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Keratin is not only a Structural Protein in Hair: Keratin-mediated Hair Growth

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Abstract

Keratin is known to be a major protein in hair, but the biological function of keratin in hair growth is unknown, which led us to conduct a pilot study to elucidate biological function of keratin in hair growth via cellular interactions with hair forming cells. Here, we show hair growth is stimulated by intradermal injection of keratin into mice, and show that outer root sheath cells undergo transforming growth factor-β2-induced apoptosis, resulting in keratin exposure. Keratin exposure appears to be critical for dermal papilla cell condensation and hair germ formation as immunodepletion and silencing keratin prevent dermal papilla cell condensation and hair germ formation. Furthermore, silencing keratin in mice resulted in a marked suppression of anagen follicle formation and hair growth. Our study imply a new finding of how to initiate hair regeneration and suggests the potent application of keratin biomaterial for the treatment of hair loss.

Introduction

Keratin is a cytoskeletal protein that forms intermediate filaments within epithelial cells and participates in maintaining the strength of the cells[1], and keratins are also the major proteins deposited inside the hair, contributing to its mechanical strength[2]. Human hair consists of three main layers: the medulla in the center of the hair, the cortex surrounding the medulla, which contains fiber mass mainly consisting of keratin protein, and the cuticle, the outer layer of the hair shaft[3]. During hair growth, dermal papilla (DP) cells secrete various paracrine factors to induce cell migration of stem cells from the bulge region of the outer root sheath (ORS) to the upper region of the follicle, and the migrated cells become transit amplifying cells, which then undergo differentiation into the matrix cells. Growth of the hair is initiated by cortical cells differentiated from matrix cells located in the follicle bulb region, and a large amount of keratin is synthesized mainly in the cortex[4–6]. Deposition and rearrangement of keratin filament is followed by the assembly of keratin-associated proteins and intracellular deposited keratin in spindle-shaped epithelial cells of the cortex, and the assembly is stabilized by the formation of inter- and intra-molecular disulfide bonds[7]. At the stages of the anagen-catagen transition of the hair cycle, apoptosis of cells appears in the epithelial strand, and then the apoptotic cells are phagocytosed by macrophages and neighboring epithelial cells, but simultaneously cellular organelles are degraded and removed. Ultimately, keratins remain the main proteins in the hair[8–11]. Such a massively deposited keratin in hair has recently been considered one of the potent biomaterials due to its good biocompatibility based on human origin and an abundant source of 300,000 tons of annually wasted hair worldwide. Keratin has been widely used for the development of various biomaterials for biomedical applications in the field of wound healing, nerve regeneration and bone regeneration[3].

In our previous study, mice models with full-thickness dorsal excisional wounds were used to assess the effect of keratin-based hydrogel on wound healing[12]. Interestingly, hair growth was found only in areas treated with keratin hydrogel with accelerated wound healing, which led us to study the biological function of hair-derived keratin in hair regeneration. In this study, keratin was extracted from human hair,

and mice model show hair follicle formation and following hair growth was promoted by intradermal injection of hair-derived keratin, and then the underlying biology of keratin to stimulate was studied by studying the interaction of keratin with DP cells and ORS cells, which are known as the main types of cells that regulate hair growth and regeneration. Hair growth was found to be modulated by keratin-mediated DP condensation and hair germ (HG) formation of ORS cells. Such DP condensation and HG formation was mediated by spatial release and deposition of keratin from the apoptotic ORS cells following TGFβ2-induced ORS cell apoptosis and caspase-mediated degradation of keratin. Our pilot study represents that keratin is not only a structural protein of hair but also a factor to induce hair regeneration.

Results

Intradermal Injection of Hair-derived Keratin Promotes Hair Growth

First, we performed *in vivo* experiments on mice to evaluate keratin-mediated hair growth by injecting hair-derived keratin into hair-removed dorsal skin area. The hairs on dorsal skin of C57BL/6 mice were removed by hair depilation cream with clipping for hair follicle synchronization, and then keratin was injected. After 14 days of keratin injection, we found that the hair growth was promoted (Fig. 1A) with higher formation of anagen follicles than in non-treated mice (Fig. 1B-D), and the sizes of hair follicles were found to be increased upon keratin injection (Fig. 1E, F). Only single injection of keratin resulted in almost equivalent or slightly higher hair growth in mice compared to minoxidil, applied every day for 14 days. Such keratin-mediated hair growth was also confirmed in a separate mouse study, and hair growth and hair follicle formation in keratin-injected C57BL/6 mice with different concentrations of 0.5 and 1.0 (w/v) % of keratin were analyzed for the number and stage of hair follicles. The promoting effect of keratin injection on hair growth was verified, and there were no significant differences in the number and stage of the formed hair follicles in mice injected with different concentrations of keratin (Supplementary Fig. 1A, B).

Keratin Induces Condensation of DP Cells and Germ Formation of ORS Cells In Vitro

To understand how injected keratin induces hair follicle formation and hair growth, we studied the extracellular interaction of keratin with DP and ORS cells, which are known to be main cells participating in hair follicle formation[4–6, 13]. The most distinct characteristic of DP cells exposed to keratin for 3 days was condensation to form spherical aggregates (Fig. 1G, H) with high expression levels of β-catenin, SOX2, CD133, and alkaline phosphatase (ALPase) (Fig. 1H), which is a molecular identity signature reflecting hair inductive property of DP cells[14,15], and high expression levels of FGF7, FGF10, and BMP6 (Fig. 1H), reflecting paracrine factors for controlling hair growth[16] (keratin-mediated condensation of DP

cells on day 1 and time-lapse images are presented in Supplementary Fig. 2A, C). However, the growth of DP cells was suppressed upon exposure to keratin (Supplementary Fig. 2B). Such keratin-mediated condensation of DP cells was observed, when cells were seeded at different cell densities, and on matrigel (Supplementary Fig. 3, 4), and no significant difference in DP cell condensation were not found when DP cells were treated with different keratin concentrations (Supplementary Fig. 5). RNA sequencing analysis showed a downregulation in expression levels of genes associated with cell division and an upregulation of mRNA-encoding proteins related to integrins, growth factors, migration, and extracellular matrix organization (Supplementary Fig. 6A, B). In addition, the effect of keratin on maintaining the DP cell condensation was analyzed. The DP cell spheroids showed higher levels of gene expression, indicating hair inductive property of DP cells as compared to a DP cell monolayer (Fig. 2A), and the spherical shape of DP cell aggregates was constantly maintained, showing high levels of molecular expression of β-catenin, SOX2, CD133, ALPase, FGF7, FGF10, and BMP6 (Fig. 2B-D) in the presence of keratin. ORS cells showed the formation of colony within a few hours of exposure to keratin and began to temporarily proliferate as early as on day 1 of cultivation, subsequently forming strand-like extended structures by day 3 (Fig. 3A, B). High β-catenin expression, known to occur during migration and further differentiation of stem cells in the ORS region[6], and a local cell population expressing P-cadherin, known to be a marker of secondary HG formation[17,18], were observed along with extended structures in keratin-treated ORS cells (Fig. 3C, low-magnification images of β-catenin and P-cadherin expression in keratin-treated ORS cells are presented in Supplementary Fig. 7). In addition, keratin-treated ORS cells showed lower expression levels of CD34 compared to untreated ORS cells, but maintained high levels of SOX9 expression (Fig. 3C). Furthermore, RNA sequencing analysis of keratin-treated ORS cells revealed upregulated mRNA expression levels of acidic hair keratins, mainly KRT31, KRT33B, KRT34, and KRT37 (Supplementary Fig. 8A). We also observed an increased molecular expression of KRT34 and β-catenin (Supplementary Fig. 8B). These findings imply hair keratin-mediated alterations in molecular and gene expression profiles indicating germ formation and further differentiation of ORS cells.

TGFβ2 induces Apoptosis of ORS cells and generates Keratin Release and Deposition, mediating Condensation of DP Cells

The findings described above demonstrated that extracellular interaction of keratin induced the condensation of DP cells and the formation of P-cadherin expressing germs of ORS cells, which led us to ask the question whether the observed pattern of the interaction of DP and ORS cells with keratin might be related to a biological event that occurs during hair cycling. During the anagen-catagen transition stage, TGFβ2 is synthesized from DP cells stimulated by dihydrotestosterone, and is spatiotemporally localized in the lower part of the hair bulb at the catagen stage, thus suppressing the proliferation of epithelial cells, but inducing caspase-mediated apoptosis[11]. Therefore, we hypothesized that exposure of keratins from apoptotic ORS cells during hair cycling might drive DP cell condensation and secondary HG formation through extracellular interaction with DP and ORS cells. To address this question, we induced

apoptosis of ORS cells by treating with TGFβ2 and characterized microenvironmental changes, such as the release or deposition of keratin. In our study, apoptosis array analysis showed upregulation of apoptosis-related markers, including Bax, caspase-3, cytochrome C, and SMAC in ORS cells treated with TGFβ2 (Fig. 4A, Supplementary Fig. 9). Extended structures composed of spindle-shaped ORS cells developed only in the presence of TGFβ2; annexin V and tunnel positive apoptotic cells were mainly found in the extended structures of TGFβ2-treated ORS cells. High expression levels of caspase-3 and massive deposition of keratin were observed along the extended structures (Fig. 4B). To determine whether the released or deposited keratin from TGFβ2-induced apoptotic ORS cells could influence DP cell condensation, the condensation activity of DP cells was tested by direct contact co-culture and culture in conditioned media. Local condensation of DP cells with the formation of spherical cell colonies was observed in the concentric region of the extended structure in TGFβ2-treated ORS cell layers (Fig. 4C, Supplementary Fig. 10A). The conditioned medium collected from TGFβ2-treated ORS cell cultures contained relatively higher levels of keratin, and the DP cell condensation to form spherical cell aggregates were distinctly improved in the DP cell culture under the conditioned media (Fig. 4D, Supplementary Fig. 10B). These results indicate that the deposition or release of keratin from TGFβ2-induced apoptotic ORS cells could be a regulator of the induction of DP cell condensation.

Keratin Release and Deposition through Caspase-6-mediated Keratin Degradation stimulates Condensation of DP Cells

Keratin fragmentation occurs during apoptosis of epithelial cells[19]; intracellular insoluble keratin is disposed during apoptosis via fragmentation into soluble fragments by caspases[20]. In our study, apoptosis array analysis showed a two-fold increase in caspase-3 expression levels in TGFβ2-treated ORS cells, and it was reported that type I keratin, including hair keratin, contains a cleavage site, VEVD, for caspase-6[21]. When hair keratin was digested with caspase-3 and caspase-6, fragmented keratin was generated only in hair keratin digested by caspase-6 (Fig. 5A), and higher gene expression and protein expression levels of caspase-6 and its cleaved form (active caspase-6) were observed in TGFβ2-treated ORS cells (Fig. 5B, C). This finding implies that the release and deposition of keratin from TGFβ2-treated ORS cells through caspase-6-mediated degradation can influence the DP cell condensation. Hence, to test this, we silenced the expression of caspase-6 gene in ORS cells and examined the levels of released keratin (Fig. 5D). We observed lower levels of released keratin in caspase-6-silenced ORS cells in the presence of TGFβ2 (Fig. 5E) and lower condensation activity of DP cells in conditioned media collected from caspase-6-silenced ORS cell culture and in co-culture on the caspase-6-silenced ORS cell layer in the presence of TGFβ2 (Fig. 5F-H). Caspase-6-silenced ORS cells developed relatively spread out structures even in the presence of TGFβ2, which were different from the extended structures of TGFβ2-treated non-silenced ORS cells. Tunnel positive apoptotic cells were found in both the spread and extended structures, and it was found that keratin deposition is influenced by caspase-6 expression levels (Fig. 5H, Supplementary Fig. 11). We then performed an immunodepletion assay to obtain direct evidence for

keratin-mediated condensation of DP cells by removing keratin from the conditioned medium of TGFβ2-treated ORS cells using a column containing anti-human type I + II hair keratin antibody-conjugated beads. The elimination of keratins in conditioned media was confirmed (Fig. 6A), and there was no substantial difference in the growth factors contained in the conditioned media before and after immunodepletion (Fig. 6B, Supplementary Fig. 12). The immunodepletion assay showed that the removal of keratins resulted in suppressed DP cell condensation (Fig. 6C), and collectively these data reflected the functional role of keratins in DP condensation.

Spatial Keratin Deposition by TGFβ2-induced Apoptotic ORS cells induces Germ Formation

Along with keratin-mediated condensation of DP cells, to understand how the release or deposition of keratin during TGFβ2-induced ORS cell apoptosis triggers germ formation, the keratin released from TGFβ2-induced ORS cells were tested for its ability to induce germ formation. Contrary to DP cell condensation, the released keratin in conditioned media was not effective in generating P-cadherin expressing cell population, which was proved by immunodepletion assay (Supplementary Fig. 13). This result prompted us to ask whether secondary HG formation in cells expressing P-cadherin is influenced by the spatially deposited keratin caused by spatiotemporal apoptosis of ORS cells. TGFβ2 expression, which was restricted to the outermost ORS cell layer in the anagen phase, was reported to be upregulated spatiotemporally in the boundary region between germinal matrix cell and the DP cell in the lower bulb region during the anagen-catagen transition[8]. Therefore, to study the spatial deposition of keratin from TGFβ2-induced apoptotic ORS cells and its effect on the germ formation of ORS cells, the time-course effect of TGFβ2 treatment on protein expression levels of caspase-6, keratin deposition, and germ formation were characterized. The extended structure progressively developed in the TGFβ2-treated ORS cell layers over the cultivation time, and the P-cadherin expressing germ was spatially developed in the TGFβ2-treated ORS cell layers (Supplementary Fig. 14). Immunocytochemical staining of the TGFβ2-treated ORS cell layers showed that a population of RUNX1 and P-cadherin-positive cells, representative markers of germ formation[10,22–24], emerged in the concentric region of the extended structure, and the caspase-6-expressing apoptotic cell population and keratin-deposited area also increased over time in the extended structure (Supplementary Fig. 14). Next, to consider the effect of spatial deposition of keratin on the formation of P-cadherin expressing germ *in vitro*, the expression of KRT31/KRT34 in ORS cells was silenced by siRNA transfections. The downregulated molecular expressions of keratin in both conditioned media and ORS cells via KRT31/KRT34-silencing were characterized (Fig. 6D). Contrary to the well-developed stranded structure in the ORS cell layers, the KRT31/KRT34-silenced ORS cells did not form an extended structure even in the presence of TGFβ2, and keratin deposition and emergence of RUNX1 and P-cadherin expressing ORS cell population were markedly suppressed in the KRT31/KRT34-silenced ORS cell culture in the presence of TGFβ2 (Fig. 6D).

In Vivo Silencing of Keratin Expression Suppresses Anagen Hair Follicle Formation and Hair Growth

Combining with all *in vitro* data from the studies of extracellular interaction of keratin, the release and deposition of keratin from TGFβ2-mediated apoptotic ORS cells have shown a pivotal role in controlling DP condensation and HG formation. Finally, to determine whether the silenced KRT31/KRT34 expression can suppress hair follicle formation and hair growth *in vivo*, the keratin expression in mice was temporarily silenced by intravascular lipofectamine-mediated delivery of KRT31/KRT34 siRNAs. RT-PCR analysis showed effective KRT31/KRT34 silencing, as indicated by downregulation of KRT31/KRT34 mRNA expressions on day 7 (Fig. 7A), and it was found that the KRT31/KRT34 silencing significantly inhibited hair growth activity compared to the control (Fig. 7B). Notably, the dysregulation of the hair follicle cycling was observed in KRT31/KRT34 silenced mice; histological analysis of hair follicle sections showed a strong suppression of the formation of anagen follicles, with no appearance of anagen follicles in 56% of skin tissue sections of KRT31/KRT34 silenced mice on day 7 (Fig. 7C). An anagen bulb containing a population of cells expressing P-cadherin was hardly seen in immunohistological sections of KRT31/KRT34 silenced mice (Fig. 7D, Supplementary Fig. 15A). In contrast, an additional injection of hair-derived keratin after KRT31/KRT34 siRNA transfection allowed the hair follicles to enter the anagen phase and regrow hair, similar to the controls. No obvious histological differences were found in the formation of hair follicles and hair growth between control skin and keratin-injected skin of KRT31/KRT34 silenced-mice after 2 weeks (Fig. 7E). Furthermore, the formation of P-cadherin-positive germs and strong expression of β-catenin were observed in the region of anagen hair follicles in sections of control skin and keratin-injected skin of KRT31/KRT34 silenced-mice (Fig. 7D, Supplementary Fig. 15A); strong staining for KRT34 was found in the ORS region surrounding the DP, which corresponds to the caspase-6-positive region (Supplementary Fig. 15B). Interestingly, it was found that the region stained positively for caspase-6, KRT34, and P-cadherin move upward into the hair shaft region of the expanded hair follicles (Supplementary Fig. 15B).

Discussion

Hair loss disorders, such as androgenetic alopecia, have been common these days, leading to the development of new pharmaceuticals for patients with hair loss. In the present study, keratin, the main structural protein in hair, is proposed as a new candidate for a therapeutic treatment of hair loss. It is shown here that an intradermal injection of human hair-derived keratin promotes hair growth with the enhanced formation of anagen hair follicle and an increase in the size of hair follicles. Hair growth is controlled by the interactions between two distinct cell types: mesenchyme and epithelial cells, while DP and stem cells from the bulge region of ORS participate in hair follicle formation[4–6, 13]. The *in vivo* injected keratin-mediated follicle formation and hair growth could be associated with keratin-mediated DP cell condensation and germ formation via cellular interaction with keratin, as evidenced by our results of strong expressions of various signature molecules, such as β-catenin and P-cadherin, highly expressed in hair follicle formation[4–6, 13], in keratin-treated DP cell and ORS cell culture. The secondary HG

expressing P-cadherin emerges at the end of the catagen and at the beginning of the telogen and leads to the first stage of hair regeneration[17,18]. The interaction of P-cadherin with β-catenin plays an important role in maintaining the anagen phase of the hair cycle[25], and the β-catenin expressing migrated cells from the bulge region of the ORS undergo temporal proliferation and further differentiation in the follicle region[6]. Keratin-treated ORS cells showed morphological change, such as spindle shape with strong expression of β-catenin and downregulated molecular expression of CD34, and a population of P-cadherin expression cells emerged. It was reported that CD34-positive stem cells convert directly into P-cadherin expressing HG cells[23]. Hair regeneration is processed by hair follicles undergoing repeated cycles of anagen (hair growth stage), catagen (regression stage), and telogen (rest stage)[26]. During the late catagen and early telogen phases, secondary HG progressively appears at the base of the follicular epithelium; at that point, HG cells form a cell cluster and become activated to begin hair regeneration[10]. During these phases, DP cells undergo condensation to form a follicular papilla beneath secondary HG. The interaction between the DP condensate and secondary HG leads to the formation of new hair follicles by enveloping the DP with the downwardly extended epithelial cells[10,22,27]. However, in spite of recent considerable progress in understanding cellular interactions to control hair growth, it is not clear how secondary HG formation and the DP condensation, the key biological events causing hair regeneration, are initiated at the beginning of a new hair cycle.

Considering *in vivo* an *in vitro* results of keratin-mediated hair growth and keratin-mediated cellular change, keratin might not be only a structural component in hair, but might have biological function in hair growth, and biological phenomena such as keratin-mediated DP cell condensation and germ formation might be closely related to a biological process that occurs during hair cycling. Hence, keratin-mediated condensation of DP cells and the formation of P-cadherin germs, assessed by *in vitro* cell study, led us to explore the biological function of keratin in hair regeneration as a pilot study. At the anagen-catagen transition stages of hair cycle, the local deposition of TGFβ2 in the lower region of the follicle is restricted due to the spatiotemporal secretion of TGFβ2 produced by DP cells[11], which is consistent with the spatial gradient of apoptosis of epithelial cells[9]. Spatiotemporally localized TGFβ2 induces apoptosis of ORS cells in the lower part of the hair bulb at the catagen stage, resulting in the expression of caspase and its-mediated the fragmentation of insoluble keratin into soluble keratin fragments[8–11,19]. Although the relationship between TGFβ2 expression and the intrinsic property of DP cells related to condensation is not well known, our data showed that TGFβ2 expression was downregulated during DP cell condensation and rapidly upregulated during the dispersion of condensed DP cells (Supplementary Fig. 16A, B). Mesenchyme condensation such as dermal condensates is promoted by BMP signaling and transient downregulation of TGF-β signaling, showing an antagonistic relationship[16,28,29]. The condensed DP cells might maintain the property of DP cells[27] to drive hair growth by releasing paracrine factors, which can induce stem cell activation and differentiation during the anagen phase, and may induce spatiotemporal apoptosis of adjacent ORS cells via increased expression of TGFβ2 during the anagen to catagen transition. In our study, it was shown that local DP cell condensation and germ formation in TGFβ2-induced apoptotic ORS cells depend on the exposure of keratin via caspase-6 expression and

consequently its-mediated keratin release or deposition, as evidenced by the suppressed DP cell condensation and germ formation in caspase-6-silenced or KRT31/KRT34-silenced ORS cell culture, even if there was no distinct difference in TGFβ2-mediated ORS cell apoptosis. Finally, it was found that the formation of anagen hair follicles and hair growth are suppressed in temporally KRT31/KRT34 silenced mice, which could be recovered by intradermal injection of additional exogenous keratin.

Taken together, the results presented in this study reveal that hair regeneration is regulated by keratin-mediated germ formation and DP condensation through biological events, including TGFβ2-induced ORS cell apoptosis, caspase-mediated degradation of keratin, and spatial release and deposition of keratin from the apoptotic ORS cells. Our pilot study indicate that keratin is not only a major structural component of hair but can also play a functional role in the induction of HG formation and DP condensation, facilitating entry into a new hair cycle. However, the precise identification of our proposed cellular function of keratin in hair growth using a proper xenograft model is necessary, which is going on as continuous study. In conclusion, considering the biological function of keratin in hair growth, our study suggests that keratin can be a potent biomaterial for developing therapeutic agents for hair loss treatment, and understanding how cellular behavior is regulated by spatiotemporal keratin release and deposition from apoptotic epithelial cells can provide additional insight into deciphering cellular interactions between epithelial cells and mesenchyme in the morphogenesis of other tissues.

Methods

Experimental Design

The aim of this study was to understand the biological function of keratin in hair growth. Firstly, to determine the activity of hair growth, back-skin hairs in mice were removed, and hair follicle formation and hair regrowth was evaluated after intradermal injection of hair-derived keratin (Fig. 1A-F). Next, to define cellular interaction of keratin with major cells participating in hair growth, cellular behavior such as DP cell condensation and germ formation was studied by treating keratin in *in vitro* DP cell and ORS cell culture (Fig. 1G-H, Fig. 2 and Fig. 3). From the results (Fig. 1-3), it was hypothesized that keratin-induced hair growth could be closely related to a biological cascade happened during hair cycle with regards to the exposure of keratin from TGF-b2-induced apoptotic ORS cells at stages of anagen-catagen transition. To characterize keratin release or deposit from apoptotic ORS cells, apoptosis of ORS cells and its following keratin release and deposit from TGF-b2-treated ORS cells were evaluated, and then DP cell condensation was observed by the direct co-culture with TGF-b2-treated ORS cells and the DP cell culture in conditioned medium collected from TGF-b2-treated ORS cell culture to evaluate the effect of released or deposited keratin from apoptotic ORS cells on DP cell condensation (Fig. 4). Following DP cell condensation induced by released and deposited keratin from apoptotic ORS cells, apoptosis-related caspase 3 and caspase 6 expressions and caspase-mediated keratin degradation were characterized, and the release and deposit of keratin through caspase-mediated keratin degradation in TGF-b2-treated apoptotic ORS cells and its effect on DP cell condensation were evaluated by *in vitro* siRNA-mediated silencing of caspase 6 mRNA expression in TGF-b2-treated apoptotic ORS cells (Fig. 5). To confirm the

effect of keratin released from TGF-b2-treated apoptotic ORS cells on DP cell condensation, the keratin were eliminated from conditioned medium collected from TGF-b2-treated cell culture by immunodepletion, and DP cell condensation was evaluated with DP cell culture in the keratin-eliminating conditioned medium collected from TGF-b2-treated apoptotic ORS cells (Fig. 6A-C). In company with proving the effect of keratin released from TGF-b2-treated apoptotic ORS cells on DP cell condensation, mRNA expressions of keratin 31 (KRT31) and keratin 34 (KRT34) were silenced in ORS cells by *in vitro* KRT31/KRT34 siRNA transfection, and then P-cadherin expressing germ formation of ORS cells was observed in KRT31/KRT34-silenced ORS cell culture in the presence of TGF-b2 to evaluate the effect of keratin spatially deposited from TGF-b2-treated apoptotic ORS cells on germ formation (Fig. 6D). Finally, to study the role of keratin in hair follicle formation and hair growth *in vivo*, KRT31/KRT34 silencing was processed by Invivofectamine KRT31/KRT34 siRNA transfection to mice (Fig. 7).

Cell Culture

Human outer root sheath cells (ORS; CEFO, CB-ORS-001) and human dermal papilla cells (DP; CEFO, CB-HDP-001) were purchased and expanded in each human outer root sheath cell growth medium (CEFO, CB-ORS-GM) and human dermal papilla growth medium (CEFO, CB-HDP-GM) at 37°C in a humidified atmosphere containing 5 % CO_2 according to the manufacturer's instructions. Cultures were fed every two days and passaged by treatment with 0.25 % trypsin/EDTA (Gibco, 25200056), and the expanded DP cells within 5 passages and ORS cells within 3 passages were used in this study.

Human Hair Keratin Extraction

Human hair keratin was extracted by slightly modified Sindai method, as reported previously [12], and kindly provided by Gapi Bio. Detail methods of microwell fabrication are available in Supplementary Methods.

Human Hair Keratin-mediated Hair Growth Test in Mice

For *in vivo* studies, male C57BL/6 mice were used, which were purchased from YoungBio (Samtako, 1404957265). The mice were housed under controlled condition at a temperature of 23 ± 2°C, humidity of 50 ± 5%, and light-dark cycle of 12 h. Mice were provided with a laboratory diet and water *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee of Konkuk University (KU18159, KU19066), and procedures on animals were performed in accordance with the relevant guidelines and regulations. The hair on the dorsal skin of mice was shaved repeatedly using an electric clipper to synchronize the hair follicle cycle. Before treatment, the dorsal hair was completely removed using the commercial hair removal cream Veet® (Reckitt Benckiser, 62200809951).

To examine the hair growth promoting effect of keratin, 1.0 (w/v)% keratin in phosphate buffered saline (PBS; Gibco, 10010023) was used. 10-week old mice were shaved repeatedly to synchronize the hair cycle and randomly assigned to three groups: Neg. Con group; 3% Minoxidil group with daily topical application of 100 μL of 3% Minoxidil (Minoxyl® 3%; Hyundai Pharm, Co., Seoul, Korea); 1.0 (w/v)% Keratin group with intradermal injection of 100 μL of 1.0 (w/v)% keratin once. The mice were sacrificed after 2 weeks.

Detail methods of the hair growth promoting effect of keratin according to keratin concentration are available in Supplementary Methods.

Interaction Assay of DP Cells with Keratin

DP cells were seeded at a density of 2×10^4 cell/cm^2 on 12 well and 6 well non-treated tissue culture plate (SPL LIFE SCIENCES, 32012, 32006). The DP cells were adjusted to be stable for 1 day in human dermal papilla growth medium (CEFO, CB-HDP-GM) at 37°C in a humidified atmosphere containing 5 % CO_2 prior to keratin treatment. After 1 day of adjustment, DP cells were cultured in human dermal papilla growth medium containing 1.0(w/v)% keratin or not. The morphological change of DP cells in the presence of keratin was observed under inverted fluorescent microscopy (Olympus IX71), and the number of condensed DP cell aggregates was counted. Cell proliferation upon keratin treatment was measured using Cell Counting Kit-8 (Dojindo Molecular Technologies, CK04-20). DP cells were seeded on 12 well non-treated tissue culture plate (SPL LIFE SCIENCES, 32012) at a seeding density of 1×10^4 cells/cm^2, and cultured in human dermal papilla growth medium (CEFO, CB-HDP-GM) containing 1.0(w/v)% keratin or not in a humidified atmosphere of 5% CO_2 at 37°C, and the medium was refreshed every two days. At specific time points (1, 3 and 5 days), each well had 10 μL of the Cell Counting Kit-8 solution added and then was incubated at 37°C for 2 h. Cell proliferation assays were performed in a 96-well plate reader by measuring the absorbance at a wavelength of 450 nm.

For DP cell condensation assay according to different cell seeding density, DP cells were seeded at a seeding density of 5×10^3 cell/cm^2, 1×10^4 cell/cm^2 and 2×10^4 cell/cm^2 on 6 well non-treated tissue culture plate (SPL LIFE SCIENCES, 32006). The DP cells were adjusted to be stable for 1 day in human dermal papilla growth medium (CEFO, CB-HDP-GM) at 37°C in a humidified atmosphere containing 5 % CO_2 prior to keratin treatment. After 1 day of adjustment, DP cells were cultured in human dermal papilla growth medium containing 1.0(w/v)% keratin or not. The number of condensed DP cell aggregates was counted using inverted fluorescent microscopy (Olympus IX71).

Detail method of interaction assay of DP cells with keratin on Matrigel is available in Supplementary Methods.

Interaction Assay of ORS Cells with Keratin

ORS cells were seeded at $2x10^4$ cell/cm^2 on 12 well and 6 well tissue culture plate (SPL LIFE SCIENCES, 30012, 30006). The ORS cells were adjusted to be stable for 1 day in human outer root sheath cell growth medium (CEFO, CB-ORS-GM) at 37°C in a humidified atmosphere containing 5 % CO_2 prior to keratin treatment. After 1 day of adjustment, ORS cells were cultured in human dermal papilla growth medium containing 1.0(w/v)% keratin or not. The morphological change of ORS cells in the presence of keratin was observed under inverted fluorescent microscopy (Olympus IX71) and time-lapse images were captured. Cell proliferation upon keratin treatment was measured using Cell Counting Kit-8 (Dojindo Molecular Technologies, CK04-20). ORS cells were seeded on 12 well tissue culture plate (SPL LIFE SCIENCES, 30012) at a seeding density of 1×10^4 cells/cm^2, and cultured in human outer root sheath cell growth medium (CEFO, CB-ORS-GM) containing 1.0(w/v)% keratin or not in a humidified atmosphere of 5% CO_2 at 37°C, and the medium was refreshed every two days. At specific time points (1, 3 and 5 days), each well had 10 µL of the Cell Counting Kit-8 solution added and then was incubated at 37°C for 2 h. Cell proliferation assays were performed in a 96-well plate reader by measuring the absorbance at a wavelength of 450 nm.

RNA Extraction and Sequencing

To perform transcriptome sequencing (RNA-Seq) analysis of DP cells and ORS cells, total RNA was extracted from the ORS and DP cells in the absences of keratin and in the presence of keratin. Detail methods of RNA extraction, sequencing and differential gene expression analysis are available in Supplementary Methods.

DP Cell Spheroid Formation and Maintenance assay of the replated DP Cell Spheroids

For DP cell spheroid formation, cell spheroids as a micro tissue unit were generated by docking DP cells into polyethylene glycol (PEG) microwell array with 450 µm in diameter. PEG microwells were fabricated by microfabrication procedures, reported previously[30]. Detail methods of DP cell spheroid formation and maintenance assay are available in Supplementary Methods.

TGFb2-mediated ORS Cell Apoptosis and Co-culture with DP Cells

ORS cells were seeded at $2x10^5$ cell/cm^2 on 12 well tissue culture plate (SPL LIFE SCIENCES, 30012) to make confluent ORS cell layer. The ORS cells were adjusted to be stable for 1 day in human outer root sheath cell growth medium (CEFO, CB-ORS-GM) at 37°C in a humidified atmosphere containing 5 % CO_2. After 1 day of adjustment, ORS cells were cultured in human dermal papilla growth medium containing 100ng/ml TGFb2 (PeproTech, 100-35B) for 5 days, and the media was refreshed every day. Detail methods of DP cell condensation in direct co-culture of DP cells and TGFb2-treated ORS cells, and under conditioned media from TGFb2-treated ORS cell layer are available in Supplementary Methods.

Immunodepletion Study

To study the role of keratin released from TGFb2-induced apoptotic ORS cells in DP condensation and germ formation of ORS cells, the released keratin in conditioned media from TGFb2-treated ORS cell layer culture was removed by immunodepletion method. First, antibodies-conjugated beads were prepared as follows; 150ml of nProtein A Sepharose (GE Healthcare, 17528001) was incubated with 400ml of guinea pig anti-Type I+II Hair Keratins antibody (PROGEN, GP-panHK) or guinea pig normal IgG (Sigma-Aldrich, I4756), as another negative control, for 18 h at 4°C. Non-specific binding was prevented with blocking buffer containing 1% bovine serum albumin (BSA; Sigma-Aldrich, A9418) in TBS (Tris-Buffered Saline; Biosesang, TR2005-000-74) with 0.1% Tween 20 (Duchefa Biochemie, P1362.1000) for 3 h at 4°C. The conditioned media were collected from TGFb2-treated ORS cell layer cultured for 5 days, and 30ml of the conditioned media were mixed with 75ml antibodies-conjugated beads, and then incubated with gentle shaking overnight at 4°C. After incubation, antibodies-conjugated beads were removed by passing the mixture through a Centrifuge Columns (Thermo Scientific, 89898). Detail methods of DP cell condensation and P-cadherin expressing germ formation of ORS cells under keratin-removed conditioned media are available in Supplementary Methods.

Caspase-3 and Caspase-6-mediated Hair Keratin Digestion Assay

1(w/v)% hair keratin was dissolved in the reaction solution composing of 50mM HEPES (Gibco, 15630-080), 50mM NaCl (JUNSEI CHEMICAL, 19015-1250), 0.1% CHAPS (Sigma-Aldrich, C3023), 10mM EDTA (Sigma-Aldrich, 03609), 5% glycerol (SAMCHUN CHEMICALS, G0274) and 10mM DTT (Sigma-Aldrich, 43815) at pH 7.2. 5U/ml Casase-3 (Enzo, ALX-201-059) or 5U/ml Casase-6 (Enzo, ALX-201-060) was added to the reaction solution containing hair keratin and incubated at 37°C for 0, 1, 3 and 24 hrs. After the reaction, samples were denatured on 70°C for 10 min in LDS sample buffer (Invitrogen, B0007). Equal amounts of denatured samples were loaded in pre-casted 4-12% Bis-Tris Plus Gels (Invitrogen, NW04120BOX), and the electrophoresis was done by running at 200 V for 22 min. The gel was rinsed three times with distilled water for 5 min each and stained by SimplyBlue SafeStain (Invitrogen, LC6060). After 1 hr of staining, the gel was rinsed using distilled water until the background was removed thoroughly, and then images of the gel was obtained using a commercialized scanner (Canon, TS8090).

In Vitro Caspase-6 Gene Silencing Study

To evaluate the effect of caspase-6 mediated keratin degradation during TGFb2-induced ORS cell apoptosis on keratin release or deposition and DP condensation, caspase-6 gene expression in ORS cells was silenced by caspase-6 siRNA transfection. Detail methods of *in vitro* caspase-6 gene silencing study are available in Supplementary Methods.

In Vitro KRT31/KRT34 Gene Silencing Study

To evaluate the effect of KRT31/KRT34 gene silencing during TGFb2-induced ORS cell apoptosis on keratin release or deposition and germ formation of ORS cells, KRT31 and KRT34 gene expressions in ORS cells were silenced by KRT31/KRT34 siRNA transfection. Detail methods of *in vitro* KRT31/KRT34 gene silencing study are available in Supplementary Methods.

Apoptosis and Growth Factor Antibody Array

TGFb2-induced ORS cell apoptosis was evaluated by comparative analysis using human apoptosis antibody array (Abcam, ab134001), and the comparative analysis of growth factors present in the conditioned medium collected from TGFb2-treated ORS cell culture in immunodepletion study were done using human growth factor antibody array (Abcam, ab134002) according to manufacturer's instructions. Detail methods of apotosis array and growth factor antibody array analysis are available in Supplementary Methods.

Western Blot Analysis

Molecular expressions of KRT34 and b-catenin in hair keratin-treated ORS cells, keratin content at protein level in conditioned medium collected from TGFb2-treated ORS cell culture, the keratin content in keratin-removed condition medium collected from TGFb2-treated ORS cell culture in immunodepletion study, keratin content at protein level in conditioned medium collected from KRT31/KRT34-silenced ORS cell culture or molecular keratin expression in KRT31/KRT34-silenced ORS cell, molecular expressions of caspase 6 in TGFb2-treated ORS cells and keratin content at protein level in conditioned medium collected from caspase 6-silenced ORS cell culture were evaluated by western blot analysis. Detail method of western blot analysis is available in Supplementary Methods.

Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)

The gene expressions indicative of DP cell's intrinsic property and TGFb2 gene expressions of DP cell spheroids and the replated DP cell spheroids were evaluated by RT-qPCR. Detail method of RT-qPCR analysis is available in Supplementary Methods.

Indirect Enzyme-Linked Immuno-Sorbent Assay (ELISA)

The molecular expressions indicative of DP cell's intrinsic property from the replated DP spheroids cultured in the presence of keratin were evaluated by ELISA. Detail method of ELISA is available

in Supplementary Methods.

In Vivo KRT31/KRT34 Gene Silencing Study

To confirm the effect of keratin on hair growth, KRT31/KRT34 were silenced by lipofectamine-mediated delivery of KRT31/KRT34 siRNAs. First, Invivofectamine complex for KRT31/KRT34 siRNA delivery was prepared as follows; siRNAs of KRT31 (Bioneer, 16660-1), KRT34 (Bioneer, 16672-1) and negative control (Bioneer, SN-1003) were purchased, and siRNAs of KRT31 and KRT34 were dissolved in RNase-free water as each 24 mg/ml concentration respectively. The two solutions, KRT31 siRNA and KRT34 siRNA, were combined as 1:1 volume ratio to be 12 mg/ml of final concentration. 12 mg of negative control siRNA was also dissolved in 1 ml of RNase-free water. siRNAs-Invivofectamine (Thermo Fisher Scientific, IVF3005) complex was prepared by the manufacturer's instruction, and 0.5 mg/ml of complex was prepared finally prior to injection to mice. For in vivo study, 6-week-old mice were shaved repeatedly to synchronize the hair cycle and randomly assigned to three groups: Con group with IV injection of 200 µl of negative control siRNA injection; siRNA group with IV injection of 200 µL of KRT31/KRT34 siRNA injection; siRNA+Keratin group with KRT31/KRT34 siRNA injection (IV, 200 µl) and intradermal injection of total 100 µl of keratin a day after first siRNA injection. For each group, mice were sacrificed at either day 7 or day 14. Pictures of the back skin were taken at day 3, 7, 10, and 14 to examine the hair growth. The silencing of KRT31/KRT34 gene expressions was confirmed by RT-qPCR. Detail method of RT-qPCR analysis is available in Supplementary Methods..

Histological Analysis

The skin tissues were fixed with 10% neutral-buffered formalin (BBC Biochemical, 0141). The tissues were embedded in paraffin and sectioned at 4 µm thickness, followed by staining with hematoxylin and eosin for histological analysis. The number of hair follicles in each cycle and diameter of anagen hair follicles were quantified in multiple fields on perpendicular sections at ×100 magnification.

Immunocytochemical and Immunohistochemical Staining

Detail methods of immunocytochemical and immunohistochemical staining are available in Supplementary Methods.

Statistical Analysis

All values obtained from *in vitro* and *in vivo* analysis are presented as the mean ± standard deviation (SD). Statistically significant differences were identified by two-sided Student’s t-test or one-way ANOVA parametric test. A P-value of less than 0.05 was considered significant.

Declarations

Data Availability

The transcriptome sequencing data (RNA-Seq) have been deposited at NCBI GenBank under BioProject ID PRJNA576064 (BioSample SAMN12924151 - SAMN12924158), and data are available in the private reviewer link, https://dataview.ncbi.nlm.nih.gov/object/PRJNA576064?reviewer=foebp22iqlsa9tg7mvrerhdbm6

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Author Contributions

S.Y.A. performed most of the experiments and wrote the paper. S.Y.K. extract and purified human hair-derived keratin. S.Y.V. extract and purified human hair-derived keratin and analyzed gene expressions using real time-qPCR. E.J.C. carried out *in vivo* silencing experiment and histological analysis. H.J.K. carried out *in vivo* mouse experiment and histological analysis. J.H.L. carried out RNA sequencing and data analysis. S.W.H. and I.K.K. discussed results of the experiments and commented on the manuscript. C.K.L. carried out *in vivo* mouse experiment and histological analysis. Y.S.H. and S.H.D. directed the project, and Y.S.H. drafted the manuscript with input from all authors.

Competing Interests

The authors declare that they have no conflict of interest.

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Figures

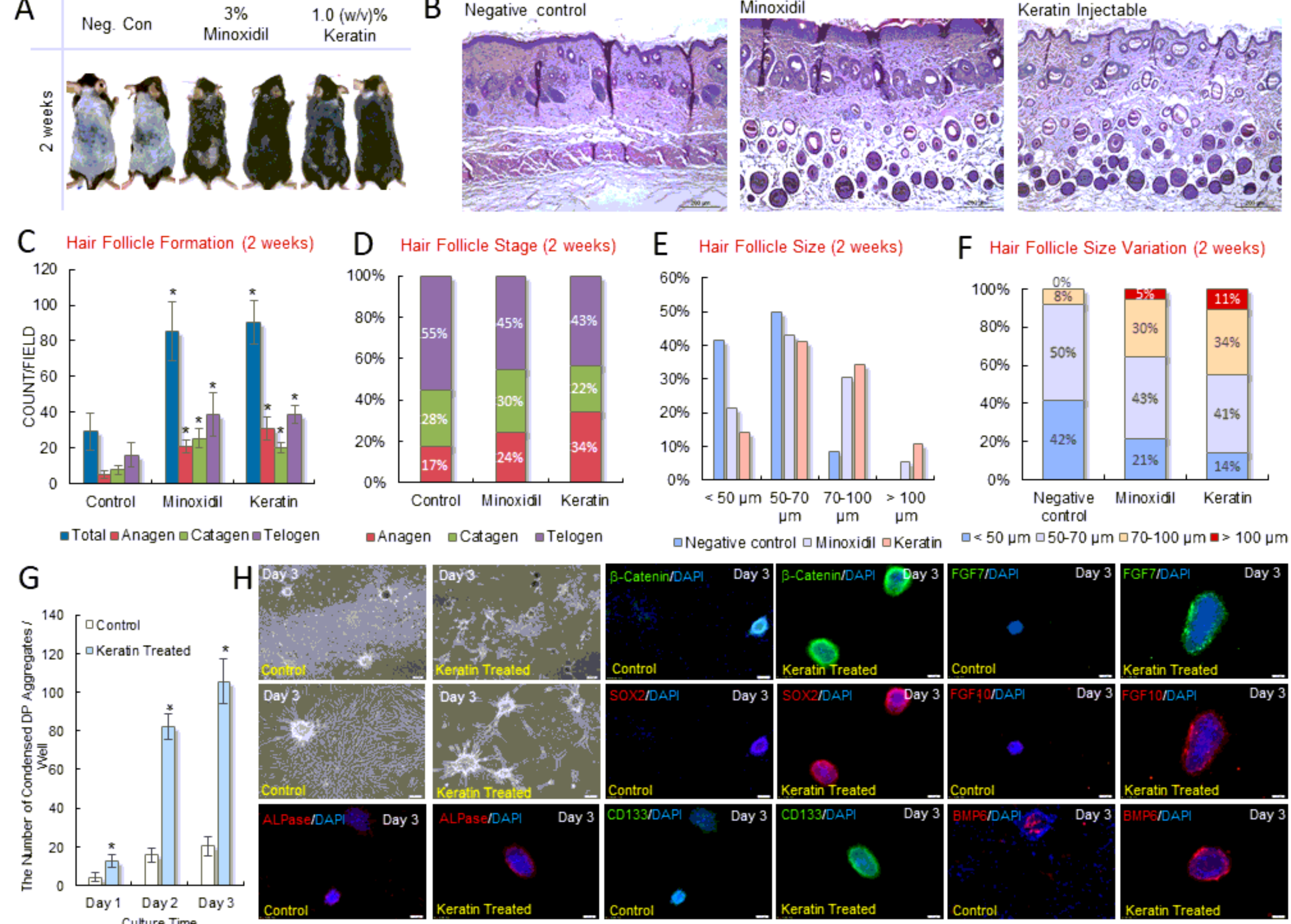

Figure 1

Intradermal injection of human hair-derived keratin induced hair follicle formation and improved hair growth, and in vitro keratin treatment induced DP condensation. A: Images of hair growth on the back skin of mice at 2 weeks after intradermal injection of keratin. B: Histological images of the back skin sections of mice at 2 weeks after intradermal injection of keratin. Scale bars, 200μm. C-F: Graphical representation and quantification of hair follicles; hair follicle formation (C), hair follicle stage (D), hair follicle size (E) and hair follicle size variations (F) in skin sections of mice (n = 16 sections, in 4 mice; mean ± standard deviation (s.d.)). *P,0.01, indicates a significant difference between control group and experimental groups. G: Graphical representation of DP cell condensation in the presence of keratin. *P,0.01, indicates a significant difference between control and keratin treated. (n=6; mean ± standard deviation (s.d.)); Control, non-treated DP cell; Keratin Treated, DP cells in the presence of keratin. H: Condensation of DP cell by immunofluorescent staining of in the presence of keratin; 4',6-diamidino-2-phenylindole (DAPI), blue; SOX2, alkaline phosphatase (ALPase), FGF10, BMP6, red; β-catenin, CD133 and FGF7, green. Scale bars, 100μm.

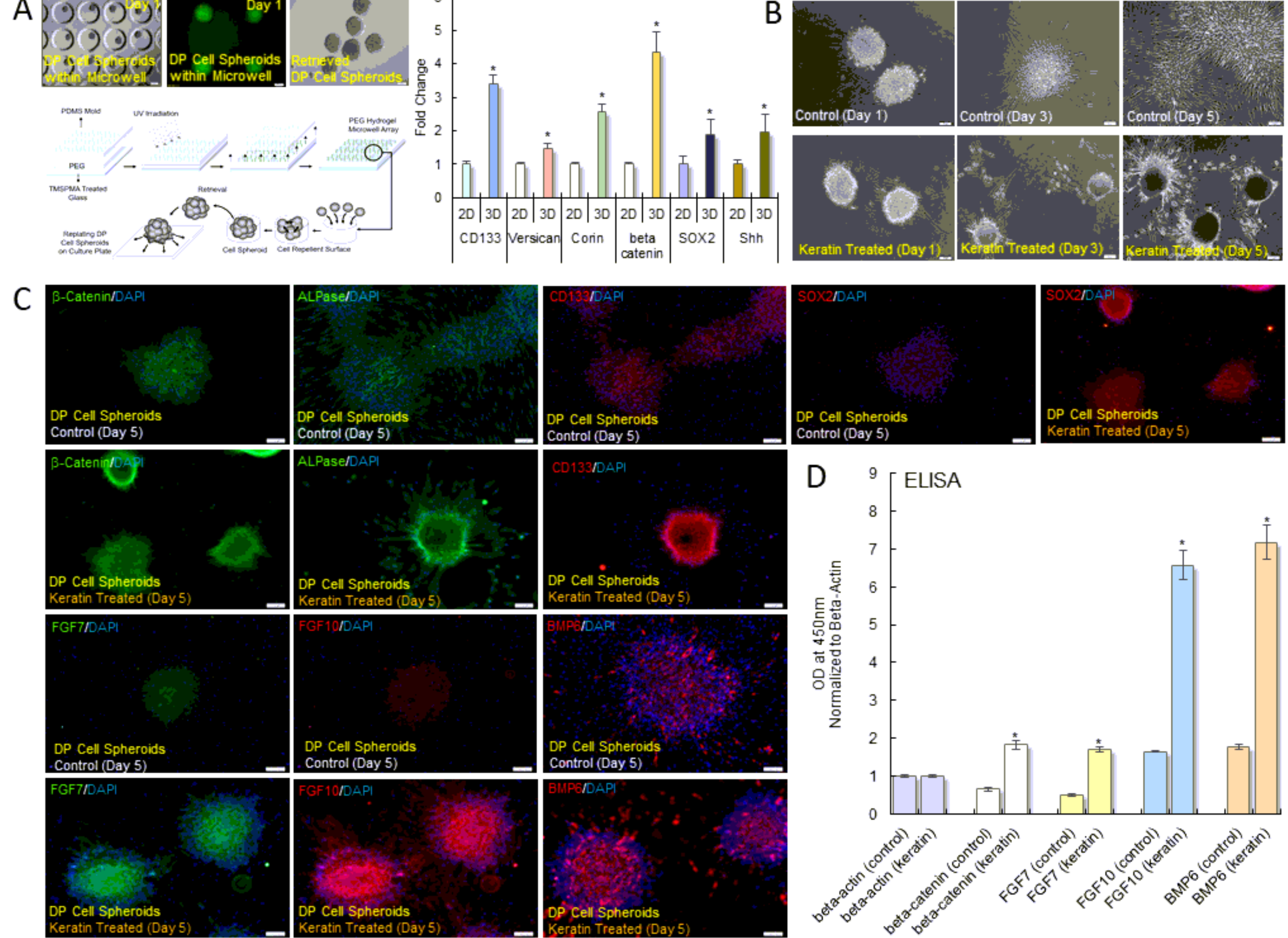

Figure 2

Hair keratin treatment retained DP cell condensation with upregulated expressions of DP cell property-related molecular markers. A: Schematic illustration of microwll-mediated DP cell spheroid formation and images of DP cell spheroids within microwells and retrieved DP cell spheroids from microwells. Graphical representation of DP cell property-related gene expressions of DP cells and DP cell spheroids. *P,0.01, indicates a significant difference between DP cells and DP cell spheroids; 2D, DP cells; 3D, DP cell spheroids. Scale bars, 100μm. (n=4; mean ± standard deviation (s.d.)). B: Images of replated DP cell spheroids in the presence of keratin by observation using a light microscope. Scale bars, 100μm. C: Images of replated DP cell spheroids in the presence of keratin by immunofluorescent staining; DAPI, blue; SOX2, CD133, FGF10, BMP6, red; β-catenin, ALPase, FGF7, green. Scale bars, 100μm. D: Graphical representation of DP cell property-related molecular expressions of replated DP cell spheroid culture in the presence of keratin; ELISA. *P,0.01, indicates a significant difference between control and keratin treated. (n=3; mean ± standard deviation (s.d.)).

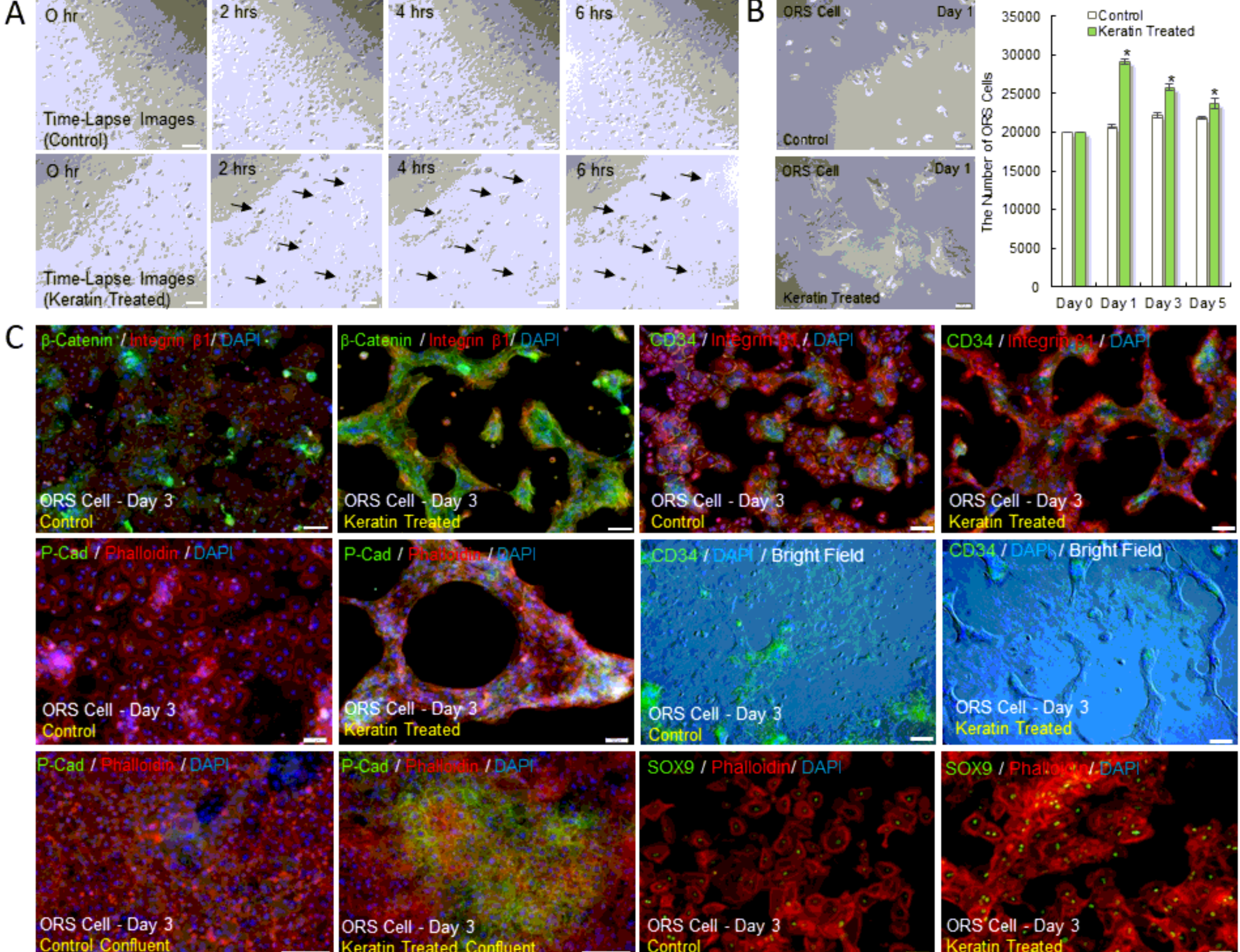

Figure 3

Hair-derived keratin induced colony formation and P-cadherin expressing germ formation of ORS cells. A: Time-lapse images of ORS cells in the presence of keratin; black arrows indicate colony formation. Scale bars, 100μm. B: Image of ORS cells and quantification of ORS cell growth in the presence of keratin. *P,0.01, indicates a significant difference between control and keratin treated. Scale bars, 100μm. (n=6; mean ± standard deviation (s.d.)); ORS, outer root sheath. C: In vitro germ formation of ORS cell by immunofluorescent staining of in the presence of keratin; DAPI, blue; phalloidin, integrin β1, red; P-cadherin, CD34, β-catenin, SOX9, green. Control confluent, ORS cell culture at confluent cell density under ORS culture medium; Keratin treated confluent, ORS cell culture at confluent cell density in the presence of keratin. Scale bars, 100μm.

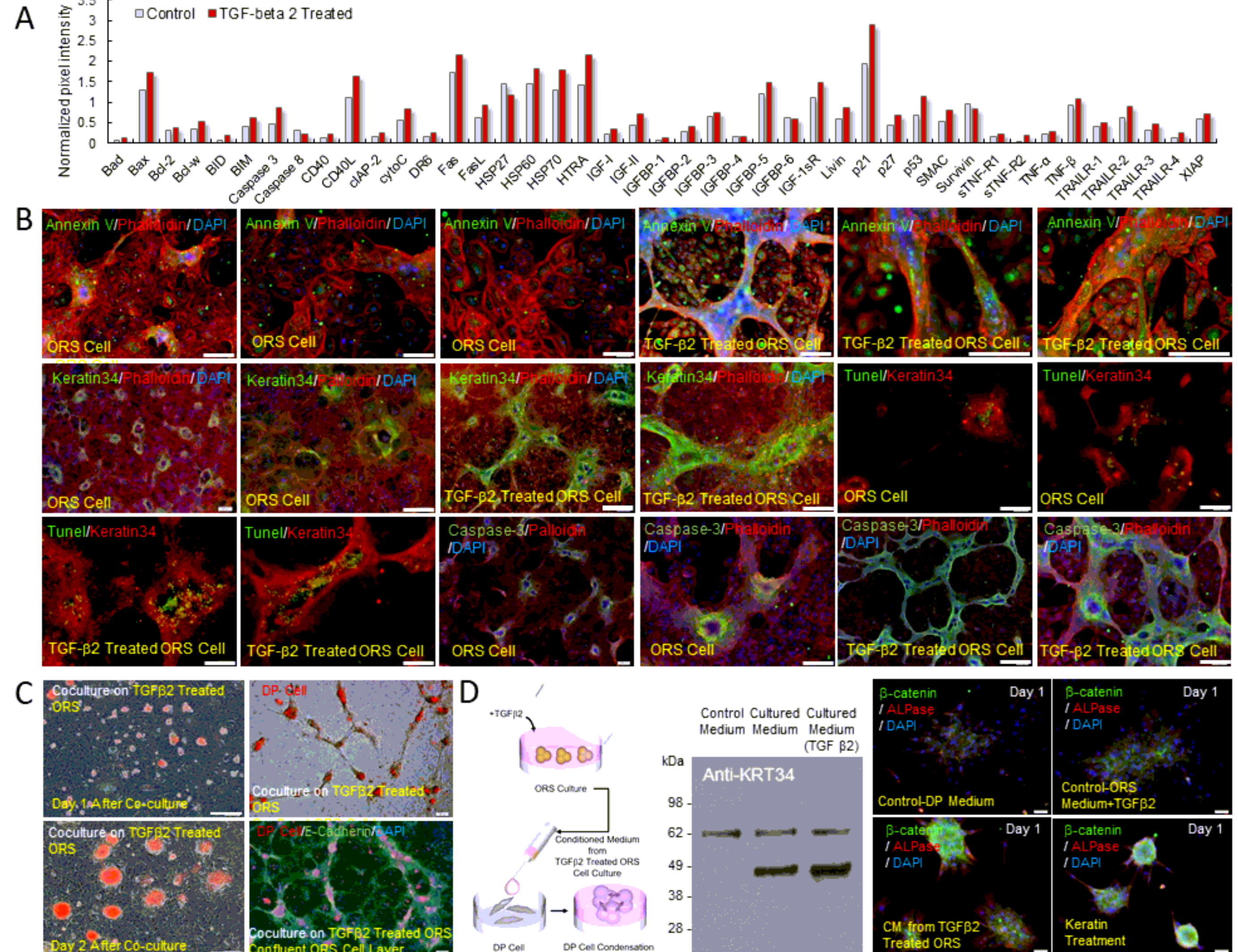

Figure 4

TGF-β2 induced apoptosis of ORS cells, keratin exposure was generated from TGFβ2-induced apoptotic ORS cells, and keratin release from TGFβ2-mediated apoptotic ORS cells induced DP cell condensation. A: Graphical quantification of apoptosis array of ORS cells and TGFβ2-treated ORS cells. B: TGFβ2-induced apoptosis and it's following keratin exposure of ORS cells by immunofluorescent staining; DAPI, blue; phalloidin, keratin 34, red; annexin V, keratin 34, tunel, caspase 3, green. Scale bars, 200μm. C: Images of DP cell condensation on TGFβ2-treated ORS cell layers. Co-culture of cell tracker-treated DP cells (red) on TGFβ2-treated ORS cell layers. Immunofluorescent image; E-cadherin, green; DAPI, blue. Scale bars, 200μm. D: DP cell condensation under conditioned medium collected from TGFβ2-treated ORS cell culture. Western blot image of released keratin 34 from ORS cell culture and TGFβ2-treated ORS cell culture. Immunofluorescent image; ALPase, red; β-catenin, green; DAPI, blue; Control-DP medium, DP culture medium; Control-ORS Medium-TGFβ2, ORS medium including TGFβ2; CM from TGFβ2 treated ORS, conditioned medium collected from TGFβ2-treated ORS cell culture; Keratin treatment, DP medium containing 1(w/v)% keratin. Scale bars, 50μm.

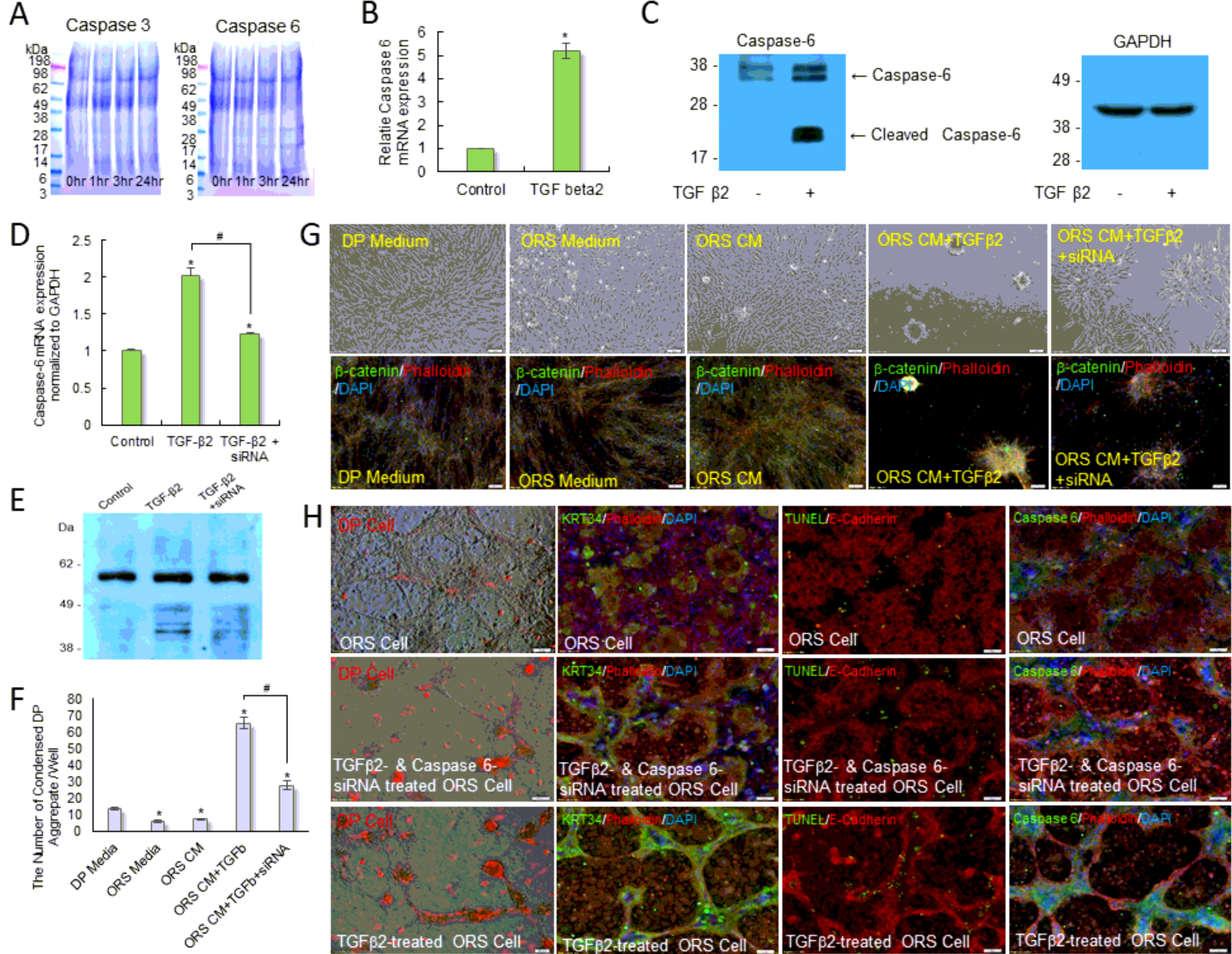

Figure 5

Caspase 6-mediated keratin degradation and DP cell condensation activity in conditioned medium collected from caspase 6-silenced ORS cell culture in the presence of TGFβ2. A: SDS-PAGE images of caspase 3- and caspase 6-mediated keratin degradation. B: Caspase 6 mRNA expression in TGFβ2-treated ORS cells by real time-qPCR. P,0.01, indicates a significant difference between ORS cells (control) and TGFβ2-treated ORS cells (TGFβ2). n=4; mean ± standard deviation (s.d.)). C: Western blot images of caspase 6 expression in TGFβ2-treated ORS cells. D: Caspase 6 mRNA expression in ORS cell culture in the presence of TGFβ2 and caspase 6-silenced ORS cell culture in the presence of TGFβ2 by real time-qPCR; Control, ORS cells; TGFβ2, ORS cell culture in the presence of TGFβ2; TGFβ2+siRNA, caspase 6-silenced ORS cell culture in the presence of TGFβ2. *P,0.01, indicates a significant difference between control and experimental group. #P0.01, indicates a significant difference between ORS cell culture in the presence of TGFβ2 and caspase 6-silenced ORS cell culture in the presence of TGFβ2. n=5; mean ± standard deviation (s.d.)). E: Western blot image of keratin in conditioned medium collected ORS cell culture in the presence of TGFβ2 and caspase 6-silenced ORS cell culture in the presence of TGFβ2. F: DP cell condensation activity in conditioned medium collected from caspase 6-silenced ORS cell culture in

the presence of TGFβ2; DP medium, basic DP medium; ORS Medium; basic ORS medium; ORS CM, conditioned medium collected from ORS cell culture; ORS CM+TGFβ2, conditioned medium collected from ORS cell culture in the presence of TGFβ2, ORS CM+TGFβ2+siRNA, conditioned medium collected from caspase 6-silenced ORS cell culture in the presence of TGFβ2. *P,0.01, indicates a significant difference between DP cell culture in DP medium and DP cell culture in other culture media and conditioned media. #P0.01, indicates a significant difference between DP cell culture in conditioned medium collected from ORS cell culture in the presence of TGFβ2 and DP cell culture in conditioned medium collected from caspase 6-silenced ORS cell culture in the presence of TGFβ2. n=4; mean ± standard deviation (s.d.)). G: Images of DP cell condensation in conditioned medium collected from caspase 6-silenced ORS cell culture in the presence of TGFβ2, and DP cell condensation by immunofluorescent staining; phalloidin, red; β-catenin, green; DAPI, blue; Scale bars, 100μm. H: Images of DP cell condensation on caspase 6-silenced ORS cell layer in the presence of TGFβ2. Co-culture of cell tracker-treated DP cells (red) with TGFβ2-treated ORS cell layers. Scale bars, 200μm. Immunofluorescent image of caspase 6-silenced ORS cells in the presence of TGFβ2: Phalloidin, E-cadherin, red; KRT34, tunel, caspase 6, green; DAPI, blue; Scale bars, 100μm.

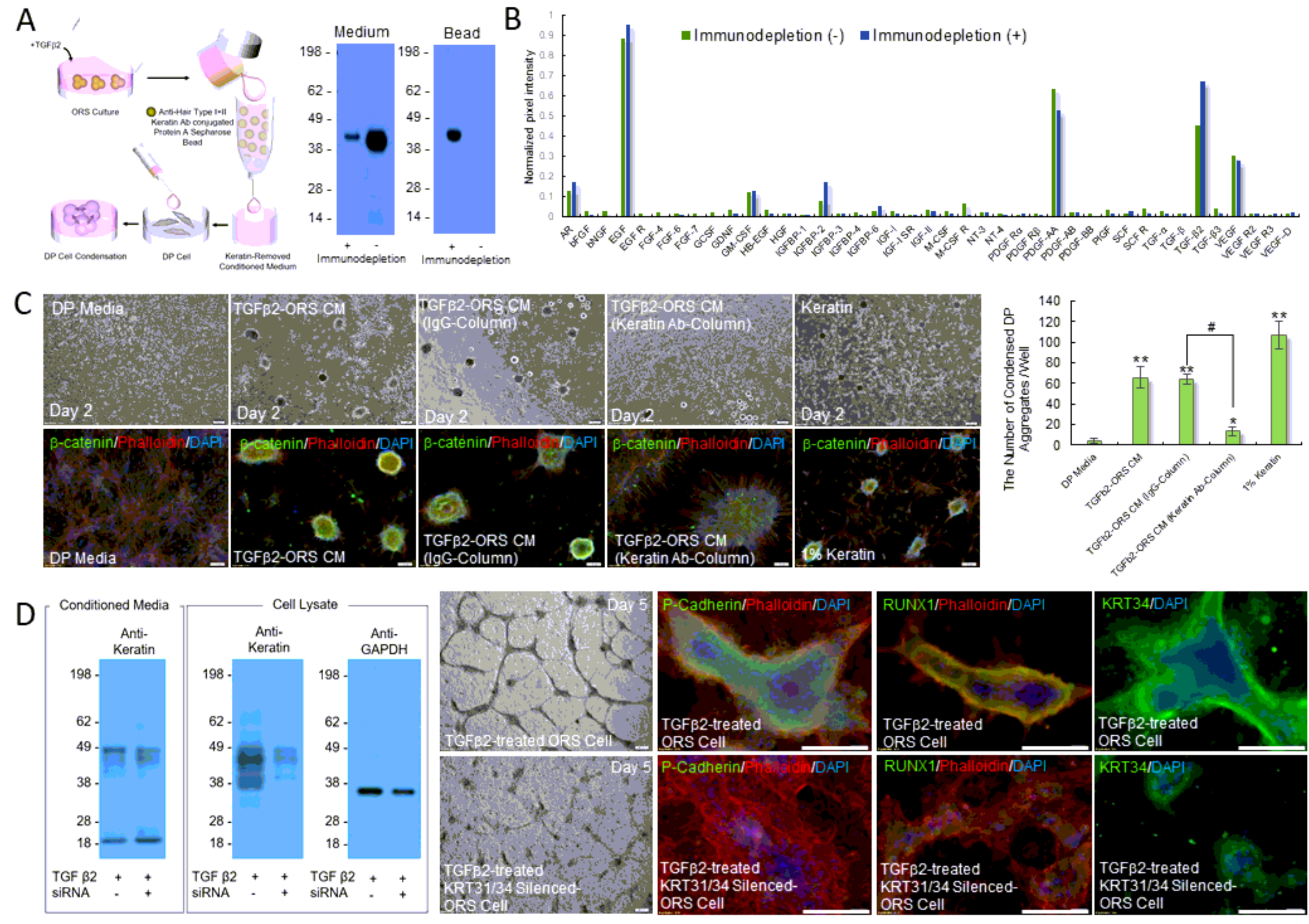

Figure 6

DP cell condensation activity in keratin-eliminated conditioned medium of TGFβ2-treated ORS cell culture using a column containing anti-human type I+II hair keratin antibody-conjugated beads, and germ formation in ORS cell culture and KRT31/KRT34 silenced ORS cell culture in the presence of TGF-β2. A: Western blot image of the keratin-eliminated conditioned medium and the keratin-bound beads. B: Graphical quantification of growth factor content of the conditioned medium and the keratin-eliminated conditioned medium collected from TGFβ2-treated ORS cell culture using antibody array for growth factors. C: DP cell condensation activity in keratin-eliminated conditioned medium of TGFβ2-treated ORS cell culture using a column containing anti-human type I+II hair keratin antibody-conjugated beads. Immunofluorescent image; phalloidin, red; β-catenin, green; DAPI, blue; DP media, DP culture medium; TGFβ2-ORS CM, conditioned medium collected from TGFβ2-treated ORS cell culture; TGFβ2-ORS CM (IgG-column), conditioned medium collected from TGFβ2-treated ORS cell culture and then treated with normal IgG-conjugated beads; 1% Keratin, DP medium containing 1(w/v)% keratin; TGFβ2-ORS CM (keratin Ab-column), keratin-eliminated conditioned medium collected from TGFβ2-treated ORS cell culture. Graphical representation of DP cell condensation activity. *P,0.05 and **P,0.01, indicate a significant difference between DP media and experimental groups. #P0.01, indicates a significant difference between TGFβ2-ORS CM (IgG-column) and TGFβ2-ORS CM (keratin Ab-column). Scale bars, 100μm. (n=4; mean ± standard deviation (s.d.)). B: Images of DP cell condensation in the keratin-eliminated conditioned medium collected from TGFβ2-treated ORS cell culture; DP media, DP culture medium; TGFβ2-ORS CM, conditioned medium collected from TGFβ2-treated ORS cell culture; TGFβ2-ORS CM (IgG-column), conditioned medium collected from TGFβ2-treated ORS cell culture and then treated with normal IgG-conjugated beads; TGFβ2-ORS CM (keratin Ab-column), keratin-eliminated conditioned medium collected from TGFβ2-treated ORS cell culture; 1% Keratin, DP medium containing 1(w/v)% keratin. Scale bars, 200μm. D: Germ formation of ORS cells and KRT31/KRT34 silenced ORS cells. Western blot image of keratin content of conditioned media and cell lysates from negative control siRNA-transfected ORS cell culture in the presence of TGFβ2 and KRT31/KRT34 silenced ORS cells culture in the presence of TGFβ2. Image of ORS cells and KRT31/KRT34 silenced ORS cells in the presence of TGFβ2. Germ formation of ORS cells and KRT31/KRT34 silenced ORS cells in the presence of TGFβ2 by immunofluorescent staining; phalloidin, red; P-cadherin, RUNX1, KRT34, green; DAPI, blue. Scale bars, 200μm.

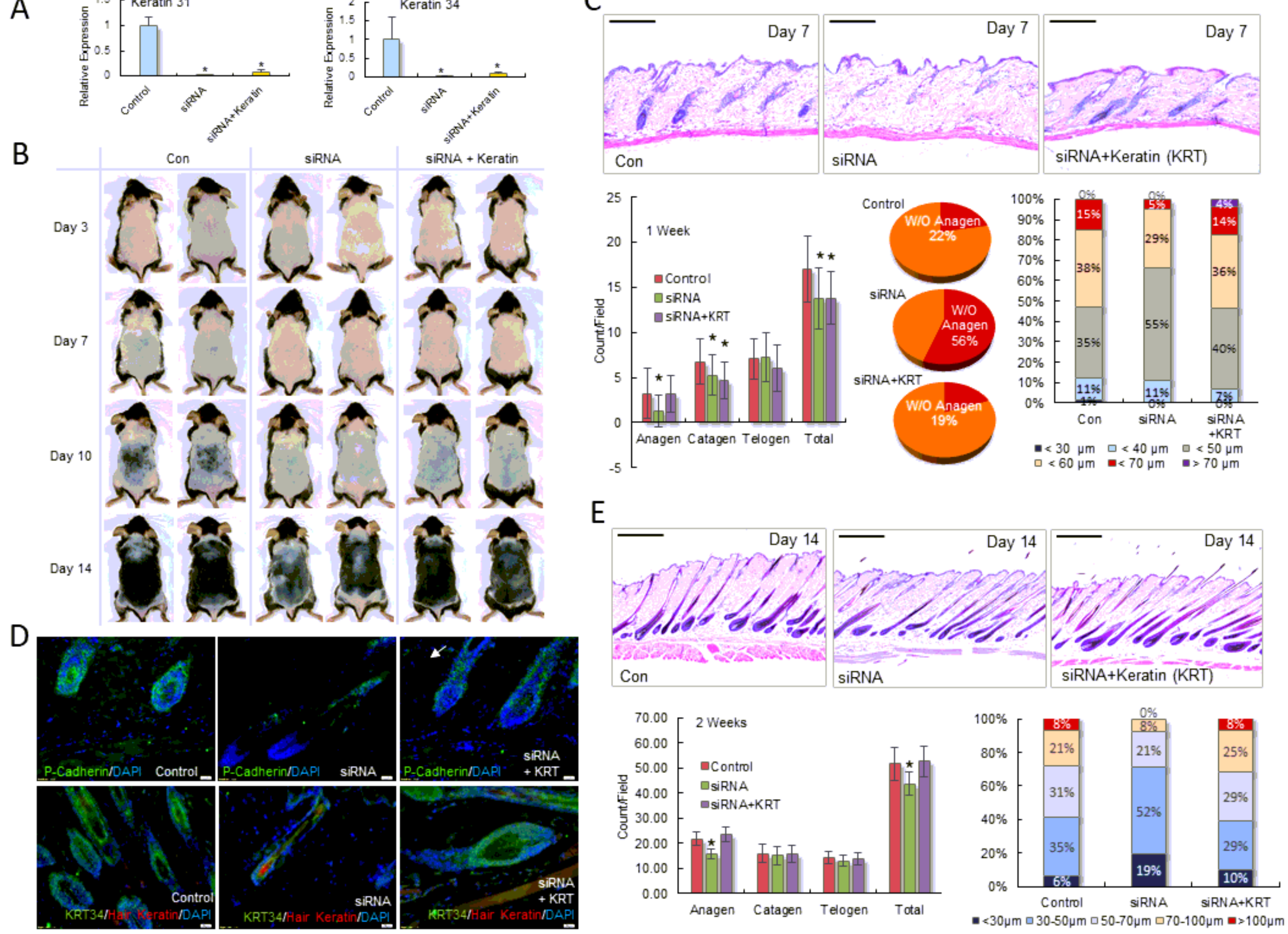

Figure 7

Temporal silencing of KRT31/KRT34 expressions in vivo suppressed hair growth. A: Graphical representation of KRT31 and KRT34 mRNA expressions in mice on day 7 after KRT31/KRT34 silencing. Control, mice injected with negative control siRNA-loaded lipofectamine; siRNA, KRT31/KRT34 silenced mice; siRNA+KRT, KRT31/KRT34 silenced and hair keratin injected mice; *P,0.05, indicates a significant difference between control and experimental groups. (n=4, in 4 mice; mean ± standard deviation (s.d.)). B: Images of hair growth on the back skin of mice on day 3, day 7, day 10 and day 14 after KRT31/KRT34 siRNA transfection. C: Histological images of the back skin of mice on day 7 after KRT31/KRT34 siRNA transfection and intradermal injection of keratin. Graphical representation and quantification of hair follicle formation in skin sections of mice (n = 36 sections, in 8 mice; mean ± standard deviation (s.d.)). *P,0.01, indicates a significant difference between control group and experimental groups. Scale bars, 200μm. D: Immunohistochemical images of the back skin of mice on day 7 after KRT31/KRT34 siRNA transfection and intradermal injection of keratin; type I+II hair keratin, red; P-cadherin, KRT34, green; DAPI, blue. Scale bars, 20μm. E: Histological images of the back skin of mice on day 14 after KRT31/KRT34 siRNA transfection and intradermal injection of keratin. Graphical representation and quantification of

hair follicle formation in skin sections of mice (n = 23 sections, in 5 mice; mean ± standard deviation (s.d.)). *P,0.01, indicates a significant difference between control group and experimental groups. Scale bars, 20μm.

Supplementary Files

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- SupplementaryInformation.pdf