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Original Article

IGF-1R deficiency in human keratinocytes disrupts epidermal homeostasis and stem cell maintenance

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ABSTRACT

Background: Epidermal stem cells (ESCs) are keratinocytes that reside in the basal layer of the epidermis and mediate epidermal homeostasis. Insulin-like growth factor 1 (IGF-1) signaling through its receptor (IGF-1R) has been identified as an important regulator in rodent skin development and differentiation. However, the role of IGF-1/IGF-1R signaling in human keratinocytes is not yet well understood.
Objective: This study aimed to clarify the role of IGF-1/IGF-1R signaling in human epidermal homeostasis.
Methods: IGF-1R specific knockout (KO) HaCaT keratinocytes were generated by CRISPR-Caspase-9-mediated non-homologous end joining frame-shift mutations. Further, the behavior of these keratinocytes in epidermal homeostasis was investigated using reconstructed epidermis and human skin equivalents.
Results: IGF-1R KO HaCaT keratinocytes were successfully established and produced thin epidermis in three-dimensional culture models. Keratin10-positive cells were frequently found in the basal layer of the reconstructed epidermis.
Conclusions: IGF-1/IGF-1R signaling was demonstrated to play a key role in maintaining human epidermal homeostasis. This method provides a new framework to investigate gene function in human epidermal homeostasis.

1. Introduction

To maintain healthy skin and establish barrier function, epidermal stem cells (ESC) must maintain long-term proliferative capacity and generate a differentiated progeny. However, epidermal thickness decreases and wound healing becomes delayed with aging [1,2], which may be caused by a decline in ESC function.

Several studies have focused on paracrine signals from the mesenchymal (dermal) layer as a factor that maintains ESC function *in vitro* [3]. Several cytokines, including osteopontin [4], angiopoietin-1 [5], platelet factor-4 [6], periostin [7], tumor necrosis factor-stimulated gene 6 protein [8], insulin-like growth factor I (IGF-1) [9], and connective-tissue growth factor [10] are released from the mesenchymal layer; however, details of their effect on human epidermal homeostasis have not been fully elucidated.

IGF-1 is one of the dermis-derived cytokines and is a well-known growth factor that promotes the proliferation and differentiation of various cells [11,12]. It mediates signals by binding to its receptor IGF-1R, which belongs to the tyrosine kinase family of growth factor receptors, and strongly activates phosphatidylinositol-3-kinase signaling. Blood IGF-1 levels and its expression by fibroblasts decrease with aging [13,14].

IGF-1/IGF-1R signaling plays a particularly important role during development. IGF-1 knockout (KO) in mice is lethal, resulting in death immediately after birth, with animals exhibiting skin abnormalities, including a thinner and disrupted epidermis with impaired barrier function and hair formation [15]. Furthermore, epidermis-specific conditional IGF-1R KO mice exhibit a thinner epidermis, suggesting that IGF-1/IGF-1R signaling is important for maintaining epidermal basal cells [16]. In addition, IGF-1 signaling may affect the epidermis in humans because patients with hyposecretion, such as Laron syndrome (a primary IGF-1 deficiency and growth hormone resistance), are not only short in stature [17] but also display early aging phenotypes such as wrinkles [18].

However, the effects of gene ablation are dependent on the genetic background of the mice [15], and not all growth factor signals are known to be functionally identical in murine and human cells

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[19]. Therefore, an experimental system to investigate gene function and the role of growth factor signals in human cells is required.

Three-dimensional (3D) culture models are extensively used to study human skin, and these have two types: reconstructed epidermis (RE) and cultured human skin equivalents (HSEs), prepared by seeding keratinocytes on a filter membrane and on a collagen gel layer containing dermal fibroblasts, respectively [20]. Although the experimental suppression of some genes has been reported in 3D human skin models [21], complete suppression is difficult to achieve in three-dimensionally proliferating cells using small molecules and siRNAs because of their stability and inefficiency.

To overcome these difficulties in human skin models and elucidate the function of IGF-1/IGF-1R signaling in epidermal homeostasis, IGF-1R KO human keratinocytes were generated using the CRISPR-Caspase-9 (Cas9) genome editing technology [22] and human RE and HSEs 3D culture skin models were developed.

Accordingly, IGF-1R-specific KO cells were successfully generated without inserting any drug resistance genes. Epithelialization using established KO human keratinocytes in 3D cultures suggests that the loss of IGF-1/ IGF-1R signaling causes a collapse of skin thickness and disruption of stem cell homeostasis.

2. Materials and methods

2.1. Cells

The human lung adenocarcinoma line, A549, was cultured in minimum essential medium (Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS; Gibco). Primary human neonatal epidermal keratinocytes (HEKs; Gibco) were cultured in EpiLife containing human keratinocyte growth supplement (Thermo Fisher Scientific). The immortalized HaCaT keratinocyte cell line and human dermal fibroblast cells (HDFs; Gibco) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and low glucose (1000 mg/mL, Sigma–Aldrich) containing 10% FBS and 1% an antibiotic mixture (5 mg penicillin/mL, 5 mg streptomycin/mL, and 10 mg neomycin/mL; Invitrogen). All cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.2. Keratinocyte proliferation and migration assay

HEKs were plated into 96-well plates in triplicate at approximately 8000 cells per well and cultured. After 24 h, cells were treated with various cytokines (100 ng/mL, R&D Systems) and then quantified after 72 h using a Cell Counting Kit-8 kit (Dojindo Laboratories) Migration assay was performed using the CytoSelect 96-Well Cell Migration Assay kit (Cell Biolabs). HEKs were seeded in the upper chamber in triplicate at 50,000 cells per well, and media containing 150 μL of various cytokines (1 μg/mL) were added to the wells of the feeder tray. After 48 h, migrating cells that had passed through the polycarbonate membrane were quantified according to the manufacturer's instructions.

2.3. CRISPR-Cas9 system

IGF-1R KO gene was achieved using a GeneArt CRISPR Nuclease Vector kit (Life Technologies). A target sequence was inserted into the CRISPR nuclease vector containing orange fluorescent protein (OFP), and the vector was transduced into One Shot Top 10 (Life Technologies). The plasmid containing the CRISPR nuclease construct was purified using a Qiafilter Plasmid Maxi kit (Qiagen), and A549/HEK/HaCaT cells were transfected with the vector using TransIT-LT1 (Mirus) according to the manufacturer's protocol. The sham control cells established by introducing GeneArt CRISPR Nuclease Vector without gRNA. OFP-positive cells were sorted using a BD fluorescence-activated cell sorting (FACS) Aria III Cell-Sorting System (BD).

2.4. Mismatch-specific endonucleases cleavage assay (CEL I assay)

Genomic modification consisting of single base mismatches or small insertions or deletions (indels) were detected by the CEL I assay using a GeneArt Genomic Cleavage Detection kit (Life Technologies) according to the manufacturer's protocol. Primer pairs used for polymerase chain reaction (PCR) analysis were 5′-GCATCGACATCCG-CAACGAC-3′ and 5′-GGACACCGCATCCAGGATCA-3′.

2.5. Sequencing

Genomic DNA was used for PCR amplification of target sites to verify the presence of indels and mutations. PCR products were sequenced directly using the primers 5′-GCATCGACATCCGCAAC-GAC-3′ and 5′-GGACACCGCATCCAGGATCA-3′. The products were cloned into a T-vector for sequencing using TOPO TA Cloning kit (Invitrogen). After blue/white selection using X-Gal/IPTG (QARTA Bio), plasmids were purified using a Plasmid Plus Purification kit (Qiagen). Sanger sequencing was performed using T3/T7 primers according to the manufacturer's protocol. DNA sequencing was performed at Fasmac Co., Ltd. In general, total 20–25 clones were sequenced for each cloned cell line.

2.6. Western blotting

To detect IGF-1R, 5×10^5 keratinocytes cells were lysed in the radioimmunoprecipitation assay buffer. Equal amounts of proteins were dissolved in sodium dodecyl sulfate (SDS) sample buffer (BIO-RAD) and separated by SDS polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes (ATTO) and, after blocking with Polyvinylidene Difluoride Blocking Reagent (Toyobo), the membranes were probed with a primary antibody against IGF-1R (Zi001, 1:2000, Life Technologies) overnight at 4 °C. After washing with 0.1% Tween-20 in phosphate buffered-saline (PBS), the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse antibody (1:10000, GE Healthcare) for 1 h at room temperature. Further, the membranes were washed with 0.05% Tween-20 in PBS, treated with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) for detection of HRP for 5 min, and analyzed with a LAS3000 (Fujifilm) or WSE-6100H (ATTO) imager.

2.7. Preparation of reconstructed epidermis (RE) and human skin equivalents (HSEs)

Dermal equivalents consisting of type I collagen and fibroblasts were prepared as described in previous studies [23,24] with some modifications. Briefly, DMEM containing 10% FBS, 0.1% acid-soluble bovine type I collagen (Koken), 250 μM 2-*O*-alpha-D-glucopyranosyl-L-ascorbic acid (Wako Pure Chemical Industries), and 1×10^5 HDFs/mL were prepared on ice and poured into a 6-well culture dishes (5 mL/well). The collagen gels were allowed to contract for 2–3 days and used as dermal equivalents. REs and HSEs were cultured in an assay media (J-TEC), it made based on previous work by Simon and Green [25], consisting of 5% FBS and physiologically active components, such as insulin, to activate IGF-1/IGF-1R signaling. We have not added external IGF-1. The genetically modified keratinocytes in assay media (2×10^5 cells/0.2 mL) were poured into a 9-mm glass ring (Iwaki) placed on the dermal equivalents. After 24 h, the glass ring was removed, and the culture was lifted to the air–liquid interface. Under these conditions only the keratinocyte layer was exposed to air to form cornified epithelium. The medium was changed every 2 days and, 14 days

after inoculation with keratinocytes, HSEs were harvested and processed for histological analysis.

2.8. Histological and immunohistological analysis of RE and HSEs

For histological analysis, RE and HSEs were fixed with 4% paraformaldehyde in 0.1-M phosphate buffer (pH 7.4) for at least 24 h and then embedded in paraffin. The sectioned samples (4 μm thickness) were stained with hematoxylin–eosin. For immunostaining, samples were fixed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 15 min, and frozen-samples were sectioned (10 μm thickness) using Optimal Cutting Temperature compound (Sakura Finetek). Epidermal differentiation was confirmed by immunofluorescence staining for keratin 14 (Biolegend) and keratin 10 (Abcam) with Alexa488-goat anti-rabbit Immunoglobulin G (IgG) or Alexa647-Goat anti-chicken IgG (Thermo Fisher), respectively. In addition, samples were counterstained with DAPI.

2.9. Measurement of epidermal thickness in HSEs

Epidermal thickness was measured in hematoxylin–eosin-stained sections using the FIJI software (NIH) by averaging the values at 3 points in each section [20].

2.10. Colony forming efficiency assay

One hundred keratinocytes were cultured as described previously [26]. Cultures were fixed in 3.7% buffered formaldehyde and stained with 1% Rhodamine B (Wako Pure Chemical Industries), and keratinocyte colonies were calculated using binary images.

3. Results

3.1. IGF-1 enhances keratinocyte proliferation and migration and plays a key role in the epidermal–dermal interaction

To identify the dermis-derived factors that regulate epidermal keratinocytes, first, the effects of dermis-derived growth factors and cytokines on keratinocyte behavior were assessed. Twelve proteins were selected, each was individually added to the culture medium, and their effects on keratinocyte proliferation and migration were evaluated. Among these factors, osteopontin and IGF-1 were both found to enhance keratinocyte proliferation (Fig. 1A), whereas transforming growth factor-β1 decreased proliferation (Fig. 1A). Cell migration assays showed that osteopontin, angiopoietin, platelet factor-4, IGF-1, and platelet-derived growth factor-BB all enhanced keratinocyte migration (Fig. 1B). Among these dermis-derived factors, only IGF-1 considerably increased both proliferation and migration of epidermal keratinocytes. These data strongly suggest that IGF-1 is a dermal factor that regulates both proliferative and migratory keratinocyte behaviors; thus, dermal IGF-1 and its receptor IGF-1R, expressed by epidermal keratinocytes [27], mediate the epidermal–dermal interaction. The IGF-1/IGF-1R signaling can also contribute to the proliferation of HaCaT keratinocytes [28].

3.2. Targeting the IGF-1R locus in human keratinocytes using the CRISPR-Cas9 genome editing system

The function of IGF-1/IGF-1R signaling in the epidermis has been examined in genetically engineered rodent models [29]; however, it has not been well studied in human keratinocytes

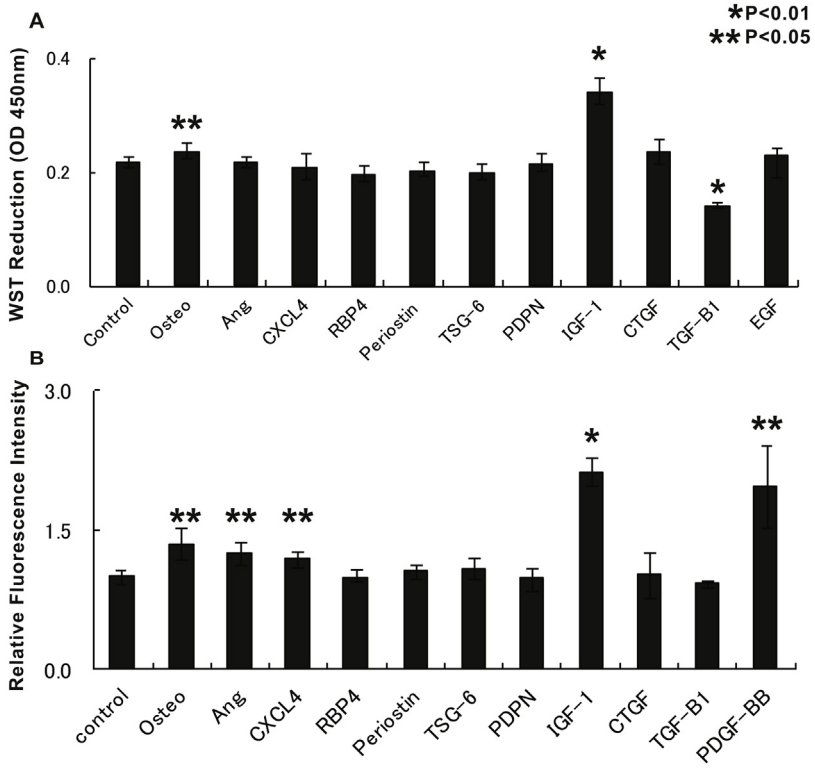

Fig. 1. Effects of cytokines on keratinocyte proliferation and migration in monolayer culture.
A: Number of HEKs after 72 h of culture in the presence of various cytokines was estimated using the Cell Counting Kit-8. Values represent the means of absorbance at 450 nm ± SD. *$p < 0.01$, vs. control. B: Relative fluorescence intensity of HEKs migrating through an 8 μm pore chamber after 48 h of culture in the presence of various cytokines. Values represent means ± SD. *$p < 0.01$ and **$p < 0.05$ vs. control (normalized to 1).Osteo: Osteopontin, Ang: angiopoietin-1, CXCL4: chemokine (C-X-C motif) ligand 4, RBP4: Retinol-Binding Protein 4, TSG-6: tumor necrosis factor-stimulated gene 6, IGF-1: insulin-like growth factor-1, CTGF: connective-tissue growth factor, TGF-β1: Transforming Growth Factor-β1, EGF: Epidermal Growth Factor, PDGF-BB: Platelet-Derived Growth Factor.

because appropriate experimental models are unavailable. Therefore, this study aimed to generate IGF-1R knockout (KO) human keratinocytes using genome editing technology. Two gRNAs targeted to Exon 1 within the IGF-1R locus were designed and inserted into a plasmid that encodes the crRNA, tracrRNA, and Cas9 mRNA (Fig. 2A). To confirm the functionality of this CRISPR-Cas9 system with respect to targeting the IGF-1R locus, first, the plasmid was transduced into A546 carcinoma cells. More than 30% of the treated cells were transduced, as evidenced by the number of OFP-positive cells (Supplementary Fig. 1A). The targeted IGF-1R locus was amplified by PCR and analyzed using a mismatch detection assay (CEL I assay), which confirmed successful genome editing (Supplementary Fig. 1B). Furthermore, direct sequencing of the PCR product indicated that the mutation in the IGF-1R locus occurred around the protospacer adjacent motif (PAM) sequence that was targeted by the designed gRNA (Supplementary Fig. 1C). Therefore, collectively, these data establish the function of the CRISPR-Cas9 system for targeting the IGF-1R locus.

Further, these methods were used to generate IGF-1R null human keratinocytes. The expression plasmid was transduced into HEKs and HaCaT cell lines [30] using lipofection, resulting in 3.2% (gRNA-1) and 4.0% (gRNA-2) of the HEKs cell and 2.4% and 3.1% of the HaCaT cells to be OFP-positive (Fig. 2B). The transduced HEK and HaCaT cell populations were then isolated using FACS based on OFP expression; successful genome editing of the IGF-1R locus was confirmed by CEL I assay (Fig. 2C). Enriched OFP-positive HEKs did not proliferate sufficiently and were not assessed any further. These HaCaT cells retained their differentiation ability [30] and possessed a heterogeneous proliferative potential comparable to normal human keratinocytes [26]. Therefore, these cells were used to investigate the role of IGF-1/IGF-1R signaling in human keratinocytes.

3.3. Establishment of IGF-1R-null human keratinocytes using the genome editing technology

To confirm that genome editing of the IGF-1R locus prevents IGF-1R protein expression, its expression was examined in cloned, transduced A549 cells (Supplementary Fig. 1D). IGF-1R was not detected in several isolated clones, indicating that IGF-1R KO cells were indeed established using this method (Supplementary Fig. 1E). Further, the isolation of IGF-1R KO human keratinocytes was attempted. First, individual OFP-positive HaCaT cells were seeded using limited dilution into micro-well plates (96 wells × 5 = 1440 wells), and 225 proliferative clones were identified (Fig. 3A). All isolated clones were examined using the CEL I assay (Supplementary Fig. 2), and genome editing was detected in only 24 clones (Fig. 3B). Furthermore, the expression of IGF-1R protein in CEL I assay-positive clones was analyzed by Western blotting, and five clones were identified to be true IGF-1R KO HaCaT cells (Fig. 3C). One clone (#2-1-8) was selected, and the sequence analysis revealed a 1-base insertion and 4-base deletion in each allele of the IGF-1R locus around the PAM sequence, respectively (Fig. 3D). IGF-1R expression was not recovered after three serial

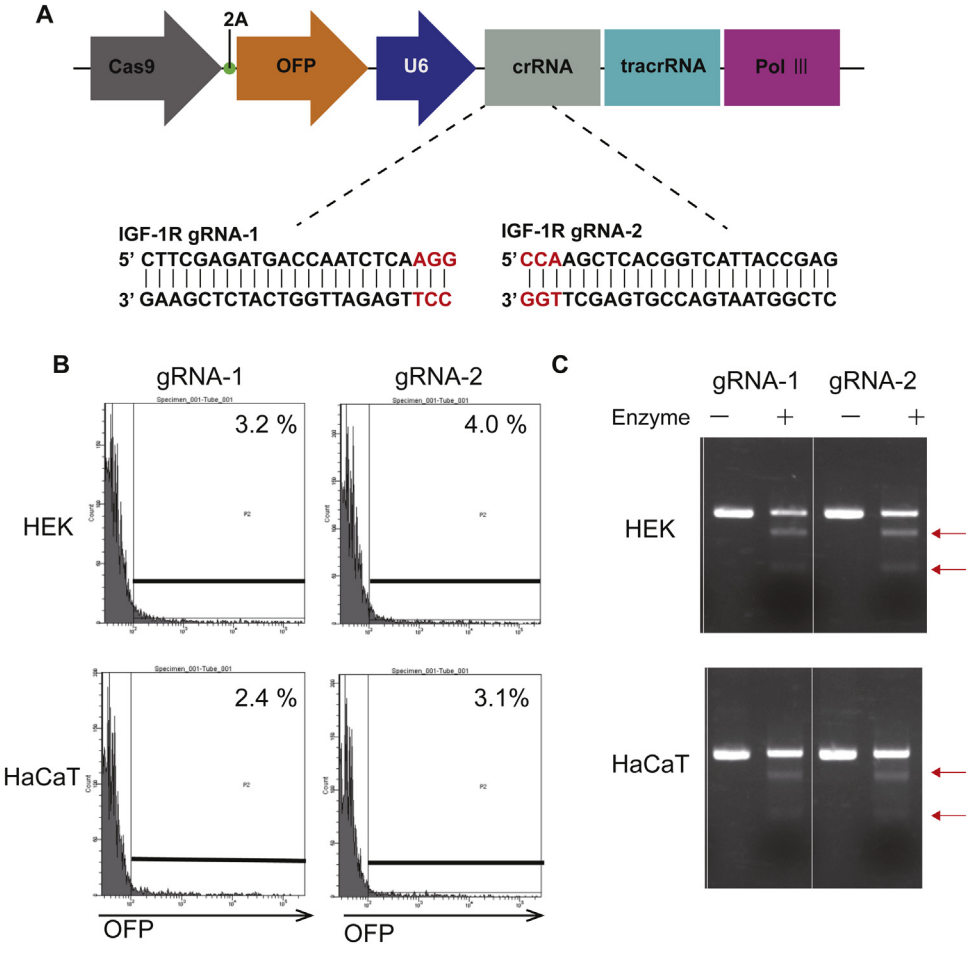

Fig. 2. CRISPR-Cas9 based genome editing of keratinocytes.
A: Diagram of the gRNA DNA plasmid. Two gRNAs were synthesized against IGF-1R Exon 1 B: Efficiency of lipid-mediated transfection of HEKs and immortalized HaCaT keratinocytes. C: Gene mutations were detected in FACS-enriched OFP-positive cell transfected with gRNA-1 or 2 in HEK and HaCaT cells. The enzyme was surveyor nuclease.

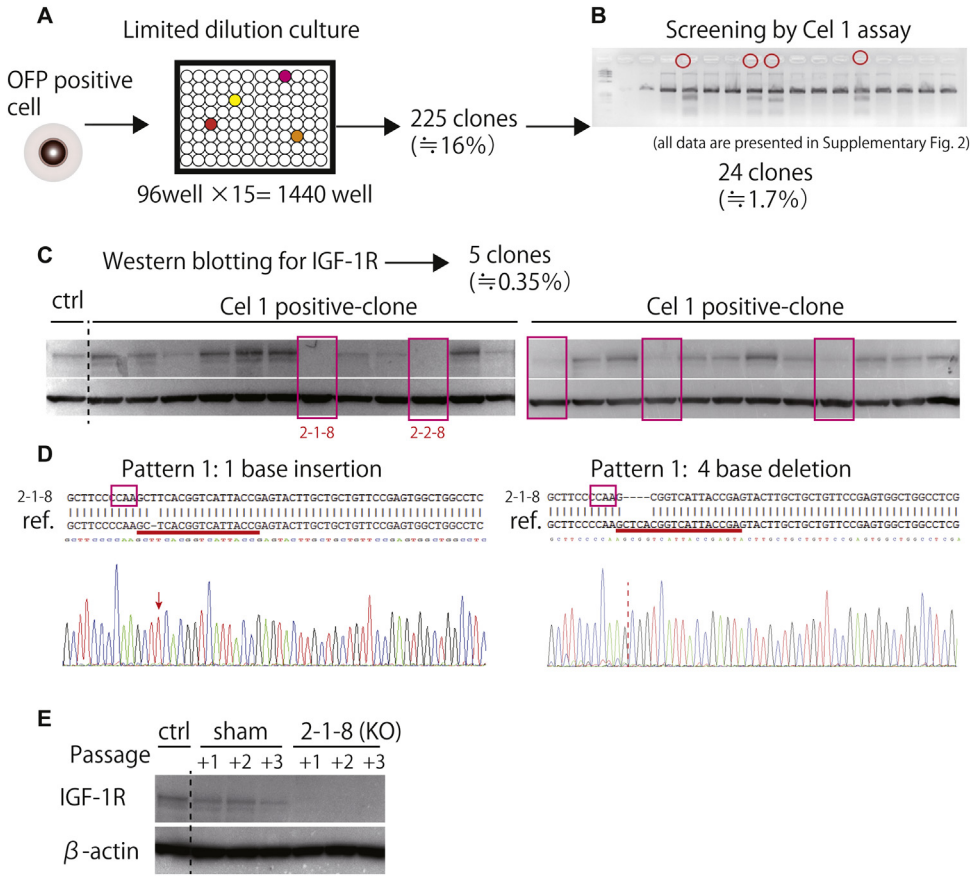

Fig. 3. Establishment of IGF-1R KO keratinocytes.
A: Limited dilution culture of FACS-sorted OFP-positive HaCaT cells. B: Primary screening of gene-edited cell lines by CEL I mutation assay. A representative gel image of a CEL I cleavage assay screening of gRNA/Cas9 transfected HaCaT clones is shown. (data are presented in Supplementary Fig. 2) C: Secondary screening of gene-edited cell lines by Western blotting against IGF-1R (upper lane) and against β-actin as a loading control (lower lane). D: DNA sequence of the IGF-1R KO clone #2-1-8. The wild type reference sequence is shown below the mutant sequences, and the gRNA2 target sequence is indicated by the red line. The protospacer adjacent motif (PAM) is indicated by the box. E: Western blotting against IGF-1R (upper lane) and loading control (β-actin; lower lane) after serial passages (1 passage/week) over 3weeks.

passages (Fig. 3E). Collectively, these data demonstrate that IGF-1R KO HaCaT cells were successfully established using the CRISPR-Cas9 genome editing technology.

3.4. IGF-1R deficiency in human keratinocytes causes epidermal hypoplasia

HaCaT cells can differentiate and generate stratified epithelia when cultivated under the appropriate conditions [30]. Therefore, the function of IGF-1R in stratified epithelium formation was examined using IGF-1R KO HaCaT human keratinocytes. Two model systems, which are routinely utilized for investigating human epidermis formation and homeostasis, were used. The first was an RE model, which can be generated when keratinocytes are seeded on a filter membrane, whereas the second was an HSEs model, which can be prepared when keratinocytes are seeded on a type I collagen gel populated by dermal fibroblasts. Stratification was induced in both models by placing the cultures at the air–liquid interface.

RE models were first generated using control and IGF-1R KO HaCaT keratinocytes, and the structure of the reconstructed tissues was examined by histological analysis after 14 days of placing the culture at the air–liquid interface. The thicknesses of the RE models produced with KO HaCaT cells were considerably decreased (Fig. 4A) by half compared with those produced with control HaCaT cells (Fig. 4B). In addition, histological analysis revealed that the basal cells were flat and appeared to be squamous epithelium in the RE models generated from the KO HaCaT cells; however, this was not observed in the control HaCaT cells (Fig. 4A). This observation was confirmed by the detection of keratin 10 in the basal layer of the RE models generated from the IGF-1R KO HaCaT cells but not the control cells (Fig. 4C). Furthermore, HSEs models with reconstructed epidermal–dermal interaction were used. The keratinocyte IGF-1R deficiency resulted in epidermal atrophy in HSEs models (Fig. 4D and E). Collectively, these results indicate that IGF-1R signaling mediates the organization of a well-stratified epidermis by preventing ectopic differentiation of ESCs.

3.5. IGF-1R is required for the maintenance of human keratinocytes with significant proliferative capacity

IGF-1R KO HaCaT keratinocytes could not organize a normal epidermal structure, which require a balance between keratinocyte proliferation and differentiation. The analysis of the RE models resulted in the hypothesis that IGF-1R deficiency decreases the proliferative capacity of human keratinocytes. To corroborate this conjecture, a cell proliferation assay was performed with non-treated conrol and sham control HaCaT cells and two KO clones. As expected, the proliferative capacities of the two KO clones were significantly lower than those of the nontreated and control HaCaT cells (Fig. 5A). Further, a colony-formation assay was performed to evaluate the long-term proliferative capacity of keratinocytes [31].

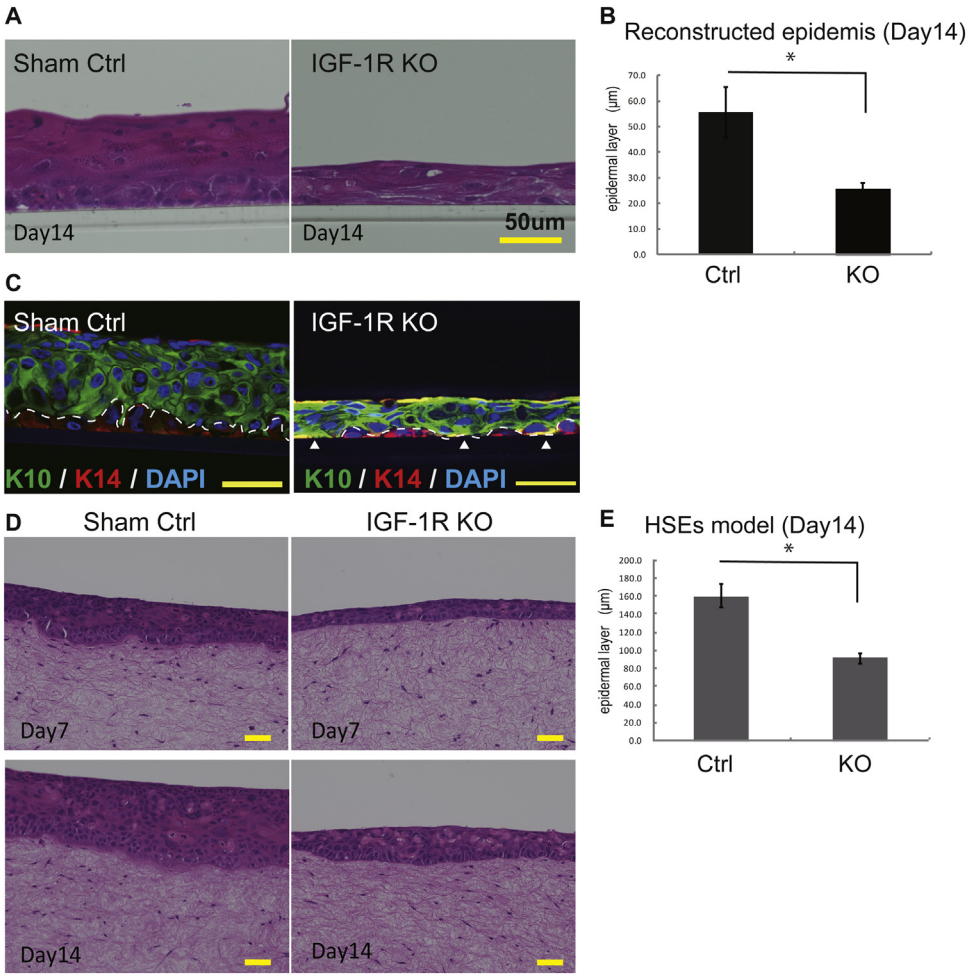

Fig. 4. IGF-1R deficiency in human keratinocytes causes epidermal hypoplasia.
A: Representative histological images of a sham reconstructed epidermis (Sham Ctrl; left) and IGF-1R KO cell (right) after 14 days of culture. B: Quantification of RE epidermal layer thicknesses. C: Representative images of immunohistochemistry for keratin 14 (K14) and keratin 10 (K10) in the epidermal layer of control RE (left) and those produced using KO cells (right). Arrowheads indicate epidermal differentiation marker K10-positive basal cells. D: Representative histological images of control (Sham Ctrl; left) and IGF-1R KO cell (right) HSEs after 7 and 14 days of culture. D: Quantification of HSEs epidermal layer thicknesses at 14 days. Scale bars =50 μm. Results are representative of four independent experiments performed in triplicate. Values represent means ± SD. *$p < 0.01$ vs. control.

Control HaCaT cells generated several large colonies with smooth peripheries (Fig. 5B and C); such colonies are derived from HaCaT keratinocytes with significant proliferative capacity [26]. However, IGF-1R KO HaCaT cells generated only small colonies (Fig. 5B and C); therefore, IGF-1R signaling is required for the long-term proliferative capacity for human keratinocytes.

4. Discussion

ESCs are keratinocytes that mediate epidermal homeostasis. Various studies have focused on the role of growth factors and cytokines in ESC maintenance through epidermal–dermal interactions, particularly in the wound-healing process [32]. In this study, IGF-1R-KO human keratinocytes generated by CRISPR-Cas9 were used to analyze IGF-1, which enhanced keratinocyte proliferation and migration, thereby demonstrating the role of IGF-1/IGF-1R signaling in human ESC maintenance and differentiation.

RE and HSEs skin models have been extensively used to investigate epidermal formation in human skin; however, it is difficult to examine the function of specific genes in such skin models because it is difficult to suppress genes during the 3D differentiation processes. In addition, siRNAs and small molecule inhibitors may affect nontarget cells in coculture systems such as HSEs and are relatively unstable and inefficient. Although IGF-1R suppression using lentiviral shRNA has recently been reported [33], complete protein suppression by shRNA has is not generally achievable.

To overcome this issue, genome-edited keratinocytes were prepared using the CRISPR-Cas9 system (Fig. 2). First, the experimental feasibility was confirmed using A549 cells, which express IGF-1R at high levels (Supplement Fig. 1). Transfection efficiency of the system was more than 30%, and mutation could be detected using the CEL I assay in the entire cell population. Forty-five colonies were selected, and five protein-deficient clones were eventually identified. In contrast, the transfection efficiencies of normal and immortalized keratinocytes (HaCaT) were as low as 3%. Mutations could not be detected when the total cell population was analyzed; however, they were analyzed using enriched OFP-positive populations (Fig. 2). Keratinocyte colonies as were selected for examination similar to A549 cells; however, KO keratinocyte clones could not be obtained because OFP-positive normal human keratinocytes did not generate colonies after cloning. Therefore, a limited dilution method (0.5–1 cells/well) was implemented to increase the number of colony acquisitions (Fig.3).

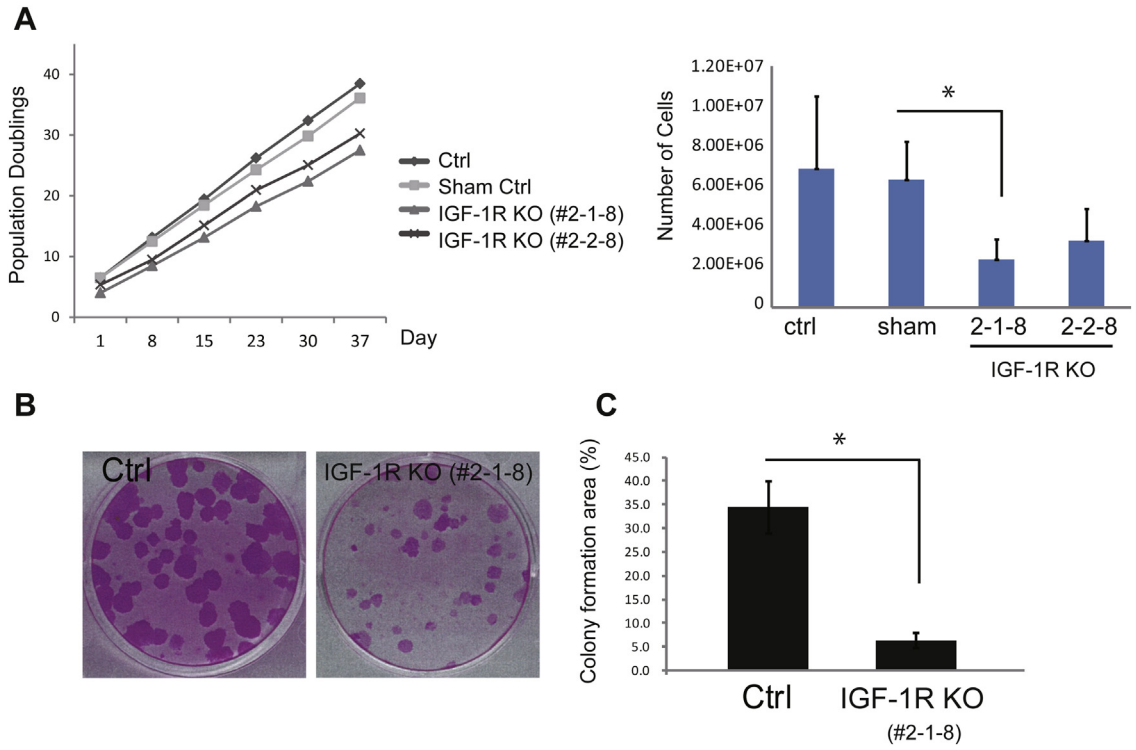

Fig. 5. Loss of IGF-1R signaling disrupts keratinocyte stem cell properties.
A: The cell numbers were counted at each passage and growth rate is represented graphically as population doubling over days in culture (left). Average cell numbers were counted for six passages (right) B: Colony formation of control (ctrl) and IGF-1R KO cells (clone #2-1-8). C: Colonies grown in 6-well plates were stained with rhodamine B and total area quantitated. Data are expressed as percentages relative to plate well area (9.6 cm^2) and represent three different experiments. Values represent means ± SD. *$p < 0.01$ vs. control.

CEL I assay was conducted to confirm successful genome editing; however, IGF-1R protein was still detected in some gene-modified clones, which were confirmed by CEL I assay, possibly because of frame-saving mutations.

The established IGF-1R KO cells exhibited lower proliferation, but these cells could be cultured for the same period as sham control cells without exhaustion (Fig. 5). Insulin/insulin receptor signaling is believed to compensate for the loss of IGF-1/IGF-1R signaling [29]. However, the thickness of the epidermis in 3D skin models produced using IGF-1R KO cells was approximately half that of those using control cells (Fig. 4). The gRNAs in this study were designed to avoid off-target gene editing as much as possible using the method described by Hsu et al. [34]. Furthermore, similar phenotypes of proliferation and thickness of RE and HSEs have been confirmed in KO strains established with gRNAs with different target positions (gRNA-1 and gRNA-2, data not shown).

This phenotype has been observed in genetically modified animals [29], suggesting that this system may be a useful tool for studying human skin. In addition, keratin 10, which is an epidermal differentiation marker, was expressed in the basal layer of RE epidermis produced with IGF-1R KO cells (Fig.4). Colony-formation assays further revealed that IGF-1R KO cells had a decreased long-term proliferative capacity (Fig. 5). These results indicate that IGF-1/IGF-1R signaling preserves the proliferative capacity of epidermal basal cells and inhibits their spontaneous differentiation; therefore, it maintains human epidermal homeostasis. Moreover, Loesch et al. have shown that IGF-1R controls DNA damage repair genes in human keratinocytes [35], therefore the ectopic differentiation in our study may caused by weakened of this repair mechanism.

This study utilized genome editing technology to clarify the role of IGF-1R signaling in human epidermal formation and homeostasis. Moreover, it provides a framework for future study of human skin biology using 3D culture systems with regard to replacement, refinement, reproducibility of experiments, and reduction of differences between species.

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Disclosure statement

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.05.001.

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