Human collagen type I-based scaffold retains human-derived fibroblasts in a patient-derived tumor xenograft mouse model

HARUKA OKAMI[1*], RYO MURANUSHI[1*], TAKEHIKO YOKOBORI[2], BILGUUN ERKHEM-OCHIR[2], GENDENSUREN DORJKHORLOO[3], TAKAOMI SEKI[1], TAKAYUKI OKUYAMA[1], RYOUSUKE FUKUSHIMA[1], SHUNSUKE KAWAI[1], KOUKI HOSHINO[1], GANTUMUR DOLGORMAA[1], KEI HAGIWARA[1], TAKAHIRO YAMANAKA[1], NORIHIRO ISHII[1], MARIKO TSUKAGOSHI[1], TAKAMICHI IGARASHI[1], AKIRA WATANABE[1], NORIO KUBO[1], KENICHIRO ARAKI[1], HIROSHI SAEKI[3] and KEN SHIRABE[1]

[1]Department of General Surgical Science, Division of Hepatobiliary and Pancreatic Surgery, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan; [2]Division of Integrated Oncology Research, Gunma University Initiative for Advanced Research, Maebashi, Gunma 371-8511, Japan; [3]Department of General Surgical Science, Division of Gastroenterological Surgery, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan

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Abstract. The present study aimed to investigate the role of a recombinant protein based on human collagen type I (RCPhC1) as a scaffold in maintaining the human tumor microenvironment within a patient-derived tumor xenograft (PDTX) model. RCPhC1, synthesized under animal component-free conditions, was explored for its potential to support the human-specific stroma associated with tumor growth. PDTX models were established using resected colorectal cancer liver metastasis specimens, and stromal cell populations from humans and mice were compared using three scaffolds: No scaffold (control), Matrigel and recombinant human collagen type I, across two passages. Specific antibodies for human Lamin B and mouse Lamin B were used for immunostaining to distinguish between human and mouse cells. Additionally, the impact of each scaffold on the invasive ability of mouse fibroblasts was assessed using an invasion assay. Patient-derived tumor tissues embedded with RCPhC1 hydrogels had significantly more human Lamin B-positive cells and fewer mouse Lamin B cells than those embedded with no scaffolds or Matrigel. The human Lamin B-positive cells in PDTX tumors with RCPhC1 hydrogels were recognized as fibroblasts. Additionally, these hydrogels significantly reduced the invasion of mouse fibroblast cell lines *in vitro* compared with Matrigel. The present study investigated RCPhC1 hydrogels as a new scaffold material for tumor engraftment in PDTX mouse models, and identified a promising experimental tool for maintaining the tumor microenvironment.

Correspondence to: Dr Kenichiro Araki, Department of General Surgical Science, Division of Hepatobiliary and Pancreatic Surgery, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan
E-mail: karaki@gunma-u.ac.jp

*Contributed equally

Abbreviations: CAFs, cancer-associated fibroblasts; CRC-LM, colorectal cancer liver metastasis; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PDTX, patient-derived tumor xenograft; RCPhC1, recombinant protein based on human collagen type I

Key words: collagen type I hydrogel, fibroblast invasion, human tumor microenvironment, PDTX model, tumor stroma

Introduction

Animal experiments have supported innovative advances in anticancer drug development. In particular, as there are no alternative methods, experimental models evaluating the safety and efficacy of anticancer drugs using carcinoma-bearing animals are highly valued as preclinical trial data (1). In the traditional model, the cell line-derived tumor xenograft model, in which cultured human tumor cell lines are transplanted into immunodeficient mice, has been frequently used. However, the patient-derived tumor xenograft (PDTX) model, in which patient-derived tumor tissue is transplanted subcutaneously or orthotopically into immunocompromised mice (NOD-SCID mice), provides an environment that more closely resembles the tumor microenvironment in the patient's body (2). The main concerns in this PDTX model are the effects of immunodeficiency in the NOD-SCID mouse model and the loss of human tumor stromal cells, including cancer-associated fibroblasts (CAFs), with PDTX tumor passaging into next-generation mice.

In the conventional PDTX model, researchers have frequently used commercially available collagen-type IV-based Matrigel as a scaffold to improve the viability of resected and PDTX tumors (3-6). Matrigel contains a variety of cell-derived growth factors as well as extracellular matrix proteins, including collagen IV. In a PDTX model of colorectal cancer liver metastasis (CRC-LM) using Matrigel, it has been

reported that tumor stroma derived from human cells were replaced early by mouse cells; however, the histological morphology and cellular function of the tumor tissues were maintained (7,8). However, Matrigel contains factors derived from mouse cells (9) and is not an animal-free component. Therefore, it is possible that an inflammatory or immune response may be induced in the transplanted tumor by the animal component. Such unintended reactions may make the obtained data difficult to interpret.

Tumor tissue contains a complex tumor microenvironment composed of cancer cells, other diverse cells, and extracellular factors in the tumor stroma. The tumor stroma has a heterogeneous composition encompassing not only CAFs and immune cells but also extracellular matrix and other diverse factors that contribute to resistance to anticancer therapy and increased cancer grade (10,11). In addition, tumor stroma serves as a niche for cancer stem cells to maintain their stemness (tumorigenic potential, self-renewal, resistance to therapy/apoptosis) (12). The extracellular matrix of the cancer stroma is mainly composed of collagen, fibronectin, hyaluronic acid, tenascins, proteoglycans, and matrix metalloproteinases. This study focused on collagen type I, which is particularly abundant (13).

The recombinant protein based on human collagen type I (RCPhC1), the focus of this study, is an innovative experimental scaffold material synthesized under animal component-free conditions (14), which is abundant in the tumor stroma and is important for tumor growth. However, the contribution of RCPhC1 as a scaffold to the maintenance of the human tumor microenvironment in the PDTX model has yet to be investigated.

Consequently, we established PDTX models using resected CRC-LM specimens and compared the number of tumor stromal human and mouse cells using control (no scaffold), Matrigel, and RChC1 as scaffold material for resected and post-transplant tumors (Passage 1 and Passage 2). Lamin B, a major component of most mammalian cells, predominantly localizes at the nuclear periphery (15). This protein is highly conserved across species, including humans and mice. Immunohistochemical staining of Lamin B1 has been reported as a valuable tool for distinguishing between human and mouse cells (16). Given its conserved nature, Lamin B1 is a characteristic marker that provides evidence of cellular origin between human and mouse cells. Therefore, we selected the immunohistochemical detected of the Lamin B to identify human-derived stromal cells in the xenograft tumors, mainly including mouse stromal components.

Materials and methods

Patient information. Three patients with colorectal cancer liver metastasis (CRC-LM) who had undergone surgery at Gunma University Hospital between 2019 and 2021 were included in the study. None of the patients had received preoperative radiation or chemotherapy. Three patients with colorectal cancer liver metastasis (CRC-LM) who had undergone surgery at Gunma University Hospital between 2019 and 2021 were included in the study. The patients consisted of a 37-year-old male (PDTX-9), a 72-year-old female (PDTX-10), and a 66-year-old male (PDTX-12). All patients provided written informed consent for our study in accordance with institutional guidelines and the principles of the Declaration of Helsinki, and the study was approved by the Institutional Review Board for Clinical Research of Gunma University Hospital (approval no. HS2018-261).

Preparation of hydrogels composed of animal-component-free RCPhC1. The solution of RCPhC1 at a concentration of 46.5 mg/ml and the solution of Tetra-PEG-OSu at 37.5 mg/ml were separately prepared using 50 mM phosphate-buffered saline (PBS). The preparation involved the following steps: 300 microliters of the filtered PEG solution were added to 300 microliters of the RCPhC1 solution in a 1:1 ratio. The combined solution was then vortexed for about ten seconds. Finally, the RCPhC1 hydrogel mixture was left to undergo gelation by heating with a water bath at 37˚C to facilitate the crosslinking reaction.

Patient-derived human tumor xenograft models of colorectal liver metastasis. All animal experiments were approved by the Institutional Animal Care and Ethics Committee of Gunma University (approval no. 18-024). Male NOD-SCID mice aged four weeks (CLEA Japan Int., Tokyo, Japan) were purchased and housed in the animal facility of Gunma University under standard conditions (12-h light/dark cycle, food and water provided *ad libitum*). All animal experiments complied with Gunma University guidelines for the care and use of laboratory animals. Mice were monitored once or twice a week for clinical signs of morbidity, including but not limited to rapid weight loss, severe lethargy, difficulty breathing, impaired mobility, or tumor burden exceeding 2,000 mm^3. One PDX12 passage2 control mouse reached the endpoint and was sacrificed. Animals reaching these endpoints were humanely euthanized using a cervical dislocation under deep anesthesia (5% isoflurane) in accordance with ethical guidelines.

Fresh CRC-LM samples were obtained from patients at the time of surgery at Gunma University Hospital. The tissues were immediately transferred to ice in a DMEM medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), supplemented with 1% penicillin/streptomycin (FUJIFILM Wako Pure Chemical Corporation) and amphotericin B (Gibco, Invitrogen, Paisley, UK). The tissues were cut into pieces of 5x5x5 mm using sterile surgical instruments and quickly grafted subcutaneously into the flank of NOD-SCID mice (Passage 1 generation: P1), embedding without scaffold or with 200 μl Matrigel™ (Corning, NY, USA) or RCPhC1. Matrigel was used at 2 dilutions in PBS cell suspension and later implanted with small tumor pieces in the mice subcutaneously. Upon growth, tumor size (mm) was measured once or twice a week in two dimensions using a slide caliper. Tumor volume was calculated using the equation (width x width x length)/2. When each xenografted tumor volume grew to >2,000 mm^3, tumors were harvested and put in transportation media for either direct propagation into a further generation (Passage 2 generation: P2) or father analyses.

Multicolor immunofluorescence staining. Paraffin-embedded blocks were cut into four μm-thick sections and mounted on glass slides. Sections were deparaffinized in xylene and dehydrated in alcohol. After rehydration through a graded series

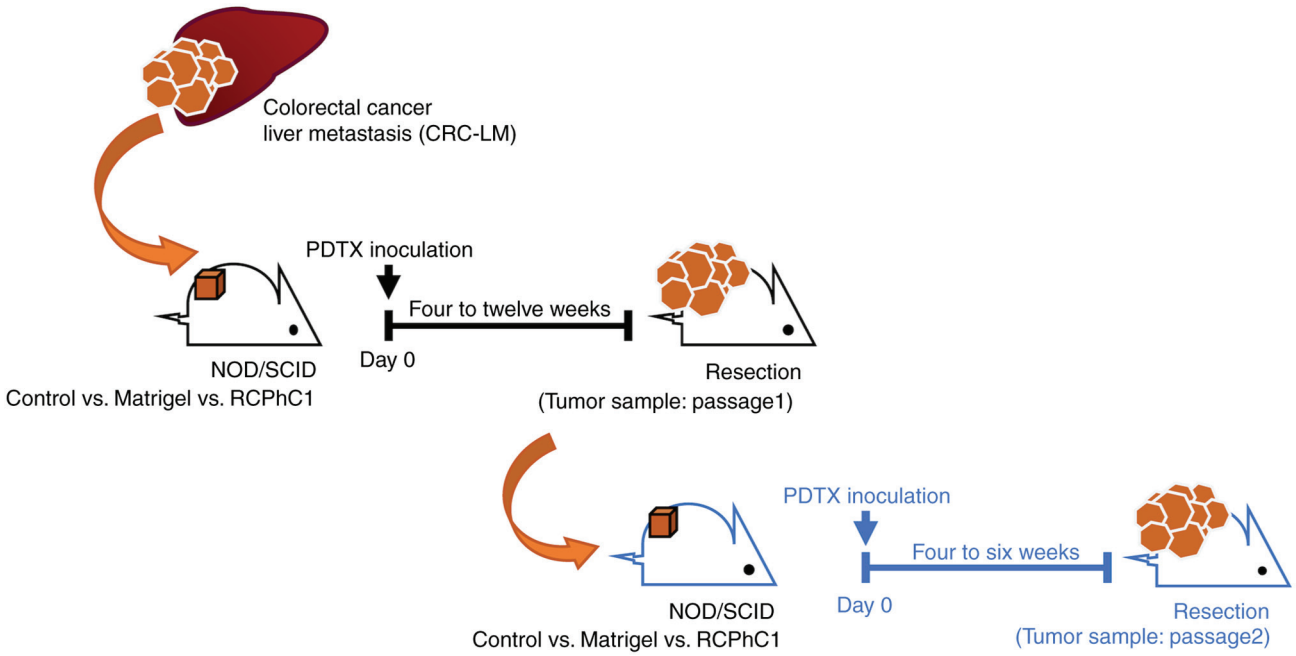

Figure 1. Experimental design of PDTX mouse model. Surgically resected specimens of CRC-LM were cut into pieces (5x5x5 mm), and transplanted into anesthetized immunocompromised mice as the control (without scaffold gel), Matrigel, and RCPhC1 hydrogels group. Tumor size was measured once or twice a week. PDTX tumors were harvested 4-12 weeks after inoculation for re-grafting in the new generation mice and further analyses. Resected tumor tissues were defined as passage1 tumor samples. Re-grafted tumors were harvested four to six weeks after inoculation for further analyses. Resected tumor tissues were defined as passage 2 tumor samples. CRC-LM, colorectal cancer liver metastases; PDTX, patient-derived tumor xenograft; RCPhC1, recombinant protein based on human collagen type I.

of ethanol treatments, antigen retrieval was performed using an Immunosaver (Nisshin EM, Tokyo, Japan) at 98-100˚C for 45 min. The endogenous peroxidase activity was inhibited by incubation with 0.3% H_2O_2/methanol for 30 min at room temperature. Nonspecific binding sites were blocked by incubation with Protein Block Serum Free Reagent for 30 min, and the sections were incubated overnight at 4˚C with the primary antibodies against mouse Lamin B1 (Sartorius Stedim Biotech, Gottingen, Germany, HS-404 003, rabbit polyclonal antibody, 1:800 dilution), human Lamin B1 (Sartorius Stedim Biotech, HS-404 013, rabbit polyclonal antibody, 1:400 dilution), and human/mouse α-SMA (Sigma Aldrich, MO, USA, A2547, mouse mAb, 1:800 dilution). Multiplex covalent labeling (human Lamin B1: Opal 690 Fluorophore, OP-001006) (mouse Lamin B1 and α-SMA: Opal 520 Fluorophore, OP-001001) with tyramide signal amplification (Akoya Biosciences, MA, USA) was performed according to the manufacturer's protocol. All sections were counterstained with DAPI, and after washing in PBS, the sections were mounted onto glass slides with a SlowFade™ Gold Antifade mountant (Thermo Fisher Scientific, Waltham, USA). All slides were examined under an All-in-One BZ-X710 fluorescence microscope (KEYENCE Corporation, Osaka, Japan).

Evaluation of stromal cell origin in PDTX tumors: human and mouse specific Lamin B staining. Human and mouse-specific Lamin B antibodies have been found useful in identifying the cell species origin in the histological samples (16). Eight fields from each sample were taken under a 40x objective lens. Human or mouse lamin B positive cells were manually counted from each PDTX tissue. The total number of stromal cells counted in the tumor tissue of each PDTX was calculated.

Cell lines. The mouse NIH-3T3 fibroblast cell was purchased from the JCRB Cell Bank (Osaka, Japan). The cell lines were cultured in DMEM (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cells were maintained at 37˚C in an atmosphere of humidified air with 5% CO_2.

Invasion assays. The membrane of upper chamber (Corning Life Sciences, Tewksbury, MA, USA) was coated with PBS (control), 100 μl Matrigel (x5 PBS dilution) or 100 μl RCPhC1 hydrogels (x2.5 PBS dilution), and 90 μl was aspirated. The upper chamber was then solidified at 37˚C for 30 min. The gel-coated chamber (8.0 μm pore size) with polyethylene terephthalate membranes were hydrated with a serum-free medium at 37˚C for 30 min. After removing the serum-free media, the upper chamber was set in the new well of the 24-well plate. NIH-3T3 cells (2x10^5 cells/well) were seeded in the upper chamber with 500 μl of FBS-free medium. The lower chamber was filled with 750 μl of complete medium with 20% FBS, and the plate was then incubated at 37˚C in a humidified atmosphere with 5% CO_2. After 72 h of incubation, the invaded cells were fixed and stained with Diff-Quik (Sysmex Corporation, Kobe, Japan). After staining, the cells that had invaded through the scaffold gels and pores in the lower membrane were counted under a microscope. Ten randomly selected fields were evaluated under an All-in-One BZ-X710 microscope (KEYENCE Corporation, Osaka, Japan).

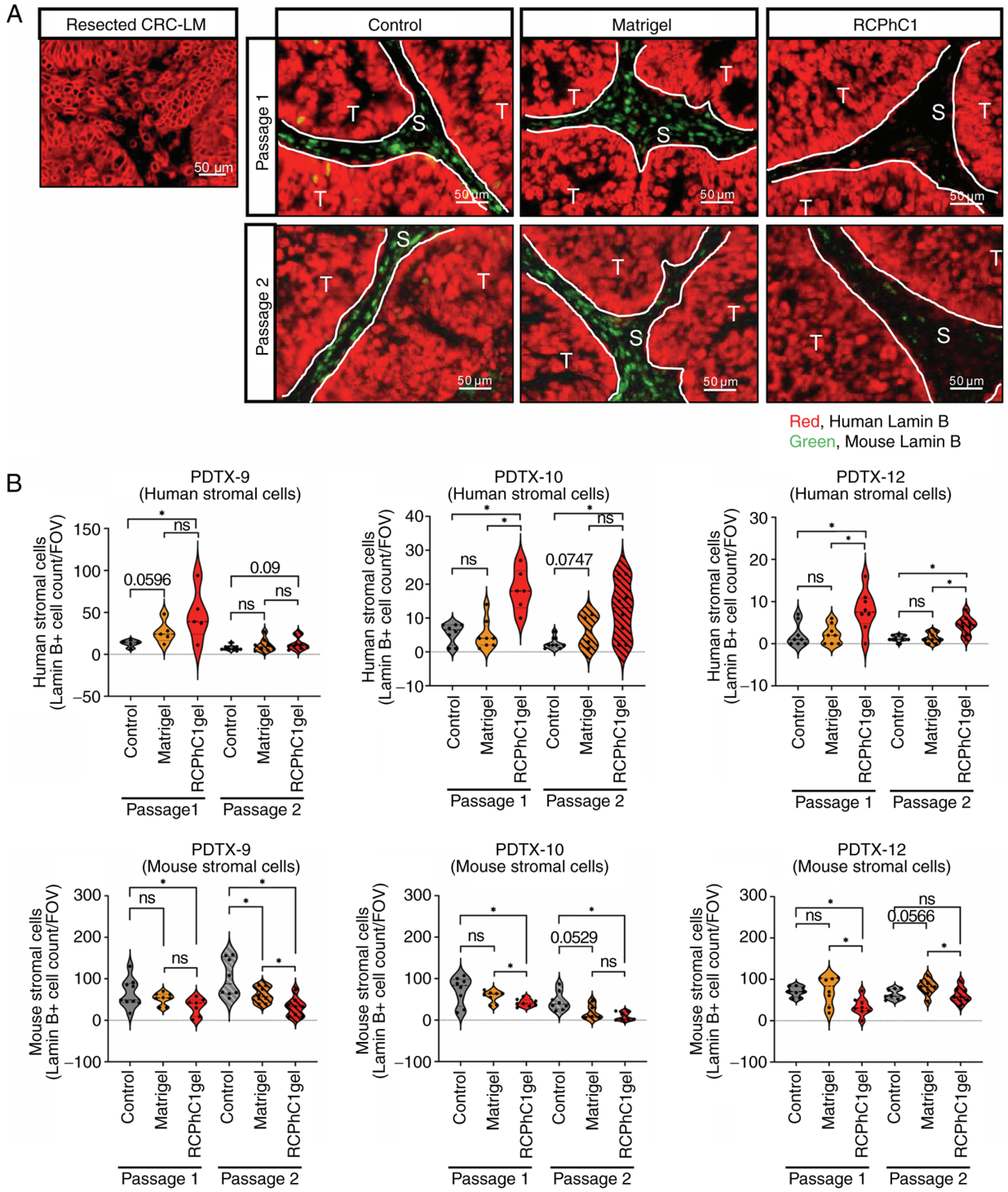

Figure 2. Immunofluorescence staining pattern of human or mouse-specific Lamin B in PDTX tumors. (A) Immunofluorescence analysis of human Lamin B (red) and mouse Lamin B (green) in the control, Matrigel, and RCPhC1 hydrogels group from PDTX-9 tumor tissues (passage1 and passage2). Scale bar, 50 μm (original magnification, x20). Left upper panel: human CRC-LM sample was used as positive control for human Lamin B (red) and negative control for mouse Lamin B (green). (B) Count data of mouse Lamin B and human Lamin B positive cells in the control (gray), Matrigel (yellow) and RCPhC1 hydrogels (red) group from tumor tissues of PDTX-9, 10, and 12 (passage1 and passage2). The black dots in the violin plots indicate mean cell number in groups. *P<0.05. ns, not significant. Lamin B-positive cell counts were analyzed independently in the control, Matrigel, and RCPhC1 gel groups of each passage using the Kruskal-Wallis test. When the Kruskal-Wallis test results were significant, Steel-Dwass multiple comparison tests were used to assess differences in each group. CRC-LM, colorectal cancer liver metastases; PDTX, patient-derived tumor xenograft; RCPhC1, recombinant protein based on human collagen type I; T, tumor cells; S, stromal cells; FOV, field of view.

Statistical analyses. All statistical analyses were performed using GraphPad Prism version 10.0 (GraphPad Software Inc., California, USA). Data for stained cell number in three groups were analyzed with Kruskal-Wallis test. When the results of the Kruskal-Wallis test were significant, Steel-Dwass multiple comparison tests were used to assess differences in stained cell number among each group. Differences were considered significant at P<0.05.

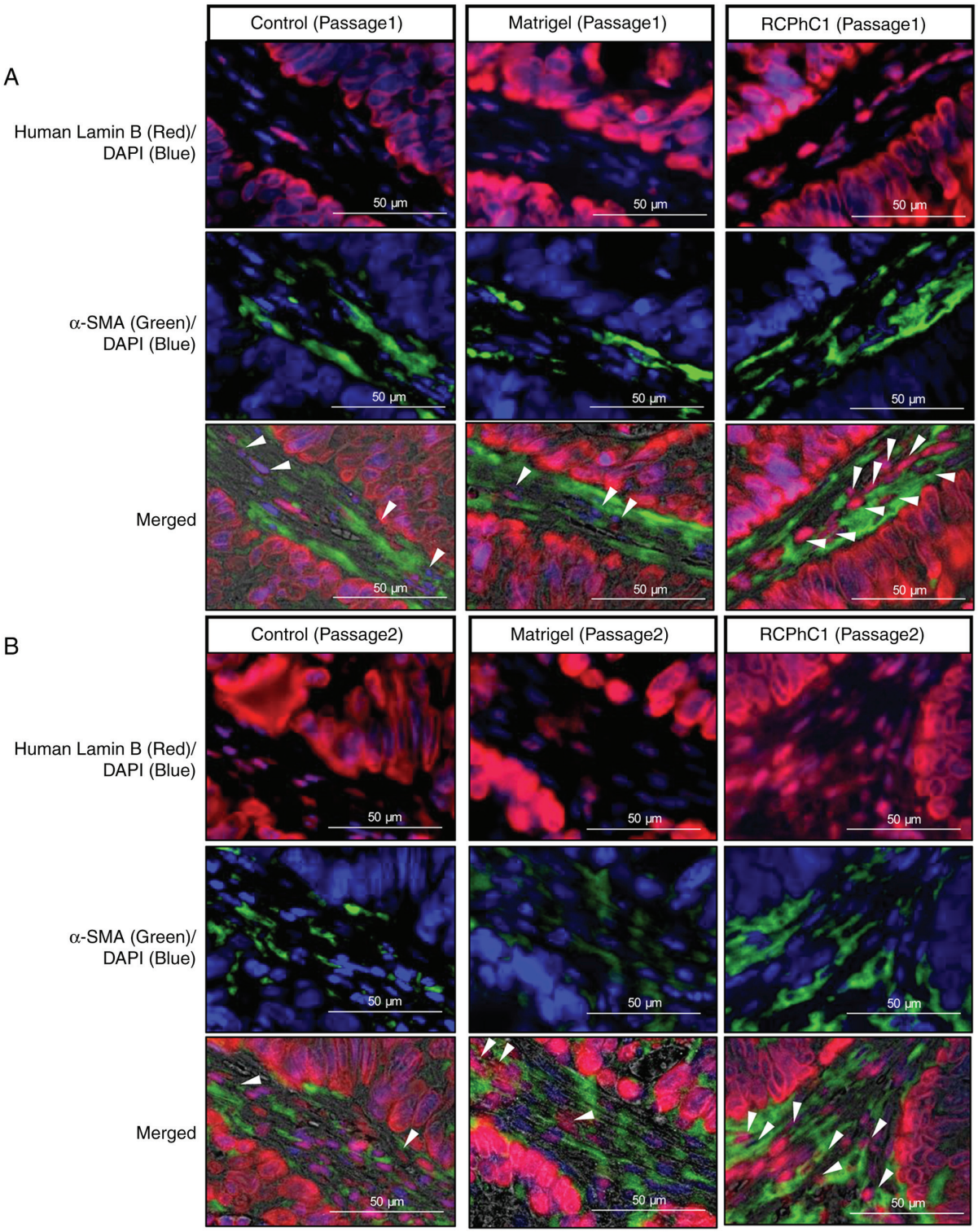

Figure 3. Evident colocalization of human Lamin B and fibroblast marker α-SMA in the PDTX tumors embedded by RCPhC1 hydrogels. Immunofluorescence analysis of human Lamin B (red) and α-SMA (green) positive cells at (A) passage 1 and (B) passage 2 in the control, Matrigel, and RCPhC1 hydrogels group from PDTX-9 tumor tissues (passage1 and passage2). Human Lamin B (red) was used as a marker for detecting human CRC-LM derived cells, and α-SMA (green) as a marker for human/mouse fibroblast cells. All sections were contrast stained with DAPI (blue). Filled arrows indicate double α-SMA and human Lamin B double positive cells as the human CRC-LM derived fibroblast cells in the control, Matrigel, and RCPhC1 hydrogels group from PDTX-9 tumor tissues (passage1 and passage2). CRC-LM, colorectal cancer liver metastases; PDTX, patient-derived tumor xenograft; RCPhC1, Recombinant protein based on human collagen type I.

Results

Human-derived cells were maintained in PDTX tissues embedded in RCPhC1 hydrogels. In this study, we planned to transplant human CRC-LM tissue into NOD-SCID mice to establish PDTX. We established and passaged PDTX without scaffold or with Matrigel or RCPhC1 hydrogels to compare the scaffold materials used for transplantation (Fig. 1). The

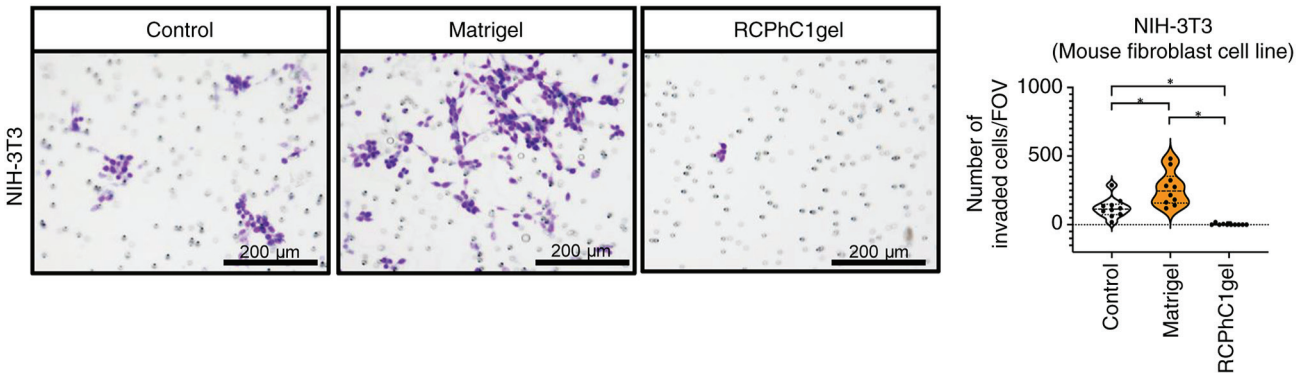

Figure 4. Invasion ability of murine fibroblast cell line NIH-3T3 against the Matrigel and RCPhC1 hydrogels. Left panel: invading cell images of NIH-3T3 cells in the control, Matrigel, and RCPhC1 hydrogels group. Cells were cultured in the chamber inserts coated with Matrigel or RCPhC1 hydrogels for 72 h in a serum-free DMEM medium. Cells that invaded the scaffold gel and migrated into the bottom chamber side with 20% FBS medium were stained using a Diff-Quik staining kit. Control group indicates the camber inserts without scaffold gel coating. Scale bar, 200 μm (original magnification, x10). Right panel: invaded cell counts of 10 fields in the control, Matrigel, and RCPhC1 hydrogels group. *P<0.05. Invaded cell numbers were analyzed independently in the control, Matrigel, and RCPhC1 gel groups using the Kruskal-Wallis test. When the Kruskal-Wallis test was significant, Steel-Dwass multiple comparison tests were used to assess differences in each group. RCPhC1, recombinant protein based on human collagen type I; FOV, field of view.

largest tumor diameters and volumes in each PDTX model were observed as follows: PDTX-9 passage 1 (20.5 mm, 1,835.1 mm^3), PDTX-9 passage 2 (15.2 mm, 706.3 mm^3), PDTX-10 passage 1 (17.2 mm, 1,513.7 mm^3), PDTX-10 passage 2 (17.9 mm, 1,119.3 mm^3), PDTX-12 passage 1 (23.4 mm, 1,858.4 mm^3), and PDTX-12 passage 2 (22.7 mm, 2,236.5 mm^3). The RCPhC1 hydrogel scaffold did not increase PDTX tumor growth or Ki67-positive tumor cells compared to the control and Matrigel (Fig. S1A and B).

Using multicolor immunofluorescence analysis, we evaluated the expression positivity of human Lamin B as red and mouse Lamin B as green in resected CRC-LM tissues and PDTX tumors (Passage 1 and Passage 2) (Fig. 2A). The number of human Lamin B-positive cells in RCPhC1 hydrogels was significantly higher than that in the controls and Matrigel at both passage 1 and passage 2 PDTX tumors (Fig. 2B). However, the number of mouse Lamin B-positive cells in RCPhC1 hydrogels was significantly lower than that in controls at all passage 1 PDTX tumors and passage two in PDTX-9 and ten tumors (Fig. 2B). The number of mouse Lamin B-positive cells in RCPhC1 hydrogels was significantly lower than that in Matrigel at passage one in PDTX-10 and 12 tumors and passage 2 in PDTX-9 and 12 tumors (Fig. 2B). These data indicated that the RCPhC1 scaffold preserved the human-derived cells in the PDTX tumor microenvironments during the passage process.

Additionally, we did not observe the distant metastasis in our CRC-LM subcutaneous PDTX models. A previous study reported that CRC PDTX tumors transplanted subcutaneously do not form distant metastases, however, do form metastases when transplanted into the colon (17). On the other hand, it has been reported that PDTX tumors of lung cancer in this study form distant metastases (18). The difference in the metastatic potential of such PDTX tumors may depend on not only the PDTX injection site but also tumor types.

RCPhC1 hydrogel preserved human-derived fibroblasts within PDTX tumors during the passage process. Consequently, human Lamin B and α-SMA double-positive cells were higher in RCPhC1 hydrogels than the control and Matrigel, suggesting that human stromal cells were maintained in PDTX tissue embedded in RCPhC1 hydrogels (Fig. 3A). There was a similar trend in passage 2 of all PDTX cases (Fig. 3B).

RCPhC1 hydrogels inhibit the invasion of mouse fibroblast cells. An *in vitro* invasion assay was performed to test whether the RCPhC1 hydrogel affected the invasion of mouse fibroblasts into PDTX tumors. As a result, the number of murine fibroblast cell line NIH-3T3 cells invading the RCPhC1 hydrogel-coated chamber was significantly lower than that in the control and Matrigel-coated ones (Fig. 4), suggesting that the RCPhC1 hydrogel can prevent murine fibroblast invasion into the PDTX tumor microenvironments.

Discussion

In this study, we established a PDTX mouse model using CRC-LM resection specimens from three cases and evaluated the scaffold-dependent maintenance function of human-derived cells during the passage process. The results show that patient-derived tumor tissues embedded with RCPhC1 hydrogels had significantly more human Lamin B-positive cells and fewer mouse Lamin B cells than PDTX tumors embedded without scaffolds or with Matrigel. The human Lamin B-positive cells in PDTX tumors with RCPhC1 hydrogels were recognized as fibroblasts. Furthermore, *in vitro* invasion assays showed that RCPhC1 hydrogels significantly inhibited the invasion of mouse fibroblast cell lines compared to Matrigel.

This study showed that RCPhC1 hydrogels had an advantage in maintaining human tumor stromal fibroblasts in the PDTX model compared to the control and Matrigel groups. Why human immune and vascular endothelial cells could not be maintained even with RCPhC1 hydrogels should be considered (Fig. S2). Ghanekar *et al* (19) attempted direct immunostaining of human CD31 in three human tumor xenografts, but did not detect human CD31, leading the authors to conclude that the endothelial cells in human HCC xenografts were of mouse rather than human origin. For this reason,

we postulated two mechanisms for entering mouse-derived cells into PDTX tumors: a direct migration/invasion route from the surroundings and a route via tumor neovasculature constructed after transplantation. We suggest that immune cells and vascular endothelial cells were not affected by the scaffold material because they are shared from the blood via the neovasculature established by the PDTX tumor (tumors that cannot construct neovasculature cannot be viable). We also speculate that migratory fibroblasts infiltrate directly into PDTX tumors from the periphery of PDTX tumors, rapidly replacing human stromal fibroblasts with mouse fibroblasts in the control and Matrigel groups; however, this phenomenon may have been inhibited in the RCPhC1 group. In fact, *in vitro* experiments in this study confirmed that RCPhC1 hydrogels inhibited the invasion of mouse fibroblasts better than Matrigel. Therefore, we hypothesize that the RCPhC1 hydrogel physically inhibits the invasion and migration of mouse fibroblasts. Furthermore, the various factors in the Matrigel may promote the invasion of host mouse fibroblasts into the tumors (9). On the other hand, such stromal fibroblasts in tumor tissues are called CAFs and have been reported to be associated with the drug sensitivity of tumor tissues. CAFs also attract attention as a cancer therapeutic target (20-22). The RCPhC1 hydrogel, which can maintain human stromal-derived CAFs, is expected to serve as a scaffold material during passaging in PDTX models to evaluate the efficacy not only of existing anti-cancer drug sensitivity but also of future CAF-targeted therapies.

Our PDTX model used immunocompromised mice, which makes it impossible to assess tumor-immune cell interactions. To solve this problem, the humanized PDTX model, in which human hematopoietic stem cells are transplanted into immunodeficient mice, has attracted attention recently (1,3,4,6). This humanized PDTX model mimics the human immune system and enables the evaluation of the drug efficacy of immunotherapy, which cannot be evaluated in the conventional xenograft model. It is expected that a new PDTX model that more closely resembles the human tumor microenvironment may be established using new scaffold RCPhC1 hydrogels, which can maintain tumor stroma, in combination with a humanized PDTX model that maintains human immune cells.

This study had some limitations. Firstly, it involved only three cases of CRC-LM, and whether the findings can be generalized to carcinomas other than CRC is unclear. Therefore, the potential of RCPhC1 hydrogels as a new scaffold material should be validated in PDTX models using extended sample size and other tumor types. Secondary, The PTX model using immunodeficient mice does not accurately reflect the relationship between tumors and immune cells in the microenvironment of human tumor tissue. Thirdly, comparative studies should be conducted to verify whether the PDTX model using RCPhC1 hydrogels or the Matrigel model accurately reflects the drug sensitivity of patients with cancer. Fourthly, RCPhC1 hydrogels physically palpate the gel itself even before PDTX tumor regrowth begins (Fig. S1A). This may be problematic for short-term drug evaluation and for recognizing mice whose transplanted tissue has failed to grow. Finally, one of the advantages of RCPhC1 hydrogel is that the gel eliminates the risk of inducing an inflammatory or immune response in the transplanted tumor due to the presence of animal components. However, in this study, we did not directly compare RCPhC1 and other animal-free scaffolds to evaluate its full potential. Further studies are needed to assess the performance of RCPhC1 hydrogel in contracts with other non-animal scaffolds.

This study investigated RCPhC1 hydrogels as new scaffold materials for tumor engraftment in PDTX mouse models. In PDTX tissues embedded with RCPhC1 hydrogels, the replacement of human-derived fibroblasts with mouse-derived fibroblasts was suppressed compared to that in the controls. Therefore, RCPhC1 hydrogels may be a promising experimental tool for maintaining the tumor microenvironment in the PDTX model.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HO, RM and KA wrote the manuscript. RM, HO and BEO collected and analyzed the image data. HO, GeD, TS, TO, RF, SK, KHo, GaD, KHa, TYa, NI, TI, AW, NK, MT and TYo analyzed and interpreted the data. HO, RM and TY drafted the manuscript. HO, TY, KA, HS and KS conceptualized the study. All authors have read and approved the final version of the manuscript. TYo and KA confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was performed in compliance with the principles of The Declaration of Helsinki. All patients were eligible for our study by the Institutional Review Board for Clinical Research of Gunma University Hospital (approval no. HS2018-261). All patients provided written informed consent for the present study in accordance with institutional guidelines and the principles of The Declaration of Helsinki. All animal experiments were approved by the Institutional Animal Care and Ethics Committee of Gunma University (approval no. 18-024).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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