



Hair Follicle and Sebaceous Gland De Novo Regeneration With Cultured Epidermal Stem Cells and Skin-Derived Precursors

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Key Words. Epidermal stem cells • Skin-derived precursors • Hair follicle regeneration • BMP4 • Sebaceous glands

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Received December 14, 2015; accepted for publication May 12, 2016; published Online First on July 25, 2016.

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1066-5099/2016/\$20.00/0

<http://dx.doi.org/10.5966/sctm.2015-0397>

ABSTRACT

Stem cell-based organ regeneration is purported to enable the replacement of impaired organs in the foreseeable future. Here, we demonstrated that a combination of cultured epidermal stem cells (Epi-SCs) derived from the epidermis and skin-derived precursors (SKPs) was capable of reconstituting functional hair follicles and sebaceous glands (SG). When Epi-SCs and SKPs were mixed in a hydrogel and implanted into an excisional wound in nude mice, the Epi-SCs formed de novo epidermis along with hair follicles, and SKPs contributed to dermal papilla in the neogenic hair follicles. Notably, a combination of culture-expanded Epi-SCs and SKPs derived from the adult human scalp were sufficient to generate hair follicles and hair. Bone morphogenetic protein 4, but not Wnts, sustained the expression of alkaline phosphatase in SKPs in vitro and the hair follicle-inductive property in vivo when SKPs were engrafted with neonatal epidermal cells into excisional wounds. In addition, Epi-SCs were capable of differentiating into sebocytes and formed de novo SGs, which excreted lipids as do normal SGs. Thus our results indicate that cultured Epi-SCs and SKPs are sufficient to generate de novo hair follicles and SGs, implying great potential to develop novel bioengineered skin substitutes with appendage genesis capacity. *STEM CELLS TRANSLATIONAL MEDICINE 2016;5:1695–1706*

SIGNIFICANCE

In postpartum humans, skin appendages lost in injury are not regenerated, despite the considerable achievement made in skin bioengineering. In this study, transplantation of a combination of culture-expanded epidermal stem cells and skin-derived progenitors from mice and adult humans led to de novo regeneration of functional hair follicles and sebaceous glands. The data provide transferable knowledge for the development of novel bioengineered skin substitutes with epidermal appendage regeneration capacity.

INTRODUCTION

Tissue engineering is emerging as a significant potential solution for tissue and organ failure, whereby tissue and organ mimics that are fully functional are implanted to replace the failed tissue and organ [1]. Adult skin consists of a keratinized stratified epidermis and an underlying layer of dermis. Hair follicles, sebaceous glands (SGs), and sweat glands, the appendages of the skin, are derived from a single layer of multipotent progenitors during skin morphogenesis [2]. In postpartum humans, however, deep injuries to the skin heal by scar formation but not by regeneration; epidermal appendages lost at the injury site do not regenerate [3].

It has been a challenge to regenerate skin appendages with defined cells. More than 10 years

have passed since the development of cultured skin substitute (CSS), which consists of cultured autologous epidermis and dermal fibroblasts. The CSS is capable of forming an epidermal layer (barrier function) but does not regenerate the appendages [4]; thereby the structure and function of the skin are not fully restored. A bioengineered skin substitute capable of regenerating the epidermis and the epidermal appendages has been an objective to achieve.

Several populations of stem cells have been identified in the skin. Epidermal stem cells (Epi-SCs) in the basal layer epidermis—which express high levels of cytokeratin (CK)5, CK14, CD29 (integrin β 1), and CD49f (integrin α 6) [5]—constantly provide new keratinocytes to the epidermis, implying great proliferation potential. Neonatal mouse

epidermal cells, but not adult epidermal cells, have been demonstrated to form *de novo* hair follicles, in the presence of dermal papilla (DP) cells [6]. Epi-SCs in the hair follicle bearing various surface markers such as CD34, Lgr5, or K15 have been shown to regenerate hair follicles when engrafted in combination with embryonic or newborn dermal cells [7–9]. In addition, follicle Epi-SCs are thought to contribute to epidermal cells when the skin is wounded [10]. The establishment of clinically applicable methods for the isolation and culture expansion of epidermal stem cells and their niche cells is essential for the development of bioengineered skin substitutes with appendage regeneration capacity.

The formation of hair follicles relies on signals derived from the dermis during skin morphogenesis [9, 11, 12]. Previous studies indicate that DP cells derived from the hair follicle are able to induce hair genesis [13–15]. However, the application of DP cells in tissue engineering has been limited by their availability. The cells are isolated from large hair follicles in the scalp, and their hair-inductive property diminishes markedly upon culture expansion [16, 17]. Intriguingly, multipotent skin-derived precursors (SKPs) have recently been isolated from the dermis of adult skin of different anatomic sites. SKPs express Sox2 and nestin and exhibit long-term proliferation potential when being cultured in suspension [12, 18, 19]. Importantly, the cells were found to incorporate into the DP and induce hair genesis when subcutaneously injected in mice [19], implying a clinically applicable cell source to induce hair follicle neogenesis.

As an essential component of the epidermal appendage, the importance of the SG has increasingly been recognized. SGs are developed from the pilosebaceous unit during hair follicle morphogenesis, when the early Sox9⁺ and Lrig1⁺ progenitor cells in the placode generate a distinct pool of stem cells shortly before the emergence of sebocytes [20, 21]. β -Catenin and peroxisome proliferator-activated receptor γ (PPAR γ) signals appear to be crucial in the genesis of SGs. Hair follicle stem cells deficient for β -catenin showed signs of massive sebocyte differentiation [22]; in contrast, PPAR γ plays a role in initiating the differentiation of sebocytes [22, 23]. It is intriguing to investigate whether cultured Epi-SCs are induced to generate SGs by modulating these signals.

In this study, we demonstrated that a combination of culture-expanded Epi-SCs derived from the epidermis and SKPs were sufficient to regenerate *de novo* hair follicles. Importantly, we showed that culture-expanded Epi-SCs derived from adult human epidermis were capable of regenerating hair follicles and hairs in the presence of culture-expanded adult human SKPs, suggesting clinically applicable cells for the bioengineering of skin substitutes with appendage neogenesis capacity. Bone morphogenetic protein 4 (BMP4), but not Wnts, sustained the hair follicle induction ability of SKPs. In addition, we showed that Epi-SCs from the epidermis differentiated into sebocytes *in vitro* and formed SGs *in vivo* upon appropriate induction. Thus our results identify clinically applicable stem cells and niche cells for *de novo* regeneration of the hair follicle and SG, suggesting a great potential to develop novel bioengineered skin substitutes with appendage regeneration capacity.

MATERIALS AND METHODS

Mice

C57BL/6 (7 weeks old) and BALB/c nu/nu mice (5 weeks old) were purchased from Guangdong Medical Laboratory Animal Center

(Guangzhou, People's Republic of China; <http://www.gdmlac.com.cn/index.php?q=en>). C57BL/green fluorescent protein (GFP) mice (6 weeks old) were obtained from Cyagen Biosciences (Guangzhou, People's Republic of China; <http://www.cyagen.com/us/en/>). The animals were maintained in a temperature controlled environment (20°C \pm 1°C) with access to food and water throughout the experiment. All animal procedures were performed with the approval of the Animal Ethics Committee of Tsinghua University.

Hair Follicle Regeneration Assay

BALB/c nu/nu mice (4–5 weeks old) were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Two symmetrical 2.5-mm-diameter full thickness skin wounds were created on the back with a skin biopsy punch as previously described [24]. We mixed 2×10^6 SKPs, or neonatal mouse dermal cells, with 1×10^6 Epi-SCs or neonatal mouse epidermal cells and encapsulated them in 10 μ l Matrigel (BD Biosciences, San Jose, CA, USA; <http://www.bdbiosciences.com/us/home>). After incubation for 30 minutes at 37°C, the cells-Matrigel was implanted into an excisional wound. The wound was then covered with Tegaderm (3M, Maplewood, MN, USA; <http://www.3m.com/>) transparent dressing, which was further covered with self-adhering elastic bandage. After 3 weeks, mice were sacrificed, the numbers of hairs were counted under a dissecting microscope, and wound tissue samples were harvested for histological analysis.

Isolation and Culture of Epi-SCs and SKPs

Neonatal mouse dorsal skin was harvested from BALB/c or C57BL/GFP mice 1–3 days after birth. After treatment with 0.3% Dispase II, the epidermis was manually removed from the tissue, from which Epi-SCs were isolated on the basis of their high adhesive property [25] and cultured in CnT-07 progenitor cell-targeted (PCT) epidermal keratinocyte medium. The dermis was digested with collagenase I to isolate SKPs, and single-cell suspensions were cultured in Dulbecco's modified Eagle's medium/F12, 3:1 supplemented with B27, 20 ng/ml epidermal growth factor, and 40 ng/ml basal fibroblast growth factor in untreated dishes. Detailed methods for the isolation of epidermal stem cells, SKPs, and dermal fibroblasts are presented in the supplemental online data.

Alkaline Phosphatase Activity

Alkaline phosphatase (AP) activity of SKPs was measured as has been previously described [26]. Briefly, SKPs were rinsed with phosphate-buffered saline (PBS) and lysed in a buffer containing 0.1% Triton X-100. After centrifugation at 4°C for 10 minutes, the upper aqueous phase was used to measure the AP activity (AP assay kit, Beyotime Biotechnology, Shanghai, People's Republic of China; <https://www.beyotime.com/index.htm>). The absorbance was read at 405 nm by using a microplate reader (BioTek, Winooski, VT, USA; <http://www.biotek.com/>) to determine the enzyme concentration. The results were normalized to the total intracellular protein content determined with the Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA; <https://www.thermofisher.com/us/en/home.html>) and expressed in nanomoles of produced *p*-nitrophenol per minute per milligram of protein (nmol/min/mg protein).

Cell Proliferation Assay

Cell proliferation was evaluated using cell-counting kit-8 (CCK-8) [27]. Cells were seeded in 96-well plates (5,000 cells per well) and incubated in growth medium supplemented with BMPs (Pepro-Tech, Rocky Hill, NJ, USA; <https://www.peprotech.com/en-US>) or Wnts (BD Biosciences) at 37°C in 5% CO₂ for 72 hours, followed by a treatment of 10 μl CCK-8 for another 3 hours. The culture was then subjected to spectrophotometric analysis with a microplate reader (BioTek) with absorbance at 450 nm. Cells cultured in the absence of CCK-8 and culture medium alone were used as controls.

BrdU Labeling and Detection

Epi-SCs were incubated with 10 μM bromodeoxyuridine (BrdU) (Sigma-Aldrich, St. Louis, MO, USA; <https://www.sigmaaldrich.com>) for 24 hours at 37°C and 5% CO₂ and then transplanted into wounds in mice. Wound tissues were harvested 15 days later, fixed in 4% paraformaldehyde at 4°C for 6 hours, washed in PBS at 4°C overnight, dehydrated in 30% sucrose, and embedded in optimal cutting temperature medium. We treated 10-μm-thick sections with 2 M HCl for 30 minutes, washed with 0.1 M borate buffer and PBS. After blocking, sections were incubated with an anti-BrdU antibody (1:100; BioLegend, San Diego, CA, USA; <http://www.biolegend.com/>) at 4°C overnight and detected with a fluorescence-conjugated secondary antibody. Samples were examined under a confocal laser scanning microscope (FV1000, Olympus, Tokyo, Japan; <http://www.olympus-global.com/en/>).

Sebocyte Differentiation and SG Formation

Epi-SCs derived from the epidermis of neonatal mice or adult human foreskin were cultured in CnT-07 PCT epidermal keratinocyte medium containing 10⁻⁶ M dexamethasone, 10 μg/ml insulin, 20 μM Rosiglitazone, and 1 μM XAV939 for 3 days to induce sebocyte differentiation. For *in vivo* SG induction, we implanted 1 × 10⁶ Epi-SCs and 2 × 10⁶ neonatal mouse SKPs in 20 μl Matrigel, or 1 × 10⁶ Epi-SCs and 2 × 10⁶ neonatal mouse dermal fibroblasts in 20 μl Matrigel without or with the supplementation of 10⁻⁵ M dexamethasone, 100 μg/ml insulin, 200 μM rosiglitazone, and 10 μM XAV939, into a 3-mm excisional wound in nude mice. Full-thickness skin tissue of the wound was harvested at 14 days and subjected to histological analysis for SGs.

Transepidermal Water Loss Assay

Excisional wounds were created on the back of nude mice. After 14 days, mice were sacrificed. Full-thickness skin samples of the wound (transplanted with neonatal mouse Epi-SCs plus dermal fibroblasts or neonatal mouse Epi-SCs plus SKPs) or unwounded area (control) on the back were carefully excised with a 2-mm biopsy punch. The tissues were placed on a tissue culture plate with the dermal side down and placed in an incubator at 37°C and 100% relative humidity for 2 hours for lipid excretion by SGs to the skin surface. The component of the lipid was analyzed using matrix assisted laser desorption/ionization (MALDI) imaging mass spectrometry. Then, the tissues were subjected to transepidermal water loss assay [28], in which the tissues were placed in a 37°C drying oven; the weight of each tissue was measured at the 0, 0.5, 1, 1.5, 2, 3, and 5 hours; and the appearance of the skin surface was imaged at 0, 1, 2, and 3 hours under a dissecting microscope.

Flow Cytometry, Fluorescent Immunohistochemistry, and MALDI Imaging Mass Spectrometry

Detailed methods for these assays are presented in the supplemental online data.

Statistical Analysis

All data were expressed as mean ± SD. One-way analysis of variance was used for data analysis, and statistical significance was defined as $p < .05$.

RESULTS

Culture-Expanded Epi-SCs and SKPs Form de Novo Hair Follicles

Epidermal stem cells are known to reside in the basal layer of interfollicular epidermis and the hair follicle bud [29, 30]. Because epidermal stem cells in the hair follicle are difficult to acquire, we investigated whether Epi-SCs in the epidermis were capable of regenerating epidermal appendages and thus could be severed as a cell source for skin bioengineering. In the epidermis of neonatal mice 3 days postborn, immunofluorescence analysis showed that cells in the basal layer were largely positive for CD49f (Fig. 1A), a feature of Epi-SCs [9]. Rapid attachment to plastic culture dishes has been recognized as a property of Epi-SCs [25]. Accordingly, we purified Epi-SCs from the epidermis of mice by selecting the cells that rapidly attached to the dish (Fig. 1B). Immunofluorescence stain showed that the cells expressed CD49f (Fig. 1C) and were positive for CK 15 (Fig. 1D). Fluorescence-activated cell sorter (FACS) analysis showed that more than 90% of the cells were highly positive for CD49f and CD29 (Fig. 1E). The cells retained adequate proliferation, but the hair neogenesis ability declined gradually along with the expression level of CD49f (Fig. 1F), with 96 ± 18 hair shafts (HS) per transplant in passage (P)0 cells and with 50 ± 10 HS in P5 cells (Fig. 1G; $n = 6$, $p < .001$). Murine SKPs have been shown previously to contribute to functional DP cells in mice [19]. Here, we examined whether SKPs were sufficient to induce Epi-SCs to form de novo skin appendages. SKPs derived from neonatal mice were culture expanded in spheroids (Fig. 1H). Immunofluorescence stain showed that the cells were positive for nestin, fibronectin, and BMP6 (Fig. 1I, 1J), which are characteristic genes expressed in SKPs [31]. When cotransplanted with freshly isolated neonatal epidermal cells into wounds in nude mice, SKPs induced hair neogenesis, with 105 ± 22 HS in P0 cells and with 21 ± 10 HS in P5 cells (Fig. 1K; $n = 6$, $p < .001$). Notably, when cotransplantation of culture-expanded Epi-SCs and SKPs derived from neonatal mice into excisional wounds in nude mice, they induced hair neogenesis, with 86 ± 24 HS in P0 cells and with 47 ± 6 HS in P3 cells (Fig. 1L; $n = 3$, $p < .05$). Black hairs were generated by culture-expanded Epi-SCs and SKPs (Fig. 1M), which remained for more than 3 weeks when the animals were sacrificed. Histological analysis showed densely populated hair follicles along with SGs in the regenerated skin tissue (Fig. 1N). The procedure of hair follicle reconstitution with cultured stem cells is illustrated in supplemental online Figure 1.

Next, we determined whether transplantation of Epi-SCs derived from adult human skin were able to generate hairs. We isolated Epi-SCs from the epidermis of adult human foreskin (average age 26 ± 5 years, $n = 7$). The foreskin was a glabrous tissue without hair follicles. Similar to Epi-SCs derived from neonatal

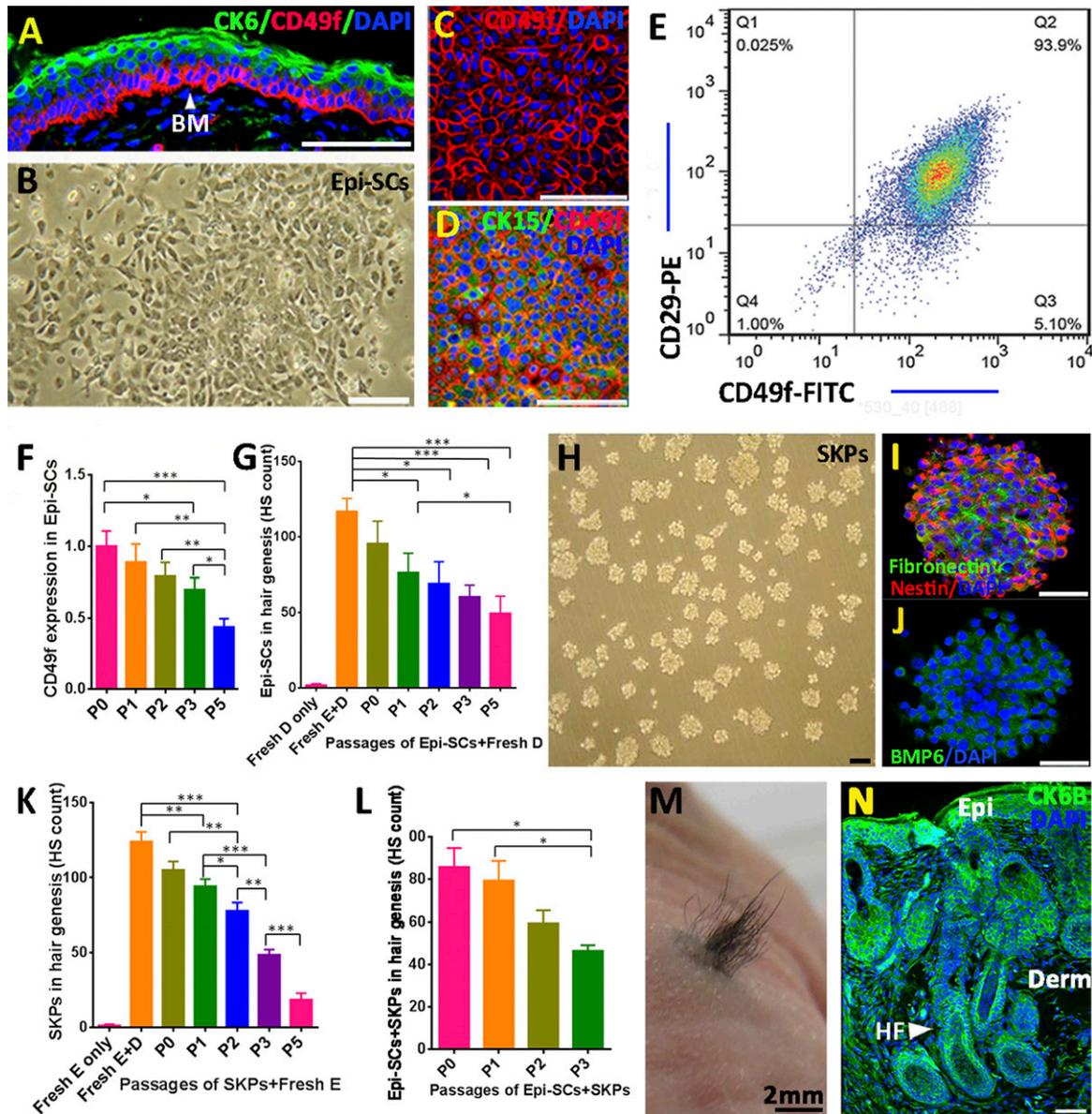


Figure 1. Hair neogenesis with cultured epidermal stem cells (Epi-SCs) and skin-derived precursors (SKPs). **(A)**: Putative epidermal stem cells residing in the basal layer of neonatal mouse epidermis expressed CD49f (red) in immunofluorescence stain, and mature keratinocytes in the top layers of the epidermis expressed cytokeratin (CK)6 (green). Nuclei were stained with 4',6-diamidino-2-phenylindole. **(B–E)**: Cultured Epi-SCs derived from neonatal mice **(B)** were positive for CD49f **(C)** and CK15 **(D)** in immunofluorescence stain; fluorescence-activated cell sorting analysis of the Epi-SCs indicated high levels of surface CD29 and CD49f **(E)**. **(F)**: The expression level of CD49f decreased progressively upon successive passages (P) in culture as determined by immunofluorescence analysis (in relation to the fluorescence intensity of P0 cells). Triple wells were used for each of the above experiments, and each experiment was repeated three times with similar results (*, $p < .05$; **, $p < .01$; ***, $p < .001$). **(G)**: Hair genesis of cultured Epi-SCs in different passages. Cultured Epi-SCs derived from neonatal mice in different passages (P0 to P5) were implanted into excisional wounds in nude mice in combination with freshly isolated neonatal dermal cells (fresh D) in Matrigel; dermal cells alone or freshly isolated neonatal epidermal cells plus dermal cells (fresh E+D) were used as controls. Hair shafts generated 20 days posttransplant were counted ($n = 6$; *, $p < .05$; ***, $p < .001$). **(H–J)**: SKPs derived from neonatal mice in spheroid culture **(H)** expressed nestin, fibronectin **(I)**, and BMP6 **(J)** in immunofluorescence analysis. **(K)**: Hair genesis of SKPs in different passages. SKPs in P0 to P5 were implanted into excisional wounds in nude mice in combination with freshly isolated neonatal mouse epidermal cells (fresh E), and freshly isolated neonatal mouse epidermal cells alone or in combination with freshly isolated neonatal mouse dermal cells (fresh E+D) were used as controls. Twenty days posttransplant, hairs generated were counted ($n = 6$; *, $p < .05$; **, $p < .01$; ***, $p < .001$). **(L–N)**: Cultured Epi-SCs and SKPs in hair genesis. Combinations of cultured neonatal mouse Epi-SCs (P0 to P3) and SKPs (P0 to P3) were engrafted into excisional wounds in nude mice, and the number of hairs generated were counted 20 days posttransplant ($n = 3$, *, $p < .05$). **(L)**. A representative image of hairs generated 20 days after a transplantation of P1 Epi-SCs and SKPs **(M)**. Immunofluorescence analysis of the skin tissue with hair genesis showed densely populated hair follicles and sebaceous glands **(N)**. Scale bars = 50 μm . Abbreviations: BM, basement membrane; BMP6, bone morphogenetic protein 6; CK, cytokeratin; DAPI, 4',6-diamidino-2-phenylindole; Derm, dermis; Epi, epidermis; Epi-SC, epidermal stem cells; FITC, fluorescein isothiocyanate; fresh D, freshly isolated neonatal dermal cells; fresh D+E, freshly isolated neonatal epidermal cells plus dermal cells; HF, hair follicle; HS, hair shafts; P, passage; PE, phycoerythrin.

mice, cultured Epi-SCs derived from human foreskin expressed high levels of CD29, CD49f, K15, and K19 (supplemental online Fig. 2), typical features of epidermal stem cells [29]. To examine their potential in hair follicle neogenesis, we cotransplanted BrdU-labeled human Epi-SCs with SKPs derived from neonatal C57BL/6 mice to nude mice, through subcutaneous injection or topical application to excisional wounds. Black hairs generated 15 days later with both administration approaches (Fig. 2A). Histological analysis showed that human Epi-SCs formed de novo hair follicles, SGs, and the epidermis, which were positive for BrdU (Fig. 2B). In addition, many cells in the newly generated structures expressed Ki67 (Fig. 2C), indicating active proliferation of the cells. Moreover, antibodies specifically targeting human CD29 and CD49f detected the presence of human epidermal stem cells in the basal layer epidermis (Fig. 2D, 2E), implying the formation of a long-term renewable structure by the human Epi-SCs. We found that Epi-SCs derived from adult human foreskin sustained sufficient hair genesis capacity after culture expansion, though the number of neogenic hairs declined with successive cell passages (73 ± 18 HS per transplant with P0 Epi-SCs and 40 ± 12 HS per transplant with P3 Epi-SCs; $n = 6$, $p < .01$), along with a decrease in the expression level of CD49 in the cells (Fig. 2F, 2G).

To further examine whether culture-expanded human Epi-SCs and SKP could form de novo hair follicles, we derived Epi-SCs and SKPs from human scalp tissue (32 ± 6 years old, female). Cultured Epi-SCs derived from the epidermis were strongly positive for CD29 and CD49f and were negative for CD200 (supplemental online Fig. 3A), which is a marker for human hair follicle stem cells similar to CD34 in mouse hair follicles [32, 33], indicating that the Epi-SCs were derived from interfollicular epidermis but not the hair follicle. SKPs isolated from the dermis of the scalp were positive for nestin and fibronectin (supplemental online Fig. 3B), characteristic genes expressed in SKPs. We transplanted a mixture of BrdU-labeled human Epi-SCs and human SKPs into excisional wounds in nude mice. After 15 days, black hairs grew out of the skin (Fig. 2H), with 33 ± 5 HS per transplantation. Histological analysis revealed BrdU-positive neogenic epidermis and hair follicles (Fig. 2I). Immunostatin with MAB1281, an antibody specifically targeting human nuclear protein, showed that the cells in the neogenic structure including the epidermis, the hair follicle with DP, and the cells in the newly formed dermis were largely of human origin (Fig. 2J).

BMP4 Sustains the Hair Inductive Ability of SKPs

There was a marked decline in the inductive ability of SKPs in hair genesis upon successive passaging in culture (Fig. 1K). Wnt and BMP pathways are crucial signals in regulating hair follicle morphogenesis and cyclic regeneration [34, 35]. Previous studies have shown that the expression level of AP largely correlates to the hair inductive ability of DP cells [17]. Here, we examined the influence of BMPs and Wnts on SKPs for their growth and AP expression in vitro and hair follicle-inductive ability in vivo. We found that supplementation of BMPs (a combination of BMP2, BMP4, and BMP6), but not Wnts (a combination of Wnt3a, Wnt5a, and Wnt10b), to the culture significantly increased the activity of AP in SKPs ($p < .001$) (Fig. 3A) but suppressed the proliferation of the cells (Fig. 3B). Among the three BMPs, BMP4 exhibited the most potent effect in increasing the activity of AP (Fig. 3C), which correlated to increased expression of AP protein levels in the cells (Fig. 3E, 3F). BMP4 at 200 ng/ml led to a

significant increase in AP activity (Fig. 3D), and the concentration was used for subsequent experiments. Hair follicle reconstitution analysis using a combination of culture-expanded SKPs, and freshly isolated neonatal mouse epidermal cells showed that treatment of SKPs with BMP4 (200 ng/ml) significantly increased their ability to induce hair neogenesis in nude mice (Fig. 3G–3I). Histological analysis indicated that EGFP-labeled SKPs contributed to the DP in the hair follicle and numerous cells in the newly formed dermis (Fig. 3J, 3K).

Epi-SCs Differentiate Into Sebocytes and Regenerate Sebaceous Glands

An efficient marker to detect SGs in immunohistochemical analysis is lacking. In this study, we showed that sebocytes expressed a high level of biotin in murine (Fig. 4A) and human skin (data not shown). Because the SG is known to be derived from the epidermal progenitors along with the hair follicle during skin morphogenesis [21], we examined whether cultured Epi-SCs derived from the epidermis were capable of differentiating into sebocytes and even forming functional SGs. On the basis of the crucial roles of Wnt and PPAR γ signals in epidermal stem cell differentiation [22, 23], we cultured Epi-SCs derived from the epidermis of neonatal mice in the presence of dexamethasone/insulin/rosiglitazone/XAV939 (DIRX), in which rosiglitazone was a PPAR γ agonist and XAV939 was a Wnt signaling inhibitor to induce their differentiation into sebocytes. After 3 days, lipid droplets appeared in the Epi-SCs, which became positive for biotin stain (Fig. 4B, 4C). Meanwhile, fluorescence-activated cell sorting analysis showed that the majority of Epi-SCs were positive for surface expression of Lrig1 (Fig. 4D), a marker of sebocyte progenitors [20, 21]. In addition, the cells showed an increase in the expression level of PPAR γ in the cytoplasm and nuclei (Fig. 4E, 4F) and a downregulation of β -catenin (Fig. 4G, 4H) after the induction. Similarly, we found that adult human Epi-SCs exhibited features of sebocytes after induction (data not shown).

To further examine whether Epi-SCs were capable of forming SGs in vivo, we transplanted a combination of neonatal mouse Epi-SCs and SKPs or neonatal dermal fibroblasts into excisional wounds in nude mice. In the presence of SKPs, the Epi-SCs generated SGs in association with hair follicles (Fig. 4I), and in the presence of dermal fibroblasts, the Epi-SCs formed SG-like structures without hair follicles (Fig. 4J). To track the Epi-SCs in vivo, we labeled the cells with enhanced GFP (EGFP lentiviruses) prior to transplantation; the results confirmed that Epi-SCs formed de novo SGs (Fig. 4K). Although Epi-SCs were able to generate SGs when cotransplanted with dermal fibroblasts (2.0 ± 0.5 SGs per microscopic field), the number of SGs was limited (Fig. 4L); however, in the presence of DIRX in the graft, the number of neogenic SGs substantially increased (10.5 ± 1.5 SGs per microscopic field; $p < .01$) (Fig. 4M, 4N).

Neogenic SGs Are Functional

SGs are specialized structures equipped for the synthesis of a broad spectrum of lipid compounds including triacylglycerols (TAG), diacylglycerols (DAG), wax esters, cholesterol esters (CEs), and free fatty acids [36]. To determine whether the neogenic SGs formed by implanted Epi-SCs were functional, we analyzed lipid substances excreted to the surface of the

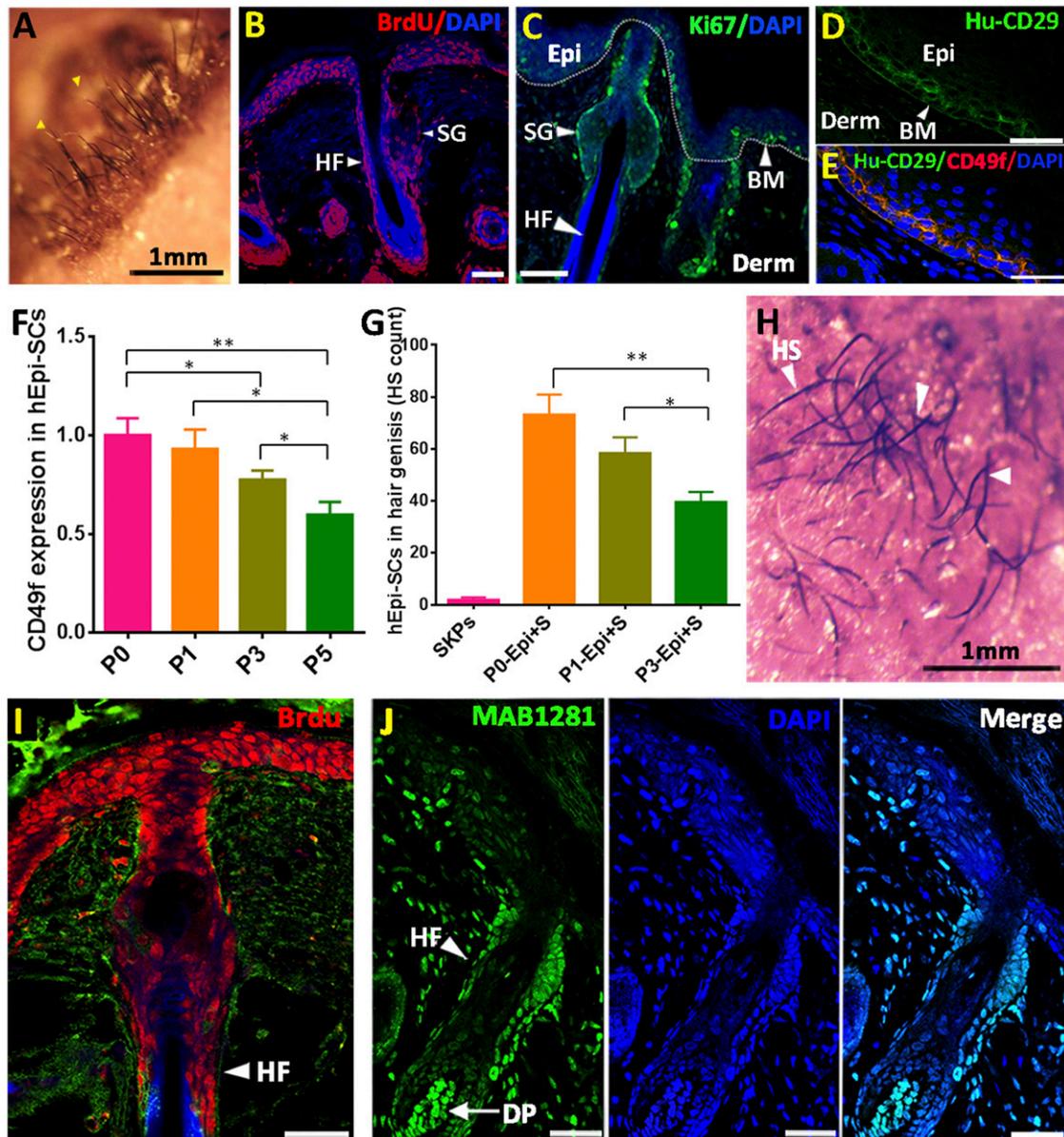


Figure 2. Cultured human epidermal stem cells (Epi-SCs) and skin-derived precursors (SKPs) form de novo hair follicles. **(A–E):** Hair follicle reconstitution. A mixture of cultured human Epi-SCs (hEpi-SCs) with cultured neonatal mouse SKPs (in passage 1) in Matrigel was implanted into excisional wounds in nude mice. A representative image at 15 days posttransplantation shows neogenic hairs **(A)**. Histological analysis of the wound at 15 days showed that hEpi-SCs that were prelabeled with bromodeoxyuridine (BrdU) formed neogenic skin and hair follicles **(B)**. Ki67⁺ cells were found in the hair follicle and epidermis of the newly formed skin **(C)**. Immunofluorescence staining with an antibody specifically against human CD29 (Hu-CD29) detected positive cells in the basal layer epidermis, suggesting that the transplanted Epi-SCs reconstituted the stem cell pool in the epidermis **(D, E)**. **(F, G):** The expression level of CD49f in hEpi-SCs decreased progressively upon culture passages as assessed by immunofluorescence analysis **(F)**, which correlated with a reduction in hair genesis ability of the cells in hair follicle reconstitution analysis **(G)** ($n = 6$; *, $p < .05$; **, $p < .01$). **(H–J):** A mixture of cultured human scalp-derived Epi-SCs, which were prelabeled with BrdU and SKPs in Matrigel, was implanted into excisional wounds in nude mice. Black hairs grew out 15 days posttransplant **(H)**. Immunofluorescence analysis of the skin tissue showed that BrdU-positive hEpi-SCs contributed to the epidermis and hair follicles in the newly formed skin **(I)**; staining with MAB1281 confirmed that hair follicles, including the DP and numerous dermal cells in the regenerated skin, were of human origin (green) **(J)**. Scale bars = 50 μm . Abbreviations: BM, basement membrane; BrdU, bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; Derm, dermis; DP, dermal papilla; Epi, epidermis; Epi+S, epidermis + SKPs; hEpi-SCs, human epidermal stem cells; HF, hair follicle; HS, hair shaft; Hu-CD 29, human CD 29; P, passage; SG, sebaceous gland; SKPs, skin-derived precursors.

newly formed skin (after transplantation of Epi-SCs and SKPs) by MALDI time-of-flight imaging mass spectrometry, in comparison with substances excreted by the normal skin, and found that they were almost identical in composition (Fig. 5A–5C), with peaks at m/z 397.3, 615.5, and 769.6

representing CEs, DAG ($\text{C}_{37}\text{H}_{68}\text{O}_5$), and TAG ($\text{C}_{47}\text{H}_{86}\text{O}_6$), respectively. Analysis of the m/z 797.7 (TAG, $\text{C}_{49}\text{H}_{90}\text{O}_6$) distribution on the surface of the newly generated skin showed that it exhibited a pattern similar to that on the normal mouse skin (Fig. 5D–5F). To examine the effect of the

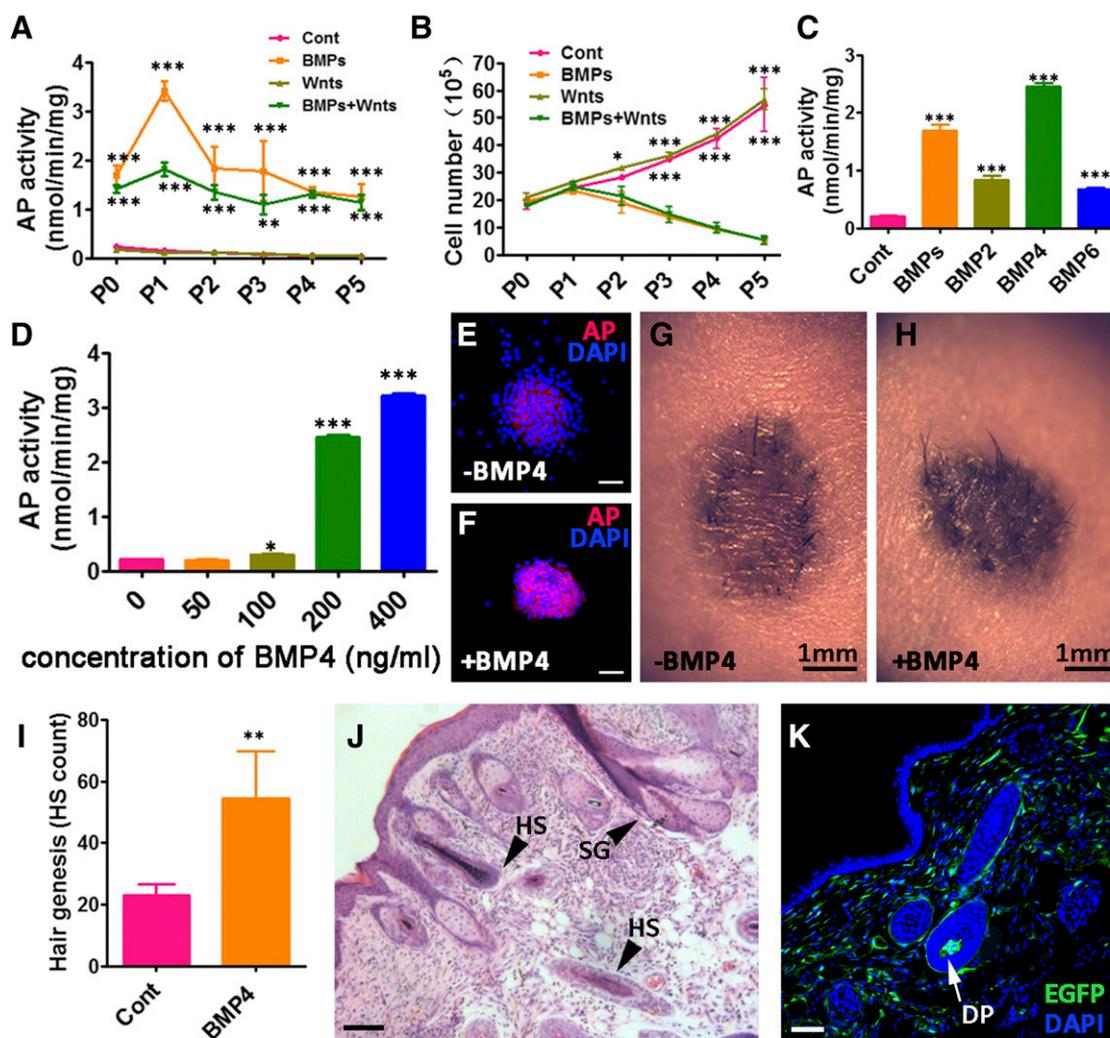


Figure 3. Effects of bone morphogenetic proteins (BMPs) and Wnts on the inductive ability of skin-derived precursors (SKPs) in hair genesis. **(A, B):** The effect of BMPs and Wnts on AP activity **(A)** and the proliferation **(B)** of SKPs. Freshly isolated SKPs (passage [P]0) were seeded in regular culture medium without (Cont) or with supplementation of BMPs, including BMP2, BMP4, and BMP6 (200 ng/ml each); Wnts, including Wnt3a, Wnt5a, and Wnt10b (200 ng/ml each); or a combination of the above BMPs and Wnts (BMPs+Wnts) and incubated for up to 5 passages (P5). AP activity **(A)** and the number **(B)** of SKPs in each passage were assessed. **(C):** The effect of different BMPs on AP activity of SKPs. SKPs were cultured in regular medium without (Cont) or with supplementation of BMP2, BMP4, and BMP6 at 200 ng/ml each or a combination of the three BMPs (200 ng/ml each) for 3 days, and the AP activity of SKPs were measured. **(D):** The effect of different concentrations of BMP4 on AP activity of SKPs. SKPs were cultured in regular medium with supplementation of different concentrations of BMP4 (0~400 ng/ml) for 3 days, and the AP activity of SKPs was measured. Triple wells were used for each of the above experiments, and each experiment was repeated three times with similar results. *, $p < .05$; **, $p < .01$; ***, $p < .001$. **(E, F):** Immunofluorescence analysis of AP in SKPs. SKPs in p3 were cultured without **(E)** or with supplementation of BMP4 at 200 ng/ml **(F)** for 3 days. The expression of AP (red) was detected under confocal microscope. Nuclei are highlighted with 4',6-diamidino-2-phenylindole (blue). **(G–I):** The effect of BMP4 on the inductive ability of SKPs in the neogenesis of hair follicles. A combination of freshly isolated neonatal mouse epidermal cells (10^5) and enhanced green fluorescent protein-expressing SKPs in p5 (2×10^5) that were pretreated without **(G)** or with **(H)** 200 ng/ml BMP4 for 3 days was engrafted into an excisional wound in Matrigel in nude mice. After 20 days, hairs grown from the wounds were photographed, and representative images are shown **(G, H)**. The number of hair shafts per wound were counted **(I)** ($n = 3$; **, $p < .01$). **(J, K):** Histological analysis of the wound tissue: hematoxylin and eosin stain showed hair follicles with hair shaft and sebaceous glands **(J)**; immunofluorescence analysis of the tissue sections showed that SKPs contributed to the dermal papilla in the hair follicle and numerous cells (green) in the dermis **(K)**. Scale bars = 50 μm . Abbreviations: AP, alkaline phosphatase; BMP, bone morphogenetic protein; Cont, without supplementation of bone morphogenetic proteins; DP, dermal papilla; DAPI, 4',6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; HS, hair shaft; P, passage; SG, sebaceous glands.

regenerated SG in protecting the tissue from water evaporation, we performed a water loss experiment. The healed skin wound with SG regeneration showed an average of $12\% \pm 1\%$ ($n = 4$) weight loss after 2 hours of air drying, in comparison with an average of $10\% \pm 0.5\%$ ($n = 4$) weight reduction in the unwounded skin tissue and an average of $35\% \pm 5\%$

weight loss in the healed skin wound without SG regeneration ($n = 4$, $p < .05$, $p < .001$) (Fig. 5G). In addition, the healed skin wound without SG regeneration showed much more evident shrinking after 2 hours of air drying (Fig. 5H), in comparison with the normal skin (Fig. 5J) or the healed skin wound with SG regeneration (Fig. 5I).

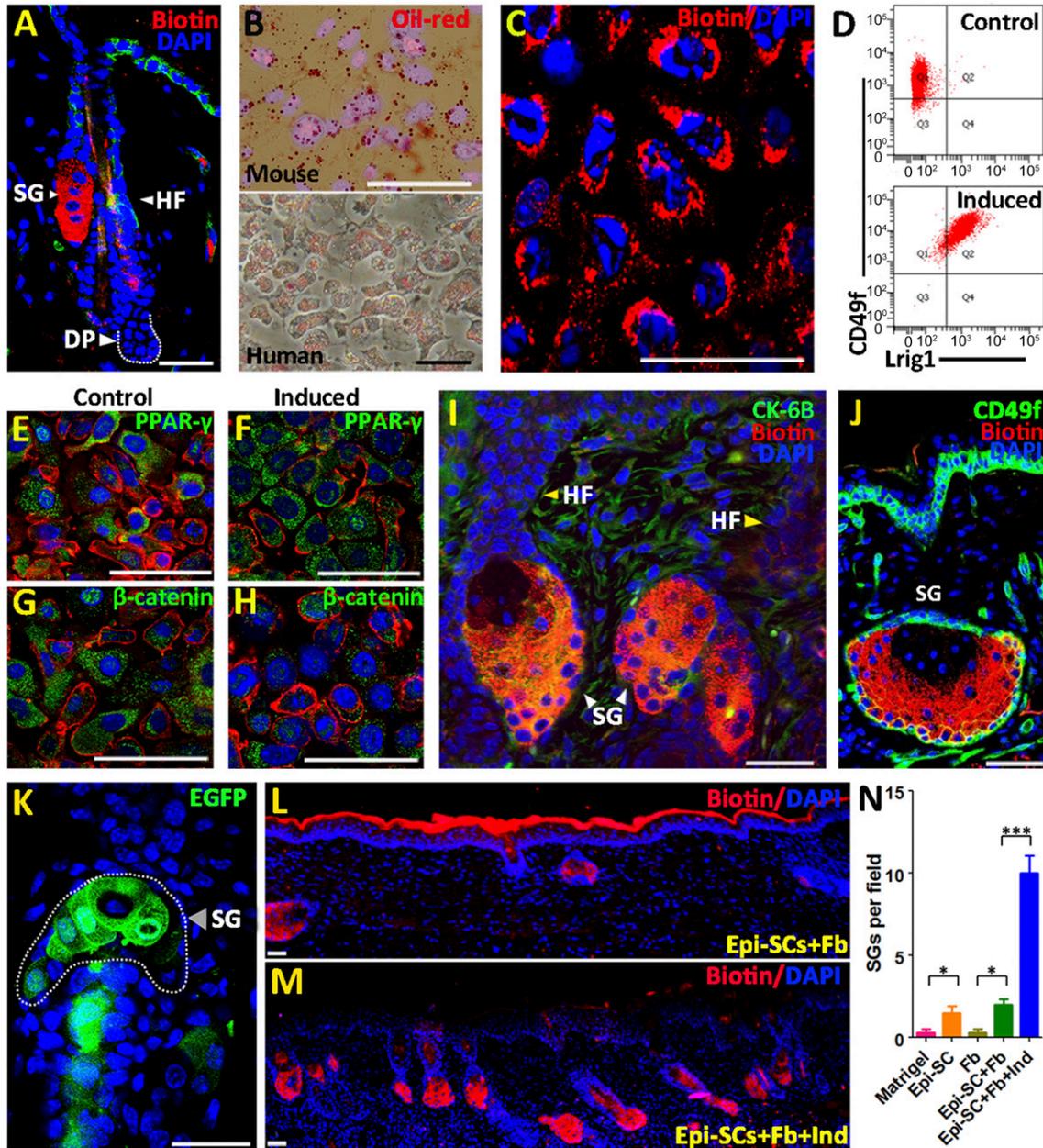


Figure 4. Epidermal stem cells (Epi-SCs) differentiate into sebocytes and form sebaceous glands (SGs). **(A):** Abundant biotin was detected in sebocytes of the SG in mouse skin after immunofluorescence stain. **(B–H):** Sebocyte differentiation. Epi-SCs derived from neonatal mouse epidermis were cultured in an induction medium containing dexamethasone, insulin, rosiglitazone, and XAV939 for 3 days; lipid droplets (red) appeared in the cells as shown after oil red stain **(B)**. Meanwhile, high levels of biotin were detected in the cells **(C)**. Fluorescence-activated cell sorting analysis indicated an increase in Lrig1 expression in the cells **(D)**. Immunofluorescence stain showed an increase in peroxisome proliferator-activated receptor γ expression **(E, F)** and a decrease in β -catenin expression **(G, H)**. **(I–N):** Formation of SGs by Epi-SCs. Epi-SCs derived from the epidermis of neonatal mice were mixed with neonatal mouse skin-derived precursors (SKPs) or neonatal dermal fibroblasts (Fb) and implanted into excisional wounds in nude mice. Fourteen days later, wound tissue sections were stained for biotin and cytokeratin (CK)6. SGs were formed in wounds received implantation of Epi-SCs and SKPs, which were in association with hair follicles **(I)**. Independent SGs in disassociation with hair follicles were found in wounds receiving implantation of Epi-SCs and dermal fibroblasts **(J)**. Cultured mouse Epi-SCs were labeled with enhanced green fluorescent protein (EGFP)-lentiviruses and then implanted into excisional wounds in nude mice with SKPs. De novo SGs expressing EGFPs were found **(K)**. **(L–N):** Neonatal mouse Epi-SCs and neonatal dermal fibroblasts were implanted into excisional wounds in nude mice in the absence **(L)** or presence **(M)** of the induction cocktail (dexamethasone, insulin, rosiglitazone, and XAV939), which significantly increased the number of SGs in the wound **(N)**. The SG number is quantified via counting of the SG structure in the tissue section of the cells' implanted area (within the wound area); the SG number is the average of more than nine sections of three mice in one group. *, $p < .05$; ***, $p < .001$. Scale bars = 50 μ m. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DP, dermal papilla; Epi, epidermis; EGFP, enhanced green fluorescent protein; Fb, fibroblasts; HF, hair follicle; Ind, induction; PPAR γ , peroxisome proliferator-activated receptor γ ; SC, stem cell; SG, sebaceous gland.

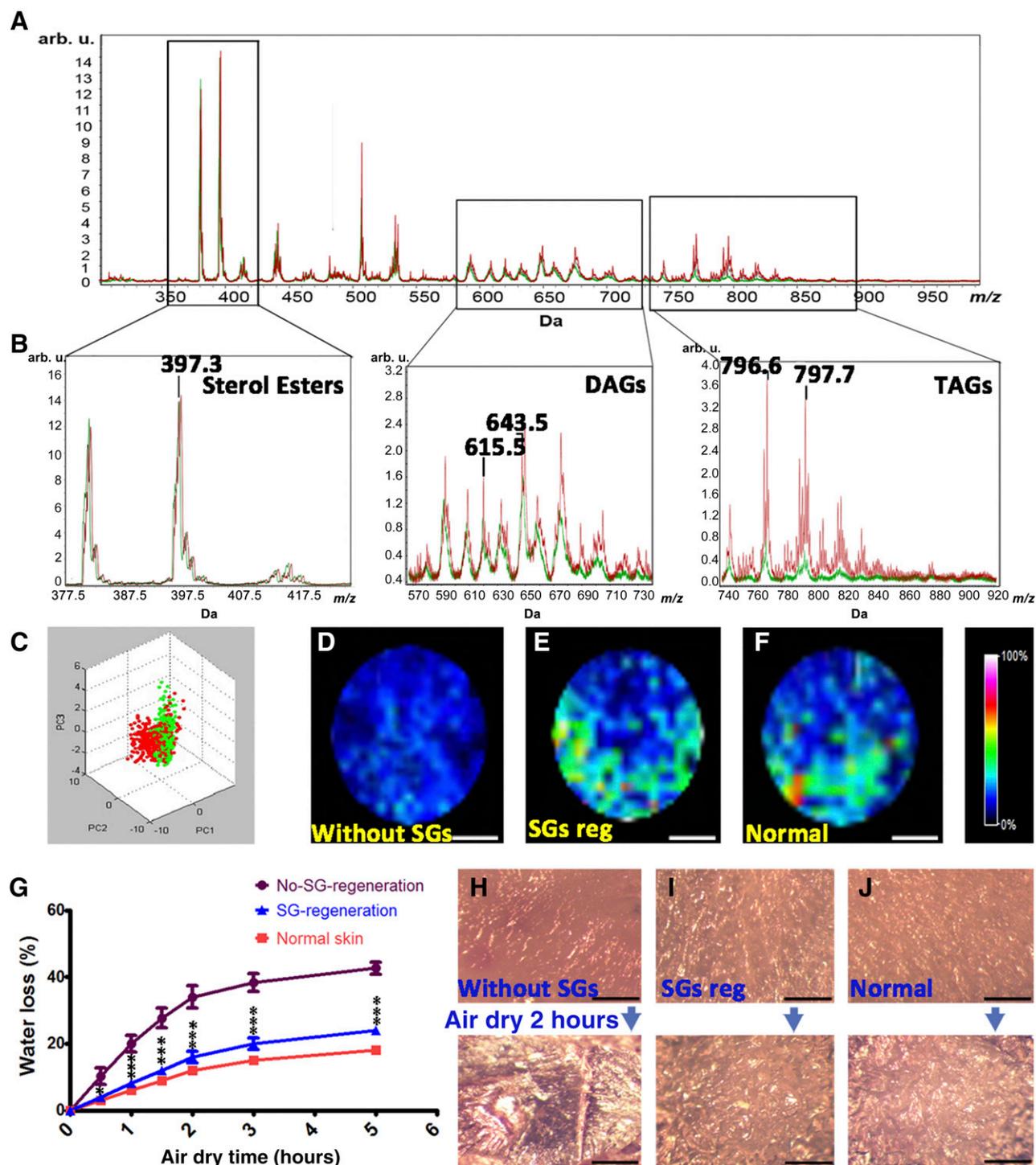


Figure 5. Regenerated sebaceous glands secrete lipids. **(A, B):** MALDI imaging mass spectrometry analysis of excretions of the normal skin indicated that the peak at m/z 397.3 corresponded to cholesterol esters **(A)** and that 615.5 and 769.6 corresponded to diacylglycerols ($C_{37}H_{68}O_5$) and triacylglycerols (TAG) ($C_{47}H_{86}O_6$), respectively **(B)**. **(C):** Principal component analysis showed a high similarity in lipid composition between samples collected from the surface of normal mouse skin and the surface of healed skin wounds, which received transplantation of Epi-SCs and SKPs. **(D–F):** Visualization of m/z 797.7 (TAG, $C_{49}H_{90}O_6$) on the normal skin or the healed skin wounds without or with sebaceous gland regeneration. **(G–J):** Water loss assay. Tissues of normal mouse skin or healed skin wound with or without SG regeneration were subjected to transepidermal water loss analysis. Weights of the tissues before and after air drying for different times were measured ($n = 4$; *, $p < .05$; ***, $p < .001$) **(G)**, and their surface appearances were photographed under a dissecting microscope **(H–J)**. Scale bars = 0.5 mm. Abbreviations: arb. u., arbitrary unit; DAG, diacylglycerols; PC, principal component, reg, regeneration; SG, sebaceous gland; TAG, triacylglycerols.

DISCUSSION

Despite the considerable achievement made in improving the quality of bioengineered skin substitutes, a skin substitute capable of regenerating functional skin appendages is lacking [37]. With the absence of epidermal appendages—which serve particular functions, including sensation, contractility, protection, waterproofing of the body's surface, and heat radiation—the practical performance of a skin substitute is significantly discounted. Generation of skin appendages such as the hair follicle has been limited by the lack of trichogenic potency in cultured postnatal cells. Therefore, it is crucial to discover stem cell progenitors that are not only capable of forming the appendages but are sufficiently available and culture expandable; Epi-SCs in the epidermis and SKPs are ideal candidates. In this study, we showed that these two types of stem cells were essential to and sufficient for regenerating the hair follicle and the SG.

We found that Epi-SCs derived from the epidermis and SKPs were highly expandable in culture. The Epi-SCs were located in the basal layer of the epidermis, and expressed K15, K19, and high levels of CD29 and CD49f, genes typically expressed in the stem cells [29]. The genes were also expressed in cultured Epi-SCs, despite the expression level of CD49f declining with successive passages, which was associated with a decrease in the number of neogenic hairs, suggesting that CD49f may serve as a marker for the hair genesis ability of cultured Epi-SCs.

As multipotent stem cells, SKPs have been isolated from the skin of different anatomical regions in mice and humans [12, 18, 19]. Impressively, when mouse or rat SKPs were injected subcutaneously alone or engrafted in combination with neonatal mouse epidermal cells, they contributed to the DP [19]. It was intriguing to investigate whether SKPs derived from adult human skin have the capacity for hair genesis. In this study, we showed that SKPs derived from adult human abdominal skin (data not shown) and scalp proliferated in suspension culture and expressed nestin, fibronectin, and BMP6, typical markers of SKPs [18, 19]. More important, culture-expanded human SKPs induced *de novo* genesis of the hair follicle and hairs when implanted in combination with culture-expanded adult human Epi-SCs, suggesting that SKPs may serve as a practical cell source for hair genesis and tissue engineering, which are advantageous in availability and expandability in comparison with DP cells.

Little has been known about the molecular niche needed for SKPs to maintain their hair inductive property. Similar to that in DP cells [17, 38], we found that the hair inductive ability of SKPs decreased with successive cell passaging in culture. Certain BMPs and Wnts have been shown previously to play a role in maintaining the inductive property of DP cells in hair genesis [17, 39, 40]. When murine DP cells were cultured in medium containing BMP2, -4, -5, or -6, their AP expression was better maintained. In the absence of BMP-induced signals, DP cells lost their signature characteristics *in vitro* and failed to generate hair follicles *in vivo* when engrafted with epithelial stem cells in mice [17]. In addition, BMP2 along with 6-bromoindirubin-39-oxime and basic fibroblast growth factor were shown to maintain the expression of DP signature genes primarily in cultured human DP cells [41]. Meanwhile, Wnt3a-treated DP cells had a higher capacity for inducing hair formation than did untreated DP cells in an engraftment study [42]. Canonical Wnts, particularly Wnt10b, was shown to maintain

AP expression in DP cells and improve their hair-inducing property in comparison with untreated DP cells in an engraftment study [43]. Inhibition of glycogen synthase kinase-3, which inhibited Wnt signaling by phosphorylating β -catenin, enhanced the expression of AP in primary DP cell culture. Meanwhile, inactivation of the β -catenin gene within DP in fully developed hair follicles resulted in dramatically reduced proliferation of the progenitors and their progeny that generated the hair shaft [40]. In this study, we examined whether BMPs and Wnts had similar effects on SKPs and found that BMP4 had a potent effect on sustaining the hair inductive ability of SKPs, suggesting a potential application of BMP4 in culture expansion of SKPs, but Wnts did not show evident effect on AP expression in SKPs.

Epi-SCs in the epidermis are cells preferable for skin bioengineering because of their advantage in availability and proliferation potential in culture. Despite several populations of stem cells in the follicle showing potency for appendage regeneration [7–9], efficient isolation and expansion regimes for these cells have not been established. Recently, Epi-SCs in the basal layer epidermis have been suggested to participate in the regeneration of the hair follicle following skin wounding in mice [44]. However, it is not known whether Epi-SCs derived from adult human epidermis have this ability, nor is it known which cells are appropriate for inducing their action in hair genesis. In this study, we showed that Epi-SCs derived from adult human forehead and scalp retained the capacity to form *de novo* hair follicles and hairs, even after culture expansion. In addition, we found that SKPs derived from adult human dermis were sufficient to induce hair follicle and hair genesis. Importantly, we demonstrated, for the first time to our understanding, that a combination of cultured adult human Epi-SCs and SKPs were sufficient to generate *de novo* hair follicles and hairs.

The SG is an important structure in maintaining skin homeostasis. In addition to its role in protecting and waterproofing the hair and skin, the SG is important for hair fiber sheath dissociation and the integrity of the hair follicle [45]. Unfortunately, little has been understood about the regeneration of the SG [46]. This is in part because of the lack of a specific marker for recognizing sebocytes. SGs are conventionally identified by the presence of ample lipid droplets in the cytoplasm. However, this feature cannot be applied to immunofluorescence analysis of the cells. In this study, we found that sebocytes in the SG expressed high levels of biotin, which made them readily distinctive from other cells in the tissue. In addition, when Epi-SCs derived from the epidermis differentiated into sebocytes, the expression level of biotin markedly increased. Thus, we identified biotin as an appropriate marker for recognizing sebocytes in the skin and in culture.

The SG is formed by the pilosebaceous unit during skin morphogenesis in association with the hair follicle. Recently, multiple stem and progenitor cell compartments in the hair follicle have been suggested to contribute to the cyclic regeneration of the hair follicle and to the continuous renewal of the SG [47]. However, stem cells in the epidermis are more desirable for a tissue-engineering purpose because of their easy availability. Because we found that Epi-SCs derived from the epidermis were capable of forming hair follicles, we further examined their potential in the genesis of SGs. On the basis of the understanding of the crucial roles of Wnt and PPAR γ signals in SG morphogenesis [22, 23, 48], we examined their benefit in the differentiation of Epi-SCs

into sebocytes and the genesis of SGs in vivo. We found that Epi-SCs derived from neonatal mouse epidermis and the epidermis of human adult foreskin differentiated into sebocytes, in the presence of dexamethasone/insulin/rosiglitazone/XAV939, in which rosiglitazone was a PPAR γ agonist and XAV939 was a Wnt-signaling inhibitor. Interestingly, when Epi-SCs were implanted in wounds in combination with SKPs, they formed SGs in association with hair follicles; however, when Epi-SCs were engrafted with dermal fibroblasts, de novo SGs formed alone, independent of hair follicles; impressively, the presence of dexamethasone/insulin/rosiglitazone/XAV939 in the graft significantly increased the number of SGs formed. These results suggest that signals from SKPs are necessary for the genesis of the hair follicle and the SG complex but may not be essential for the genesis of individual SGs. Moreover, our data indicated that the neogenic SGs were functional in excreting lipids to the skin surface and protecting the skin from water loss. Thus, our study identified clinically applicable stem cells and efficient methods for SG regeneration.

CONCLUSION

This study provided convincing data showing that a combination of cultured Epi-SCs and SKPs derived from mice and adult humans were sufficient to generate de novo hair follicles and functional SGs. Our results provide transferable knowledge for the development of novel

bioengineered skin substitutes capable of regenerating epidermal appendages.

ACKNOWLEDGMENTS

We gratefully acknowledge Bing Yu and Wugen Zhan for expert technical assistance during the course of these studies. This work was supported by grants from Natural Science Foundation of China (31371404, 31571429), Natural Science Foundation of Guangdong (2015A030311041), and Shenzhen Science and Technology Innovation Committee (JCYJ20140417115840279, GJHZ20150316160614842, and JCYJ20160301150838144).

AUTHOR CONTRIBUTIONS

Xiaoxiao Wang, Xusheng Wang: conception and design, collection and/or assembly of data, data analysis and interpretation; J.L., Y.J., E.E.T., M.C.: provision of study materials or patients; T.C., L.G., S.W., J.W., Y.C., J.G.: collection and/or assembly of data; Y.W.: conception and design, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

REFERENCES

- Langer R, Vacanti JP. Tissue engineering. *Science* 1993;260:920–926.
- Fuchs E. Scratching the surface of skin development. *Nature* 2007;445:834–842.
- Martin P. Wound healing—Aiming for perfect skin regeneration. *Science* 1997;276:75–81.
- Boyce ST, Kagan RJ, Yakuboff KP et al. Cultured skin substitutes reduce donor skin harvesting for closure of excised, full-thickness burns. *Ann Surg* 2002;235:269–279.
- Blanpain C, Fuchs E. Epidermal homeostasis: A balancing act of stem cells in the skin. *Nat Rev Mol Cell Biol* 2009;10:207–217.
- Ehama R, Ishimatsu-Tsujii Y, Iriyama S et al. Hair follicle regeneration using grafted rodent and human cells. *J Invest Dermatol* 2007;127:2106–2115.
- Jaks V, Barker N, Kasper M et al. Lgr5 marks cycling, yet long-lived, hair follicle stem cells. *Nat Genet* 2008;40:1291–1299.
- Hsu YC, Pasolli HA, Fuchs E. Dynamics between stem cells, niche, and progeny in the hair follicle. *Cell* 2011;144:92–105.
- Morris RJ, Liu Y, Marles L et al. Capturing and profiling adult hair follicle stem cells. *Nat Biotechnol* 2004;22:411–417.
- Ito M, Liu Y, Yang Z et al. Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nat Med* 2005;11:1351–1354.
- Wang X, Tredget EE, Wu Y. Dynamic signals for hair follicle development and regeneration. *Stem Cells Dev* 2012;21:7–18.
- Hunt DP, Morris PN, Sterling J et al. A highly enriched niche of precursor cells with neuronal and glial potential within the hair follicle dermal papilla of adult skin. *STEM CELLS* 2008;26:163–172.
- Ramos R, Guerrero-Juarez CF, Plikus MV. Hair follicle signaling networks: A dermal papilla-centric approach. *J Invest Dermatol* 2013;133:2306–2308.
- Jahoda CA, Horne KA, Oliver RF. Induction of hair growth by implantation of cultured dermal papilla cells. *Nature* 1984;311:560–562.
- Driskell RR, Clavel C, Rendl M et al. Hair follicle dermal papilla cells at a glance. *J Cell Sci* 2011;124:1179–1182.
- Qiao J, Zawadzka A, Philips E et al. Hair follicle neogenesis induced by cultured human scalp dermal papilla cells. *Regen Med* 2009;4:667–676.
- Rendl M, Polak L, Fuchs E. BMP signaling in dermal papilla cells is required for their hair follicle-inductive properties. *Genes Dev* 2008;22:543–557.
- Toma JG, Akhavan M, Fernandes KJ et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 2001;3:778–784.
- Biernaskie J, Paris M, Morozova O et al. SKPs derive from hair follicle precursors and exhibit properties of adult dermal stem cells. *CELL STEM CELL* 2009;5:610–623.
- Frances D, Niemann C. Stem cell dynamics in sebaceous gland morphogenesis in mouse skin. *Dev Biol* 2012;363:138–146.
- Niemann C, Horsley V. Development and homeostasis of the sebaceous gland. *Semin Cell Dev Biol* 2012;23:928–936.
- Lien WH, Polak L, Lin M et al. In vivo transcriptional governance of hair follicle stem cells by canonical Wnt regulators. *Nat Cell Biol* 2014;16:179–190.
- Schmuth M, Jiang YJ, Dubrac S et al. The-matic review series: Skin lipids. Peroxisome proliferator-activated receptors and liver X receptors in epidermal biology. *J Lipid Res* 2008;49:499–509.
- Wang X, Ge J, Tredget EE et al. The mouse excisional wound splinting model, including applications for stem cell transplantation. *Nat Protoc* 2013;8:302–309.
- Bickenbach JR. Isolation, characterization, and culture of epithelial stem cells. *Methods Mol Biol* 2005;289:97–102.
- Lyu Z, Wang H, Wang Y et al. Maintaining the pluripotency of mouse embryonic stem cells on gold nanoparticle layers with nanoscale but not microscale surface roughness. *Nanoscale* 2014;6:6959–6969.
- Shi W, Nie D, Jin G et al. BDNF blended chitosan scaffolds for human umbilical cord MSC transplants in traumatic brain injury therapy. *Biomaterials* 2012;33:3119–3126.
- Hardman MJ, Sisi P, Banbury DN et al. Patterned acquisition of skin barrier function during development. *Development* 1998;125:1541–1552.
- Blanpain C, Fuchs E. Epidermal stem cells of the skin. *Annu Rev Cell Dev Biol* 2006;22:339–373.
- Fuchs E. Finding one's niche in the skin. *Cell Stem Cell* 2009;4:499–502.
- Toma JG, McKenzie IA, Bagli D et al. Isolation and characterization of multipotent skin-derived precursors from human skin. *STEM CELLS* 2005;23:727–737.
- Terunuma A, Cross JW, Dyke M et al. Behavior of human foreskin keratinocytes expressing a hair follicle stem cell marker CD200. *J Invest Dermatol* 2008;128:1332–1334.
- Cotsarelis G. Gene expression profiling gets to the root of human hair follicle stem cells. *J Clin Invest* 2006;116:19–22.
- Plikus MV, Mayer JA, de la Cruz D et al. Cyclic dermal BMP signalling regulates stem cell

activation during hair regeneration. *Nature* 2008;451:340–344.

35 Huelsken J, Vogel R, Erdmann B et al. beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell* 2001;105:533–545.

36 Smith KR, Thiboutot DM. Thematic review series: Skin lipids. Sebaceous gland lipids: Friend or foe? *J Lipid Res* 2008;49:271–281.

37 Böttcher-Haberzeth S, Biedermann T, Reichmann E. Tissue engineering of skin. *Burns* 2010;36:450–460.

38 Higgins CA, Chen JC, Cerise JE et al. Microenvironmental reprogramming by three-dimensional culture enables dermal papilla cells to induce de novo human hair-follicle growth. *Proc Natl Acad Sci USA* 2013;110:19679–19688.

39 Shimizu H, Morgan BA. Wnt signaling through the β -catenin pathway is sufficient to

maintain, but not restore, anagen-phase characteristics of dermal papilla cells. *J Invest Dermatol* 2004;122:239–245.

40 Enshell-Seiffers D, Lindon C, Kashiwagi M et al. β -catenin activity in the dermal papilla regulates morphogenesis and regeneration of hair. *Dev Cell* 2010;18:633–642.

41 Ohyama M, Kobayashi T, Sasaki T et al. Restoration of the intrinsic properties of human dermal papilla in vitro. *J Cell Sci* 2012;125:4114–4125.

42 Kishimoto J, Burgeson RE, Morgan BA. Wnt signaling maintains the hair-inducing activity of the dermal papilla. *Genes Dev* 2000;14:1181–1185.

43 Ouji Y, Nakamura-Uchiyama F, Yoshikawa M. Canonical Wnts, specifically Wnt-10b, show ability to maintain dermal papilla cells. *Biochem Biophys Res Commun* 2013;438:493–499.

44 Rompolas P, Mesa KR, Greco V. Spatial organization within a niche as a determinant of stem-cell fate. *Nature* 2013;502:513–518.

45 Sundberg JP, Boggess D, Sundberg BA et al. Asebia-2J (Scd1(ab2J)): a new allele and a model for scarring alopecia. *Am J Pathol* 2000;156:2067–2075.

46 Zhang S, Shui G, Wang G et al. Cidea control of lipid storage and secretion in mouse and human sebaceous glands. *Mol Cell Biol* 2014;34:1827–1838.

47 Jaks V, Kasper M, Toftgård R. The hair follicle—a stem cell zoo. *Exp Cell Res* 2010;316:1422–1428.

48 Dozsa A, Dezso B, Toth BI et al. PPAR γ -mediated and arachidonic acid-dependent signaling is involved in differentiation and lipid production of human sebocytes. *J Invest Dermatol* 2014;134:910–920.



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