuccessful and they become life-threatening for the patient. Tissue engineering has emerged as a new interdisciplinary field combining scaffolds, cells and biomolecular signals towards the treatment of skin lesions. This useful strategy may contribute not only to the treatment of deep skin injuries but also to the understanding of skin regeneration.

The main goal of tissue-engineered skin grafts is to restore lost barrier function. Many dermal-epidermal composites or skin equivalents have been described for use in the clinic<sup>[2]</sup> but the inability of these skin constructs to regenerate skin appendages as hair follicles has limited their use. Although the ability to reconstitute adult skin with functional skin appendages has long been a major clinical objective, the regeneration of epidermal appendages, such as hair follicles, and sebaceous and sweat glands, is a challenge that is still pending.

Recent studies clearly demonstrate that there are

multipotent stem cells with the capability to regenerate hair follicles and sebaceous glands in adult mammalian skin and this multipotency can be maintained in cell culture<sup>[3,4]</sup>. The hair-differentiation potentiality of epidermal stem cells can be activated by inductive dermal cells. As will be extensively reviewed in this article, co-grafting of those cellular components from mice allows complete hair reconstitution<sup>[5]</sup>. The successful regeneration of hair follicles in immunodeficient mice suggests that creating human hair follicles in tissue-engineered skin grafts is feasible. However, regeneration of human hair in a similar manner has not been reported.

Skin appendages cannot be restored in healed wounds by current tissue-engineered skin grafts<sup>[6]</sup> mainly because of their limited self-regeneration capability in adults<sup>[7]</sup>, the lack of appendage structures in the human skin grafts and probably an improper molecular microenvironment. Many strategies still need to be explored, particularly enriching isolated cells with trichogenic capability, maintaining this capability during processing, and providing the cells with proper environmental signals.

The lack of skin appendages by skin wounds and by pathologies such as different kinds of alopecia not only affects the patients' psychological well-being<sup>[8]</sup>, but also endangers the inherent functions of the skin.

Skin appendages cannot regenerate on their own after damage<sup>[7]</sup>. For the fully functional regeneration of ectodermal organs, it has been proposed that a bioengineered organ could be developed by reproducing the embryonic processes of organogenesis.

This review summarizes current advances in the different experimental strategies to regenerate or neogenerate hair follicles, *in vitro* and *in vivo*, with emphasis on approaches that include the neogenesis of hair follicles in adults from isolated cells and by tissue engineering as well as an analysis on patent trends in this field.

## ANATOMY AND BIOLOGY OF HAIR FOLLICLES

The skin consists of three layers: epidermis, dermis, and hypodermis. The epidermis is in fact a multi-layered epithelium extending from the basement membrane that separates it from the dermis to the air. The dermis is located below the epidermis and is a connective tissue comprised of extracellular matrix, fibroblasts, vascular endothelial cells, and skin appendages.

Skin appendages, including hair follicles, sebaceous glands and sweat glands, are linked to the epidermis but project deep into the dermal layer. A human has around five million hair follicles with two types of hair, terminal hair (long, thick pigmented) and vellus hair (thin, unpigmented)<sup>[9]</sup>.

Hair follicles are composed of an outer root sheath

(ORS) that is contiguous with the epidermis, an inner root sheath (IRS) and the hair shaft (HS). The matrix surrounding the dermal papilla (DP), in the hair root, contains actively dividing and relatively undifferentiated cells that are essential for follicle formation.

In embryogenesis, the skin begins as a single layer of epidermal stem cells. Then, mesenchymal cells populate the skin to form the underlying collagenous dermis<sup>[10]</sup>. Human hair follicles start to develop through complex morphogenetic processes resulting from highly coordinated series of bidirectional epithelial-mesenchymal interactions<sup>[11]</sup>. Hair follicle development is initiated by the appearance of a thickening in the embryonic ectoderm called placode resulting from the condensation of the underlying mesoderm that will form the DP<sup>[12]</sup>. The DP becomes a permanent part of the follicle base<sup>[13]</sup> enveloped by the hair bulb. It is considered the commander of the hair follicle determining the hair thickness, length, and life cycle<sup>[8]</sup>. Signals from the condensed mesoderm induce the proliferation of the placode which forms mature hair follicles by a systematic series of differentiation and proliferation processes of epithelial cells. The hair follicle becomes fully mature when its bulb nears the bottom of the dermis. At this point, the proliferative cells (matrix) at the follicle base continue to divide, producing progeny cells that terminally differentiate to form the growing hair that emerges from the skin surface. The inner layers begin to differentiate into concentric cylinders to form the central HS and the surrounding channel, the IRS.

These tissues undergo continual rejuvenation and, in response to injury, they must be prepared for wound repair. The capability of the skin for maintaining tissue homeostasis, regenerating hair, and repairing the epidermis after injury resides in its stem cells.

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## HAIR FOLLICLE STEM CELLS AND THE HAIR CYCLE

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Stem cells are undifferentiated cells that are ultimately responsible for the constant renewal of the skin due to their distinguishing properties of self-renewal for the entire life span of an organism and their ability to differentiate into a variety of specialized cells.

A pool of progenitor cells is located on the basement membrane of the skin. These cells contribute to epidermal homeostasis undergoing continuous self-renewal and differentiation to keratinocytes that migrate towards the surface of the skin where they undergo terminal differentiation and maturation providing the skin's barrier properties.

Another reservoir of slow-cycling multipotent stem cells that gives rise to a range of differentiated cell types in skin is located in a specialized region of the ORS in the hair follicle, known as the bulge<sup>[3,4,14-16]</sup>. Early work showed that these slow cycling multipotent stem cells not only contribute to the growth of hair

follicles themselves and of the sebaceous glands<sup>[3]</sup> but also can be activated and migrate out of hair follicles in order to repair the damaged epithelium<sup>[17]</sup>; however, they contribute little to the turnover of the intact epidermis. In the absence of these hair follicle stem cells (HFSC), hair follicle and sebaceous gland morphogenesis is blocked, and epidermal wound repair is compromised<sup>[18]</sup>. The epithelial-mesenchymal interactions with the underlying DP play a pivotal role in embryonic hair genesis<sup>[12]</sup>, in the regulation of post-natal hair follicle cyclical activity, and in the repair of wounded skin<sup>[19-22]</sup>.

Unlike most organs, hair follicles do not reach homeostasis once they mature. Each mature hair follicle is a regenerating system, which physiologically undergoes cycles of growth (anagen), regression (catagen), and rest (telogen) numerous times in adult life<sup>[23]</sup>. In catagen, hair follicle stem cells are maintained in the bulge. Then, the resting follicle re-enters anagen (regeneration) when proper molecular signals are provided. During late telogen to early anagen transition, signals from the DP stimulate the hair germ and quiescent bulge stem cells to become activated<sup>[24]</sup>. Many paracrine factors are involved in this crosstalk at different hair cycle stages and some signaling pathways have been implicated<sup>[3,19,25]</sup>. In anagen, stem cells in the bulge give rise to hair germs, then the transient amplifying cells in the matrix of the new follicle proliferate rapidly to form a new hair filament<sup>[26]</sup>.

Even if it is generally believed that hair follicles do not form after birth in humans<sup>[27]</sup>, the formation of vellus hair follicles from the reconstituted epidermis of an abraded area of facial skin was described in 1956<sup>[28]</sup>. However, the great challenge of skin tissue engineering and skin wound regeneration remains to be its inability to reliably reconstitute skin appendages, most notably hair follicles and sweat glands. The information about the mechanisms that generate and maintain skin appendages provided by recent studies with skin-derived progenitor cells, may be the basis of new therapies that could help to overcome these limitations.

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## CAUSES OF HAIR LOSS AND REGENERATION OF THE HAIR FOLLICLE

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Hair loss responds to different causes ranging from mild traumas, such as hair fiber plucking, to severe traumas such as partial- or full-thickness skin lesions. Pathological processes, such as cicatricial or non cicatricial alopecia, can also be responsible for hair loss. After plucking, if HFSC and DP remain, a new hair filament would be spontaneously regenerated. Some label retaining studies have shown that hair follicle stem cells in the bulge remain intact after hair plucking<sup>[29,30]</sup> whereas others reported bulge stem cells suffering apoptosis after plucking which are replaced by label retaining cells coming from hair germ<sup>[31]</sup>.

It was also observed that the slow-cycling bulge cells (CD34 positive cells in mouse) are involved in normal hair homeostasis and wound healing, whereas regeneration after hair plucking involved actively cycling cells from the lower ORS<sup>[26]</sup>. However, the cell dynamics in this process is less clear than in the physiological renewal and further studies are required to understand this process.

However, when the cellular niches are completely lost, it is necessary to generate a completely new hair follicle in a process called hair follicle neogenesis.

Based on the knowledge on the epithelial and dermal cells, and their interactions, during the embryonic hair generation and adult hair cycling, different experimental approaches have been designed to regenerate hair follicles or generate new ones by the neogenesis process.

These hair regeneration and neogenesis attempts are recapitulated in this article, and can be classified into 4 categories: (1) reversion of pathological intra- and/or extra-follicular environment, for instance androgenetic alopecia (AGA); (2) regeneration of complete hair follicles from the recombination of hair follicle parts; (3) neogenesis of hair follicles from isolated cells; and (4) neogenesis of hair follicles by tissue engineering.

#### **Reversion of pathological intra- and/or extra-follicular environment**

Hair follicle cycle and growth can be affected and deregulated by paracrine factors from the follicle itself and/or from the surrounding dermal tissue, or by endocrine factors<sup>[32,33]</sup> leading to hair loss. Many factors have been described as molecular mediators of hair follicle growth<sup>[11]</sup>. Some of them, such as PDGF<sup>[33]</sup> and Wnt proteins<sup>[34]</sup>, are positive regulators while others, such as BMPs<sup>[35]</sup>, suppress hair growth. Many paracrine factors involved in the epithelial-mesenchymal crosstalk at different stages of the hair cycle, and some implicated signaling pathways, have also been studied<sup>[3,19,25]</sup>.

In addition, endocrine factors, such as sexual hormones, have both physiological and pathological effects on hair growth as in human male beard, and androgenetic alopecia respectively. Androgenetic alopecia<sup>[36]</sup> is a common, progressive disorder in which large, terminal scalp hairs are gradually replaced by smaller hairs following a defined pattern. The DP is the site through which androgens act on follicle cells by altering the regulatory paracrine factors involved in the differentiation and proliferation of HF stem cells. DPs from balding scalp have higher levels of androgen receptor and 5-alpha reductase. Dihydrotestosterone can induce DP to secrete factors including, TGF-beta1 and DKK-1 which down regulate keratinocytes growth<sup>[37,38]</sup>.

A functional crosstalk between the androgen receptor and Wnt signaling pathways in DPCs has been described in target tissues<sup>[39]</sup>. Moreover, our group has demonstrated that androgens regulate secreted factors involved in normal HF stem cell differentiation via the

inhibition of the canonical Wnt signaling system in androgen sensitive DPCs. We provided evidence that androgen activation of GSK-3 $\beta$  would be responsible for the inhibition of Wnt/ $\beta$ -catenin signaling<sup>[40]</sup>. The current pharmacological treatment for AGA includes androgen metabolism modulators, such as the 5-alpha reductase inhibitor finasteride and the antiandrogen agent flutamide. Nevertheless, the results of these treatments are not only variable and patient-dependent, but can also cause undesirable side effects. Therefore, therapies acting on specific molecular targets are necessary to treat this pathology downstream from the actual antiandrogenetic pharmaceutical modulators. The crosstalk between the androgen receptor (AR) and Wnt/ $\beta$ -catenin signaling pathways could be one alternative therapeutic target, among many others recently reviewed<sup>[41]</sup>.

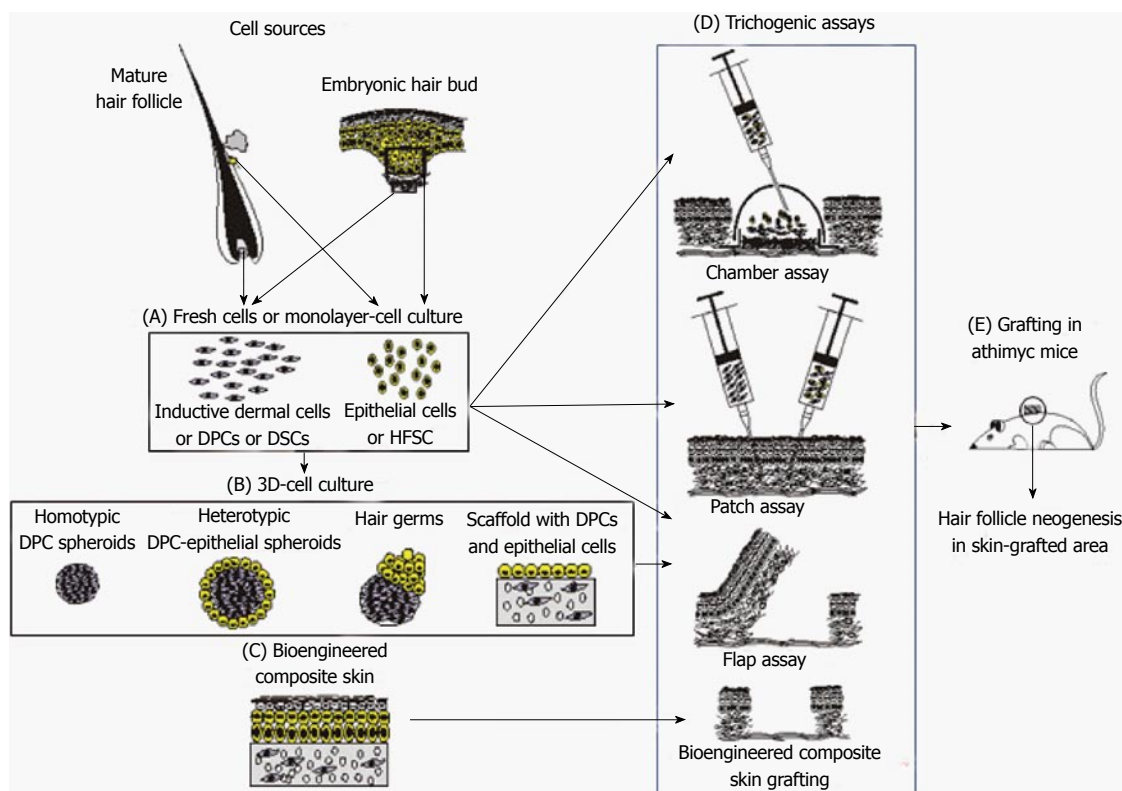
In these experimental approaches, modulation of the intra-follicular microenvironment and the extra-follicular macroenvironment could contribute to promote growth of the existing hair follicles but not to increase their number by the formation of new ones.

#### **Regeneration of hair follicles by recombination of hair-follicle parts**

Taking into account the inductive ability of DP and the current knowledge on epithelial-mesenchymal interactions, different recombination of dermal and epidermal tissue have been employed for hair follicle regeneration. In the early 1960s, researchers showed that whole follicle end bulbs and isolated whole whisker papillae (containing dermal and epidermal components) remain viable and may produce whisker hair follicles in a new site<sup>[42]</sup>. The most surprising finding was that isolated DPs (without epidermal components) were capable of inducing the generation of new hair follicles recruiting epithelial cells from the implantation sites. The same results were obtained when epidermis from different origins were transplanted together with whisker DP<sup>[43]</sup>. Regeneration of hair follicles was also observed in both mice<sup>[43-45]</sup> and humans<sup>[46]</sup> when dermal sheath tissue was used, which was sufficient to regenerate also the DP structure. After implantation, the whisker DP was capable of inducing hair follicle regeneration retaining the information to determine hair fiber type and follicle size<sup>[47]</sup>.

#### **Neogenesis of hair follicles in adult individuals from isolated cells**

Grafting of dermal-inductive tissue is limited by the fact that it is not possible to generate more hair follicles than the obtained from the donor tissues. To overcome this limitation different approaches and experimental models using freshly or cultured isolated cells from both dermal and dermal/epidermal origin were tested. Most of them involved neonatal and embryonic murine cells. One of the first models used was the chamber assay (Figure 1) in which dermal and epithelial cells are seeded inside a chamber consisting of a cylinder



**Figure 1** Hair follicle neogenesis using isolated epithelial and dermal cells. Inductive-dermal and Epithelial Cells can be obtained from adult tissue or embryonic sources. Isolated cells by enzymatic digestion of living tissues, or monolayer cell cultures (A) can be used in the trichogenic assays (D): Chamber assay; Patch assay; and flap assay. Moreover, isolated cells can be cultured in 3D-conditions, allowing them to create their own extracellular matrix as in the case of homotypic and heterotypic spheroids and hair germs (B). Also, cells can be seeded on a precast scaffold used for cellular support or used to produce a bioengineered composite skin (C). Any of these constructs can be evaluated by the trichogenic assays (D): Flap assay; and Bioengineered composite skin grafting. All these trichogenic assays are mainly performed in the back-skin of athymic mice in order to avoid immune rejections and to take advantage of its bare-skin (E). DPCs: Dermal papilla cells; DSCs: Dermal sheath cells; HFSC: Hair follicle stem cells.

inserted through a full-thickness skin lesion in mouse dorsal skin, covered by a dome. In former studies using this device in nude mice<sup>[48]</sup>, it was showed that hair buds obtained from neonatal mice combined with fresh neonatal dermal cells or with immortalized clones from vibrissae rat DPCs produced mature and cycling hair follicles. Using the same chamber assay, hair bud preparations without any additional inductive dermal component were unable to form hair follicles resulting in a scarring reparative tissue, a thin epidermis and a dermis without appendages<sup>[49]</sup>. On the other hand, the combination of hair buds with fresh dermal cell preparations or early passages of cloned vibrissae cell lines resulted in skin with normal epidermal and dermal layers.

Although these studies confirmed the trichogenic potential of dissociated dermal cells, particularly DPCs, the conditions of the chamber assay correspond to a wound healing environment and do not represent the physiological status. Other approaches allow studying the trichogenic potential of isolated cells in a more physiological environment, as is the case of the patch assay (Figure 1). This assay consists in injecting dissociated murine neonatal epidermal and dermal cells hypodermically into adult mouse skin. This system, originally described by Zheng

*et al.*<sup>[50]</sup>, showed the formation of cell clusters that resulted in "infundibular cysts" which generated hair germs, followed by pegs that grew to differentiate into cycling mature pilosebaceous units. Surprisingly, whereas patch hair follicles were in anagen phase, the host skin hair follicles were in telogen and vice versa, suggesting that follicle cycling depends on the trichogenic cells and not on the host skin hair cycling. Even when the shaft grows inside an intradermal cyst that tends to extinction by inflammatory processes, none of the other already described systems at that moment showed to be so efficient to evaluate the trichogenic potential of isolated cells. A variation of this method is called tracheal grafting method. It consists in inoculating rat tracheas with fresh or cultured keratinocytes from newborn mice together with newborn fibroblasts or cultured DPCs and implanting them subcutaneously in athymic mice for four weeks. In this assay hair follicles and sebaceous glands were generated using cultured keratinocytes from newborn mice and fresh newborn fibroblasts<sup>[51]</sup>. Although adult mice keratinocytes successfully produced a two layered epithelium, they failed to generate hair follicles or sebaceous glands<sup>[51]</sup>.

Other strategy to evaluate the hair inductive capability of dermal cells is the "flap assay" (Figure 1). This

approach originally described by Qiao *et al.*<sup>[52]</sup> consists in: (1) generating a skin flap in the dorsal skin of nude mice; (2) locating an embryonic epidermis supported by a silicone sheet in direct contact with the muscle with the basal side of the embryonic epidermis facing upwards; (3) seeding embryonic murine dermal cells or cultured DPCs onto the basal face of the graft-epidermis; and (4) pulling over the graft and suturing the flap edges. In this configuration the graft-dermal cells were layered between the graft-epidermis and the host connective tissue. When fresh embryonic dermal cells were used, abundant hair was developed four weeks after grafting. The induced hairs were oriented vertically down towards the silicone sheet. After surgical flap inversion, the formed hairs were exposed in the host skin surface and survived for more than 12 mo.

### **Neogenesis of hair follicles by tissue engineering**

Although the assays described above provided much information respect to the hair neogenesis process and the cells involved in it, the high number of cells required, the need of *in vivo* animal models and the poor results obtained with human cellular sources make these procedures not applicable for clinical uses in the near future. The development of new strategies has become necessary and begun to emerge. They are mainly focused on tissue engineering-based follicle neogenesis, including three-dimensional (3D) cell culture conditions generated by the cells themselves or by the use of biocompatible scaffolds.

Lee *et al.*<sup>[53]</sup> developed a simplified procedure to reconstitute hair-producing skin. They obtained both epidermal and dermal cells from newborn mice, and mixed them in different ratios. A high density cellular suspension was prepared in drops of minimal volume on tissue culture inserts or wells. They allowed the cells to settle until gel consistency was obtained. Alternatively, they seeded the cells on the collagen-side of the Integra<sup>TM</sup> matrix (porous matrix of cross-linked bovine tendon collagen and glycosaminoglycan and a silicone layer). Both constructs were grafted in full thickness skin wounds generated on the back of athymic mice. After grafting, the epidermal cells formed a basal layer and some epidermal microcysts could be observed. Dermal cells started to form dermal condensations adjacent to the epidermal layer or cysts. Eight days after grafting hair germ started to appear and progressed to hair peg, observing complete hair follicles four days later that kept the ability to continuously cycle for at least one year. This method would allow preparing constructs with specific sizes and shapes useful to treat alopecia by tissue implants.

Two separate reports<sup>[54,55]</sup> showed hair follicle neogenesis using hair germs obtained by the previously described "organ germ method"<sup>[56]</sup>. One of them<sup>[54]</sup> generated bioengineered hair germs mixing epithelial and mesenchymal cells derived from mouse embryos within a collagen gel drop. In these culture conditions, the two cellular types generated a structure with

two cellular layers separated by a translucent region they called hair germ. When these hair germs were transplanted ectopically into subrenal capsules, the presence of mature hair follicles was observed. These bioengineered hair follicles had a normal histological structure with the concentric epithelial layers of ORS and IRS, DP, hair matrix, and sebaceous glands. These follicular units transplanted intracutaneously in nude mice (Figure 1), were connected to the arrector pili muscle and nerve fibers and were able to produce hair shafts and cycling.

In addition, a fully functional orthotopic hair regeneration was demonstrated via intracutaneous transplantation of bioengineered hair follicle germs generated by epithelial and mesenchymal cells derived from mouse embryos<sup>[55]</sup> (Figure 1). These bioengineered hairs responded to hair cycles and had the correct structure of natural hair follicles and shafts, and the proper connections with surrounding host tissues. Qiao *et al.*<sup>[57]</sup>, using embryonic dermal cells and hair-peg-derived keratinocytes, generated cellular aggregates by hanging drop methods. These aggregates cultured in methylcellulose coated wells elongated, and after seven days, acquired hair follicle features. These structures termed "proto hairs", showed a morphological structure more developed respect the previously described "hair germs"<sup>[54,55]</sup>. They presented hair-like characteristics as an inner mass of cells similar to the DP structure surrounded by matrix-like keratinocytes and a partially keratinized substance that could represent intent to produce a hair shaft. After implantation into shallow incisions in nude mice (Figure 1), black-pigmented hair fibers were observed emerging from epidermis. These hairs persisted throughout the experimental period (6 mo) and were shown to regrow after plucking.

Although embryonic tissues are used as cellular sources, these studies showed the potentiality of dissociated cells to form hair follicle germs or proto-hairs able to generate mature hair follicles both by ectopic and orthotopic implantation. Collectively, these studies provide a proof of concept for the use of Follicular Cell Implantation to restore hair in patients suffering from hair loss.

As it was already mentioned, dermal cellular components from the skin, such as DPCs or dermal sheath cells (DSCs), have the potential to induce hair formation in *in vivo* assays and also induce *in vitro* differentiation of hair follicle stem cells to hair lineage<sup>[25,40]</sup>. Even if it is possible to isolate and culture DPCs, the inductive ability tends to fade with time in 2D cultures<sup>[6,58]</sup>. Considering that the hair follicle neogenesis requires a large number of DPCs, it is necessary to look for new strategies to maintain their inductive potential in culture in order to use them in skin bioengineering and hair follicle neogenesis.

The niche generated by the extracellular matrix (ECM) of DP probably plays a key role in keeping DPC inductive activity. Accordingly, the loss of ECM throughout culture time could be responsible for the

fading of DPC inductive properties.

DPCs are naturally aggregated at the hair-bulb base. In proper culture conditions, these cells tend to aggregate<sup>[13,59]</sup> and their inductive activity is preserved after they are grafted in this state. It has been demonstrated that it is possible to generate dermal papilla spheroidal microtissues using different methods including rotation, two-step rotation and flotation, and hanging drop<sup>[60-62]</sup>. Osada *et al.*<sup>[63]</sup> artificially prepared DPC spheres by aggregation of mouse vibrissae follicle DPCs in a round-bottom 96-well low-binding plate. These spheres expressed higher amounts of versican, an indicator of inductive capability, than DPCs in monolayer cultures. In a "patch assay" (Figure 1), together with embryonic-epidermal cells, they induced hair follicle neogenesis, keeping their inductive capability for at least twenty-six passages.

However, these methods are labor intensive and the microtissues obtained have a very variable size and cell number content, which reduces reproducibility. DPCs can self-assemble into dense spheroids when seeded on controlled biomaterial surfaces such as poly (ethylene-co-vinyl alcohol) (EVAL)<sup>[64]</sup> or polyvinyl alcohol (PVA) membranes<sup>[65]</sup>. The DPC microtissues generated on EVAL surface<sup>[64]</sup> expressed DPC-markers such as neural cell adhesion molecule (NCAM)<sup>[66]</sup> and  $\alpha$ -smooth muscle actin<sup>[67]</sup>. Alkaline phosphatase activity, which correlates with hair follicle inductive ability of DPC<sup>[68]</sup>, was higher in spheroid microtissues than in monolayer cultured cells. Finally, the spheroids mixed with newborn mouse epidermal cells and injected into the hypodermis of nude mice in a "patch assay" (Figure 1) were able to generate new hair follicles.

The use of PVA as substratum material enhanced DPC aggregation<sup>[65]</sup>, preventing cellular attachment and spreading. Using this material and reducing culture surface it is possible to control the number, size and compaction of spheroidal microtissues. DPCs quickly aggregated into a single spheroid whose diameter decreased progressively due to tissue compaction. The expression of DPC-inductive capability markers, versican<sup>[69]</sup> and alkaline phosphatase<sup>[68]</sup>, were present in spheroids of various sizes. All these experiments were made both with adult DPCs from rat vibrissae, and human scalp hairs, giving similar results. The hypodermal injection in nude mice of eighty spheroids altogether with keratinocytes from newborn mice induced HF neogenesis. These results are very promising given that transplantation of dispersed cultured human DPCs had shown very low efficiency in inducing new hair follicles<sup>[70]</sup>. Using the same approach, heterotypic folliculoid microtissues or hair follicle-like organ germs were produced using dissociated adult epithelial and mesenchymal cells by self-assembly on EVAL coated wells<sup>[71]</sup>. Keratinocytes and DPCs seeded together grew into floating or loosely attached multicellular spheroids. Histological analysis showed that the spheroids are composed by

a core of DPCs surrounded by keratinocytes. These keratinocytes expressed keratin 6, a cytokeratin preferentially expressed by ORS *in vivo*<sup>[72]</sup>, and DPCs expressed versican, a marker associated to HF-inductive ability. Using "patch assays" (Figure 1) disperse DPCs and keratinocytes did not form new hair follicles, whereas heterotypic folliculoid microtissues did. These last experiments would contribute to the development of new strategies for scalable production of organoid microtissues of epithelial organs for hair follicle neogenesis by bioengineering approaches.

Altogether, these results strongly indicate that the hair-inductive capability of DPCs can be restored by three-dimensional spheroid cultures.

### **Hair follicle neogenesis using human cells**

During embryogenesis, dermal mesenchymal cells drive HF development and a condensate of these cells remains during adult life forming hair follicle DP. These cells retain the capability to induce hair follicle regeneration and neogenesis<sup>[21,73]</sup> as reviewed by Ohyama *et al.*<sup>[5]</sup> and Yang *et al.*<sup>[6]</sup>. However, most of these experiments were performed using murine and embryonic cells, whereas DPC from human origin showed many difficulties to maintain inductive capability in culture<sup>[74]</sup>.

As discussed above, rat cultured DPCs self-aggregate within the dermis forming a papilla-like structure that synthesizes its own extracellular matrix<sup>[13]</sup>. However, such behavior was not observed with cultured human DPCs, highlighting specie-specific differences. Human keratinocytes from ORS and DPCs cultured in Matrigel™ (3D network of extracellular matrix proteins and collagen) have shown to form tubule-like structures in this skin-equivalent *in vitro*<sup>[75]</sup> and organize themselves into epidermoid cyst-like spheroids<sup>[72,76]</sup>, but did not form complete hair follicles. Similarly, Sriwiriyanont *et al.*<sup>[77]</sup>, observed neofollicle formation in nude mice grafted with engineered skin substitutes containing murine DPC and human keratinocytes in a collagen-glycosaminoglycan matrix, but not in those containing human DPC and human keratinocytes.

Ehama *et al.*<sup>[78]</sup> reported the formation of hair follicle-like structures using human primary cultures of foreskin- or adult-derived epidermal cells co-grafted with murine DPCs by a chamber assay (Figure 1). The innermost regions were similar to the hair cortex and medulla of mature human follicles. Hair shaft-like fibers occasionally emerged at the skin surface, and structures, reminiscent of the DP, at the follicle bottom were also observed. However, these structures showed no bulge region, nor all the follicular epithelial layers, and the DP-like tissue failed to express the anagen DP marker versican. These hair follicle-like structures correspond to the initial follicle formation suggesting that the differentiation process was altered.

Using the "flap assay"<sup>[52]</sup> (Figure 1), Qiao *et al.*<sup>[79]</sup> showed that DPCs from human scalp combined with epidermis from mouse embryo produced mature

hair follicles that persisted and grew. The authors reported that the inductive potential of DPCs could be maintained using keratinocyte-conditioned medium. These DPCs were able to form DP and dermal sheath *in vivo*.

Spheroids microtissues were also obtained by culturing human DPCs in a 96-well low-binding plate, and implanted intradermally into nude mice using the "patch assay" (Figure 1)<sup>[80]</sup>. This combination of human DPC spheres with murine neonatal epidermal cells generated new hair follicles. In contrast, hair follicles were never observed when monolayer DPC cultures were used.

Cultured human DPCs do not induce hair neogenesis unless changes in the culture conditions are made. In this sense, the formation of DPC-microsphere tissues has shown to improve hair-inductive properties of rodent cells as well as the expression of cell-markers associated with these properties<sup>[63-65]</sup>. Therefore, all these observations encouraged investigators to look for new strategies intended to achieve the first step toward human hair follicle neogenesis that is, providing culture conditions that confer hair-inductive ability to human DPCs. Higgins *et al.*<sup>[74]</sup> evaluated the human DPC-transcriptome observing that monolayer DPC cultures showed the most important changes immediately after early outgrowths from DP explants. These changes involved 3729 transcripts, including several involved in hair follicle development. This large number of differentially expressed transcripts shows the physiological changes that 2D culture conditions do on the molecular signature of DPCs. The generation of DPC spheroids by the hanging drop method partially restores the intact DP signature. These human-DPC spheres induced hair follicle neogenesis when placed in between foreskin epidermis and dermis and grafted onto nude mice (Figure 1). These hair follicles showed a DP and dermal sheath highly positive for alkaline phosphatase, hair specific keratin markers disposed in concentric layers and blood vessel formation around follicles. Nevertheless, neither sebaceous glands nor hair fibers emerging from the skin surface were seen. These results suggested that human DPC spheroids would be able to initiate hair follicle morphogenesis but the production of a complete hair follicle requires additional signals.

Recently, Miao *et al.*<sup>[81]</sup> produced DPC-spheroids from cultured DPCs on a Matrigel™ scaffold. As reported by Higgins *et al.*<sup>[74]</sup>, the DPC spheres were shown to restore DP-signature gene expression of NCAM, versican and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), lost during monolayer culture. The authors also claimed that these DPC-spheres, combined with hair germinal matrix cells, onto Matrigel-coated plates produced colorless fiber-like structures *in vitro*.

Other alternative to culture dermal mesenchymal cells in 3D conditions is the organotypic method consisting in culturing cells inside a scaffold. Using this method, Wu *et al.*<sup>[82]</sup> prepared a collagen gel with

human DPCs or dermal sheath cells (DSCs). This gel was seeded with keratinocytes from interfollicular skin, superior ORS or inferior ORS obtaining *in vitro* a bilayered skin. Nonetheless, only the constructs containing superior ORS keratinocytes showed hair follicle-like structures. That is, an outer root sheath, nearly ten layers of concentric epithelial cells, a middle layer IRS-like and some keratinoid substances in the center, similar to a hair fiber. When these constructs were transplanted into nude mice (Figure 1), only the organotypic cultured tissue containing superior ORS keratinocytes and DPCs exhibited hair follicle structures.

In a recent study<sup>[83]</sup>, composite skin substitutes were generated by seeding human neonatal foreskin keratinocytes onto a dermal equivalent structure cultured in air-liquid interface condition. This dermal equivalent was composed of DPCs from human scalp contained in a collagen-I gel. Eight weeks after grafted onto nude mice (Figure 1), these constructs presented hair follicles showing bulb, dermal sheath, hair matrix and cortex. Histological analysis showed concentric layers of IRS and ORS, sebaceous glands and hair shaft. Immunohistochemistry assays revealed that both epithelial and dermal cells from neo-follicles were from human origin, and that DPCs and DSCs expressed human nestin and versican.

The analysis of all these reports leads to the conclusion that hair follicle neogenesis using human epithelial and dermal cells is a very difficult task that requires special culture conditions, somehow recreating the normal or embryonic skin environment, and the use of embryonic or neonatal cells.

Very recently, we prepared in our laboratory a dermal-epidermal skin substitute by seeding an acellular dermal matrix with cultured hair follicle epithelial stem cells and DPCs, both obtained from adult human scalp<sup>[84]</sup>. These constructs were grafted into a full-thickness wound generated on nude mice skin (Figure 1). In only fourteen days, histological structures reminiscent of many different stages of embryonic hair follicle development were observed in the grafted area. These structures showed concentric cellular layers of human origin, and expressed *k6hf*, a keratin present in epithelial cells of the companion layer. Although the presence of fully mature hair follicles was not observed, these results represent, up to our knowledge, the first report showing that both epithelial and dermal cultured cells from adult human scalp in a dermal scaffold were able to produce *in vivo* structures that recapitulate embryonic hair development.

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## ANALYSIS OF PATENTS TRENDS IN HAIR FOLLICLE REGENERATION AND NEOGENESIS

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So far, we have made a concise review of the scientific



literature describing the attempts to regenerate the hair follicle. We have also mentioned a number of reviews on academic articles dealing with hair regeneration or wound induced follicle neogenesis that have been published in recent years<sup>[85]</sup>. However, reviews on tissue engineering and hair regeneration patents are less common. Many of these strategies have been combined in different methods intended for use in active applications. In this section, we review and analyze published patents on hair regeneration<sup>[86-150]</sup>, as a measure of the interest in the industry for this area of research, identifying the currently available technical developments, favorite research strategies and main points of interest. Parameters analyzed include not only chronological patent publishing trends, but also the most cited patents, top patent owners (assignees), most cited documents, and the classification of patents among technology areas according to the International Patent Classification (IPC). Moreover, hair regeneration applications that have been patented throughout the years are screened (Figure 2).

Patent search was performed using the Thomson Innovation Database, a collaboration platform for searching and analyzing global patents, integrated with analytics and workflow tools which allow access to more than 40 databases from different countries. For each particular group of hair regeneration, specific keywords (descriptors) were selected. Descriptors were chosen based on the synergy of two approaches: the search of specific keywords and concepts used in academic publications, and the selection of additional descriptors from specific patent vocabulary. Therefore, the keywords are: Stem cell; progenitor cell; precursor cell; repair; growth; wound; regeneration; neogenesis; skin; tissue engineering; dermis; epidermis; dermal; hair follicle; sebaceous gland and sebaceous unit. The presence of the chosen descriptors was checked in the title, abstract and claims of existing patents, considering documents published between years 1990 and 2014. More than forty technology patent and patent application databases were used for patent search, including the Spanish OEPM, the American USPTO, the worldwide WIPO, the European EPO, the patent offices of France, Germany, Great Britain and the Far-Eastern patent offices of Japan, China and South Korea. Besides the keywords, the search process was completed with the use of the IPC codes under which the patents of interest could be framed. The combination of the search approaches previously mentioned offered a universe of about 475 patent families, which were manually reviewed and filtered, setting aside those directed to gene therapies, mesenchymal, embryonic stem cell, osteoblast, neuron and hematopoietic cell and others. The result of this process was the identification of 65 patent families<sup>[86-150]</sup>. The selection of the document to be considered in patent trends was the first published. It is important to note that patent applications can be published in different countries under different national numbers. This is called the family of the patent. In

the search performed for the present study, care was taken to include just one patent per family.

Tables showing patents that are most frequently cited take into account all the patents in the family, since it may occur that the earliest document is not necessarily the most cited one.

### **Competitive dynamics**

The competitive dynamics indicator shows the distribution of patents among the different organizations, providing a general picture of the tissue repair regeneration industry and their relative positioning. On the basis of this information we can identify the most prolific applicants for hair regeneration technology patents in the world, by establishing the number of applications filed by each company.

Table 1 lists the applicants that have filed more than one application, which will be our "Reference Group". Most of the members of this group are manufacturing companies, indicating that this technology belongs to the industrial sector. The highest percentile is shared by a small number of applicants with five patents each, while a second group includes a wide range of companies with no clear technological leadership.

The group of companies of the first percentile shown in Table 1, are the leading hair regeneration manufacturers Shiseido Co Ltd and Aderans Research Institute, which own more than five published patents each, and which own almost 20% of all filed applications in this field.

The second percentile of institutions having one or two applications each could offer a favorable scenario for technology transfer.

It should be highlighted that there are over 20 institutions or about 40% patent applications from non-profit institutions including universities and foundations. The most prolific institutions are: Jilin University, University of Pennsylvania, University of Southern California and National Taiwan University, which gives an idea that this field is very prolific in academic institutions.

### **Technology trends**

The patent technology trends indicator introduces the serial number of patent applications showing how technology has evolved over time as well as its appropriation by companies and non-profit institutions in the field of hair regeneration. This technological area has experienced constant growth. In the 90's an average of 2.5 patent applications were filed per year, and in the first decade of the 21<sup>st</sup> century the average increased to 6 patent applications per year (Figure 2).

The years 2011 and 2012 stand out as the ones with the most patent applications, 10 and 16 respectively.

Aderans Corp Company filed most patent applications from 2005 to 2008. Shiseido and Follica, as well as the University of Pennsylvania have filed patent

**Table 1** Owners of hair regeneration patents and patent applications matching all the descriptors found in academic literature

| Assignee  | No of patent applications | Percentage |
|---|---------------------------|------------|
| Shiseido Co Ltd                                 | 5                         | 7.69%      |
| Aderans Research Institute Inc                  | 5                         | 7.69%      |
| Hospital for Sick Children                      | 2                         | 3.08%      |
| Biointegrencia Inc, phoenixbio Co Ltd           | 2                         | 3.08%      |
| Follica Inc                                     | 2                         | 3.08%      |
| Organ technologies Inc                          | 2                         | 3.08%      |
| University of Jilin                             | 2                         | 3.08%      |
| University of Southern California               | 2                         | 3.08%      |
| National University of Taiwan                   | 2                         | 3.08%      |
| Aderans Research Institute Inc, Bioamide Inc    | 1                         | 1.54%      |
| Agency for science, technology and research     | 1                         | 1.54%      |
| Alvi Armani Genomics Inc                        | 1                         | 1.54%      |
| Anticancer Inc, Li L, Yang M                    | 1                         | 1.54%      |
| Beijing Yonghe Hair Transplant Technology       | 1                         | 1.54%      |
| Biomaster KK                                    | 1                         | 1.54%      |
| Chen B, Gao Q, Zeng Q                           | 1                         | 1.54%      |
| Chinese Academy of Science Institute of Zoology | 1                         | 1.54%      |
| Total No of patent applications                 | 65                        | 100.00%    |
| No of Entities                                  | 50                        |            |

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applications in recent years.

From this general perspective, it can be inferred that the technological field is, at present time, in a developmental stage, with an annual growth rate of 10%, based on the number of patent documents filed since 2010.

From another perspective, Figure 3 shows the cumulative patent families published in the last 20 years. As it can be seen, since 2005 there has been a remarkable increase of attraction in the area: while 50% of the patents required 15 years to be generated, the remaining 50% has been filed over the past five years.

The analysis of patent application-trends shows that, at the present time, the development of hair regeneration technology is evolving positively, making this technological field attractive to companies that start producing and selling products as a result of the development of hair regeneration technology.

### **Technological leadership indicator**

The technology leadership indicator identifies specific companies with the most consolidated and developed technologies in this specific field. The Patents which have received the highest number of citations are considered to have the highest degree of technological progress or impact. The analysis of patent citations allows the identification of patents which have been most useful for later researchers and for the development of newer technologies and may illustrate the diffusion of technical knowledge and its different practical applications throughout the years.

Just as those patents that have received most citations are not always the most relevant ones, and those most relevant are not always the most cited ones; a number of empirical studies demonstrate a

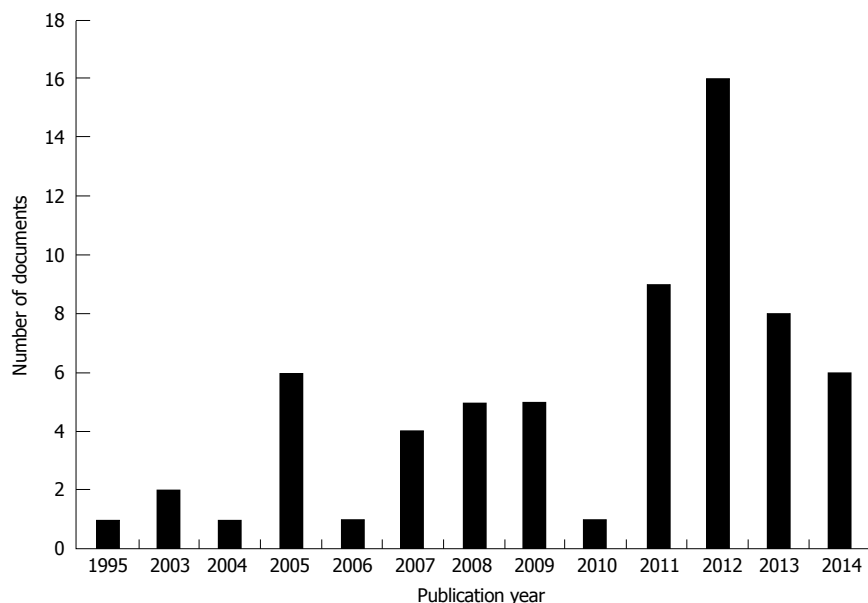
strong correlation between those variables. In Table 2 the 10 most cited institutions as references are listed.

Up to the present, New York University and Pennsylvania State University have received the highest number of citations with 29 citations which reflects the importance of the works of Lavker *et al.*<sup>[86]</sup> previously described. Following these institutions, companies like Biointegrencia Co and Phoenixbio Co Inc, and Anticancer Inc have 17 citations. The Hospital for Sick Children, Aderans Research Institute Inc, Sci Torico Innovations Inc and Trichoscience Innovations Inc are among the most innovative companies.

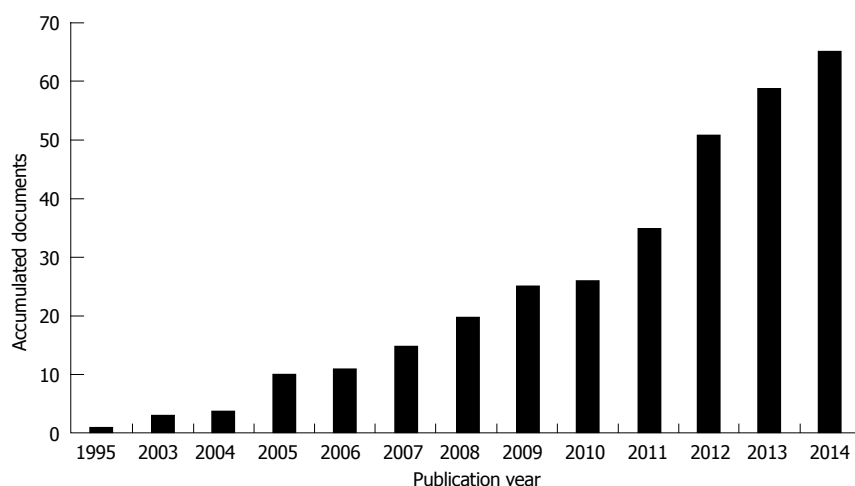
Skin substitutes were among the earliest products to be developed using principles of tissue engineering, and their success is evident in the clinical use of several commercially available products. However, skin substitutes capable of performing all the functions of normal skin are not currently available, which limits their use in patients. Hair follicle neogenesis is not observed using current skin substitutes. Thus, the efforts in hair and skin bioengineering continue to be in a leading place in regenerative medicine. Furthermore, hair restoration is one of the fastest growing areas of cosmetic therapies for both men and women.

Hair follicles are important not only for appearance, skin hydration, barrier formation, and protection against pathogens but also in wound healing. As hair follicles store epidermal stem cells, skin with hair follicles heals faster. In addition, bulge stem cells are less susceptible to loss through minor trauma and damage through ultraviolet light. Thus, treatments that involve neogenesis of normal hair follicles would find much wider application for restoring normal skin function and appearance.

Methods and compositions capable of generating morphologically-correct, fully-developed human hair



**Figure 2** Evolution of the number of patent applications related to hair regeneration (time frame 1990-2014). Own investigation using patent data base Thomson Innovation.



**Figure 3** Evolution of accumulated patent families. Tendency line. Own generation based on patent databases.

follicles, useful for treating conditions such as full- or partial-thickness skin loss, wounds, burns, scars, and hair loss have been developed in order to fulfill this necessity. In this analysis, only patents aiming to hair follicle regeneration or neogenesis have been selected. As reviewed, it has been determined that both epithelial cells and mesenchymal cells are essential for hair follicle regeneration. The regeneration of chimeric hair follicles comprised of mouse DPC and human epithelial cells has been shown to be possible, however it is still not possible to regenerate completely human hair follicles. One of the reasons for this is the difficulty to obtain an adequate number of human DPCs having the ability to induce hair follicle formation to be used for transplantation. Consequently, most of the patents filed in this field provide cellular compositions capable of hair neogenesis and regeneration or methods for

isolating progenitor skin cells as well as the culture conditions that will allow them to keep their inductive properties to promote hair neogenesis or regeneration.

The first most cited patent listed in Table 2 (WO1995001423), published in 1995, described a method of culturing and modulating subpopulations of follicular keratinocytes from upper portions of hair follicle to be used for identifying agents which stimulate hair growth or prevent hair loss and useful for follicular reconstruction and transplantation as well as wound coverage in burns and skin ulcer<sup>[86]</sup>.

In 2005 a patent application (WO2005053763A1) developed a method for transplanting the dermal papilla or dermal papilla cells and outer skin epidermis tissue or epidermal cells to hairless scalp to regenerate hair<sup>[94]</sup>.

Different tissue engineered-hair follicle grafts were also patented; a scaffold constructed to mimic

**Table 2 Most cited patents from 1990 to present**

| Publication number | Title  | Assignee  | Inventor   | Publication year | Count of citing patents | Ref.  |
|--------------------|--|---|--|------------------|-------------------------|-------|
| WO1995001423A1     | Methods of culturing and modulating the growth of hair follicular stem cells | New York State Univ, Pennsylvania Univ                    | Lavker RM, Sun T, Yang J   | 1995             | 29                      | [86]  |
| WO2005053763A1     | Hair growth method   | Biointegrance Inc, Phoenixbio Co Ltd                      | Matsunaga M, Matsunaga MC, Shimada T, Shimada TC, Toyoshima K, Toyoshima KC, Yoshizato K | 2005             | 17                      | [94]  |
| WO2003024406A2     | Nestin-expressing hair follicle stem cells                                   | Anticancer Inc  | Li L, Lingna L, Meng Y, Ri R, Yang M   | 2003             | 17                      | [87]  |
| WO2005071063A1     | Methods of making and using skin-derived stem cells                          | Hospital For Sick Children                                | Biernaskie J, Fernandes K, Fernandez K, Mckenzie I, Miller F, Miller FD                  | 2005             | 16                      | [91]  |
| WO2002060396A2     | Hair follicle neogenesis by injection of follicle progenitor cells           | Aderans Res Inst Inc, Bioamide Inc                        | Barrows TH   | 2002             | 16                      | [88]  |
| US20050214344A1    | Tissue engineered biomimetic hair follicle graft                             | Aderans Res Inst Inc                                      | Barrows T, Barrows TH, Cochran S, Cochran SA, Marshall B                                 | 2005             | 15                      | [90]  |
| US20070092496A1    | Method of delivering cells to the skin                                       | Aderans Res Inst Inc                                      | Du X, Stenn K, Stenn KS, Washenik K, Washenik KJ, Zhang Y, Zheng Y                       | 2007             | 10                      | [99]  |
| WO2003104443A2     | Hair follicle mesenchymal stem cells and use thereof                         | Torico Sci Innovations Inc, Trichoscience Innovations Inc | Hoffmann R, Mcelwee KJ   | 2003             | 9                       | [89]  |
| US20070122387A1    | Hair grafts derived from plucked hair  | Aderans Res Inst Inc                                      | Barrows TH, Cochran SA, Marshall B, Schlicher R, Su Y                                    | 2007             | 9                       | [105] |
| WO2007062386A2     | Hair follicle graft from tissue engineered skin                              | Aderans Res Inst Inc                                      | Barrows TH, Macintyre P, Washenik KJ   | 2007             | 8                       | [98]  |

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the architecture of the native hair follicle and for percutaneous implantation facilitating the follicle neogenesis process<sup>[90]</sup> (US20050214344), a hair graft comprising plucked hairs having adhered epidermal stem cells and follicular dermal cells for implantation into wounded skin<sup>[105]</sup> (US20070122387A1) and a hair graft comprising a scaffold, and tissue engineered epidermal and dermal layer with hair follicle progenitor cells (WO2007062386A2)<sup>[98]</sup>.

Several methods for isolating skin-derived precursor stem cells from hair follicles or dermal papillae as well as the culture conditions or the delivery into the skin of these cells in order to induce hair growth are also among the most cited patents (WO2002060396A, WO2005071063A1 and US20070092496A1)<sup>[88,91,99]</sup>.

Finally, among the most cited patents are those describing methods for isolating hair follicle mesenchymal stem cells useful for treating alopecia and gene therapy (WO2003104443A2)<sup>[89]</sup> or useful for hair loss, burns or skin replacement as well as for treating neurological or degenerative disorders (WO2003-024406A2)<sup>[87]</sup>.

The rest of the patents analyzed whose content is not mentioned, can be found in the References section<sup>[86-150]</sup> at the end of this review.

## CONCLUSION

Although a successful strategy to generate human hair

follicles from adult cells has not yet been reported, the results presented in this review suggest the issues that need to be addressed before success can be achieved. Perhaps the most important of those issues is to provide cells with a three-dimensional structure that simulates the natural scaffold. Herein the cells should develop and interact among themselves or with other cell types in the epithelial-mesenchymal interactions. Improving culture conditions that allow the expansion of these cells without losing their natural properties, as well as selecting the appropriate epithelial stem cells, should give us the tools needed to face the challenge of regenerating human hair follicles.

These efforts in hair and skin bioengineering are also visible in the growing number of patent applications filed during the last decade, indicating that this technological field is not only attractive to academic research but also to the companies that own almost half of these patents.

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