

Article

Xenograft-Derived Human Breast Cancer Organoids Can Form Chimeras with Host Mouse Mammary Epithelial Cells Which Promote Tumor Cell Proliferation

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Abstract

Breast cancer progression and treatment responsiveness are significantly influenced by the tumor microenvironment. Therefore, transplantation into the mammary fat pad is widely employed to establish a mouse xenograft model of breast cancer. This study reports chimeric organoids derived from breast cancer xenografts composed of human and mouse cells. During passaging of an organoid line derived from breast cancer xenografts, characteristic cell clusters composed of smaller cells were observed. Immunostaining with a mouse-specific antibody revealed that the smaller cells were mouse cells composed of luminal- and basal-like cells. Chimeric organoids were observed in four of the six xenograft-derived organoid lines. Organoids composed solely of human cells rapidly diminished after passaging, with chimeric and mouse-cell-only organoids becoming predominant. When human breast cancer cells were co-cultured with mouse mammary epithelial cells, chimeras were frequently observed. The PCNA positivity rate in breast cancer cells within chimeras was higher than that in breast cancer cells within organoids composed solely of human cells. These findings indicate that xenograft-derived breast cancer organoids frequently contain mouse cells and that mouse mammary epithelial cells promote the proliferation of human breast cancer cells.

Keywords: breast cancer; xenograft; organoid; chimera



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1. Introduction

Breast cancer is one of the most common malignant tumors globally [1]. Tumor progression and treatment responsiveness are significantly influenced by the tumor cells themselves and by the tumor microenvironment (TME) [2,3]. As components of the TME, cancer-associated fibroblasts, immune cells, and normal mammary epithelial cells have been reported to influence tumor growth and treatment outcomes [4–6].

In a conventional two-dimensional adherent culture, the in vitro culture of patient-derived breast cancer cells is generally difficult. Advances in organoid culture technology

have made it possible to culture organoids in breast cancer. Breast cancer organoids established from patient-derived tumors accurately reproduce the three-dimensional structure, molecular profile, and drug responsiveness of a patient's tumor, leading to a rapid expansion of their research applications in recent years, including breast cancer [7].

Patient-derived organoids and xenografts (PDXs) are widely used as preclinical models to reflect the biological characteristics of tumors [7–9]. When establishing mouse xenograft models for breast cancer, the mammary fat pad transplantation method is widely employed, with the expectation that the TME will promote breast cancer cell proliferation and enhance the success rate of the PDX models [10]. Cancer organoids can be established from PDX models, thereby enabling the construction of a “shuttle experimental system” between *in vivo* and *in vitro* settings, which overcomes the low-throughput performance of PDX models [11,12].

In developmental studies, when mammary glands are reconstituted *in vivo* from breast stem cells, mouse mammary epithelial cells are pre-cleared from mouse fat pads to distinguish them from transplanted cells. However, when creating breast cancer PDX models, the fat pads are not necessarily cleared. Therefore, organoids from xenograft tumors may retain mouse-derived mammary epithelial cells. The presence of mouse-derived epithelial cells may complicate the interpretation of the experimental results and compromise the reliability of drug testing using organoids. However, no studies have systematically analyzed the presence of mouse mammary epithelial cells within PDX-derived organoids.

This study revealed that breast cancer organoids established from PDX tumors generated by transplantation into non-cleared fat pads frequently contained mouse-derived mammary epithelial cells. We demonstrated that mouse mammary epithelial cells isolated from xenograft tumor-derived organoids and normal mouse mammary epithelial cells promoted the proliferation of human tumor cells when co-cultured. These findings highlight the importance of evaluating the coexistence of host mammary epithelial cells in organoid cultures derived from orthotopic transplant tumors.

2. Materials and Methods

2.1. Organoid Preparation and Culture

This study was approved by the ethics committees of Kyoto University (R1575, G1481, R2220), the National Institutes of Biomedical Innovation, Health, and Nutrition (206-04), and Osaka International Cancer Institute (1004075009) and informed consent was obtained from all participating patients. The patient clinical information is shown in Table S1. The organoids were prepared from xenografts using the cancer tissue-originated spheroid (CTOS) method [13], embedded in a growth factor-reduced (GFR) Matrigel® basement membrane matrix (Corning Inc., Corning, NY, USA) and cultured in human breast cancer organoid medium [14] (Table S2).

2.2. Mouse Mammary Organoid Preparation and Culture

Mouse mammary organoids were generated from the fourth mammary fat pad in the same manner as specified by the CTOS method, embedded in GFR Matrigel®, and cultured in human breast cancer organoid medium [14] (Table S2). Mouse spheroids (isolated from M63 organoids) were cultured under the same conditions as the mouse mammary organoids. M63 mouse cells were purified by treatment with diphtheria toxin (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) (Figure S1), which acts specifically on human cells [15].

2.3. Animal Experiments

All animal studies were approved by the Institutional Animal Care and Use Committee of Kyoto University (18564); the National Institutes of Biomedical Innovation, Health, and Nutrition (DS18-086R21); and the Osaka International Cancer Institute (1002035111). Mice were housed under standard specific pathogen-free conditions. Organoids were suspended in a 1:1 mixture of medium and Matrigel (Corning) and injected into the non-cleared mammary fat pads of female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice for M63 using a 27-gauge needle (Terumo, Tokyo, Japan). For the other lines, tumor fragments were transplanted into the non-cleared mammary fat pads of female Super SCID mice (C3H/HeJ/NOs-scid), followed by subcutaneous transplantation of 5 mm³ pieces into the flank of female NOD/SCID mice for passaging.

2.4. Co-Culture of Human Breast Cancer Cells with Mouse Cells

Breast cancer organoids and mouse spheroids (derived from M63 cells) or mouse mammary organoids were dissociated and mixed in a single test tube at a ratio of 3:2. After incubating with TrypLE™ Express enzyme (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 7 min, the cells were pipetted using a P200 pipette to disperse them into cell clusters comprising few cells. These clusters of cells were embedded in Matrigel GFR and cultured in human breast cancer organoid medium. Organoids were harvested after 6 days of culture and subjected to further analysis. Mouse cells from M63 breast cancer xenograft-derived organoids were purified by exposing isolated mouse cells to 200 ng/mL diphtheria toxin for 7 days to eliminate human cells.

2.5. Morphological Analyses

Following passage of the organoids, they were stained with Hoechst 33342 (1 µg/mL; Thermo Fisher Scientific) and the size of individual cells was assessed under a brightfield microscope (Leica Microsystems, Wetzlar, Germany). Immunofluorescence staining of formalin-fixed, paraffin-embedded PDX and organoid samples was performed using previously reported methods [16]. Hematoxylin and eosin (HE) staining was performed as previously reported [13]. The primary antibodies used were as follows: anti-cyclophilin A (Cell Signaling Technology, Beverly, MA, USA; Cat#51418, 1:100), anti-p63 (Santa Cruz Biotechnology, Dallas, TX, USA; Cat#sc-25268, 1:100), anti-cytokeratin 5 (CK5; Abcam, Cambridge, MA, USA; Cat#ab52635, 1:200), anti-cytokeratin 8 (CK8; DSHB, Iowa city, IA, USA; 1:100), anti- α -smooth muscle actin (α SMA; Sigma-Aldrich, St Louis, MO, USA; Cat#A2547, 1:200), and anti-PCNA (Cell Signaling Technology, Cat#2586, 1:2400). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific). Images were acquired using a BX50 microscope equipped with CellSens standard imaging software (version 4.4.1) (Olympus, Tokyo, Japan).

2.6. Evaluation of the PCNA-Positive Rate of Human Cells in Organoids

After performing immunohistochemistry, the total number of nuclei and PCNA-positive nuclei were counted in each organoid to calculate the PCNA-positive rate. Human cells were identified based on their cyclophilin A-negative status.

2.7. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 10.5.0 (GraphPad, San Diego, CA, USA). Student's *t*-test was used for single comparisons. When the data did not follow a normal distribution, the Mann–Whitney U test was used. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Organoids Established from Patient-Derived Breast Cancer Xenografts Contained Mouse Cells

During the passage of M63 xenograft-derived organoids, two distinct clusters comprising cells of differing sizes were observed, and Hoechst 33342 staining revealed that the large cells were not cell doublets (Figure 1A). Each cluster was isolated, embedded in Matrigel, and cultured. Both cell types formed spheroids with no morphological differences (Figure 1B, brightfield). Microscopically, spheroids composed of large cells exhibited more abundant cytoplasm than those composed of small cells (Figure 1B, HE). Immunohistochemical staining using a mouse-specific anti-cyclophilin A antibody [17] revealed that spheroids composed of large cells were negative for cyclophilin A, whereas those composed of small cells were positive (Figure 1B, mCyclophilin A). The small cells were either CK8+ or CK5+/p63+/αSMA- (Figure 1C). Next, we confirmed that the cells prepared from normal mouse mammary tissue could be cultured as organoids in the culture medium used in this study (Table S2, Figure 1D). The mouse mammary-gland-derived organoids were either CK8+ or CK5+/p63+/αSMA+ (Figure 1D). CK5+/p63+/αSMA- cells positive for mouse cyclophilin A were considered mouse basal-like epithelial cells. Thus, it is possible that mouse mammary gland-derived cells exist within M63 organoids, albeit at a lower level of differentiation than that of normal cells.

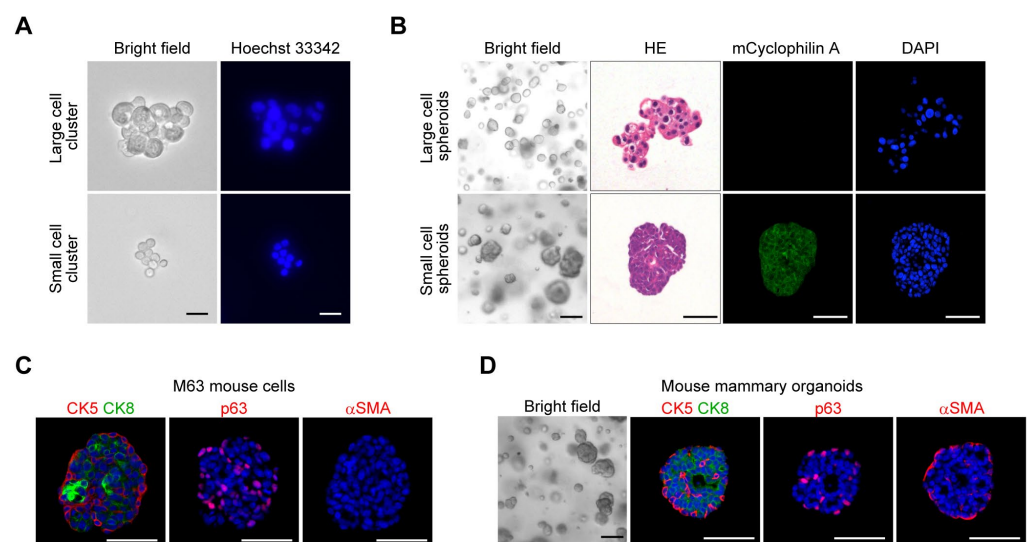


Figure 1. The presence of mouse cells in organoids derived from xenografts. (A) During the passing process of M63 breast cancer organoids, cell clusters of differing sizes were observed. The upper panels show large cell clusters, while the lower panels show small cell clusters. Left panels: bright-field images; right panels: Hoechst 33342 staining. Scale bar: 20 μ m. (B) Microscopic and immunofluorescence (IF) staining images of spheroids derived from large (upper panels) and small (lower panels) cell clusters. mCyclophilin A (green): staining with a mouse-specific anti-cyclophilin A antibody; 4',6-diamidino-2-phenylindole (DAPI, blue). Scale bars: 100 μ m (brightfield), 50 μ m (hematoxylin and eosin [HE], mCyclophilin A and DAPI). (C) IF staining of serial sections of spheroids derived from mouse cells isolated from M63 breast cancer organoids. Nuclei were counterstained with DAPI (blue). Scale bar, 50 μ m. (D) Microscopic and IF staining images of mouse mammary organoids. IF images were obtained from serial sections. Nuclei were counterstained with DAPI (blue). Scale bar: 100 μ m (brightfield), 50 μ m (IF staining).

3.2. Incidence of Chimeras in Breast Cancer Xenograft-Derived Organoids

When chimeric organoids established from xenograft tumors were stained with a mouse-specific antibody, the human breast cancer cells were found to be surrounded by a monolayer of mouse cells (Figure 2A, left), embedded within a thick layer of mouse cells (Figure 2A, middle), or present as islands within mouse cells (Figure 2A, right). As all

chimeras in the passaged organoids were enveloped by mouse cells, their chimeric nature could be readily identified using thin sections. Next, we assessed the chimeric fraction of the six xenograft-derived organoids by combining the other five PDXs. Chimeras were confirmed in four out of six lines (67%) (Figure 2B). Analysis of the chimeric ratios in each passage of a breast cancer organoid line revealed that human-derived organoids rapidly diminished after the first passage. By the third passage, organoids composed solely of mouse cells or chimeras (Figure 2C). At passage 0, mouse cells in the chimeras partially adhered to human cells (Figure 2D,E). However, with subsequent passages, the proportion of mouse cells increased and were present surrounding the human cells (Figure 2E). These results demonstrated that breast cancer organoids derived from xenografts that had been once transplanted into mouse mammary fat pads frequently formed chimeras of human breast cancer cells and mouse cells.

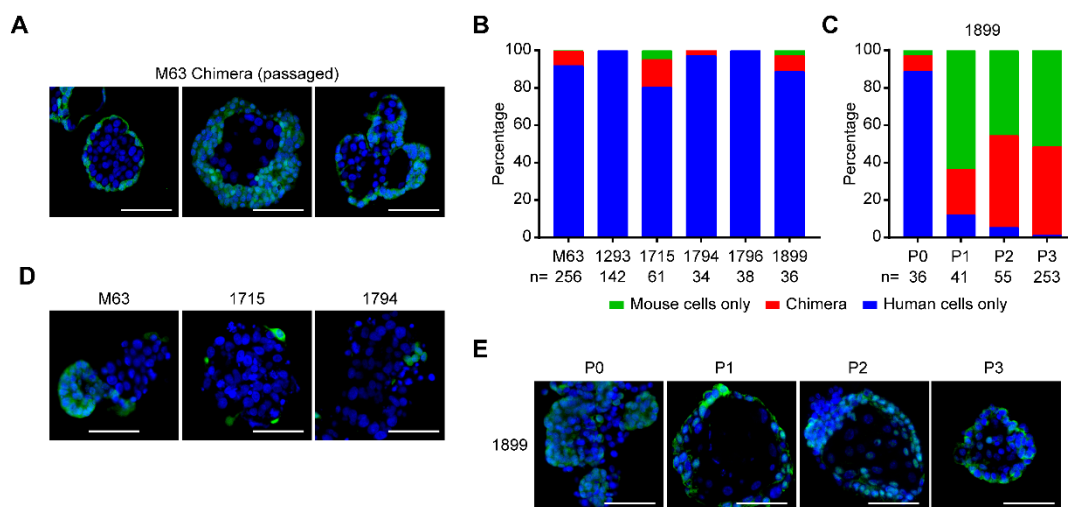


Figure 2. Chimera formation in breast cancer xenograft-derived organoids. (A) Three chimeric patterns observed in multiple-passaged M63 breast cancer organoids. (B) Percentage of chimeras in primary breast cancer organoids derived from six mouse xenograft lines. Human only, blue; mouse only, green; chimera, red. (C) Percentage of chimeras per passage in 1899 breast cancer organoids. (D) Representative IF image of chimeras in primary xenograft-derived organoids. (E) Representative IF image corresponding to (C). Blue, DAPI; green, mouse cyclophilin A in panels (A,D,E). Scale bars: 50 μ m in panels (A,D,E).

3.3. Human Breast Cancer Cells Showed Enhanced Proliferation When Co-Cultured with Mouse Mammary Epithelial Cells

Next, we analyzed the interactions between human breast cancer cells and M63 mouse cells. M63 mouse cells were purified by treatment with diphtheria toxin, which specifically eliminates human cells [15] (Figure S1). For human breast cancer cells, organoids prepared from patient tumors and subsequently passaged in vitro only were utilized [14]. When human breast cancer cells were co-cultured with M63 mouse cells, approximately 50% of the spheroids formed chimeras (Figure 3A). Next, the PCNA positivity rate of human breast cancer cells was evaluated and compared to that in co-cultures and pure breast cancer organoid cultures (monoculture). The PCNA positivity rate was significantly higher in human breast cancer cells in the chimeras than in organoids containing only human breast cancer cells cultured across all six lines examined (Figure 3B,C). In addition, the PCNA positivity rate in the spheroids composed of human cells alone in co-culture was also significantly increased compared to that in the organoids in monoculture (Figure 3D). These results suggest that mouse cells in M63 organoids promote the proliferation of human breast cancer cells, with humoral factors potentially contributing to this process.

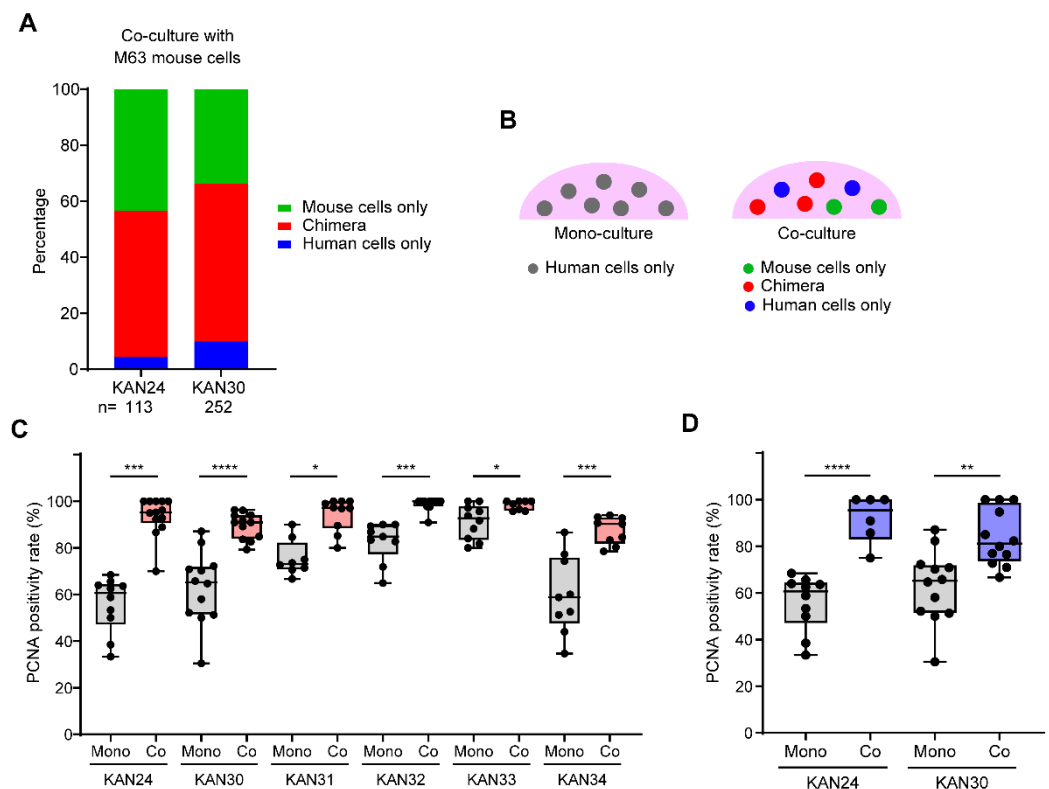


Figure 3. The proliferation of human breast cancer cells is promoted by co-culture with M63 mouse cells. (A) Percentage of chimeras generated by co-culture of breast cancer organoids and M63 mouse cells. Human only, blue; mouse only, green; chimera, red. (B) Schematic patterns of organoids or spheroids in monoculture or co-culture. (C) Comparison of PCNA positivity rates in human breast cancer cells between the mono-cultured organoids (Mono) and the chimeras in co-culture (Co). (D) Comparison of PCNA positivity rates in human breast cancer cells between the mono-cultured organoids (Mono) and the spheroids with only human cells in co-culture (Co). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3.4. Proliferation of Human Breast Cancer Cells Is Promoted by Co-Culture with Normal Mouse Mammary Epithelial Cells

Since M63 mouse cells originate from the tumor microenvironment, they may have already been educated by cancer cells. Therefore, we investigated whether similar effects could be elicited in normal mouse mammary epithelial cells. When human breast cancer organoids were co-cultured with normal mouse mammary epithelial cells, approximately 50% of the spheroids formed chimeras (Figure 4A). The PCNA positivity rate was significantly higher in human breast cancer cells in the chimeras than in organoids containing only human breast cancer cells (Figure 4B,C). In addition, the PCNA positivity rate in the spheroids composed of human cells alone in co-culture was also significantly increased compared to that in the organoids in monoculture (Figure 4D). These results are mostly similar to the results using M63-derived mouse cells, suggesting that even normal mouse epithelial cells can affect the growth of breast cancer cells in organoids.

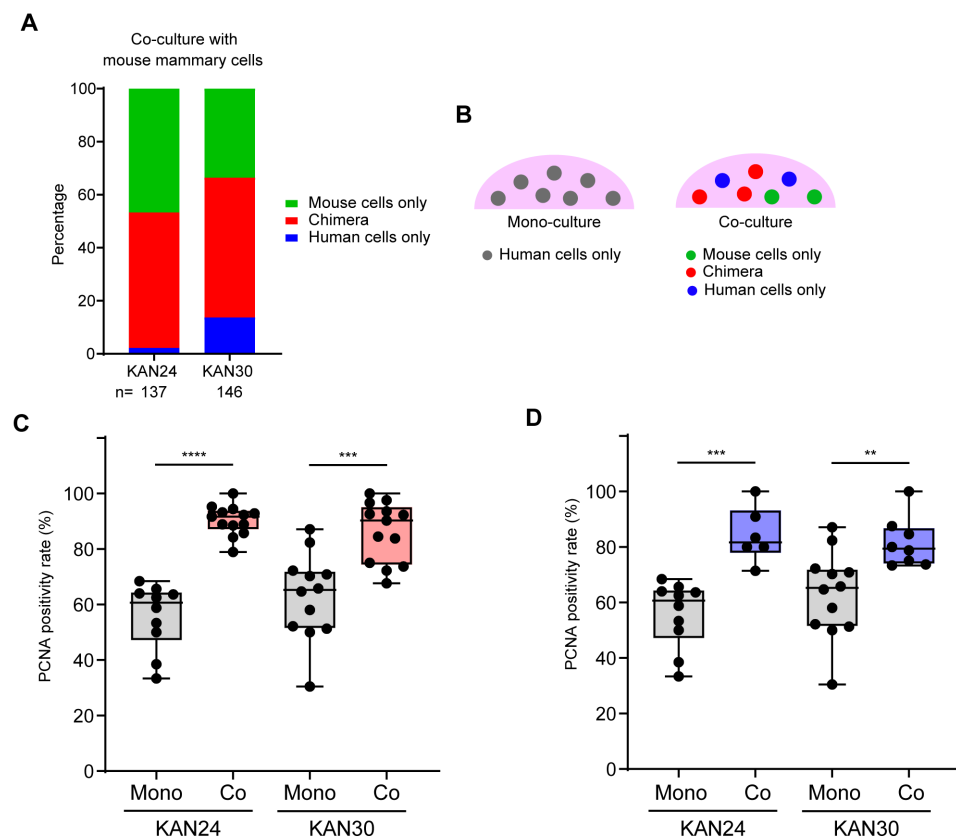


Figure 4. The proliferation of human breast cancer cells is promoted by normal mouse mammary epithelial cells. (A) The percentage of chimeras generated by co-culture of breast cancer organoids and mouse mammary epithelial cells. Human only, blue; mouse only, green; chimera, red. (B) Schematic patterns of organoids or spheroids in monoculture or co-culture. (C) Comparison of PCNA positivity rates in human breast cancer cells between the mono-cultured organoids (Mono) and the chimeras in co-culture (Co). (D) Comparison of PCNA positivity rates in human breast cancer cells between the mono-cultured organoids (Mono) and the spheroids with only human cells in co-culture (Co). ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

4. Discussion

In this study, we demonstrated that mouse-derived mammary epithelial cells formed chimeras with human breast cancer cells in organoids established from xenografts created by transplanting human breast cancer organoids or tissues at least once into the non-cleared mammary fat pads of mice. Furthermore, we demonstrated that these mouse epithelial cells promote the proliferation of human breast cancer cells. These findings demonstrate that host-derived mammary epithelial cells can persist into organoid cultures and highlights their functional significance.

Organoids derived from normal mouse mammary glands contained CK5, p63, and α SMA-positive myoepithelial cells. However, CK5/p63-positive cells isolated from mouse cell spheroids derived from M63 chimeric organoids were α SMA-negative and were likely to be basal-like cells. This suggests that the mammary epithelial cells of mice may have been influenced by tumor cells, leading to changes in their properties. These findings support previous reports that the loss of α SMA-positive myoepithelial cells is associated with tumor progression and epithelial–mesenchymal transition [18,19].

Previous studies have demonstrated that normal cells adjacent to cancer cells are significantly affected by the cancer cells [20]. Furthermore, altered normal-like cells may influence cancer cells. In this study, the proportion of PCNA-positive cells was significantly increased in chimeras of human breast cancer cells and mouse mammary epithelial cells

compared to that in organoids containing human breast cancer cells alone. Furthermore, in co-culture with mouse mammary epithelial cells, the PCNA-positive rate significantly increased, even in spheroids derived solely from human breast cancer cells. This suggests that mouse mammary epithelial cells promote the proliferation of human breast cancer cells through direct cell–cell contact and humoral factors. These findings support previous reports that normal mammary epithelial cells influence tumor cell proliferation via exosomes [6], growth hormones [5], and chemokines [4].

Previous studies reported that organoids generated from patient-derived xenografts (PDXs) often contain host-derived cells from the mouse environment [21]. The phenomenon of chimera formation observed in the mouse models in this study may occur similarly when organoids are prepared directly from patients with breast cancer. However, whilst chimeras containing mouse cells can be identified using species-specific antibodies, in chimeras with human cells, distinguishing normal cells from cancer cells is not straightforward, as there are no common markers to differentiate the two cell types. Normal human mammary organoids can be maintained under the same culture conditions as human breast cancer organoids. The absence of a mouse cell niche may lead to increased cell death in addition to reduced proliferative potential, as shown in this study. Furthermore, repeated passaging leads to the predominance of cells derived from normal mammary tissues. Therefore, when conducting drug sensitivity testing using human breast cancer organoids, it is desirable to use primary organoids whenever possible to faithfully replicate the patient's cancer.

This study revealed that in chimeras, cancer cells exhibit a characteristic structure in which they are surrounded by the host mammary epithelial cells. In ductal carcinoma in situ (DCIS), abnormal cells are surrounded by normal ductal cells and do not infiltrate the surrounding tissue. However, all organoid lines used in this study were derived from invasive cancers and formed characteristic structures, even in artificially generated chimeras. In other words, this structure might be an inevitable outcome of the interaction between cancer and the normal epithelium. Models demonstrating this structure may be applicable as novel research tools for elucidating direct interactions between normal mammary epithelial cells and breast cancer cells.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/organoids5010006/s1>. Figure S1: Purification of mouse cells using diphtheria toxin. Table S1: Clinical information. Table S2: Culture medium composition [22–29].

Author Contributions: Conceptualization, C.S., H.U., K.O., J.K. and M.I.; Methodology, C.S., H.U., R.C., J.K., K.O. and M.I.; Validation, H.U. and C.S.; Formal analysis and data interpretation, H.U., T.N. (Takahiro Nakayama), K.Y., A.K. and M.I.; Investigation, H.U., C.S., H.E. and T.N. (Taisei Nomura); Resources, T.N. (Takahiro Nakayama), K.Y., T.N. (Taisei Nomura) and A.K.; Data curation, H.U. and C.S.; Writing—original draft preparation, H.U. and M.I.; Writing—review & editing, H.U., C.S., J.K., K.O., R.C., H.E., T.N. (Takahiro Nakayama), K.Y., A.K. and M.I.; Visualization, H.U. and M.I.; Supervision, J.K. and M.I.; Project administration, M.I.; Funding acquisition, M.I. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the ethics committees of Kyoto University (R1575, 26 June 2018; G1481, 2024.10.25; R2220, 2 December 2019), the National Institutes of Biomedical Innovation, Health, and Nutrition (206-04, 11 July 2019) and Osaka International Cancer Institute (1004075009, 7 April 2010) for studies involving humans. The animal study protocol was approved by the Institutional Animal Care and Use Committee of Kyoto University (18564, 28 June 2018), the National Institutes of Biomedical

Innovation, Health, and Nutrition (DS18-086R21, 10 October 2009) and the Osaka International Cancer Institute (1002035111, 27 April 2010) for studies involving animals.

Informed Consent Statement: Written informed consent has been obtained from the patients to publish this paper.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: J.K., K.O., R.C. and M.I. are members of the Department of Clinical Bio-Resource Research and Development at Kyoto University, which is sponsored by KBBM, Inc. H.U. and C.S. are employees of KBBM, Inc. The other authors declare no conflicts of interest. This work was supported in part by a collaborative grant from KBBM-Kyoto University.

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