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Original Article

Establishment of reproducible method for production of cultured epidermal sheets with controlled melanocyte density

Mai Mori [a, b, 1], Chie Kobori [a, c, *, 1], Ryo Takagi [a, d], Moeka Tani [a], Hidenori Akutsu [a, b], Akihiro Umezawa [a]

[a] Center of Regenerative Medicine, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
[b] Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8510, Japan
[c] Department of Plastic and Reconstructive Surgery, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan
[d] Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, TWIns, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

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ABSTRACT

Introduction: Vitiligo, the most common human pigmentary disorder, significantly impacts patients' quality of life through its profound psychological and social consequences. The condition's complex pathogenesis and substantial effect on patients' well-being underscore the critical need for advanced therapeutic approaches. Treatment for vitiligo by autologous cultured epidermal transplantation has been developed, and the efficacy of this treatment has been determined. However, there is concern that differences in postoperative pigmentation of affected areas may occur due to difficulty in adjusting melanocyte content. This study aimed to develop a reproducible method for fabricating cultured epidermal sheets containing melanocytes for use in clinical applications.

Methods: Human epidermal keratinocytes and melanocytes derived from skin tissues of volunteer donors were prepared under different culture conditions and the proliferation of keratinocytes and melanocytes was determined. The density and percentage of melanocytes in cultured epidermal sheets were evaluated by DOPA staining and flow cytometry. Moreover, a novel clinical-grade melanocyte culture medium with specific supplements was developed that avoids bovine pituitary extract containing unknown animal-derived unknown materials.

Results: The density of melanocytes was higher when melanocytes were seeded first, as opposed to the simultaneous seeding of melanocytes and keratinocytes. The best condition in the present study for preparing cultured epidermal sheets with high melanocyte density was to seed melanocytes at 1.0×10^4 cells/cm^2 on Day 1, followed by feeder cells on Day 2 and keratinocytes at 8.0×10^4 cells/cm^2 on Day 3. The optimal culture medium for melanocytes was determined to contain 10 nM aMSH, 10 nM endothelin-1, and 10 μg/mL FGF-2 as culture supplements.

Conclusions: The results of this study establish a foundation for the development of cell therapy effective for vitiligo.

1. Introduction

Human epidermis is composed of 95 % keratinocytes and the remainder consists of melanocytes, Langerhans cells, and Merkel cells. Melanocytes play an important role in protecting the body from harmful ultraviolet rays by synthesizing and transferring melanin to keratinocytes. Vitiligo, affecting approximately 0.1 % of the global population, is the most common human acquired pigmentary disorder. It significantly impacts patients' quality of life, causing severe psychological distress, especially when it involves the exposed skin, such as on the face or hands [1–3]. The complex

Abbreviations: MCs, Melanocytes; KCs, Keratinocytes.

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* Corresponding author. Center of Regenerative Medicine, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan.

E-mail address: iida-c@ncchd.go.jp (C. Kobori).

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[1] Mai Mori and Chie Kobori contributed equally to this work.

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and unclear etiology of vitiligo has hindered the development of definitive therapies. The current first-line treatment includes medical therapies such as psoralen, ultra-violet A, and immunomodulating agents. However, these approaches are often less effective for vitiliginous lesions on areas like the back of the hands, eyelids, or around the mouth. In such cases, surgical interventions are considered, including transplantation of cultured epidermal autograft [4].

Our team previously employed cultured epidermal autograft transplantation for vitiligo treatment with favorable outcomes. However, inconsistent results in some cases led us to hypothesize that the unreproducible ratio of melanocytes to keratinocytes in the grafts might be responsible for the variable pigmentation outcomes.

To address this issue, we developed a novel approach involving separate isolation and culture of melanocytes and keratinocytes from donor skin, followed by co-culture with controlled cell ratios. This method successfully yielded reproducible and high-density melanocyte-containing epidermal sheets. However, it necessitated the development of a new melanocyte culture medium, as the commonly used MGM4 (Lonza, Switzerland) contains bovine pituitary extract (BPE), which is inconsistent with the Standard for Biological Ingredients because it contains unknown animal-derived material.

Therefore, this study aimed to develop a reproducible method for fabricating cultured epidermal sheets with controlled melanocyte density, as well as to establish a BPE-free culture medium suitable for melanocyte proliferation and maintenance. We anticipate that these advances will enhance the stability and clinical applicability of cultured epidermal autograft transplantation, potentially broadening its use in vitiligo treatment.

2. Methods

2.1. Preparation of epidermal cell suspension

Subcutaneous tissue was removed by scissors from skin specimens, and the skin specimen was disinfected with 10 % povidone-iodine (MEIJI, Japan) and washed with Dulbecco's Phosphate-Buffered Saline (DPBS, Thermo Fisher Scientific, US). The epidermis and dermis were shredded into approximately 5 mm squares and soaked in Disperse I (FUJIFILM, Japan) overnight at 4 °C to separate the epidermis from the dermis. Separated epidermis was treated with 0.25 % Trypsin-EDTA (Thermo Fisher Scientific) at 37 °C for 20 min under 5 % CO_2 and then separated into residue and cell suspension by a 100 μm filter. Epidermal cell pellets were obtained by centrifugation of the cell suspension at 190×*g* for 5 min. The cell suspension was made in KCM (keratinocyte culture medium [5]) supplemented with 10 μM Rho kinase inhibitor Y-27632 (FUJIFILM) for keratinocyte culture and in MGM4 (melanocyte culture medium, Lonza, Switzerland) for melanocyte culture [6].

2.2. Culture of keratinocytes

Mouse fibroblasts were incubated with MEM-α (Thermo Fisher Scientific) containing 10 μg/mL mitomycin C (Nacalai tesque, Japan), 100 units/mL Penicillin Streptomycin (Pen-Strep, Thermo Fisher Scientific), and 0.25 μg/mL Fungizone (Bristol Myers Squibb, Japan) at 37 °C for 2 h under 5 % CO_2. Then, the cells were treated with 0.25 % Trypsin EDTA at 37 °C for 1 min under 5 % CO_2, and the collected cell suspension was centrifuged at 190×*g* for 5 min to remove the supernatant. The cells were suspended in MEM-α containing 10 % FBS (Thermo Fisher Scientific), 100 units/mL Pen-Strep, and 0.25 μg/mL Fungizone, and viability of the cells were measured using Vi-CELL XR (Beckman Coulter). The cells were then seeded at 2.0 × 10^4 cells/cm^2 and cultured at 37 °C under 5 % CO_2 to serve as feeder layers for keratinocytes. The next day, epidermal cells were seeded at 1.0 × 10^4 cells/cm^2 in feeder cells seeded culture dishes and cultured in KCM medium +10 μM Y-27632 at 37 °C under 5 % CO_2. Medium was changed every three to four days. After approximately one week, when the cells became confluent, they were treated with 0.25 % Trypsin-EDTA for 10 min at 37 °C and 5 % CO_2. The collected cell suspension was centrifuged at 190×*g* for 5 min, the supernatant was removed, suspended in KCM medium +10 μM Y-27632, and seeded at 1.0 × 10^4 cells/cm^2 and passaged as keratinocytes.

2.3. Culture of melanocytes

Epidermal cells suspended in MGM4 medium were seeded at 4.0 × 10^4 cells/cm^2 on culture dishes previously coated with 0.25 μg/cm^2 Laminin-511 (Nippi, Japan) and incubated at 37 °C under 5 % CO_2. Medium was changed every three to four days. On day 14 of the culture, the cells were treated with 0.25 % Trypsin-EDTA for 3 min at 37 °C under 5 % CO_2 in order to detach only melanocytes from other cells and centrifuged at 1000 rpm for 5 min. After removal of the supernatant, the cells were suspended in MGM4 medium and seeded whole without counting the number of viable melanocytes in the first passage. Approximately 14 days after passages, the cells were treated with 0.25 % Trypsin-EDTA for 3 min at 37 °C, 5 % CO_2, and the cell suspension was centrifuged at 190×*g* for 5 min. Then the supernatant was removed and the cells suspended in MGM4 medium and seeded at 2.0 × 10^4 cells/cm^2 for passaging.

2.4. Co-culture of melanocytes and keratinocytes

The seeding densities of melanocytes and keratinocytes were 0.1–5.0 × 10^4 cells/cm^2 and 1.0 to 8.0 × 10^4 cells/cm^2. When seeding melanocytes, culture dishes were laminin-coated in advance, and mitomycin C treated feeder cells were seeded one day before seeding keratinocytes. When melanocytes and keratinocytes were seeded simultaneously, the cells were cultured in KCM medium +10 μM Y-27632 (Fig. 1A). When melanocytes were seeded prior to keratinocyte seeding, they were cultured in MGM4 medium, and when feeder cells were seeded, they were cultured in MEM-α containing 10 % FBS, 100 unit/mL Pen-Strep and 0.25 μg/mL Fungizone (Fig. 1B and C). Co-culture of keratinocytes and melanocytes were cultured in KCM medium +10 μM Y-27632 and medium was changed every three to four days until confluent.

2.5. Medium examination

Melanocytes were cultured with MGM4 or TIVA [7] medium supplemented with 10 nM aMSH (Sigma-Aldrich), 10 nM Endothelin-1 (FUJIFILM), 10 μg/mL FGF-2 (KAKEN PHARMACEUTICAL, Japan). Melanocytes, cultured in MGM4 medium, were suspended with 2.0 × 10^4 cells/cm^2 on the dishes coated with 0.25 μg/cm^2 Laminin-511. Four dishes were prepared on each medium and cell counts of melanocytes were performed on Day 4, 7, 10, 13. The ingredients for MGM4 and TIVA are provided in Table 1.

2.6. DOPA staining

Cells were washed with DPBS, fixed with 4 % paraformaldehyde (Nacalai tesque) for 10 min at room temperature, and washed three times with DPBS. The cells were soaked in 1 mL of 0.1 M PB (MUTO PURE CHEMICALS, Japan) for 10 min at room temperature. The cultured epidermal sheets were then light-

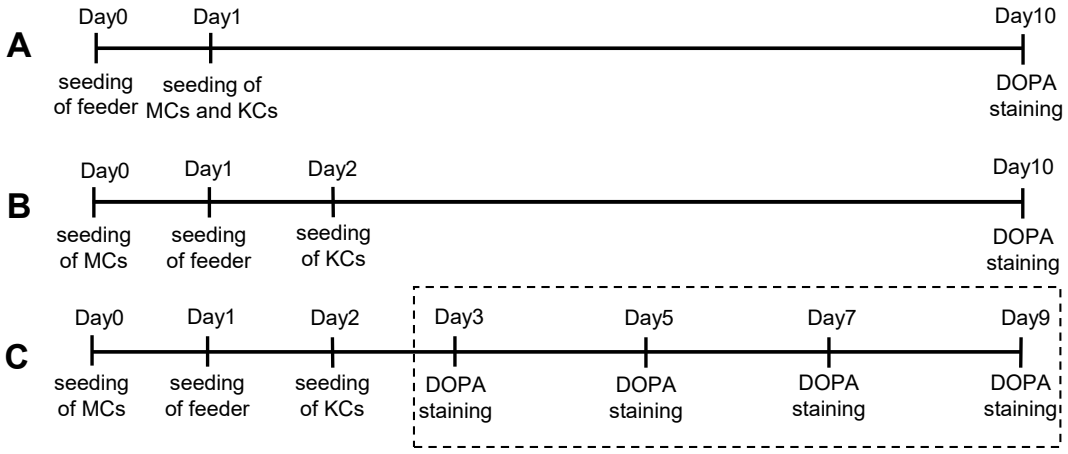

Fig. 1. Seeding order of melanocytes, feeder cells and keratinocytes in co-culture. A. Seeding melanocytes (MCs) and keratinocytes (KCs) simultaneously. **B**. Seeding melanocytes 2 days before keratinocytes. **C**. Seeding melanocytes 2 days before keratinocytes and observing melanocytes with DOPA staining on Day 3, 5, 7, 9.

Table 1
The ingredients in MGM4 and TIVA.

Ingredients	Control (MGM4)	TIVA
Basal medium	MBM4 [Lonza]	Ham's F12 [FUJIFILM]
Hormon		
Hydrocortisone	Hydrocortisone [Lonza]	None
Insulin	Insulin [Lonza]	None
Triiodothyronine	None	None
Growth factor		
FGF-2	hFGF-B[Lonza]	None
cAMP inducer		
IBMX	None	0.1 mM IBMX [Nacalai tesque]
Na3VO4	None	1 μM Na3VO4 [Selleck]
dbcAMP	None	1 mM dbcAMP [Selleck]
Cholera toxin	None	None
Animal materials		
FBS	0.5 % FBS [Lonza]	10 % FBS [Gibco]
BPE	BPE [Lonza]	None
Others		
PMA	PMA [Lonza]	50 ng/mL PMA [Selleck]
CaCl2	CaCl2 [Lonza]	None
Antibiotics		
Penicillin-streptomycin	None	1 % penicillin-Streptomycin [Gibco]
Gentamicin/Amphotericin B	GA-1000 [Lonza]	None

shielded and stained with 0.2 % L-DOPA (FUJIFILM) dissolved with 0.1 M PB overnight at 37 °C under 5 % CO_2. The stained cells were then washed with DPBS and observed in four fields of view under a phase contrast microscope, and the number of melanocytes per unit area was calculated from the average number of melanocytes in each field of view.

2.7. Flow cytometry

Cells were immersed in Fixation/Permeabilization solution (Becton Dickinson, US) for 20 min at 4 °C and washed with 1× BD Perm/Wash buffer prepared by diluting BD Perm/Wash (Becton Dickinson) with MilliQ water. The cells were then treated with primary antibody diluted in 3 % FBS/PBS for 30 min on ice and washed with 3 %. After washing with 3 % FBS/PBS, the cells were treated with a secondary antibody diluted in 3 % FBS/PBS on ice for 30 min. After washing with 3 % FBS/PBS, the cells were analyzed using a Cell Sorter SH800 Series (SONY, Japan). In this study, we analyzed Cytokeratin10 (CK10) expressed in keratinocytes in the spinous layer, Cytokeratin14 (CK14) expressed in keratinocytes in the basal layer, and tyrosinase related protein-1 (TRP-1) expressed in melanocytes. The antibody information is provided in Table 2.

2.8. Immunocytochemical analysis

Cells were fixed with 4 % paraformaldehyde for 10 min at 4 °C. After washing with DPBS, the membrane was permeabilized with 0.1 % Triton X-100 (Nacalai tesque) for 10 min, and the cells were shaken at room temperature for 30 min using a non-specific reaction blocking reagent. After washing with DPBS, the primary antibody solution diluted with 1 % BSA (MERCK, Germany) was added to the cells, and the cells were shaken at 4 °C overnight. Thereafter, the cells were washed with

Table 2
Antibody list.

Antibody list		
Primary antibody		
CK10	905403	(Biolegend)
CK14	ab7800	(Abcam)
TRP1	ab235447	(Abcam)
DAPI	D523	(Dojindo)
Secondary antibody		
Goat anti-mouse IgG2a AF594	A21135	(Invitrogen)
Goat anti-rabbit IgG(H + L) AF488	A11008	(Invitrogen)

DPBS and shaken at 4 °C for 30 min in the dark using a secondary antibody diluted 1/500 with 1 % BSA and DAPI diluted 1/1000. After washing with DPBS, a mounting medium was added, a cover glass was placed on the specimen, and the specimen was observed using a confocal microscope. The antibody information is provided in Table 2.

2.9. Quantitative reverse transcriptase-PCR (qRT-PCR)

Total RNA was prepared by using ISOGEN (Nippon Gene, Japan) and PCR Inhibitor Removal Kit (Zymo Research, USA). The RNA was reverse transcribed to cDNA by using Superscript III Reverse Transcriptase (Invitrogen, USA) with ProFlex PCR System (Applied Biosystems, USA). qRT-PCR was performed on QuantStudio 12 K Flex (Applied Biosystems, USA) using a Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, USA). Expression levels were normalized with the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences are shown in Table 3.

2.10. Mouse experiments

Anesthesia was induced with 3 % Isoflurane with a vaporizer for small animals, and a nose cone was used to maintain anesthesia during the procedure. The cultured epidermis sheets at day 5 were implanted under the skin on the back of SCID beige mice, and silicone sheets were placed over the muscle to prevent integration with the muscle. Local anesthesia was not used because the procedure was restricted to skin manipulation. The animals were observed on the warming mat until the anesthesia wore off sufficiently and then returned to their cages. Their skin was examined two weeks after the operation. Euthanasia was performed with an overdose of isoflurane.

2.11. Statistical analysis

We analyzed all data using Microsoft Excel 2019 and values were expressed as mean and standard deviation. We conducted paired t-tests to compare mean values and considered that P value of less than 0.05 in a two-tailed test was a significant difference.

3. Results

3.1. Optimal co-culture conditions for melanocytes and keratinocytes

We determined the best seeding procedure and optimal densities of melanocytes and keratinocytes in order to obtain cultured epidermal sheets with high-density melanocytes. The density of melanocytes was higher when melanocytes were seeded first compared to the simultaneous seeding of melanocytes and keratinocytes (Fig. 2A). When seeding in sequence, the optimal melanocyte seeding density was 1.0×10^4 cells/cm^2. Below 1.0×10^4 cells/cm^2, melanocyte density increased significantly, but above 1.0×10^4 cells/cm^2, melanocyte density did not change (Fig. 2B).

Table 3
Primer list.

Primer	
GAPDH	CGACCACTTTGTCAAGCTCA
	AGGGGTCTACATGGCAACTG
MITF	GAAATCTTGGGCTTGATGGA
	TCTCTTTGGCCAGTGCTCTT
Tyrosinase	CTCAAAGCAGCATGCACAAT
	CCATGTAGGATTCCCGGTTA
TRP1	CCGAAACACAGTGGAAGGTT
	TGTCCAATAGGGGCATTTTC

To investigate melanocyte trends after co-culture, melanocytes were examined using DOPA staining on Days 3, 5, 7, and 9 of co-culture (Fig. 1C). The melanocytes maintained their function, but the number of melanocytes decreased over the time (Fig. 2C and D). On the other hand, no change was observed when the number of days between melanocyte and keratinocyte seeding were extended (data not shown). To shorten the period of co-culture, meaning the period of melanocyte decline, we increased the keratinocyte seeding density and produced a high density of melanocytes by seeding keratinocytes at 8.0×10^4 cells/cm^2 (Fig. 2E).

The cultured epidermal sheets, prepared according to the optimal conditions described above, were analyzed by flow cytometry and immunocytochemical staining. The percentage of melanocytes in the cultured epidermal sheets was 14 % (Fig. 2F). Immunocytochemical analysis revealed that both the keratinocyte marker CK14 and the melanocyte marker TRP-1 were positive in the cultured epidermal sheets (Fig. 2G). TRP1-positive melanosomes were observed in the cytoplasm of keratinocytes near the dendrites of melanocytes, indicating that melanocytes maintained their function, melanin delivery, in cultured epidermal sheets.

To investigate effectiveness and safety, the sheets of keratinocytes alone and those co-cultured with melanocytes were implanted under the skin of two mice for each condition (Fig. 2H). Both sheets were integrated with the skin at two weeks, and no inflammation or tumorigenesis was observed with HE staining (Fig. 2I).

3.2. Producing a BPE-free melanocyte medium

To create a BPE-free melanocyte medium, we compared MGM4, a commercial product, with a medium to which we added various reagents. Melanocytes did not increase in number in MGM4 without BPE or PMA (Fig. 3A). When one, two, or all three reagents (aMSH, Endothelin-1, and FGF-2) were added to the TIVA medium, the highest proliferation rate was observed in the medium with all three reagents (TIVAMEF). TIVAMEF melanocytes had more and longer dendrites and significantly higher proliferative capacities than MGM4 melanocytes (Fig. 3B). The doubling time of TIVAMEF and MGM4 were 4.9 ± 1.8 days and 11 ± 5.0 days, respectively, and there was a significant difference ($p < 0.001$).

Gene expression levels in TIVAMEF melanocytes were measured by qRT-PCR and compared to MGM4 melanocytes. MITF, which is characteristic of melanocytes, was high, and tyrosinase and TRP1 were also expressed, similar to MGM4 melanocytes (Fig. 3C). This pattern of gene expression was stable over a long period of time (p18, i.e., 120 days), which indicated that the TIVAMEF was an appropriate environment for melanocytes.

4. Discussion

Our study has identified optimal conditions for preparing cultured epidermal sheets with high melanocyte density, demonstrating that sequential seeding of melanocytes, feeder cells, and keratinocytes yields superior results compared to simultaneous seeding. Specifically, seeding melanocytes at 1.0×10^4 cells/cm^2 on Day 1, followed by feeder cells on Day 2, and keratinocytes at 8.0×10^4 cells/cm^2 on Day 3 produced the highest melanocyte density. This finding challenges the conventional simultaneous seeding approach [8], and provides a novel perspective on optimizing melanocyte density in cultured epidermal sheets.

Sequential seeding is advantageous because melanocytes bind more loosely to the culture surface than keratinocytes, so as many melanocytes as possible should be seeded and attached first to the culture surface. Indeed, after seeding and propagation of

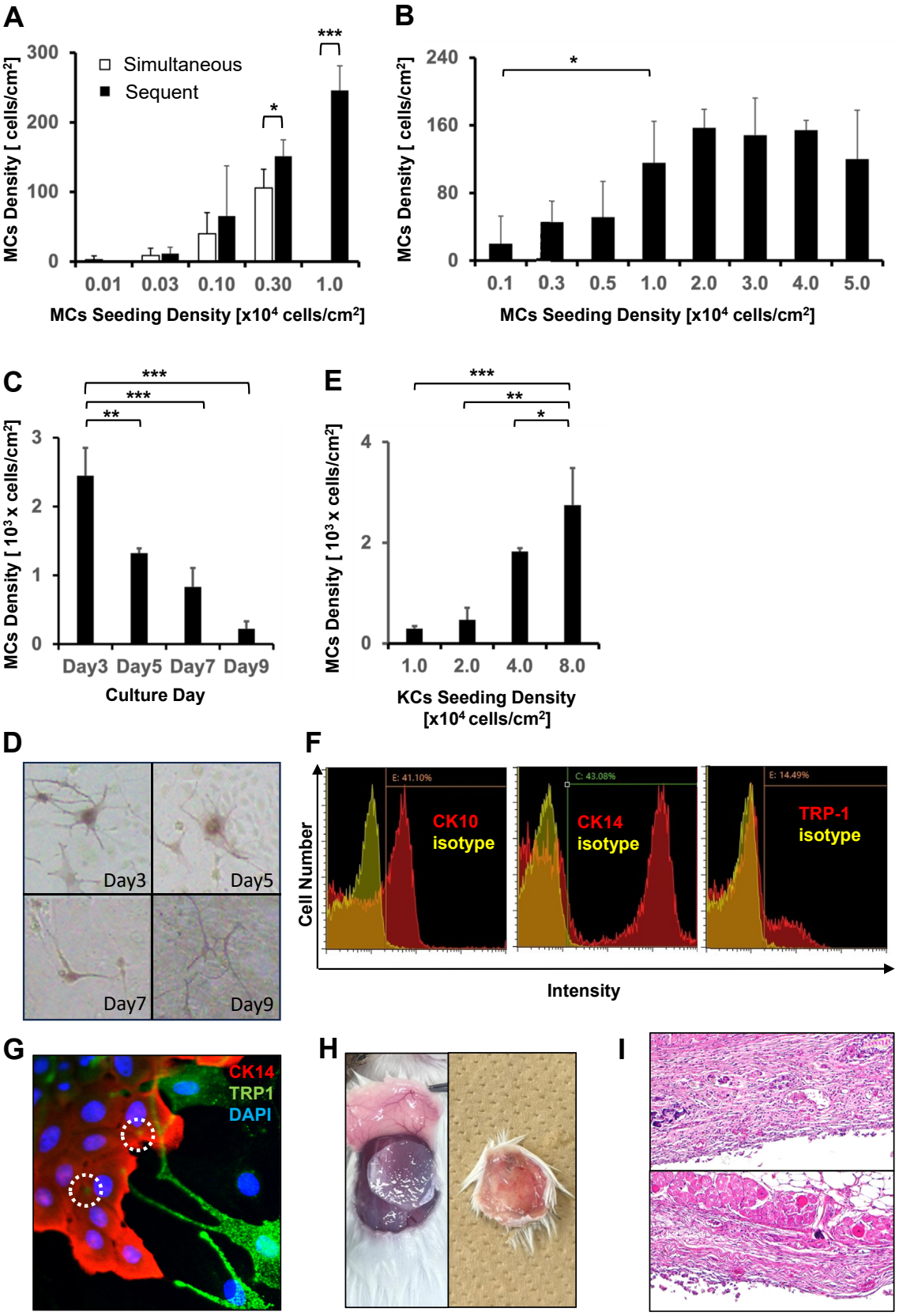

Fig. 2. Investigation of optimal co-culture conditions. A. Comparison of simultaneous and sequential seeding of melanocytes and keratinocytes (*$p < 0.05$, ***$p < 0.001$). **B**. Comparison of final melanocyte density by melanocyte after sequential melanocyte seeding density (*$p < 0.05$) **C**. Changes in melanocyte density during co-culture (**$p < 0.01$, ***$p < 0.001$). **D**. DOPA (3,4-dihydroxyphenylalanine) reaction of melanocytes on Day3 (left upper), 5 (right-upper), 7 (left-lower), 9 (right-lower) of co-culture. **E**. Changes in the final melanocyte density with various keratinocyte seeding densities. Expression levels were calculated from the results of triplicate technical experiments and the charts represent the average ± standard deviation (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **F**. Flow cytometric analysis of melanocytes and keratinocytes with antibodies against CK10, CK14, and TRP1.

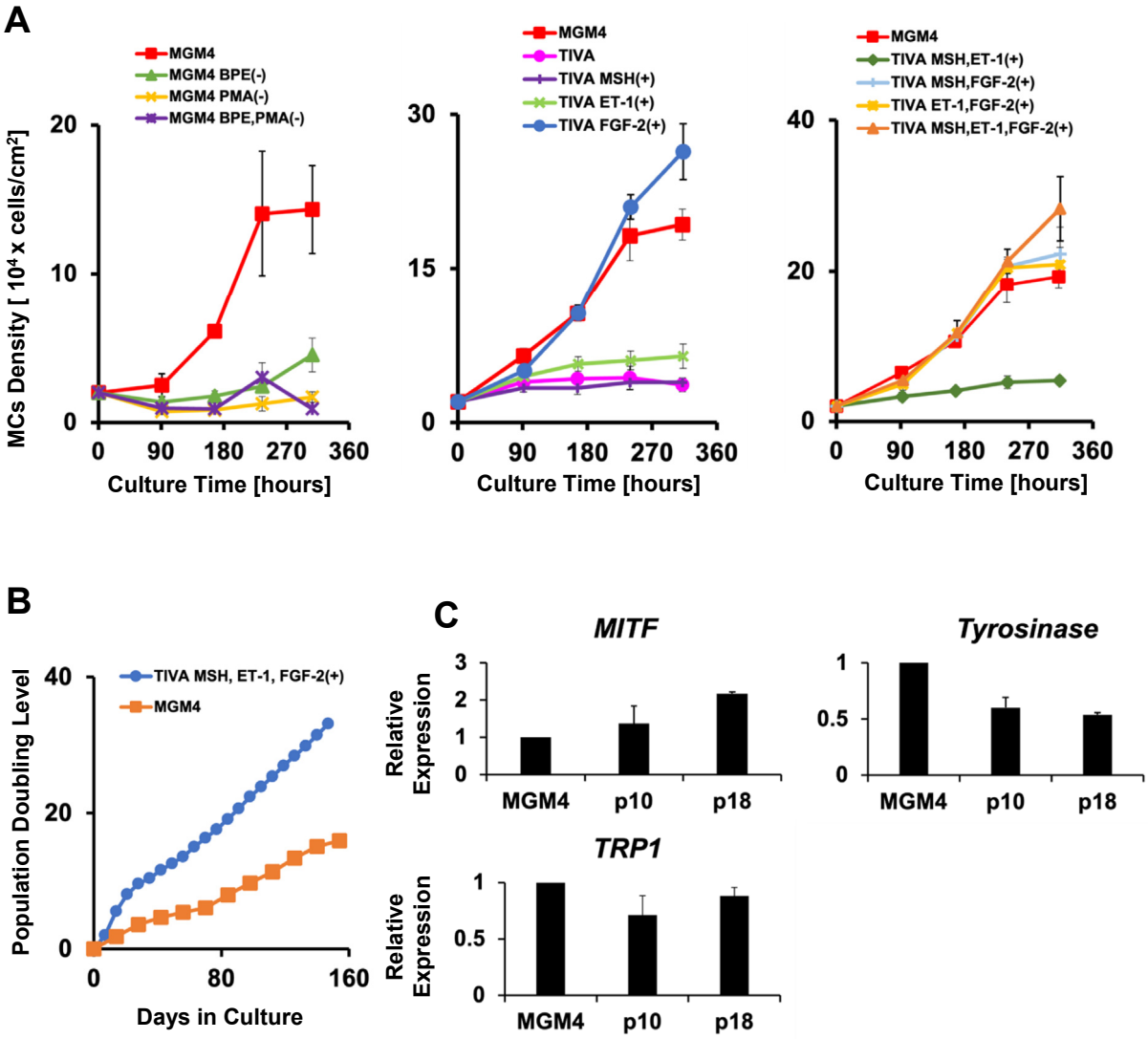

Fig. 3. Proliferative capacity of melanocytes in each medium. A. Comparison of melanocyte proliferation capacity in each culture medium. Expression levels were calculated from the results of triplicate experiments and the charts were drawn as the average ± standard deviation. **B.** Population doubling of melanocytes in MGM4, the medium with TIVA, and all three reagents (TIVAMEF). "Population doubling" indicates the cumulative number of divisions of the cell population. **C**. Quantitative RT-PCR analysis of genes for MITF, Tyrosinase, TRP1 of melanocytes in MGM4 and passage 10 and 17 of TIVAMEF. The expression levels were normalized by expression of GAPDH. The expression levels of melanocytes in MGM4 were designated as 1.0. Expression levels were calculated from the results of triplicate technical experiments and the charts are drawn as averages ± standard deviation.

keratinocytes, most melanocytes gradually detached after nine days. Melanocyte adhesion was reported to be facilitated by keratinocyte-derived Lmn-332 [9], but our results are not consistent with this finding. We considered that while keratinocytes strongly adhered via hemidesmosomes, the presence of Lmm-332 increased not only adhesion but also the migratory capacity of melanocytes.

Our method resulted in cultured epidermal sheets containing 17 % melanocytes, which is significantly higher than the 0.86 % previously reported [4]. However, it is important to note that this density is still approximately one-third of the in vivo melanocyte concentration on the basement membrane. This discrepancy highlights the need for further optimization to achieve physiological melanocyte densities in cultured epidermal sheets. Because increasing the seeding density of melanocytes did not change the final density in this study, alternate culture conditions need to be investigated. Since keratinocytes did not proliferate in a simple mixed medium for melanocytes and keratinocytes (data not shown), KCM, a medium specifically for keratinocytes, was used in this study. In the future, culture conditions that allow both cells to proliferate need to be investigated so that the sheets have long-term stability.

In addressing the clinical applicability of our approach, we developed a bovine pituitary extract (BPE)-free medium supplemented with α-melanocyte-stimulating hormone (αMSH), Endothelin-1, and fibroblast growth factor-2 (FGF-2). This

41 % of cells were positive for CK10, 43 % for CK14 and 14 % for TRP1. **G.** Immunocytochemical analysis of melanocytes and keratinocytes with antibodies against CK14 (red) and TRP1 (green). Nuclei were counterstained with DAPI (blue). White dotted circles showed the transferred melanosomes. **H.** Gross appearance of transplanted epidermal sheets. The sheets were affixed over the rubber and transplanted subcutaneously (left). Two weeks later, the sheets were assimilated with the subcutaneous tissue (right). **I.** Histological analysis of keratinocytes alone (upper) and co-cultured sheets (lower) by H.E. stain. The upper side of the photo is the skin side. There should have been a cell sheet at the lower edge, but it was assimilated into the surroundings and was no longer discernible.

formulation not only maintained melanocyte proliferation but also demonstrated superior growth potential compared to the commercial MGM4 medium. Interestingly, while FGF-2 alone produced sufficient growth in our study, contrary to previous reports [10], the combination of all three factors yielded optimal results, consistent with earlier findings. It is worth noting that the new medium induced morphological changes in melanocytes, particularly increased dendritic formations, likely due to αMSH [11]. However, the expression of melanocyte-specific genes remained comparable to cells cultured in MGM4, suggesting that the fundamental melanocyte characteristics were preserved despite the morphological alterations. For clinical applications, a medium without BPE is required, as was used in the present study. Future studies include producing cell sheets using this medium, and its safety will be determined in animal experiments.

5. Conclusion

This study presents significant advances in preparing cultured epidermal sheets for vitiligo treatment. We established an optimized sequential seeding protocol that produces cultured epidermal sheets with significantly higher melanocyte density than previous methods. Additionally, we developed a novel BPE-free culture medium that effectively supports robust melanocyte proliferation and maintenance, potentially enhancing the clinical applicability of this approach. These findings contribute to the foundation for development of more effective cell therapies for vitiligo. Future research should focus on further increasing melanocyte density to match physiological levels and evaluating the long-term stability and efficacy of grafts produced using these methods in clinical settings. Additionally, investigating the functional implications of the observed morphological changes in melanocytes cultured in the new medium will be crucial for ensuring the safety and efficacy of this approach in vitiligo treatment.

Ethical statement

The institutional Review Board approved all experiments using human tissues at National Center for Child Health and Development. We obtained informed consent from all participants or all parents whose participants were under 18 years old. Human cells in this study were utilized in full compliance with the Ethical Guidelines for Medical and Health Research Involving Human Subjects (Ministry of Health, Labor, and Welfare (MHLW), Japan; Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan).

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

MM, CK, and RT designed experiments. MM, CK and MT performed experiments. MM, CK, and RT analyzed data. MM, CK and MT contributed reagents and materials. MM, CK, RT, HA, and AU discussed the data and manuscript. MM, CK and RT wrote this manuscript. The authors read and approved the final manuscript.

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Declaration of competing interest

AU is the associate editor of this journal but was not involved in the peer review or decision-making of this manuscript. The other authors declare no conflict of interest regarding the work described herein.

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