Development of a Novel *In Vivo* Cancer Model Using Cell Sheet Engineering

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Abstract. *Aim: Standard in vivo cancer models entail injecting single cancer cells, but this technique is not always successful. We developed a novel cancer cell sheet by using temperature-responsive polymer poly(N-isopropyl acryl amide)-coated plates, which allow controlled attachment and detachment of living cancer cells via simple temperature changes. Materials and Methods: Four human cancer cell lines were used to make cell sheets. The cancer cell sheets were subcutaneously transplanted into nude mice and compared regarding their tumor-forming ability with the conventional cell suspension technique. Results: Human cancer cell sheets were successfully transplanted into nude mice. The cancer cell sheets resulted in stable engraftment and showed a higher tumor volume determined by total flux with the IVIS® imaging system. Conclusion: Novel cancer cell sheets are useful tools to make in vivo cancer models in mice for the assessment of anticancer therapeutics.*

Cancer is one of the most common causes of death worldwide. Recently, molecular and genetic studies on cancer have led to the development of a variety of targeted drugs. However, these studies focus on cancer cells, molecules and genes, and do not investigate solid cancerous tissues, which usually exist *in vivo*. Evaluating cancer as a solid tissue *in vitro* or *in vivo* could provide important clinical information with considerable applications. Therefore, we aimed to develop a novel model of cancer tissue for assessing solid tissue instead of cultured single cells (1). Anticancer drug screening tests have been performed by transplanting human malignant tumors in mice. Growth of human cancer in athymic nude mice was reported for the first time in 1969. Animal models of human cancer are expected to play a central role in the *in vivo* pre-clinical studies on anticancer drugs. A common strategy for creating such models is to inject cultured cells and ascites or to graft a small piece of cancerous tissue into nude mice. Typically, grafts are transplanted subcutaneously (*s.c.*). When implanted *s.c.*, it is easier to measure tumor size because it develops close to the surface of the skin. Transplantation of a variety of human cancer grafts is possible by this method. However, determining the graft size can be quite tricky, as successful transplantation depends on the graft size. In the case of transplantation of ascites cell suspension, there is no guarantee that cancerous tissue will be formed, even after the amount of cells has been determined experimentally. Currently, there is no stable carrier for cancerous cells (2-6). We have developed a cell sheet engineering technique involving culture plates pre-coated with temperature-responsive polymers from which cultured cells can be harvested as a sheet. Cell sheet engineering involves pre-coating culture plates with temperature-responsive polymers. Poly(N-isopropylacrylamide) (PIPAAm) (7) is a temperature-responsive polymer that becomes hydrated and dissolves in aqueous solutions at temperatures below 20˚C. At higher temperatures, it becomes a turbid precipitate due to dehydration. PIPAAm contains hydrophilic amide groups and hydrophobic isopropyl groups as side-chains. At low temperatures, hydrogen bonds form between water molecules and the amide groups (hydration). Hydrogen bonds also form between water molecules and the isopropyl groups; as a result, PIPAAm adopts a clustered structure due to hydrophobic hydration and has an unstable form with stretched polymer chains. If the temperature is then elevated, Brownian movements of the water molecules are intensified. At the lower limit of critical solution temperature, the water molecules surrounding the isopropyl groups of the hydrated structure collapse and the hydrophobic isopropyl groups aggregate due to hydrophobic interactions. Thus, the entire

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polymer chain aggregates and precipitates. A few clinical studies using pre-coating culture plates with temperature-responsive polymers have used oral epithelial cell sheets (8), corneal epithelial cell sheets (9) and myocardial cell sheets (10). When this technology is used to generate a tumor-bearing animal model, the cancer cell sheets are formed through intracellular adhesion and the formation of a stratified structure is mediated by the extracellular matrix, enabling the transplantation of the cancer cell sheets both subcutaneously and in organs such as the liver. In the present study, we generated cancer cell sheets and transplanted them into mice to establish solid tumor-bearing animals. This method, involving transplantation, is rather novel and effective because it ensures more stability than conventional methods.

Materials and Methods

Cancer cell lines. Cells from the following human cancer cell lines were initially cultured on Falcon 3003 dishes (ϕ, 100 mm): HCT-116(-luc2) (human colon cancer cell line including luciferase 2; Summit Pharmaceuticals International Corporation (SPI), Tokyo, Japan); Panc-1 (human pancreas cancer cell line; American Type Culture Collection (ATCC), Manasas, VA, USA); Li-7 (human liver cancer cell line; Riken, Ibaraki, Japan); MKN74 (human gastric cancer cell line; Riken). The culture medium was then removed with a Pasteur pipette and the cells were washed with phosphate-buffered saline (PBS; Gibco, Carlsbad, CA, USA) (15 ml). After removing the PBS using a Pasteur pipette, the cells were incubated for 5min in a 1.5-ml solution containing 0.25% trypsin and 2.65 mM Na_4 ethylenediamine tetraacetic acid (Na_4EDTA). After confirming that the cells were detached from the culture dish, 15 ml of culture medium (RPMI1640 added to 10%FBS, McCoy's 5a and Dulbecco's modified Eagle medium [DMEM]) was added; cell suspensions were formed by pipetting into another dish. The resulting suspensions were centrifuged at 800 rpm for 5 min and the supernatants were removed to prepare cell suspensions for the experiments.

Construction of cancer cell sheets. To create the cancer cell sheets, cancer cell lines were seeded in temperature-responsive culture dishes (UpCell 3.5cm dish, CellSeed, Tokyo, Japan) at 1×10^4 cells/cm^2 and were incubated at 37˚C under 5% CO_2 for 72 h. To elucidate the adhesion and proliferation patterns of human cancer cells cultured on temperature-responsive culture dishes, the cancer cells were also seeded in35-mm diameter tissue-culture polystyrene (TCPS) culture dishes at the same density and were incubated in the same conditions. Time-course observations were conducted at 5 randomly selected locations on each dish and the adhesion and proliferation patterns of the cells were evaluated. The following equation was used to calculate the cell adhesion rate (%): (cells/cell seeding density(cm^2)/(cells/cm^2) ×100. We also tried to add layers of cancer cell sheets to create three-dimensional (3-D) cancer tissue *in vitro*. Two or four cancer cell sheets were layered *in vitro* and a pathological examination was performed. When the cancer cells reached confluence in the UpCell culture dishes, they were harvested as cell sheets by low-temperature treatment at 20˚C under 5% CO_2 for 20 min to remove them from the temperature-responsive culture dishes. Then, cancer cell sheets were harvested from the TCPS dishes.

Transplantation of cancer cell sheets into nude mice. Subcutaneous transplantation of HCT-116(-luc2) cancer cell sheets into nude mice was conducted using the following method. The mice were held-down for 5 min to make an incision in the dermis for transplantation of the flap, which was done using a cell shifter. After that, the incision was sutured with a nylon thread. Cell suspensions were seeded in 35-mm diameter temperature-responsive culture dishes and TCPS dishes at 1×10^4 cells/cm^2. Cells were cultured at 37˚C under 5% CO_2. After 4 days in culture, the cells reached confluence. The periphery of the cultured cells was then severed with a needle and low-temperature treatment was performed at 20˚C under 5% CO_2 for 1 h. Then, the cancer cell sheets were harvested using a cell shifter for support. Nude mice (7 weeks, female; CLEA Japan, Tokyo, Japan) were first anesthetized with 4% isoflurane (0.25 l/min), which was followed by 2% isoflurane for maintaining anesthesia. The dorsal skin was disinfected with 70% alcohol and the midline dorsal skin was incised and detached using straight surgical scissors. The transplant was placed under the skin so that it could adhere to the subcutaneous tissue and was left to rest for 3 min. Finally, the incision was sutured with 4-0 nylon thread. To compare the tumor growth of cancer cell sheets with the standard protocol, cancer cell suspensions were prepared using the method described above for injection. Cells were seeded in 35-mm diameter temperature-responsive culture dishes at 1×10^4 cells/cm^2 and were cultured for 3 days at 37˚C in 5% CO_2. The culture medium was removed using a Pasteur pipette and the cells were washed with 2 ml of PBS. After removing the PBS, cells were incubated for 5 min in a 1.5-ml solution of 0.25% trypsin and 2.65 mM Na4EDTA. After the cells had detached, 2 ml of Hanks' balanced salt solution was added and a cell suspension was prepared by pipetting. Next, 200 μl of the cell suspension (6×10^6 cells) was transplanted into the midline of the back of nude mice by subcutaneous injection. These animal experiments were carried out in accordance with ethical guidelines relevant for performing the experiment.

Haematoxylin and eosin (H&E) staining of in vivo-growing tumors. Cancer tissue, 4-μm thick, was cut on a microtome (HM335E, Microm GmbH, Germany). Slides were dried for 15 min at 42˚C on a slide warmer (Slide warmer SW85, Adamas Instrument B.V., Rhenen, Netherlands). In our department, H&E staining was performed according to the standard procedure using a Leica Multistainer (ST5020, Leica,Wetzlar, Germany). Slides were de-waxed and rehydrated with successive applications of xylene, 100% alcohol, 70% alcohol and tap water. Haematoxylin (Mayer's, Klinipath, Benelux) was applied for 4 min; this was followed by a 20-s differentiation in ammonia after which eosin (Eosin Y A+B, Klinipath, Benelux) was applied for 20 s.

Measurement of in vivo growing tumor size. *In vivo* tumor growth was measured based on tumor size. Tumor size was calculated as the product of the long axis, short axis and height. Tumor volume was calculated by considering tumors to be of elliptic cylinder or ellipsoid shape. The standard method is to assume an elliptical shape but, with cell sheet transplantation, the general shape for different tumors differs.

Evaluation of in vivo growing tumor volume measured by IVIS imaging sytem. The IVIS® imaging system (Perkin Elmer, Waltham, MA, USA) monitors gene expression and protein behavior *in vivo*, but it does not involve killing of the experimental animal (11). IVIS

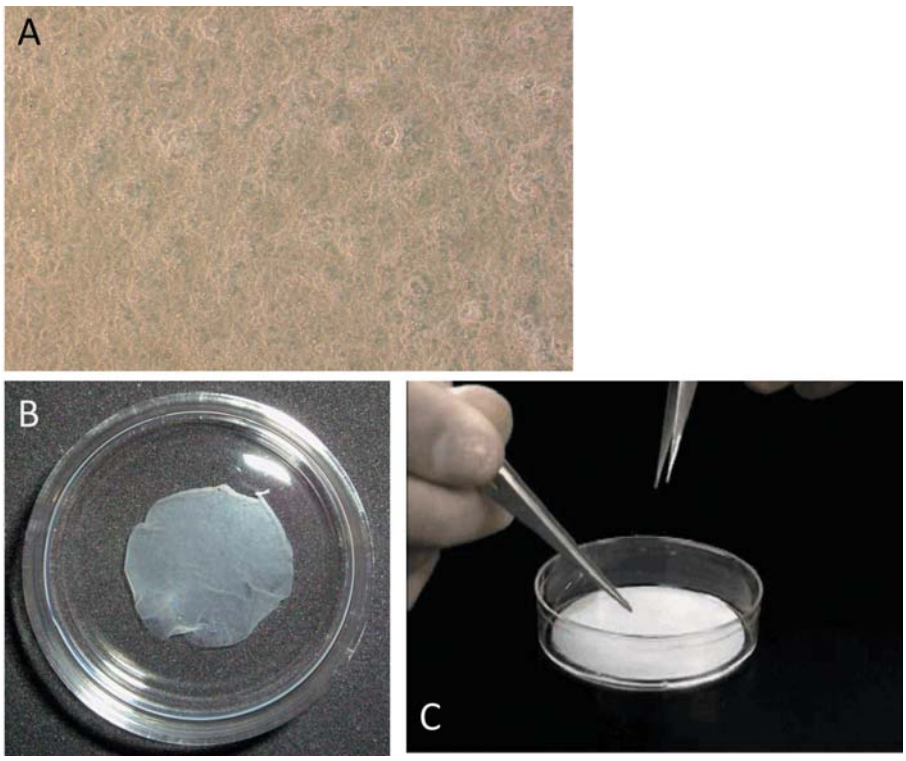

Figure 1. *Making HCT-116 cancer cell sheet with a temperature-responsive dish. A. The HCT-116 cancer cells grew in the same manner as when cultured on tissue-culture polystyrene (TCPS) culture dishes. B. When the dish was subjected to low-temperature (20˚C) treatment, the cancer cell sheet detached from the temperature-responsive culture dish as a sheet. C. The cancer cell sheet was picked up using a cell shifter.*

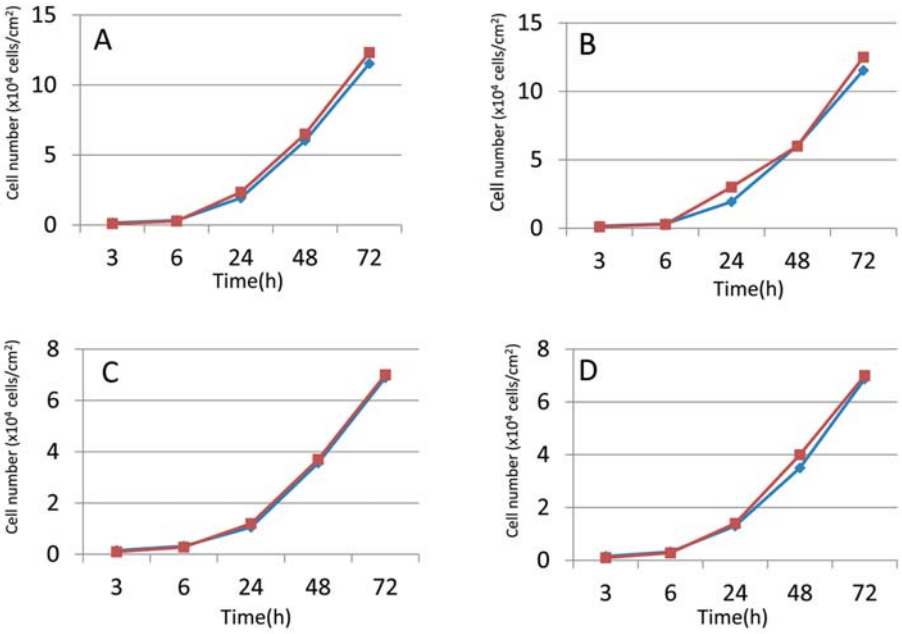

Figure 2. *Number of cancer cells cultured in vitro with UpCell or tissue-culture polystyrene (TCPS) culture dishes. The cancer cell numbers after in vitro culture were similar for the 4 cancer cell lines employed. A. HCT-116 (human colon cancer). B. Panc1 (human pancreas cancer). C. Li-7 (human liver cancer). D. MKN74 (human gastric cancer). Proliferation of the four cancer cell lines on PIPAAm-grafted (■) and control TCPS (◆) dishes. The four cancer cell lines were plated onto culture dishes at a density of 1×10^4 cells/cm^2. Cell numbers were counted by microscopic examination at 3, 6, 24, 48 and 72 h after seeding in the dish.*

has been used to assess biological responsiveness at the gene and protein levels. More recently, IVIS has been used to perform non-invasive *in vivo* imaging for observing the inside of an animal's body by tagging genes and proteins with light-emitting markers. This study used IVIS to observe tumor volumes over time by using the light-emission properties of a luminescent enzyme, luciferase. Increases and decreases in cancer cell numbers can be quantified on the basis of light intensity. It is also possible to quantify the expression of disease-related genes in animal models of disease. The intensity of a light source in an organism is calculated based on measurements made at the surface of the body using a charge-coupled device (CCD) camera. Physical quantities (photons/s) are used in the calculations. Measurements made with IVIS reflect light intensity, which is positively correlated with the number of cancer cells, considered to be sufficiently quantifiable. HCT-116 human colon cancer cells were labeled with luciferase (HCT-116-luc2). Time-course observations were conducted on days 0, 1, 3, 5, 7, 9, 12, 14, 19, 28 and 35 after transplantation using the IVIS system. The luminous flux from HCT116-luc2 was used for the evaluation.

Results

Successful construction of the cancer cell sheet. We attempted to create cancer cell sheets with 4 cancer cell lines and succeeded *in vitro* culture with these cancer cell lines (HCT-116, Panc-1, Li-7 and MKN74) in this study (Figure 1). These cancer cell sheets showed similar cell proliferation patterns on temperature-responsive culture dishes with similar behavior on TCPS dishes (Figure 2). There were no differences in the rate of cell adhesion to the surface of either type of dish.

Pathological findings of the multi-layer cancer cell sheets. The cancer cell sheets were collected and stained by H&E. A single cancer cell sheet did not consist of a single layer of cells (Figure 3-A). It was easy to layer 2 or 4 cancer cell sheets over each other to create multi-layer cancer tissues *in vitro* (Figure 3B and C). Thus, *in vitro* 3-D culture of cancer cells seemed to be possible to be made easily by using cancer cell sheets.

Macroscopic and pathological findings of in vivo growing tumors. Tumor tissues were extracted from the mice in the fourth week and histochemical analyses were performed (Figure 4). We performed H&E staining to compare the tissue morphology of tumors formed from the transplantation of cell sheets and those formed from the transplantation of cell suspensions. The directions of tumor growth were founded to differ between the two groups. The tumor made by *s.c.* injection existed in the subcutaneous areas. In contrast, the tumor made by cancer cell sheet permeated through the basement membrane.

In vivo tumor growth of HCT-116 cells by measurement of the size. The tumor size transplanted into nude mice by the cell suspension or cancer cell sheet were measured with vernier calipers on every few days. *In vivo* tumor growth did not exhibit significant differences in both groups (Figure 5). However, there were gross differences because the cancer cell sheets invaded into the mouse body. It seemed to be difficult to measure the protuberance of the transplanted cancer tissue from the surface of the mice. Therefore, the real volume of the tumor should be determined by measuring the total flux of the IVIS imaging system.

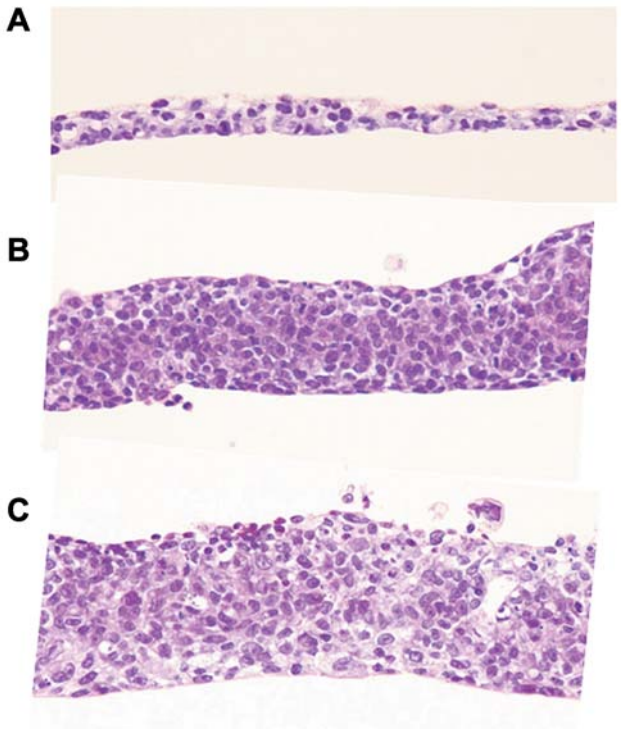

Figure 3. *Pathological findings of the multi-layer cancer cell sheets. HCT-116 cancer cells were stained with hematoxylin–eosin (H&E) (×100). A. Single cancer cell sheet. B. Two-layer cancer cell sheets. C. Four-layer cancer cell sheets.*

In vivo tumor growth by measurement with the IVIS imaging system. The IVIS imaging system was used to measure tumor growth *in vivo* with HCT-116-luc2 (Figure 6). There were no significant differences in tumor growth between the two groups measured by average radiance (data not shown). In contrast, there were significant differences in tumor growth measured by the total luminous flux (Figure 7). Thus, the IVIS imaging system was a useful tool to measure *in vivo* tumor volume made by transplantation of cancer cell sheets.

Discussion

Investigations on anticancer agents are extremely time- and cost-intensive. In general, they can take over 10 years and cost 500 million dollars from the time of completion of

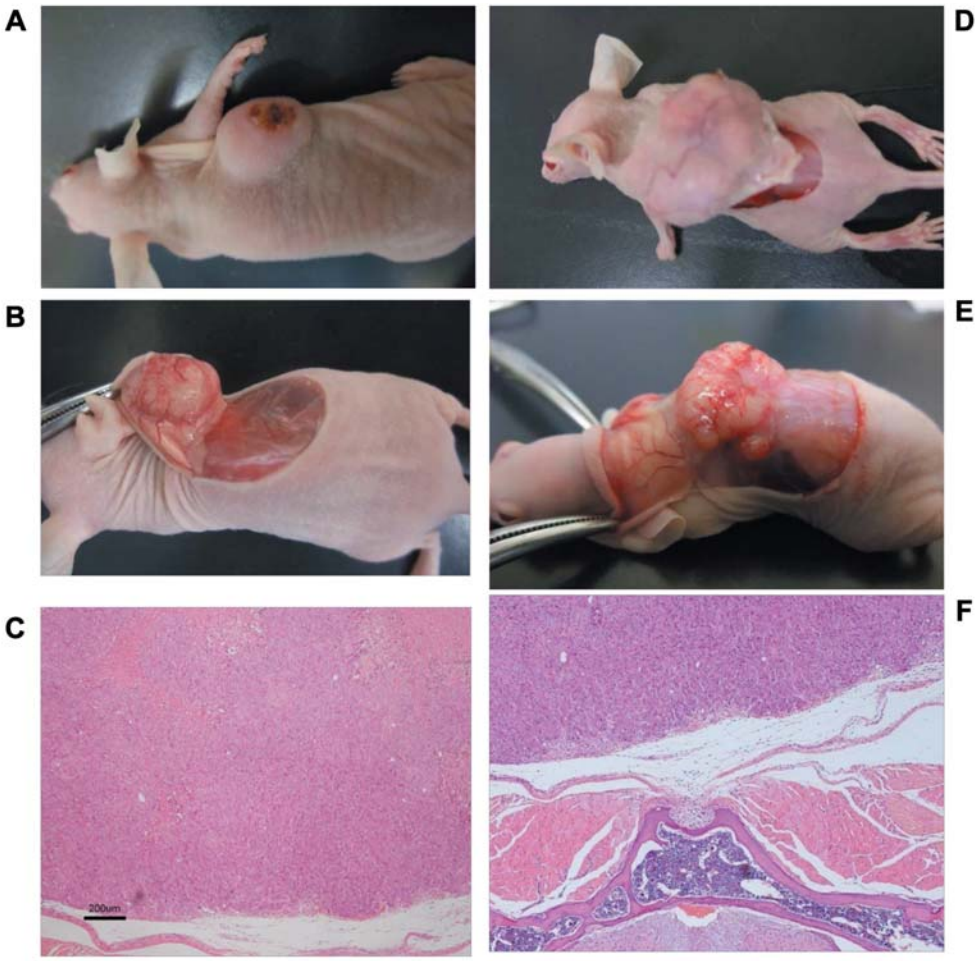

Figure 4. *Macroscopic and pathological findings of in vivo growing HCT-116 cells transplanted by cancer cell sheet or s.c. injection. A. Gross appearance of a mouse s.c. injected with cancer cells. B. Gross appearance of the tumor after skin incision. C. Pathological findings of the tumor. D. Gross appearance of the mouse transplanted with the cancer cell sheet. E. Gross appearance of the tumor after skin incision. F. Pathological findings of the tumor.*

phase III clinical trials until approval. From 1945 to 1969 anticancer drugs were screened in mice transplanted with cells from the fast-growing murine leukemia cell lines P388 and L1210. However, with murine leukemia models, the correlation between anticancer effects and therapeutic efficacy is limited to leukemia, lymphomas and some highly-proliferative solid cancers. In 1969, a study used athymic nude mice to investigate the growth of human cancers. Since then, nude mice or severe-combined immunodeficiency (SCID) mice deficient in functional T or B cells that have been transplanted with human cancers have been used for the pre-clinical testing of anticancer drugs *in vivo*. In general, grafts of several square millimeters in size, cultured cells or ascitic fluids containing cancer cells are transplanted into nude or SCID mice. These are generally transplanted under the skin, which makes it possible to evaluate the therapeutic efficacy of anticancer drugs by using slide calipers to measure the tumor diameter from outside the skin. This method allows the transplantation of various types of human cancers. However, such an approach has certain limitations. For instance, the central portion of the tissue graft may become necrotic if the graft is too large. Conversely, the transplantation may fail if the graft is too small. In addition, to control for the subsequent tumor growth, the size of the transplanted tissue must be uniform across experiments. When transplanting cell suspensions, such as ascitic fluids, all the cells may not grow in the target location and cancerous tissue may not always form, even if uniform amounts of cells are transplanted.

Okano *et al*. (12) established a method for harvesting cultured cells in a non-invasive manner using low-temperature treatment without the help of proteases such as trypsin. This method employs electron-beam irradiation to covalently immobilize PIPAAm to commercial polystyrene

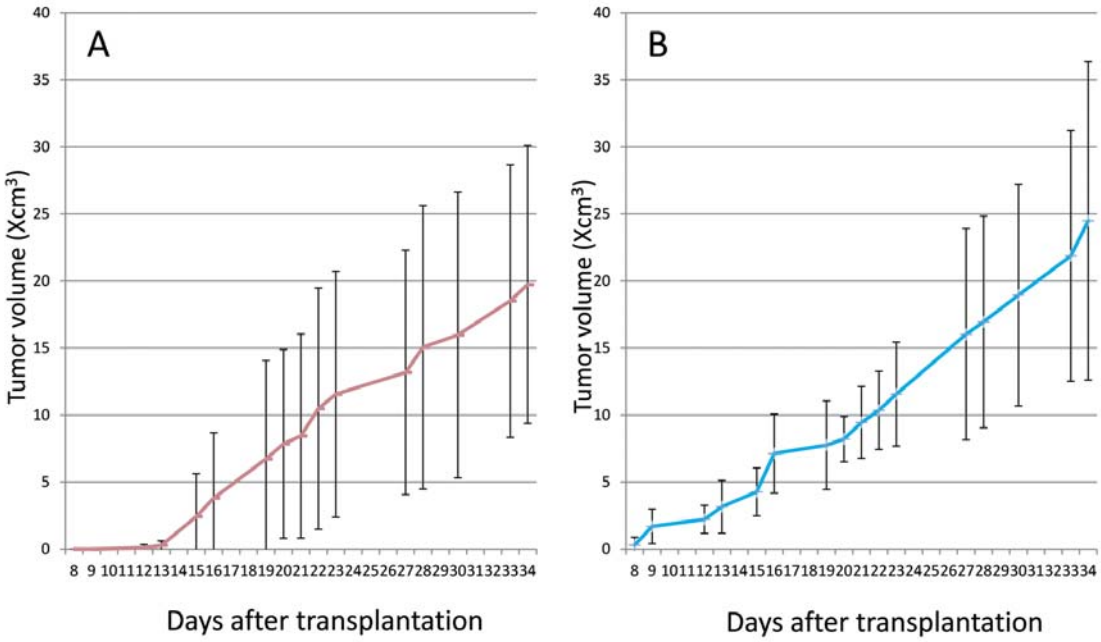

Figure 5. *In vivo tumor growth of HCT-116 measured by size. A. A HCT-116 cell sheet was transplanted into the dorsal subcutaneous tissues of nude mice (n=6). B. HCT-116 cell suspensions (6×10^6 cells) were transplanted into the midline of the back of nude mice by s.c. injection (n=6). Tumor sizes were assessed periodically for 5 weeks with vernier calipers. Tumor volume was calculated by considering tumors to be of elliptic cylinder or ellipsoid shape. Elliptic cylinder: $V=\pi/4\times abc$; Ellipsoid: $V=\pi/6xabc$ (a: diameter, b: a short through, c: thickness).*

culture dishes (temperature-responsive culture dishes). Because the method is non-invasive and does not degrade any molecules on the cell membrane or the extracellular matrix (ECM), including proteins attached to the cell, there is little damage to the cells and cellular function can be preserved. In addition, low-temperature treatment alone allows layers of confluent cells to be harvested as single-layer sheets while maintaining intracellular adhesion. The ECM, which maintains intracellular adhesion, can be preserved at the bottom surface of the sheets when the cells are harvested, thus allowing for the construction of multilayer sheets. Oral epithelial cell sheets, corneal epithelial cell sheets, and myocardial cell sheets are already being applied clinically. Herein, we demonstrated that culturing cancer cells on temperature-responsive culture dishes, until they reach confluence and detaching sheets of cells using low-temperature treatment, is an effective method for harvesting and transplanting cancer tissue. Sheets of HCT-116 cells cultured on temperature-responsive culture dishes were *s.c.* transplanted in nude mice and their ability to form cancerous tissue *in vivo* was tested. As a control, cancer cell suspensions harvested by trypsinization were transplanted using a conventional *s.c.* injection method. The formation and proliferation of cancerous tissue were compared between the methods. H&E staining confirmed the characteristics of the tumors in mice transplanted with cell suspensions and in those transplanted with cancer cell sheets. Notably, the mouse models differed with regard to the site of tumor extension. While the tumors grew subcutaneously and were surrounded by a thin fibrous capsule in mice transplanted with cell suspensions, there was partial invasion of the bones in mice transplanted with cancer cell sheets. This was clearly visible macroscopically. In mice transplanted with cell suspensions, the tumors could be removed as well-circumscribed masses. In contrast, in mice transplanted with cancer cell sheets, the tumors did not extend outwards from the transplant site; instead, they infiltrated deeply. In all cases, tumor tissues could only be extracted by cutting the spine.

The results of the present study strongly suggest that tumor-bearing animals, that are more stable and larger, can be obtained with cancer cell sheet transplantation than with conventional methods. The cancer cell sheets could be transplanted on the target organs such as liver. Therefore, a cancer cell sheet transplantation method would be expected to provide an efficient model for screening anticancer agents in future. We are now trying to transplant cancer cell sheets on the liver to create a novel liver cancer model. In addition, it could be easy to layer the cancer cell sheets for making the tumor tissues *in vitro*. The 3-D structure and extracellurar

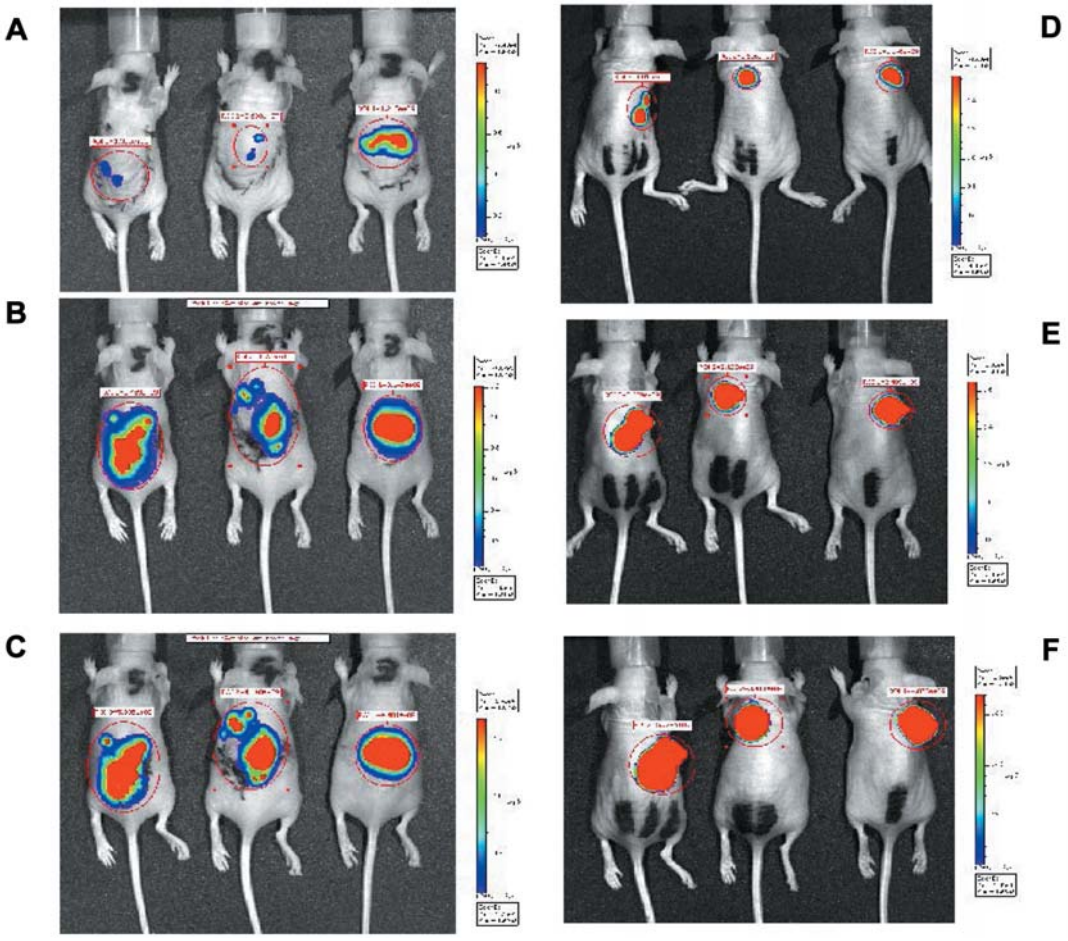

Figure 6. *IVIS imaging system used to assess tumor growth in vivo. Panels A, B and C present mice transplanted with a HCT-116-luc2 cancer cell sheet over a period of time. D, E and F present mice injected with HCT-116-luc2 cancer cells. Images for the 3rd, 12th and 28th day are shown. The intensity of the light source is indicated. The number of cancer cells labeled with luciferase is correlated to the intensity of the light.*

matrix of cancer cultures are useful in anticancer drug studies (13, 14). In order to make more layers, it would suffice to add any vascular formation (15). Hence, we are working on creating 3-D cancer tissue *in vitro* for further use with our novel technology (16).

Conclusion

We present a novel method for creating *in vivo* tumor models in mice by using cell sheet engineering. We succeeded to create cancer cell sheets and transplant them into nude mice to create tumor tissues *in vivo*. Our method is convenient and yields better results than simple transplantation of cell suspensions: the tumor volume was bigger and the cancer cells showed stable engraftment *in vivo*. Cell sheet engineering would be a useful tool to make a novel cancer model for the assessment of anticancer therapeutics in the future.

Conflicts of Interest

This work has been supported by the Global-COE Project, Japan. Yamato M is a scientific consultant for CellSeed Inc., the stakeholder of the company and inventor of cell-sheet-related patents. The other authors have no competing interests.

Ethical Conduct of Research

The authors state that they have obtained appropriate institutional review board approval for all animal experimental investigations.

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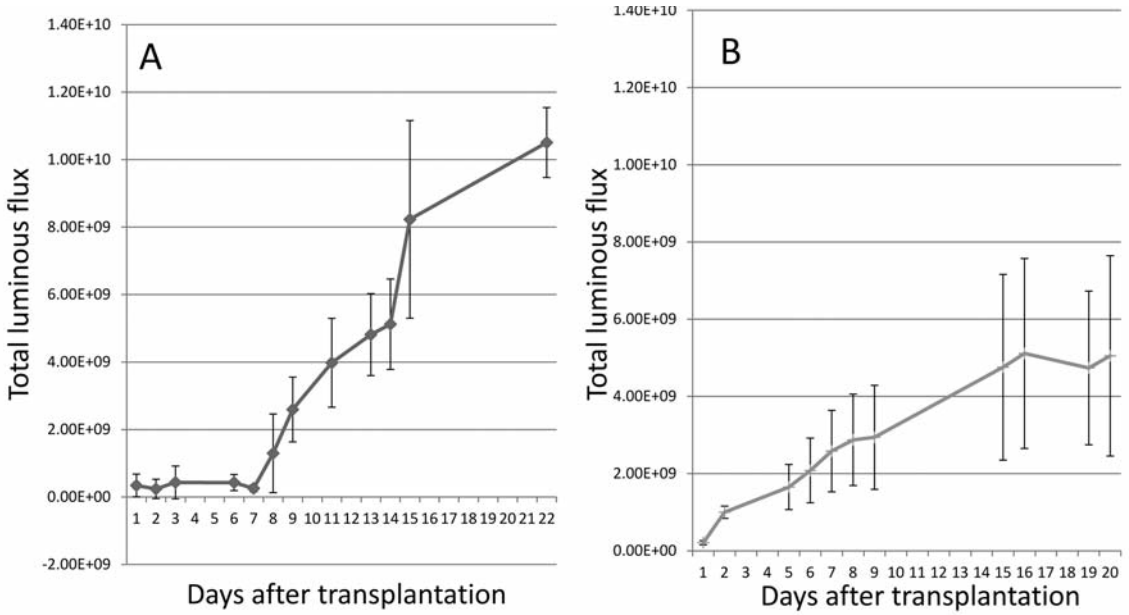

Figure 7. *In vivo tumor growth of HCT-116-luc2 measured by total luminous flux with the IVIS imaging system. A. Total luminous flux of the mice transplanted with HCT-116-luc2 cancer cell sheet (n=15). B. Total luminous flux of the mice transplanted with HCT-116-luc2 cell injection (n=15). The intensity of the light indicates the percentage of emitted light (cells). By quantifying the light flux, we are able to quantify all of HCT-116-luc2 cells.*

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