의학박사 학위논문

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a new method for trichogenecity test

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이 논문을 의학박사 학위논문으로 제출함

2019년 4월

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백승환의 박사 학위논문을 인준함

2019년 7월

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Skin equivalent assay:

a new method for trichogenecity test

by

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A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science (Dermatology) at Seoul National University College of Medicine

April, 2019

Approved by thesis committee

July, 2019

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ABSTRACT

Skin equivalent assay:
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Hair follicle reconstitution requires highly organized epithelial-mesenchymal interactions. Skin equivalents containing the epidermal and dermal cells with hair reconstitution capacity can reproduce these processes, but have not been established. This study was conducted to develop a hair follicle-producing three-dimensional (3D) skin equivalent assay using neonate mouse epidermal and dermal cells. A skin equivalent comprised of mouse dermal cells (MDCs) embedded in type I collagen and overlaid with mouse epidermal cells (MECs) was used. MDCs were mixed with type I collagen and cultured for 7 days. One day after adding MECs on top, the composites were grafted onto nude mice. MDCs cultured on a two-dimensional (2D) plate for 7 days and mixed

with MECs as a negative control, and freshly isolated MDCs and MECs mixture (chamber assay) as a positive control were also grafted. Six weeks after grafting, regenerated hair follicles were analyzed. Our 3D skin equivalent culture assay reproducibly regenerated hair follicles, while MDCs precultured in the 2D model with MECs did not. Compared to the chamber assay, which produced randomly oriented hair follicles, nearly all regenerated hair follicles in our assay extruded through the skin and numerous regenerated hair follicles were higher than those in the chamber assay. Several representative genes associated with hair induction showed higher expression in our assay than in the 2D model. When Wnt3a was added, the number of regenerated hairs increased. Organized hair follicle regeneration was accomplished using our assay. This approach can be applied to assess a test agent with hair growth-promoting effects.

Keywords: Skin equivalent, hair reconstitution assay, hair induction

Student Number: 2016-30574

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INTRODUCTION

The hair follicle is mini-organ that serves protective roles against physical and immunological insults.[1, 2] It also provides a reservoir of stem cells that can differentiate into hair follicle cells or epidermal cells.[3, 4] Therefore, bioengineering of the hair follicle is important for developing treatments for permanent hair loss as well as physiologic and enhanced regenerative therapies for skin defects.[5]

Complete regeneration of the hair follicle structure is difficult due to three-dimensional structural complexity of hair follicle.[6] Many *in vitro* models of hair follicle have been introduced and range from monolayer cell culture of hair follicle cells to sphere culture and sandwich models. [7-10] These *in vitro* models are sometimes useful, but they do not reproduce the fully functional hair, so there are limitations that they cannot deal with the questions about hair cycle.

In vivo state, hair follicles continue to receive blood supply and signals from nearby cells. Because these factors are critical for hair follicle development, complete reconstitution of hair follicles *in vitro* is hard to achieve. Thus, various *in vivo* hair

follicle reconstitution assays have been developed.

Hair follicle reconstitution from disassociated cells requires epithelial mesenchymal interactions.[11-13] Neonate human foreskin keratinocytes and neonate mouse epidermal cells (MECs) can be used as an epithelial cell source, whereas human dermal papilla (DP) cells or neonate mouse dermal cells (MDCs) can be used as a mesenchymal cell source.

The chamber assay and the patch assay are representative methods for reconstructing the hair follicle structure *in vivo*.[12] In the chamber assay, a sterile silicon chamber is implanted on the back of immune suppressed mouse and cell mixture of MECs and MDCs is spread into the chamber to achieve hair reconstruction. [14-16] The diameter of the chamber is 1 ~ 1.5 cm and the required numbers of the MECs and MDCs are both 1.0×10^7. The average cell yield per one neonate mouse skin is about 1.0×10^7 MECs and 1.0×10^7 MDCs, thus to perform one chamber assay one neonate mouse should be sacrificed. While requiring a large number of cells, the chamber assay has the advantage of high quality of regenerated hair follicles.

In the patch assay, 1.0×10^6 MECs and 2.0×10^6 MDCs mixture is injected subcutaneously on immune suppressed

mouse.[17, 18] Then, a cyst is formed at the injection site, and hair regeneration occurs inside the cyst. This method has a strength in that it requires small number of cells and is relatively simple to perform.

In addition, the planar assay, which has been developed recently, produces a self-organized new born skin organoid using MDCs and MECs and reconstructed skin formed a large number of hair follicles.[19] Recently, a comparison of these newborn skin organoids with those prepared using adult mouse dermal and epidermal cells revealed that suppressing epidermal differentiation is a critical point enabling hair regeneration of adult cells.[20]

In the general artificial skin making process, fibroblasts are used to form the dermis layer, while keratinocytes are used to form the epidermal layer.[21] After maturation of the composite, fibroblasts undergo collagen remodeling and keratinocytes are differentiated and stratified, resulting in skin that appears normal. However, no skin appendage structure such as hair follicles has been produced.

Several studies have been conducted to make a hair follicle structure in skin substitutes using trichogenic cells. Leiros et al. generated the construct using human hair follicle stem cells and

DP cells with a porcine dermal acellular matrix, resulting in embryonic hair bud-like structures when grafted onto nude mice.[22] Dong et al. observed the regeneration of hair follicles on *in vivo* grafts using mouse vibrissae DP cells precultured in Wnt1a-overexpressing bone-marrow mesenchymal stem cell-conditioned media and mouse keratinocytes.[23] A method of preparing a skin equivalent with MDCs and MECs for use in hair reconstitution assays has not been established.

The dermal part of a hair follicle consists of the DP and dermal sheath (DS).[24] The cells in DP and DS have the ability to induce hair follicles in hair reconstitution assays and express specific markers.[25] Although most markers are not well known for their function, some representative markers have been studied how cell characteristics vary with marker expression. Alkaline phosphatase (AP) is a classic marker of DP cells and is also expressed in proximal DS.[26] During *in vitro* culture of DP cells, hair inductivity is known to decrease after subculture, as is AP expression.[27] Hey1, versican are also markers for hair inductivity.[28, 29] CD133 is a marker of a hematopoietic stem cell that is expressed in the stage 3-4 hair follicle during development. CD133 positive dermal cells are known to induce hair neogenesis 10 times more than

unsorted dermal cells.[30] CD133 and Tbx18 are known be markers of DP progenitor cells.[30, 31] Sox2 is one of the highest-expressed transcription factors in the DP cells and is known to control the migration of hair shaft progenitors.[32]

In this study, we generated and characterized a three-dimensional (3D) skin equivalent system that produces hair follicles by using MDCs and MECs. We also examined the changes in the hair inducing marker genes (AP, hey1, versican, CD133, Tbx18, and Sox2) in this system and tested the effect of hair-growth promoting compounds.

MATERIALS AND METHODS

1. Cell isolation

Trichogenic neonate MDCs and neonate MECs were isolated as described previously with some modifications (Figure 1A).[16] Briefly, neonatal mice [C57BL/6J] (The Jackson Laboratory, Bar Harbor, ME, USA) were sacrificed within the first 24 h after birth. After sterilization with betadine and 70% ethanol, the trunk skin was harvested. Epidermal and dermal layers were separated by floating on 1.5 U/mL Dispase II (Roche Applied Science, Indianapolis, IN, USA) in PBS at 37° C for 1 h. The white epidermal layer was peeled off, centrifuged for 5 min, and incubated in 0.25% trypsin (Invitrogen, Carlsbad, CA, USA) for 10 min at 37° C. Cell suspensions were filtered through 40-㎛ strainers (FALCON, Durham, NC, USA). The dermis was digested in 0.35% collagenase I (Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37° C for 1 h. The cell pellets were resuspended in Dulbecco's Modified Eagle's medium (Sigma, St. Louis, MO, USA). Samples were filtered sequentially through 100-㎛ strainers

(FALCON). This study was approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University Hospital (IACUC number: 16-0013-C2A0).

2. Generation of 3D skin equivalents

3D skin equivalents were constructed using MECs and MDCs (Figure 1B). Briefly, collagen matrices were prepared by mixing the listed components in order: 10× MEM (minimum essential medium containing Earle's salts; Sigma; cat. no. M0275), 200 mM L-glutamine (Invitrogen, cat. 25030-081), fetal bovine serum (FBS; Hyclone, Logan, UT, USA; cat. SH30071), 71.2 mg/mL NaHCO3 (Sigma, cat. no. S5761), and type I collagen (Nitta Gelatin, Osaka, Japan; cat. no. 160927). A total of 5×10^5 MDCs were mixed with a prepared collagen matrix and the mixture was gently pipetted to evenly incorporate MDCs into the collagen matrix. The initial collagen concentration was 2.55 mg/ml in 3D skin equivalent. This cellular mixture was added to 3-μm porous polycarbonate membrane inserts (Millicell, cat. no. PITP01250, Millipore, Billerica, MA, USA). Dermal construct formation was carried out on ice to avoid warming of the collagen, and then the

construct was gently transferred to a 37℃ incubator for 30 min for gelatinization. The dermal construct was incubated for 7 days to allow for gel contraction. Fibroblast culture medium, prepared as described previously,[21] was changed every 2 days. Initially, the diameter of the insert was 12 mm. The diameter of the established skin equivalent is 7 mm. When we examined the changes in the number of MDCs during the incubation period in collagen gel for 7 days, we found there was no increase in the number of cells (N = 6) (Figure 2). To investigate the effects of Wnt3a (R&D Systems, Minneapolis, MN, USA; cat. no. 1324-WN-010/CF), we added 10 ng/mL Wnt3a to this medium. After 7 days of gel contraction, freshly isolated 1×10^6 MECs were added to each construct with epidermalization I medium which was prepared as described previously.[21] After maturation for 24 h, the 3D skin equivalent was grafted onto nude mice.

3. Grafting 3D skin equivalent on nude mice

For grafting of the 3D skin equivalent, we used a modified method of Lee et al (Figure 1C).[19] We used 8-week-old female Balb/C nude mice (Charles-River Laboratories,

Sulzbach, Germany) as recipients. After making a wound with a diameter of 1.5 cm on the back of nude mice, a sterile silicon chamber was inserted. Because our 3D skin equivalent was jellified, it was picked up with small forceps and grafted through a small hole on the chamber (N = 5). MDCs cultured on a two-dimensional (2D) plate for 7 days (2dMDC, 5×10^5 cells) mixed with freshly isolated MECs (fMECs, 1×10^6 cells) (negative control, N = 5) and a mixture of freshly isolated MDCs (fMDCs, 5×10^5 cells) and fMECs (1×10^6 cells) (chamber assay, positive control, N = 5) were also grafted. To determine the effects of Wnt, we transplanted 3D skin equivalents treated with Wnt3a at 10 ng/mL (N = 4) and those without (N = 4). After 2 weeks, the chamber was removed, and the graft site was harvested at 6 weeks after transplantation. The procedural details on dressing are presented in figure 3.

4. Hair count assay

Six weeks after grafting, regenerated hair follicles were analyzed (Figure 1D). Gross and dermoscopic evaluation was carried out (Dermlite FOTO, 3Gen, San Juan Capistrano, CA, US). Under microscopy, we manually counted the number of

regenerated hairs and compared the results among the study groups.

5. Histology

To investigate the orientation and density of the hair follicles, the tissues at the grafted site were analyzed histologically as described previously.[33] The skin tissues were fixed in Fekete' s acid-alcohol-formalin overnight and then transferred to 70% ethanol until trimming. The tissues were embedded in paraffin and sectioned at 4 μm both horizontally and vertically. They were stained with hematoxylin and eosin.

6. Hair-inducing marker gene expression - real time PCR

Total RNA was isolated from the 3D dermal constructs, MDCs cultured in 2D for 7 days, and freshly isolated MDC using RNAiso Plus reagent (Takara Bio). To investigate the effects of Wnt3a, we isolated total RNA in Wnt treated 3D dermal constructs and those without Wnt treatment. Approximately 2 μg of total RNA was used for cDNA synthesis using a First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA)

according to the manufacturer's instructions. Quantitative estimation of mRNA expression was performed by real-time polymerase chain reaction by using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex Taq (Takara Bio, Inc.), according to the manufacturers' instructions. All experiments with SYBR Green were performed in triplicate and were independently repeated 13 times. The primers used in this study are as follows: m36b4 (forward, TGG GCT CCA AGC AGA TGC; reverse, GGC TTC GCT GGC TCC CAC), AP (forward, CAG GTC CCA CAA GCC CGC AA; reverse, CCC GGT GGT GGG CCA CAA AA), Hey1 (forward, ACC TTC CAG TGG CTC CTC; reverse, TTT AGT GTC CGT CAG AAG AGA G), versican (forward, GAG CCA GAC ATC GCA GAA GA; reverse, TTC ATC GTC AGA ACC TCC CA), CD133 (forward, TCC CAC TTG ATG CCA CTG CCA A; reverse, ATT CCG CTC CCA GCT GAG CG), Tbx18 (forward, ATG GCC TCC AGA ATG CGT ATG; reverse, TGT CCC CCA TCA AGC CTG TT), Sox2 (forward, ACT GGC AAG ACC GTT TTC GTG GT; reverse, ACC AAC GAT ATC AAC CTG CAT GGA CA).

7. Statistical analysis

Statistical analyses were performed using SPSS version 22 software (SPSS, Inc., Chicago, IL, USA). Analysis of variance and Student's *t*-test were used to determine statistical significance. *P*-values < 0.05 were considered significant.

RESULTS

1. Number of regenerated hairs

Six weeks after grafting, minimal hair regeneration was achieved in the negative control group (2dMDC−fMEC mixture) and a small number of hairs was regenerated in the positive control group (fMDC−fMEC mixture). In contrast, our 3D skin equivalent system uniformly reproduced a large number of hairs (Figure 4A, B).

The numbers of regenerated hairs were 255 ± 61 (Mean ± SD), 61 ± 23 (Mean ± SD) and 1.8 ± 4 (Mean ± SD) in 3D skin equivalent group, positive control group and negative control group, respectively. The 3D skin equivalent system showed more significant hair regeneration than that by the positive and negative controls ($P < 0.01$) (Figure 4C). In histologic section, hair density was higher in the 3D skin equivalent group than in the positive control group (Figure 4D).

2. Hair−inducing marker gene expression

We compared the expression of representative genes associated with hair induction between 2D−cultured MDCs (2D

MDC), 3D-cultured MDCs (3D MDC), and fMDCs (Figure 4E).

2.1. 2D MDC versus 3D MDC

AP, Hey1, and versican are correlated with hair-inducing properties.[29] Expression of these genes was higher in the 3D skin equivalent group than in the matched 2D culture samples ($P < 0.01$). However, the expression of versican was similar ($P = 0.86$).

CD133 and Tbx18 are markers of DP precursors.[30, 31] Both were higher in the 3D skin equivalent group than in the 2D culture group ($P < 0.01$).

Sox2 is one of the most expressed transcription factors in the DP cells.[32] The expression of Sox2 was higher in the 3D skin equivalent group than in the 2D culture group ($P < 0.05$).

2.2. 3D MDC vs fMDC

Because fMDCs are one of the most potent trichogenic cells, fMDCs showed the highest expression levels of hair-inducing marker genes. The expression of AP, Hey1, and Tbx18 was higher in fMDC than 3D MDC. However, the expression of versican, CD133 and Sox2 was comparable between two groups.

3. Hair orientation

When tissues at the transplantation site were investigated from the dermal side, we observed that the direction of the hair differed between the 3D skin equivalent group and chamber assay group. In our 3D skin equivalents, hair growth towards the dermal side was minimal, whereas in chamber assay tissue, we observed many hairs growing horizontally across the dermis (Figure 5A).

Histological analysis results revealed normal hair regeneration in both the vertical and transverse sections of the 3D skin equivalent group. In the chamber assay group, distorted hairs were observed in the vertical section and oval shapes of regenerated hairs, indicating that the regenerated hairs were not vertically oriented, were observed in the transverse section (Figure 5B).

Characterization of the regenerated hair in the skin equivalent assay confirmed that the regenerated hair had normal dermal papilla and a normal sebaceous gland structure (Figure 5C).

4. Wnt3a effect on 3D skin equivalent

Wnt signals are an essential component of hair development and regeneration.[34, 35] Several Wnts are known to be involved in the physiology of hair follicles, among which Wnt3a has been shown to be highly effective in increasing hair inductivity of the DP cells.[36] To examine the effect of a hair growth-promoting agent in our 3D skin equivalent assay, we added Wnt3a and investigated the number of regenerated hairs.

The number of regenerated hairs was significantly increased in the group treated with 10 ng/mL Wnt3a compared to the group without Wnt3a treatment ($P < 0.05$) (Figure 6A). The numbers of regenerated hairs were 255 ± 27 (mean ± SD) and 361± 88 (mean ± SD) in the untreated and 10 ng/mL Wnt3a treated group, respectively (Figure 6B). When we compared the changes in the number of MDCs in the group with and without Wnt, the number of cells was similar between two groups ($P = 0.356$, N=6). The expression of Hey1 and Tbx18 was higher in Wnt treated group than the group without Wnt treatment ($P < 0.01$) (Figure 7).

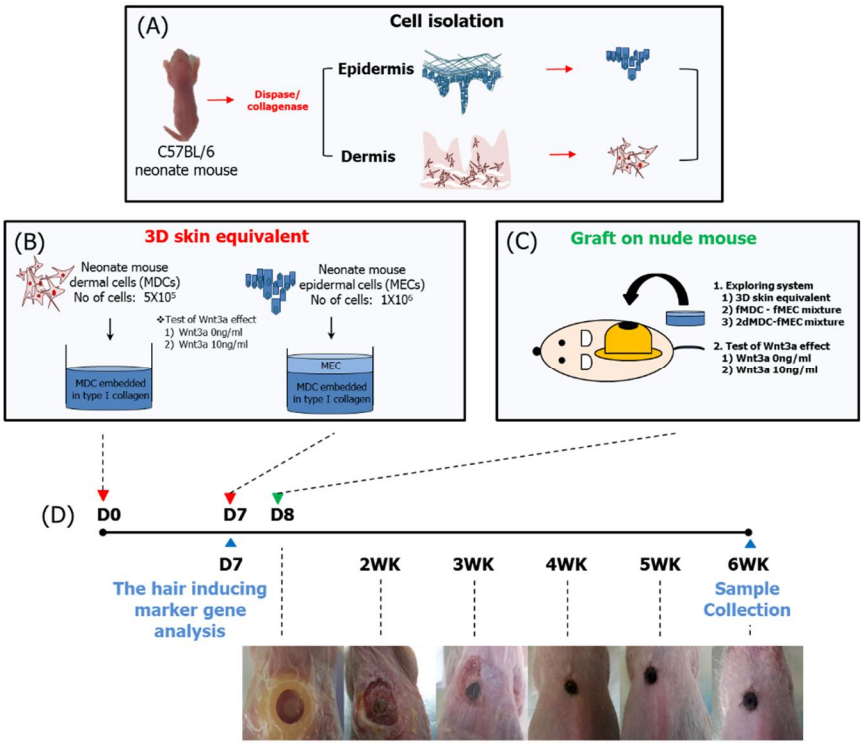

Figure 1. Scheme of the present study.

Neonate mouse dermal cells (MDCs) and neonate mouse epidermal cells (MECs) were isolated from newborn C57BL/6J mice (A). A total of 5 × 10^5 MDCs were mixed with type I collagen and cultured for 7 days. After collagen contraction, 1 × 10^6 MECs were added to the dermal construct. When testing the effect of Wnt, Wnt3a was added to the culture medium of the dermal construct (B). One day after adding MECs, the composites were grafted onto nude mice (N = 5). The mixture of MDCs cultured in 2D plate for 7 days (2dMDCs) and freshly isolated MEC (fMECs) (negative control, N = 5) and freshly

isolated MDCs (fMDCs) and fMECs mixture (positive control, N = 5) were also grafted. In a further experiment to see the Wnt effect, we grafted 3D skin equivalents treated with Wnt3a (N = 4) and without (N = 4) (C). Six weeks after grafting, graft site tissues were harvested and formed hair follicles were analyzed. In marker gene analysis, we compared the dermal construct, 2dMDCs and fMDCs (D).

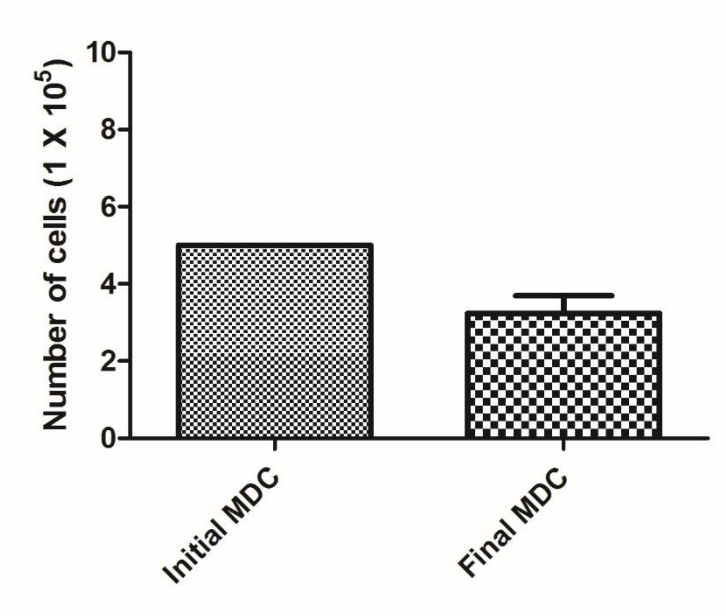

Figure 2. The changes in the number of MDCs in the dermal construct.

During the incubation period in collagen gel for 7 days, there was no increase in the number of MDCs. (N = 6)

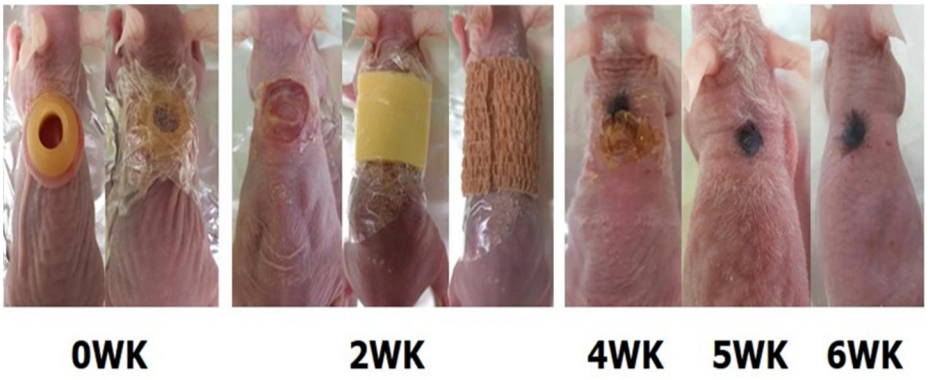

Figure 3. The dressing procedures in skin equivalent assay.

On the day of transplantation (0 weeks), we attached a Tegaderm ™ Film (3M Health Care, St. Paul, Minn., USA) on the chamber to moisturize the gel and let it settle stably on the back of the mouse. Two weeks after the transplantation, the chamber was removed and Medifoam (Ildong Pharmaceutics, Seoul, Korea) was applied inside out onto the back, and the tegaderm was attached onto it. Finally, we wrapped the transplanted area with Coban (3M Health Care, St. Paul, MN, USA). Four weeks after transplantation, all the dressing materials were removed. The regenerated hair follicles were analyzed histologically 6 weeks after the transplantation.

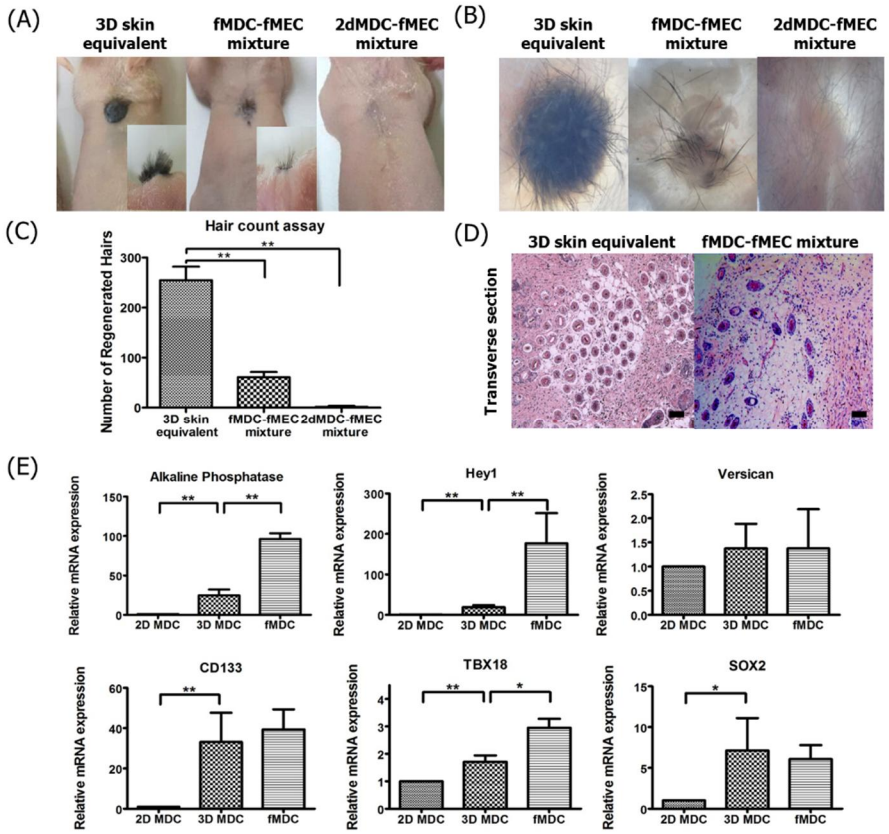

Figure 4. Regenerated hairs of the 3D skin equivalent and positive and negative controls.

At 6 weeks after transplantation, nearly no hair regeneration in the 2dMDC-fMEC mixture (N = 4) and a small number of disoriented hair regeneration in fMDC-fMEC mixture (N = 5) were observed in gross analysis (A) and dermoscopic view (B). In 3D skin equivalents (N = 5), a uniform and large amount of hair regeneration was observed. The number of regenerated hairs was higher in the 3D skin equivalent than in the 2dMDC-fMEC mixture and fMDC-fMEC mixture ($P < 0.01$) (C).

Histological analysis revealed higher hair density in the 3D skin equivalent than in the fMDC−fMEC mixture (scale bar, 100 μm) (D). Several representative marker genes (Alkaline phosphatase, Hey1, CD133, Tbx18, and Sox2) were highly expressed in the 3D skin equivalent compared to in 2dMDCs. The expression of versican, CD133, and Sox2 was comparable between fMDC and 3D skin equivalent (E). * $P < 0.05$, ** $P < 0.01$.

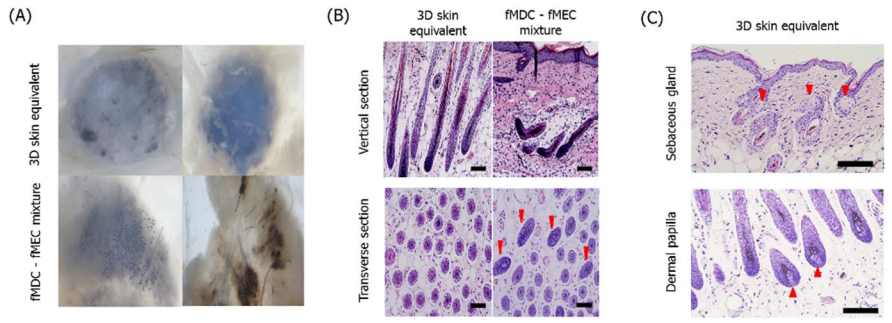

Figure 5. Hair orientation.

The tissues of the grafted site were examined by dermoscopy from the dermal side. Whereas hairs oriented horizontally across the dermis were observed in the chamber assay group (fMDC-fMEC mixture), hairs growing toward the dermis were not observed in the 3D skin equivalent (A). In the 3D skin equivalent, normal-looking hairs were observed in both vertical and transverse sections. In the chamber assay (fMDC-fMEC mixture), aberrant oriented hairs were observed in the vertical section and oblique-cut hairs (arrowhead) were observed in the transverse section (scale bar, 100 μm) (B). The regenerated hairs showed a normal hair structure including sebaceous gland and dermal papilla (arrowhead) (scale bar, 100 μm) (C).

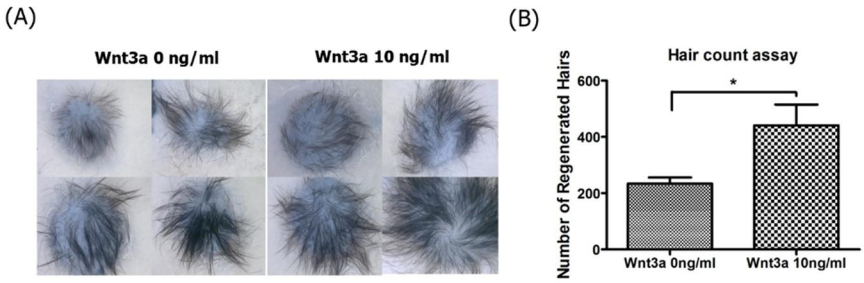

Figure 6. Effect of Wnt3a on 3D skin equivalent.

Wnt3a was added during generation of the 3D skin equivalent. Wnt3a increased hair regeneration 6 weeks after grafting of the equivalent on nude mice (dermoscopic view) (A). The number of regenerated hairs was higher in the 10 ng/mL Wnt3a-treated group than in the untreated group ($P < 0.05$) (B). $* P < 0.05$.

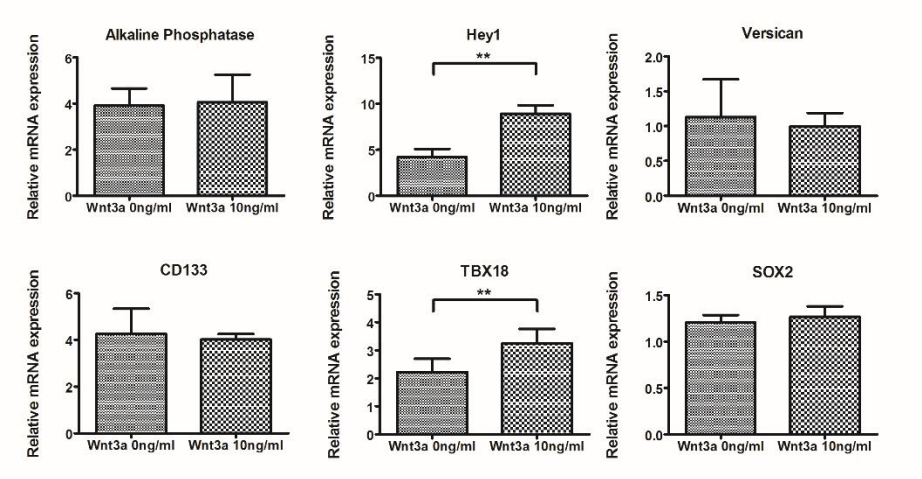

Figure 7. Hair-inducing marker gene analysis between skin equivalent with and without Wnt3a treatment.

The expression of Hey1 and Tbx18 was higher in Wnt3a-treated group than the group without Wnt3a treatment. ** $P < 0.01$.

DISCUSSION

In the present study, we produced a skin equivalent system using trichogenic cells and confirmed that hair reconstitution occurs in this model when transplanted *in vivo*. Compared to the widely-used chamber assay, regenerated hairs were more in number and well-oriented in the present system. As a test model of a hair-growth promoting agent, our skin equivalent showed reliable results with increased hair regeneration following Wnt3a treatment.

Skin equivalents have various applications, including therapeutic and research purposes.[37] In regenerative medicine, skin equivalents are used to treat skin defects caused by trauma, burns, and diseases to provide a protective barrier. Previously, many skin equivalents were constructed for therapeutic purposes, some of which are commercially available.[38] In the research field, skin equivalents have been made to test for skin irritation to replace animal testing. To date, the EpiDerm and EPISKIN models showed an acceptable specificity and sensitivity for skin irritation potential and experimental protocols are being evaluated to achieve better

predictability.[39] Skin equivalents prepared in our study can be used to test hair-inducing agents. It is also possible to evaluate the hair-inducing properties of cells by making similar skin equivalents with different cells.

There are two representative types of hair reconstitution assays: the patch assay and chamber assay.[12, 16-18, 40] These assays can effectively be applied to investigate follicle reconstitution, but each has distinct and different advantages and limitations. The patch assay is the simplest method and requires fewer cells, but produces low-quality and low-density hair shafts because it regenerates hair in the cyst structure. As the hairs pass through the hair cycle, the cyst becomes inflamed and fibrosis occurs over the long term.[40] While the chamber assay produces hair shafts of high quality and density, it requires a large number of cells.[16] In this study, we revealed that the regenerated hairs formed across the dermis horizontally in the chamber assay, which is consistent with the histologic findings of Lichti et al. who first described this assay.[14] Our assay is advantageous because it showed better hair shaft quality and density compared to the chamber assay. Furthermore, the number of cells used was smaller than that used by the chamber assay.[16]

The recently developed planar assay is very similar to the skin equivalent assay among all the published hair regeneration systems. In the planar assay, the mixture of epidermal and dermal cells was used as the starting cell population embedded in the collagen gel. In the current system, dermal cells were embedded in the collagen for 7 days, followed by seeding of epidermal cells. The planar assay is advantageous in that it can be performed in a flexible size and shape and is easy to carry out.[19, 20] Our method has the advantage in part to perform quantitative research procedures, as, while planar assay achieves many hair regeneration with a large number of cells, our assay shows consistent hair follicle reconstitution with a specified number of cells.

It is important that the orientation of regenerated hair in the hair reconstitution assay penetrates the skin surface as in a normal hair shaft. In the patch assay, disoriented hairs are not physiologic, decreasing the quality of the regenerated hairs.[40] According to the Lee at al., randomly mixed MECs and MDCs can self-organize and eventually form well-oriented hairs, when the slurry of MECs and MDCs was grafted onto immune-deficient mice.[19] Similar processes may occur at the implanted site in the chamber assay. However, in our study, we

observed aberrantly oriented hair in the chamber assay, which may affect the quality of regenerated hair. The better orientation of regenerated hairs in our system may be related to the layered structure of the epidermis and dermis, which was pre-generated *in vitro*.

The levels of hair-inducing marker genes were increased in our 3D skin equivalent compared to in 2D culture. It is well-known that the *in vivo* characteristics of DP cells are maintained by cell aggregation in sphere culture.[41-44] DP cell aggregation can sustain DP function by providing a similar microenvironment of DP cells *in vivo*.[12] Our skin equivalent granted MDCs a microenvironment analogous to that *in vivo*, which may have increased the hair induction properties.

No changes in the number of MDCs during incubation period in the collagen for 7 days would have been related to the relatively hypoxic state. Because the media in contact with oxygen was not directly in touch with the MDCs, and MDCs were surrounded by collagen. Hypoxic status is known to inhibit cell proliferation in many cell types, including embryonic fibroblasts and embryonic stem cells.[45-47] In addition, hypoxia is one of the features of the stem cell niche and is known to help maintain the undifferentiated state of the stem-

progenitor cells.[47, 48] In line with these studies, the induction capacity of MDCs in the current system would be increased, with static cell proliferation.

The fMDCs showed higher expression of genes of hair inductivity, including AP and Hey1, than 3D MDCs, and the number of regenerated hair of the chamber assay using fMDC was lower than that of the skin equivalent assay using 3D MDCs. Usually, the camber assay with 20 mm diameter chamber uses 1×10^7 MDCs and 1×10^7 MECs to achieve dense hair regeneration.[40] Even with the high trichogenic capacity of each fMDC, 5×10^5 fMDCs would be insufficient for the epithelial–mesenchymal interactions across the whole wound area (15 mm diameter of chamber in our experiment). In our approach, more hair regeneration could occur due to the increased epithelial–mesenchymal interactions within the structured skin equivalent system.

Previously developed hair reconstitution assays have a determined the number of cells required to achieve proper hair regrowth.[40] In a preliminary study, we mixed various numbers of MDCs with collagen. In the preparation of artificial skin, when the number of dermal fibroblasts was increased, collagen contraction became severe and the area of artificial

skin decreased.[21] Therefore, by adding more MDCs than we established in the preliminary study, although the number of cells was large, the skin equivalent area itself decreased and the number of regenerated hairs was reduced. Based on these results, we determined the appropriate number of MDCs (data not shown).

One limitation of this study was that the skin equivalents could not reproduce hairs *in vitro*. Hair follicles receive oxygen and nutrients from the blood and paracrine signals from surrounding cells *in vivo*. These factors are known to be critical for hair follicle development and cycling.[24] Previous studies failed to regenerate hair *in vitro* because these conditions were not fully mimicked.[40] Further studies are needed to mimic the *in vivo* state. This may be achieved by using microfluidic devices or by making an organoid using a 3D printer to stimulating the *in vivo* state.

In summary, we developed a protocol for generating a 3D equivalent system using MDCs and MECs that could reconstitute hair follicles when grafted on immune-deficient mice. Several hair-inducing marker genes (AP, hey1, CD133, Tbx18, and Sox2) were expressed at higher levels in our system compared to in the 2D culture model. The quality and

density of regenerated hair was better than those of the chamber assay method using the same number of cells. Treatment with Wnt3a during preparation of the dermal layer in our system increased the number of regenerated hairs.

This study showed that our skin equivalent can be used as a hair reconstitution assay. Our system showed promising hair regeneration compared to existing hair reconstitution assays by replicating the *in vivo* orientation of epithelial and mesenchymal cells while preparing the construct. This suggests that the epithelial–mesenchymal interaction required for hair regeneration is further enhanced by using our system. Further studies are needed to investigate the hair growth–promoting effect of various agents in this system.

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국 문 초 록

인공피부 모낭 분석법:

발모성 평가를 위한 새로운 방법의 개발

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모낭 재구성에는 고도로 조직화 된 상피-중간엽 상호 작용이 필요하다. 모낭 재구성 능력을 가진 표피 세포 및 진피 세포를 함유하는 인공피부는 모낭 재구성 과정을 재현 할 수 있지만, 아직 제작 방법이 확립되어 있지 않다. 이 연구는 신생아 마우스의 표피 세포와 진피 세포를 이용하여, 모낭을 생성하는 3차원 인공피부를 개발하기 위해 수행되었다.

I 형 콜라겐을 포함한 기질에 마우스 진피 세포 (Mouse dermal cells: MDCs)를 혼합하여 진피 조직을 제작하고, 마우스 표피 세포 (Mouse epidermal cells: MECs)를 상부에 첨가하여 표피 조직을 완성한 인공피부를 제작하였다. MDCs는 I 형 콜라겐을 포함한 기질에 3차원으로 7 일간 배양 하였다. 상부에 MECs를 첨가 한 지 하루 후, 인공피부를 누드 마우스에 이식시켰다. 2 차원으로 7 일간 배양 한 MDCs와 신선하게 분리한 MECs를 이용한 체임버 분석법을 음성 대조군으로, 신선하게 분리한 MDCs와 MECs를

이용한 체임버 분석법을 양성 대조군으로 사용하였다. 이식 6 주 후에 재생된 모낭을 분석하였다.

우리의 인공피부 모낭 분석법은 재현적으로 모낭을 재생 시켰다. 이에 반해 2차원 배양된 MDCs와 MECs 를 이용한 체임버 분석법은 모낭 재생이 이루어지지 않았다. 모발 방향성이 무작위적인 모낭을 생성하는 체임버 분석법에 비해, 인공피부 모낭 분석법에서 거의 모든 재생 모낭은 모발 방향성이 증가되었고, 재생된 모낭의 수도 체임버 분석법보다 높았다. 모발 유도와 관련된 몇 가지 대표적인 유전자의 발현은 인공피부 모낭 분석법에서 2차원 배양보다 더 높았다. 모발 성장 증가 물질에 대한 반응을 보기 위하여 Wnt3a를 인공피부 제작 시 첨가 하였을 때, 재생 된 모낭의 수가 증가하였다.

인공피부 모낭 분석법은 기존의 모낭 분석법에 비하여, 모발 방향성과 재생된 모낭의 수가 증가하는 것을 관찰 할 수 있었다. Wnt3a 첨가 시 재생된 모낭의 수가 증가 되었다는 점에서, 이 분석법은 모발 성장 촉진 효과가 있는 시험 약제를 평가하는 데 적용 할 수 있을 것이다.

주요어: 인공피부, 모낭 재구성 분석법, 모낭 유도
학번: 2016-30574