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Transwell-Hypoxia Method Facilitates the Outgrowth of 3D-Printed Collagen Scaffolds Loaded with Cryopreserved Patient-Derived Melanoma Explants

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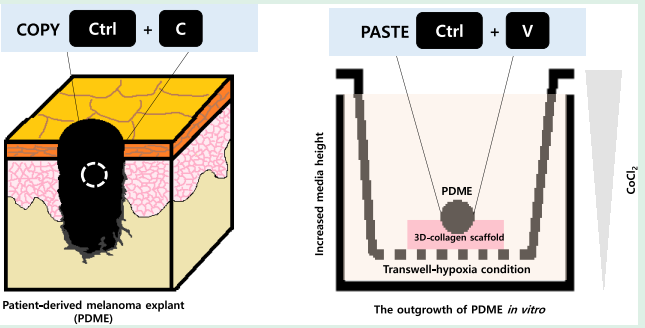
ABSTRACT: A previous study from our laboratory demonstrated the effects of *in vitro* three-dimensional (3D)-printed collagen scaffolds on the maintenance of cryopreserved patient-derived melanoma explants (PDMEs). However, it remains unknown whether 3D-printed collagen scaffolds (3D-PCSs) can be harmonized with any external culture conditions to increase the growth of cryopreserved PDMEs. In this study, 3D-PCSs were manufactured with a 3DX bioprinter. The 3D-printed collagen scaffold-on-frame construction was loaded with fragments of cryopreserved PDMEs (approximately 1−2 mm). 3D-PCSs loaded with patient-derived melanoma explants (3D-PCS-PDMEs) were incubated using two types of methods: (1) in transwells in the presence of a low concentration of oxygen (transwell-hypoxia method) and (2) using a traditional adherent attached to the bottom flat surface of a standard culture dish (traditional flat condition). In addition, we used six different types of media (DMEM high glucose, MEM α, DMEM/F12, RPMI1640, fibroblast basal medium (FBM), and SBM (stem cell basal medium)) for 7 days. The results reveal that the culture conditions of MEM α, DMEM/F12, and FBM using the transwell-hypoxia method show greater synergic effects on the outgrowth of the 3D-PCS-PDME compared to the traditional flat condition. In addition, the transwell-hypoxia method shows a higher expression of the MMP14 gene and the multidrug-resistant gene product 1 (MDR1) than in the typical culture method. Taken together, our findings suggest that the transwell-hypoxia method could serve as an improved, 3D alternative to animal-free testing that better mimics the skin's microenvironment using *in vitro* PDMEs.



KEYWORDS: *in vitro three-dimensional culture system, 3D-printed collagen scaffolds, patient-derived melanoma explants, transwell-hypoxia method, 3D cancer model*

INTRODUCTION

For decades, the research and development of new anticancer drugs has required the extensive use and disposal of animals for testing, with costs reaching approximately $2.6 billion from discovery to marketing approval.[1] Unfortunately, around 60% of new pharmaceutical compounds fail in randomized controlled phase II trials.[1] The reasons for this are the subject of much speculation, but the major causes include the limitations of two-dimensional (2D) *in vitro* and *in vivo* models within the field of research work.[2] In general, traditional 2D *in vitro* culture systems such as immortal cancer cell lines have been used as an accessible, easily usable set of biological models to search for a molecule or compound targeted in cancer research and biomarker discovery.[2] Although many drugs show promising results in 2D *in vitro* systems, most of them do not perform well in clinical trials because existing models cannot mimic the human-specific tumor microenvironment (HSTME).[2] To overcome these limitations, recent studies have introduced breakthrough technologies, including combinations of three-dimensional (3D) culture systems loaded with patient-derived tumor explants (PDTEs).[3−7] However, these models still raise questions about how accurately explant culture condition *in vitro* platforms mimic the specific characteristics of the HSTME for specific tumor types.

The HSTME consists of the dynamic interplay between tumor, stroma, and a mixture of immune and nonimmune cells.[8,9] The biochemical and physical characteristics of these components also contribute to controlling cancer cell differentiation, proliferation, angiogenesis, invasion/extravasation, cancer stem cell maintenance, and immunosurveillance evasion.[5,6] However, these complex interactions are not

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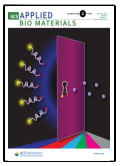

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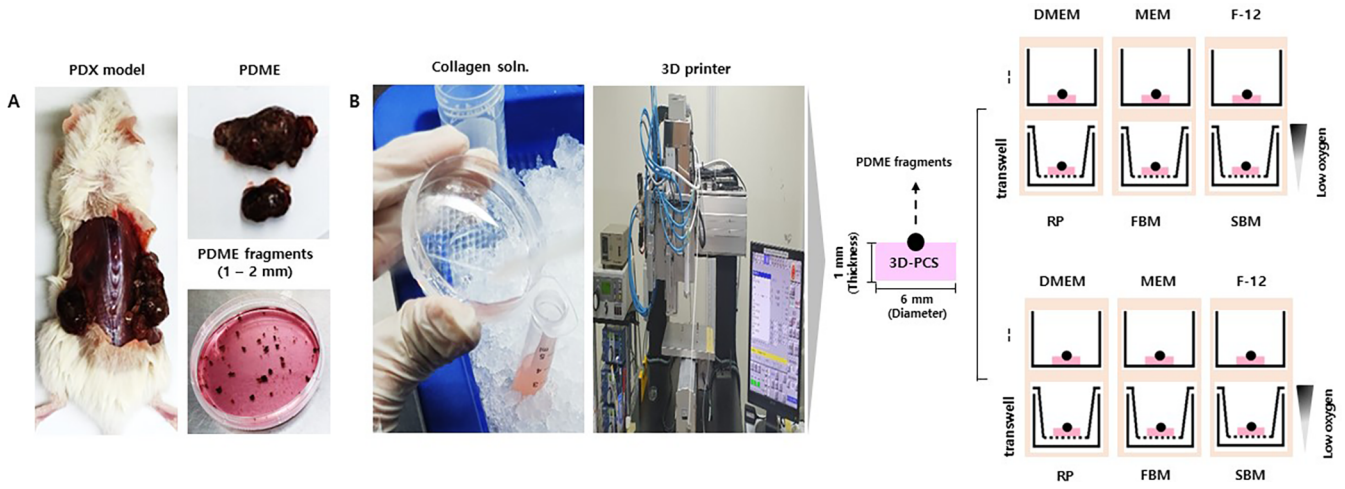

Figure 1. Workflow schematic of the preparation and cultivation of 3D-PCS-PDMEs. (A) Human PDME fragments are generated in the PDX model, creating tumor size tissue that is then divided into smaller fragments for culture growth. (B) The PDME fragments are loaded in 3D-PCSs and placed in transwells with or without hypoxia in six types of culture media: DMEM, DMEM high glucose; MEM, MEM α; F-12, DMEM/F-12; RP, RPMI1640; FBM, fibroblast basal medium; and SBM, stem cell basal medium.

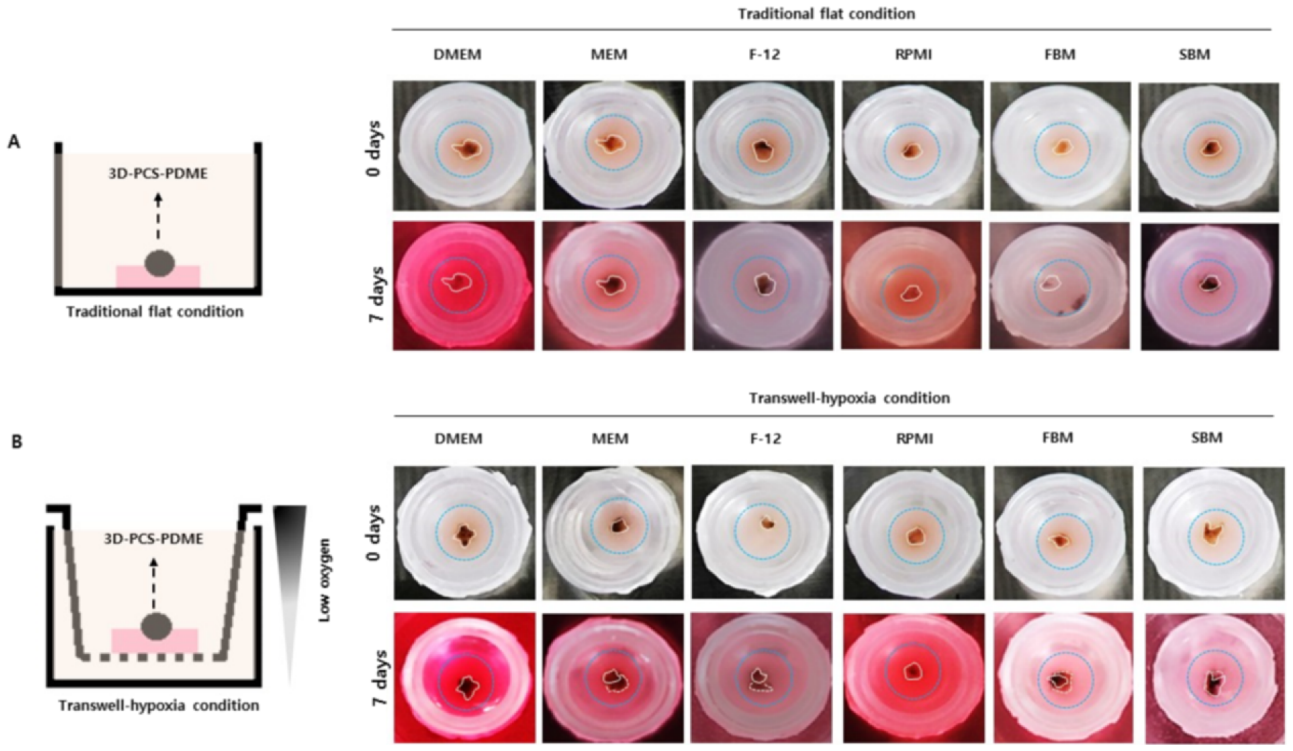

Figure 2. The outgrowth onto the 3D-PCS-PDMEs in the traditional flat condition and transwell-hypoxia method with six different types of media. (A) Design of the culture environment on 3D-PCS-PDME cultivation using a transwell system-hypoxia method or traditional flat condition. (B) Representative images showing the morphologic changes of 3D-PCS-PDMEs with traditional flat condition or transwell-hypoxia method in the indicated media at 0 and 7 days. After manufacture of 3D-PCS-PDMEs, they were incubated with different media in a transwell system with hypoxia for 0 and 7 days. The 3D-PCSs are encircled with a blue dash line. The PDME fragments are indicated with a white dashed line. The expanded area of PDME is marked with a white solid line.

captured in traditional monolayer cell cultures. PDTEs can serve as a suitable means of imitating the HSTME architecture in *in vitro* models.[3−7] Indeed, PDTEs can more directly evaluate a drug's efficacy as a fresh tumor sample derived from several biopsy procedures without deconstruction/reconstruction.[3−7] Recent studies report that the PDTE model serves as a suitable platform for preclinical studies, anticancer drug, and biomarker discovery.[3−7] PDTEs consist of the *ex vivo* culture of biopsy derived from freshly resected human tumors that retain the histological characteristics of original tumors in the body.[3−7] Despite its many advantages, the PDTE *in vitro* model still requires improvement in terms of longevity, renewal of the HSTME, tissue preparation, and supplements for the culture media.

In the case of cutaneous melanoma (CM), 3D cultures developed to investigate HSTME reprogramming are better

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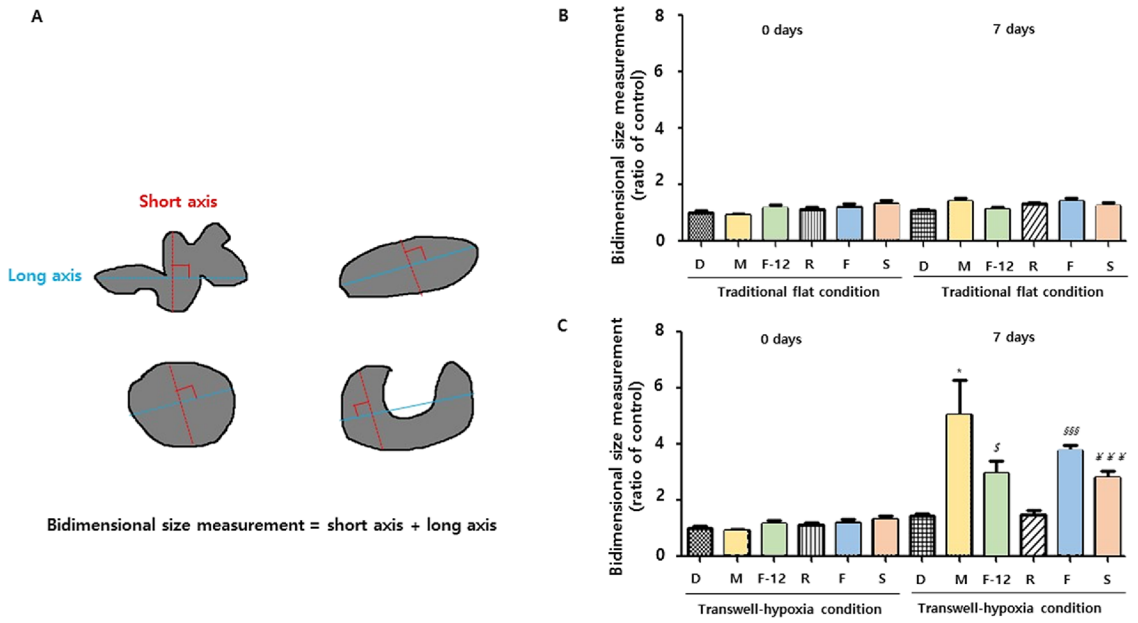

Figure 3. The bidimensional size measurement for determining 3D-PCS-PDMEs in 2D-cultured and transwell-hypoxia method. (A) Overview of schematics for size criteria of the outgrowth of 3D-PCS-PDMEs using WHO guidelines. (B, C) Bar graph indicating the bidimensional change in the PDME size for each culture medium after 0 and 7 days of incubation. D (DMEM), M (MEM α, yellow), F-12 (DMEM/F12, green), R (RPMI1640), F (FBM, blue), and S (SBM, red). (*, $) $p < 0.05$ or (¥¥¥, §§§) $p < 0.001$ versus each control group using Student's *t*-test.

suited than 2D cultures for mimicking HSTME complexity *in vitro*.[10] Specifically, melanoma metabolic changes to the HSTME markedly contribute to tumor development and immune escape.[10] Although a previous study from our laboratory has reported that 3D-printed collagen scaffolds (3D-PCSs) retain the stable expression of melanoma biomarkers in cryopreserved patient-derived melanoma explants (PDMEs) under long-term culture,[11] it remains unclear whether the external culture method raises any combinational effects in PDME-linked HSTME renewal. Therefore, this study aimed to build up alternative methods used in external supporters with a hypoxia condition and optimization of the culture medium for expanding the outgrowth of the 3D-PCS loaded with patient-derived melanoma explant (3D-PCS-PDME) *in vitro* system.

MATERIALS AND METHODS

Reagents and PDME. Cobalt chloride ($CoCl_2$), MTT, and DMSO (dimethyl sulfoxide) were purchased from Sigma (St. Louis, MO, USA). MS collagen (type 1 atelo-collagen from porcine skin) was obtained from MS Bio, Inc. (Gyeonggi, Rep. of Korea). Dulbecco's modified Eagle's medium (DMEM) high-glucose media, Dulbecco's modified Eagle medium/nutrient mixture F-12(DMEM/F-12), and RPMI1640 formulations were purchased from Welgene Inc. (Gyeongsangbuk-do, Rep. of Korea). Minimum Essential Medium (MEM) α, fibroblast basal medium (FBM), and stem cell basal medium (SBM) were purchased from Thermo Fisher Scientific (Rockford, IL, USA). 1−2 mm PDME fragments were obtained from patient-derived melanoma xenograft (PDX) models, which were sourced from Seoul St. Mary's Hospital (Seoul, Rep. of Korea), as described in our prior study.[11]

Fabrication for the 3D-PCS-PDME and Its Cultivation. 3D-PCS-PDMEs were prepared, as described previously.[11] To manufacture uniform and stable 3D-PCSs, we utilized the extrusion-based 3D printing method using an X Printer (T&R Biofab Co., Ltd., Gyeonggi-do, Rep. of Korea). The experimental culture environment of 3D-PCS-PDMEs consisted of two different cases: (1) the traditional flat condition as a general 2D culture method (Figure 1A) and (2) a hypoxia condition supported by 6-well plates with an increased media height (total volume 7 mL, predicted $pO_2 < 6.2\%$) in the presence of $CoCl_2$ (50 μM).[12−14] Each medium was not changed for 7 days (Figure S1 in the Supporting Information). For the culture treated under the traditional flat condition serving as the control group, the 3D-PCS was placed on the floor of the cell culture dish (Figure 2A). In the case of the transwell system-hypoxia condition, the 3D-PCS-PDMEs were added on top of the transwell membrane in the upper chamber and then incubated with different growth media (Figure 1B). We used six types of growth media: DMEM high glucose with 10% FBS, MEM α with 10% FCS, DMEM/F-12 with 5% FBS, RPMI1640 with 5% FBS, FBM, and SBM. 2 mM L-glutamine and 1% PS were added to each medium. The samples were incubated at 37 °C in a 5% CO_2 incubator.

The Viability Assay and Assessment of PDME Outgrowth in 3D-PCS-PDMEs. The samples were cultured in different growth media for 7 days. A standard criterion for assessing the outgrowth of PDMEs in 3D-PCS-PDMEs is that all sites of PDMEs be measurable in at least one dimension (Figures 2 and 3A). We modified the World Health Organization-response evaluation criteria (RECIST) to standardize tumor measurements using imaging to assess the tumor response in clinical trials, as indicated below.[15,16]

Bidimensional size measurement (size criteria) = short axis (a) + long axis (b)

$$1 - [(A' \times B') + (a' \times b')]/[(A \times B) + (a \times b)] \times 100(\%)$$

$$(\text{by WHO} - \text{RECIST})$$

The thickness and diameter of the samples were measured with a ruler. After incubation, the metabolic activity of 3D-PCS-PDMEs was assessed using an MTT assay. The samples were incubated for 2 h in each medium with MTT solution. The incubated culture medium was then removed, and the MTT was extracted with DMSO for 1 h at RT. We used a negative control that had only DMSO without 3D-PCS-PDMEs. Absorbance was determined at 590 nm using an ELISA reader (EMax; Molecular Devices, Sunnyvale, CA, USA).

Live and Dead Staining. To determine the viability of 3D-PCS-PDMEs, a double staining kit of live and dead cells was used, consisting of propidium iodide (PI) and calcein-acetoxymethyl (AM), as previously described.[11] The samples were placed in the PI and

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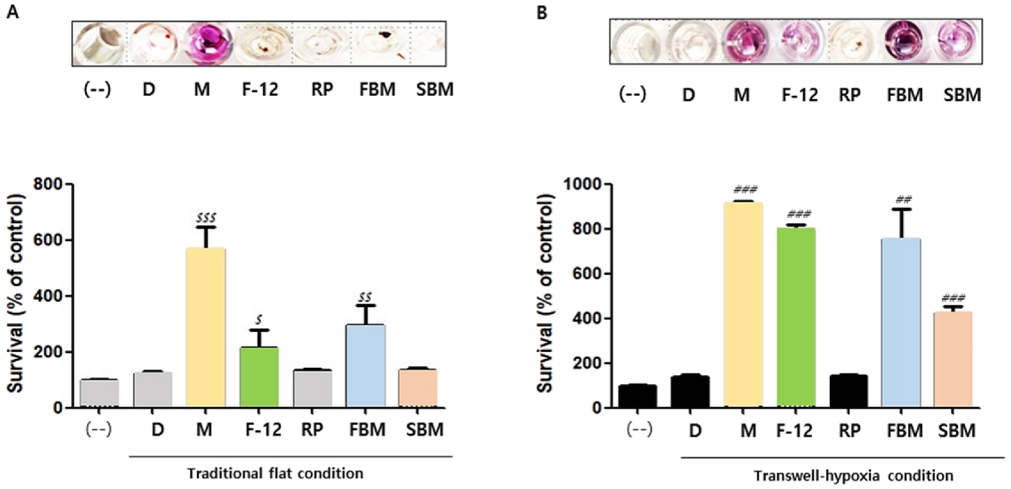

Figure 4. The applicability of different media and culture environments for maintaining the 3D-PCS-PDMEs. (A) Traditional culture method. (B) Method with transwell-hypoxia. After 7 days of incubation, the metabolic activity of the indicated groups was determined using an MTT assay. ($, #) $p < 0.05$, ($$, ##) $p < 0.01$, or ($$$, ###) $p < 0.001$ versus the DMEM group using Student's *t*-test.

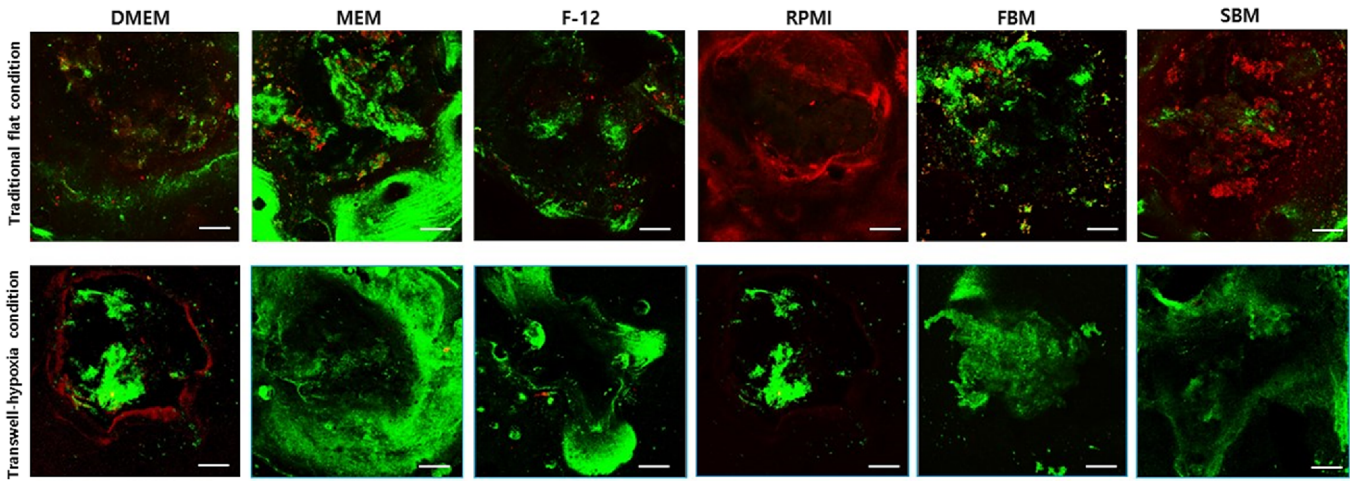

Figure 5. Comparison of different media and culture environments on the viability of 3D-PCS-PDME cultivation. After incubation with different media and/or the upper chamber for 7 days, the samples were labeled using a live and dead kit. Confocal live images independently indicating live (green) and dead (red) 3D-PCS-PDMEs. Scale bar = 5 mm.

calcein-AM mixture and then incubated at 37 °C in a 5% CO_2 incubator for 30 min. Live images were acquired using an Olympus FV1200 confocal microscope with 473 and 559 nm laser lines.

Quantitative Reverse-Transcription PCR (qRT-PCR). The cDNA of the samples was synthesized using a qRT-PCR assay kit. We used SYBR Green qPCR Master Mix and the appropriate primers (Applied Biosystems) to amplify the target gene expression, which was then analyzed on a StepOnePlus real-time PCR system (Applied Biosystems). The relative gene expression from all data was obtained using the ΔCt method with normalization versus actin, as previously described.[17] The primers used were human MMP14 [forward, 5′-TCA AGG AGC GCT GGT TCT G-3′; reverse, 5′-AGG GAC GCC TCA TCA AAC AC-3′], human MDR1 [forward, 5′-TGG TTC AGG TGG CTC TGG AT-3′; reverse, 5′-CTG TAG ACA AAC GAT GAG CTA TCA CA-3′], and human actin [forward, 5′-CAC CAA CTG GGA CGA CAT GG-3′; reverse, 5′-CTC TGT TGG CCT TGG GGT-3′].

Statistical Analysis. SPSS software ver. 17.0 (SPSS, Chicago, IL, USA) and GraphPad Prism software (GraphPad Software, San Diego, CA, USA) were used to perform the statistical analysis. Student's *t*-tests (for comparisons of two groups) were used for the statistical analyses. Data are expressed as means ± standard error of the mean (SD). A value of $p < 0.05$ was considered significant. ($, #, §, ¥) $p <$ 0.05−0.01, ($$, **, ¥¥) $p <$ 0.01−0.001, and ($$$, ###) $p < 0.001$ vs corresponding controls. All the error bars represent the standard deviation of three or more biological replicates.

RESULTS

Optimization Strategy for Preserving the High Efficiency of the 3D-PCS-PDME Outgrowth *In Vitro* System. We investigated whether external culture conditions affect the establishment of the PDME-HSTME. As shown in Figure 1, the commercial transwell system-hypoxia culture method applied in this study consisted of three steps: (1) preparation of PDME fragments derived from PDX models, as previously described,[11] (2) manufacture of the 3D-PCS using a 3DX bioprinter, and (3) a transwell-hypoxia condition with a culture medium. In general, isolation of cells from specific regions of 3D cultures is possible but leads to a lower number of generated tissue-derived cells, while a low-throughput method such as enzymatic digestion can lead to the destruction of the HSTME.[18,19] To protect and induce HSTME's renewal, the present method did not use a digestion solution for obtaining smaller PDME fragments.[11] The hypoxia condition

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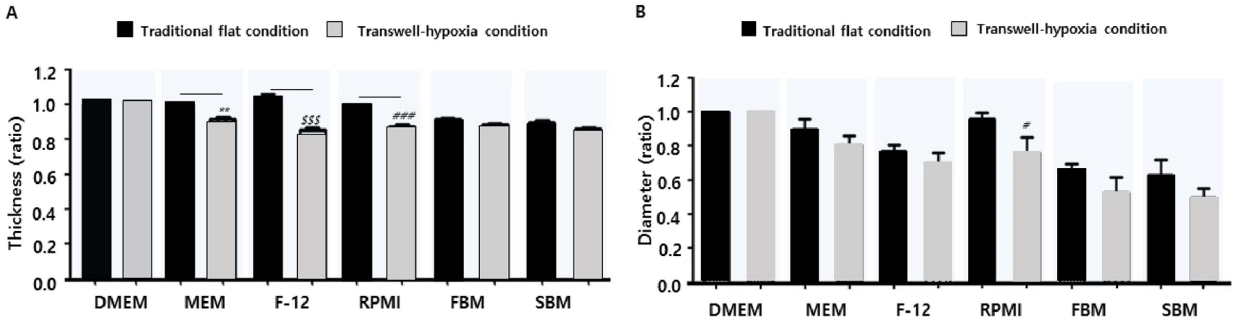

Figure 6. The turnover of morphological metrics in 3D-PCS-PDME cultivation in the presence or absence of different media and culture environments. (A) 2D adherent condition. (B) With an upper chamber. After 7 days, the diameters of the groups were measured with a ruler. (#) $p < 0.05$, ($$, **) $p < 0.01$, or ($$$, ###) $p < 0.001$ versus the DMEM group using Student's *t*-test.

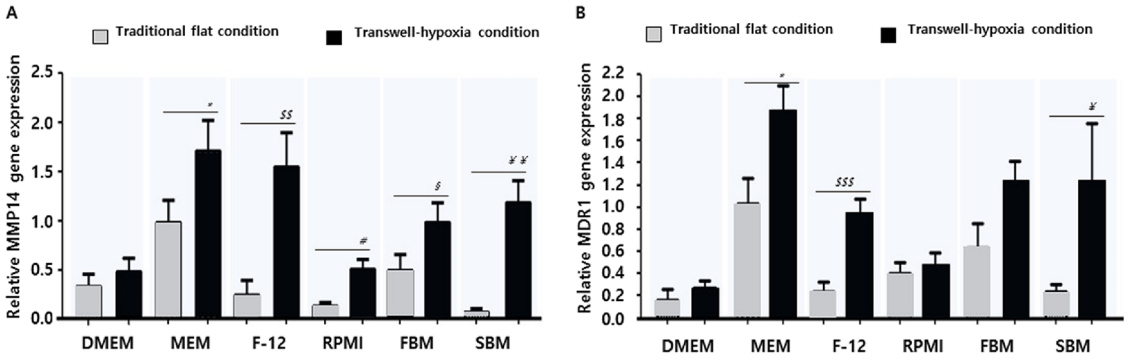

Figure 7. MMP14 and MDR1 expression correlates with the outgrowth of 3D-PCS-PDMEs in the presence or absence of different media and culture environments. (A, B) qRT-PCR bar graph to quantify the expression of the MMP14 gene (A) and MDR1 gene (B) in each group as a ratio difference compared to the corresponding control. ($, #, §, ¥) $p < 0.05$, ($$, ¥¥) $p < 0.01$, or ($$$) $p < 0.001$ versus the DMEM group using Student's *t*-test.

was induced with an alternative method that utilizes $CoCl_2$ without a hypoxia chamber.[12−14,20] In the case of the transwell-hypoxia method, the 3D-PCS-PDMEs were incubated with an increased media height in the presence of 50 μM $CoCl_2$ for 7 days (Figure S1). CellROX deep red is a fluorogenic probe for measuring cellular hypoxia conditions in live imaging.[21] Confocal live images demonstrated that hypoxia was effectively induced (Figure S2). Calcein-AM dye is useful for staining live cells treated under normoxia—the traditional flat condition—and transwell-hypoxia-exposed 3D-PCS-PDMEs. Under the transwell-hypoxia method, high levels of calcein-AM-stained live cells were observed (Figure S2). Therefore, this strategy made it possible to promote the outgrowth of 3D-PCS-PDMEs.

Morphological Effect Comparison between the Transwell-Hypoxia and Traditional Flat Conditions for Promoting 3D-PCS-PDME Outgrowth. In this study, the 3D-PCS technique required only a small quantity of PDMEs (<2 mm) when we generated 3D-PCS-PDMEs for the PDME outgrowth *in vitro* (Figures 1 and 2). To improve PDME outgrowth *in vitro*, we tested two methods: (1) use of the traditional flat condition and (2) the addition of transwells and hypoxia exposure. Different morphological outgrowths of PDME were observed under the transwell-hypoxia condition that were not evident under the traditional flat condition (Figure 2). According to the World Health Organization-based response evaluation criteria in solid tumors (WHO-RECIST),[15,16] PDME outgrowth measured according to bidimensional sites of tumor growth chosen as target parts can be measured in a reproducible fashion. Based on WHO-RECIST, we assessed PDME outgrowth under the traditional flat condition and the transwell-hypoxia condition with six different types of culture media. Bidimensional size measurement showed greater outgrowth of PDME in the transwell-hypoxia condition with MEM α, DMEM/F12, FBM, and SBM when compared to 3D-PCS-PDMEs incubated under the traditional flat condition (Figure 3). Consistent with our findings, live/dead staining and an MTT assay also confirmed the enhancement of cell viability and metabolic activity in transwell-hypoxia-exposed 3D-PCS-PDMEs (Figures 4 and 5). The results indicate that the transwell-hypoxia method could be considered to have a combinational effect on the PDME outgrowth in the 3D-PCS.

Collagen Retention and Matrix Metalloproteinase 14 Gene Expression in 3D-PCS-PDMEs between the Transwell-Hypoxia and Traditional Flat Conditions. Extensive collagen remodeling is linked to the growth of cancer.[22,23] A recent paper reported that mice lacking matrix metalloproteinase 14 (MMP14) in stromal fibroblasts (MMP14 $Sf^{-/-}$) reduced melanoma growth and increased skin stiffness.[23] In addition, a previous study demonstrated that multidrug-resistant gene product 1 (MDR1) was expressed in human melanoma stem cells.[24] If the transwell-hypoxia method positively impacts the outgrowth of the PDME in the 3D-PCS, the effects of the transwell-hypoxia method on the 3D-PCS-PDME *in vitro* could affect collagen remodeling as well as the expression of MMP14 and/or MDR1 at the mRNA level. To test this, we measured the diameter and thickness of 3D-PCS-PDMEs under the traditional flat condition and the transwell-hypoxia condition after 7 days of incubation in

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different culture media. The expression of the MMP14 gene was determined by qRT-PCR analysis. The thickness of the 3D-PCS under the transwell-hypoxia condition was lower than that in the traditional flat condition (Figure 6A). On the contrary, the diameter showed no significant differences between the traditional flat condition and the transwell-hypoxia condition (Figure 6B). Correlating with this observation, there were differences in the expression level of the MMP14 and MDR1 between the traditional flat condition and the transwell-hypoxia condition (Figure 7). Therefore, the collagen changes, MMP14 expression, and MDR1 expression in 3D-PCS-PDMEs can serve as biomarkers for determining an outgrowth of PDME *in vitro*. These results provide evidence for the application of the transwell-hypoxia method as an effective option for the 3D culture of explants derived from cancer patients.

DISCUSSION

This study focused on the optimization of 3D-PCS-PDME cultivation to induce an HSTME reprogramming response from the culture condition. In general, 2D culture methods cannot simulate natural HSTME reprogramming due to the lack of cellular cell−cell communication and networking (cell−cell and cell−matrix).[2] To overcome this, the direction of research and development in *in vitro* cell culture settings of HSTME reprogramming is moving toward 3D culture systems that involve the design and/or manufacture of 3D scaffolds as 3D bioprinted extracellular matrices, organoid culture, or spheroid culture. Recent papers demonstrate how 3D bioprinting can be applied in developing patient-related tumor microenvironment models *in vitro* and *ex vivo*.[4−8,11] Among them, we previously generated 3D-PCSs from cryopreserved PDMEs using a 3D bioprinting technique.[11] This resulted in improvements in the maintenance of cryopreserved PDMEs in a 3D culture system for 21 days.[11] Despite the positive effects of 3D-PCSs on the homeostasis of the PDMEs, there is a clear need to perform additional studies that mimic intact melanoma. Decoding CM complexity and progression requires a more detailed understanding of the interplay between the tumor microenvironment and several types of cells, including keratinocytes, cancer-associated fibroblasts (CAFs), fibroblasts, adipocytes, immune cells, and components of the extracellular matrix, and tumor-specific physicochemical properties.[10,25−27]

To represent and study this interplay, we developed a 3D *in vitro* melanoma-HSTME model that integrates the complex networks among nutrient-oxygen microenvironment, culture method, and melanoma-related cells more effectively. Furthermore, our experimental protocol does not involve any chemical, enzymatic, or mechanical digestion in the manufacture of PDMEs (Figure 1). This strategy has the advantage of being less damaging to the characteristics of the PDMEs and thus can more accurately stimulate the HSTME renewal of the PDME *in vitro*. Indeed, the vast heterogeneity within the cell types of the HSTME crucially impacts treatment responses.[8,10,26] The composition of the medium is an essential key for maintaining the complex tumor *in vitro*.[11,19,25−27] In the case of melanoma biopsy, short- and long-term cultures of malignant melanoma derived from different specimens have been cultured in DMEM/F12, RPMI, and DMEM with 10−15% FBS for many basic studies and for evaluation of the precision of anticancer drugs.[9,18,19,25] A previous study focused on the phenotypic diversity of PDME populations in stem cell medium.[26] When PDMEs derived from melanoma at clinical stages III and IV were grown in SBM, these melanoma populations exhibited diverse morphology and frequency of CD133-positive cells, which are known to be a biomarker of CSCs.[26] Other papers have shown that CAFs, which can make up most of the tumor mass, affect progression, metastasis, and resistance to therapy.[10,27] Such research on identifying and optimizing cell culture media *in vitro* has been responsible for the main productivity and homeostasis gains in batch-fed cell culture-based biomanufacturing. According to a design-of-experiment approach, researchers seek to determine which additives and feeds best suit the cell culture process. The best cell media for long-term incubation of *in vitro* 3D cancer models will depend on the specific application and research goals. Taking these efforts and research context into account, we selected six types of culture media (DMEM, MEM α, F12, RPMI, FBM, and SBM) after fabrication of 3D-PCS-PDMEs for expanding the outgrowth of PDMEs. In addition, we used a commercial transwell system with inserts that can serve as external supports in mimicking the flexible and permeable 3D architecture culture platform. Commercial transwell inserts are one of the most common tools used in tissue culture platforms to evaluate drug screening, cell migration, invasion, and barrier formation.[28,29]

Many researchers have also reported the importance of hypoxia in regulating invasiveness, angiogenesis, vasculogenic mimicry, and response to therapy in the CM progression.[20,30] Furthermore, many cells in the human body are strongly involved in hypoxia.[20,30] Although many papers mention the importance of hypoxia in CM progression, a few papers that studied the potential hypoxia-dependent drivers using 3D spheroids did not use PDEs.[20,30] In consideration of these studies, we established an *in vitro* alternative method of manufacturing a 3D PDME culture using commercial transwell inserts with an increased media height in the presence of $CoCl_2$ to induce hypoxia. We compared the morphology of 3D-PCS-PDME cultivation between the traditional flat condition and the transwell-hypoxia method with six types of media. The outgrowth of PDMEs under the transwell-hypoxia condition with five types of media was greater than that under the traditional flat condition. To further confirm these findings, the cell viability of 3D-PCS-PDMEs for each group was assessed by a live and dead staining assay. The results show that the transwell-hypoxia condition method exhibited significantly more surviving cells in 3D-PCS-PDMEs than under the traditional flat condition across four media types. Consistent with the live and dead staining assay results, the MTT assay shows that the transwell-hypoxia method especially promotes the metabolic activity of PDME on 3D-PCS in MEM α, DMEM/F-12, FBM, and SBM compared to the traditional flat condition, which supports previous findings that *ex vivo* tumor culture systems that apply a classic 2D monolayer method prefer two types of medium (i.e., DMEM/F-12 and MEM α).[9,18,19,25] Therefore, MEM α, DMEM/F-12, FBM, and/or SBM media in the transwell-hypoxia-exposed 3D-PCS-PDME condition may play a role in standardizing cell responses such as survival and growth of PDME.

A solid tumor itself is also made up of the tumor parenchyma and stroma.[8,10] In particular, the tumor stroma can serve as a vital key for maintaining tumor parenchymal growth and invasiveness and is considered a supporter of the tumor parenchyma.[8,10] Simultaneously, cancer cells can traverse individual ECM composition/organization or regulate

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the expression of matrix remodeling enzymes as MMP through active as well as passive mechanisms.[22,23] Individual ECM components also provide spatially contextual cellular signaling and a reservoir of growth factors in the HSTME.[22,23] Although the tumor stroma microenvironment is relatively understudied in CM, numerous studies have underscored the intricate relationship between the stromal microenvironment and the complex ECM-MMP processing for melanoma growth.[22,23] For example, a recent study using dermal fibroblasts derived from MMP14 $Sf^{-/-}$ mice demonstrated that MMP14-collagen processing in stromal fibroblasts impacts melanoma growth.[23] Specifically, MMP14 deletion in fibroblasts generates a collagen-rich stiff matrix.[23] It is well known that collagen impacts the modulation of proteolysis of ECM proteins and the deposition of ME reprogramming with ECM associated with a variety of proteolytic enzymes such as MMP in healthy skin and tumors.[23] Furthermore, additional biomarker candidates should be identified if 3D-PCS-PDME models are to serve as a tool for drug screening with superior performance in comparison with 2D models as an alternative to animal-free testing. A previous study found that a subpopulation of $MDR1^{+}$ cells is located in primary melanoma cells.[24] In light of such findings, we investigate how the traditional flat condition and transwell-hypoxia methods might affect collagen retention as well as MMP14 and MDR1 gene expression after 7 days of incubation. Contrary to expectations, there was no dramatic difference in the diameter of the 3D-PCS between the two methods, although the transwell-hypoxia method showed a much higher expression of the MMP14 and MDR1 genes compared to the traditional flat condition. These results indicate that MMP14 and MDR1 gene expression can be used as biomarkers for determining the growth and overall condition of the 3D-PCS-PDMEs in an *in vitro* system. Although further study is required to better understand the bidirectional communication between the resident tumor cells and ECM cultivation and to improve 3D PDME-HSTME renewal models, the transwell-hypoxia method might be suitable for stimulating HSTME renewal.

CONCLUSIONS

The combination of the transwell-hypoxia method and MEM α, DMEM/F-12, FBM, and/or SBM media could serve as an improved, 3D alternative to animal-free testing that better mimics the skin's microenvironment using *in vitro* PDMEs in which a higher expression of the MMP14 gene and MDR1 gene was found than that in the traditional flat condition.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsabm.2c00710.

Representative transwell-hypoxia method for promoting the efficiency of the 3D-PCS-PDME outgrowth *in vitro* system and validation of hypoxia (PDF)

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Y.-M.J. and S.W.J. wrote the manuscript, handled the work for the manuscript, and conceived and designed the research. Y.-M.J. and M.J.P. performed statistical analysis of data and performed all experiments. W.-Y.S.Y., S.W.J., and Y.-M.J. handled funding and supervision. C.H.B., M.J.P., and Y.-M.J. were responsible for 3D-PCS-PDMEs and PDMEs. All authors reviewed and approved the final manuscript.

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Notes

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ABBREVIATIONS

AM, calcein-acetoxymethyl; CM, cutaneous melanoma; 3D-PCSs, 3D-printed collagen scaffolds; 3D-PCS-PDMEs, 3D-PCSs loaded with patient-derived melanoma explants; FBM, fibroblast basal medium; HSTME, human-specific tumor microenvironment; MDR1, multidrug-resistant gene product 1 MDR1; MEM α, Minimum Essential Medium α; MMP14, matrix metalloproteinase 14; PDME, patient-derived melanoma explants; PDTE, patient-derived tumor explants; PI, propidium iodide; SBM, stem cell basal medium

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