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REGULAR ARTICLE

Cell biological profiling of reprogrammed cancer stem cell-like colon cancer cells maintained in culture

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Abstract

Cancer stem cells (CSCs) are specific targets for therapeutic applications, but the rarity of CSCs within tumors makes the isolation of CSCs difficult. To overcome these problems, we generated CSCs in vitro using established reprogramming techniques. We transduced four previously established reprogramming factors, Oct3/4, Sox2, Klf4, and L-myc, into the colon cancer cell lines LoVo and OUMS-23, and investigated the biological characteristics of these lines. Tra-1-60+ cells were obtained from reprogrammed induced pluripotent stem (iPS) cell-like colonies and showed CSC properties, including colony formation, maintenance of colonies by repeated passages, and feeder cell dependency, as well as increased expressions of CSC markers such as CD133 and ALDH1. The CSC-like cells showed increased chemoresistance to 5-fluorouracil and elevated tumorigenicity upon transplantation into kidneys of immune-deficient mice. These tumors shifted to a poorly differentiated stage with many atypical cells, cytoplasmic mucin, and focal papillary components, with demonstrated dedifferentiation. The principal component analysis from DNA microarrays showed that though both cell lines moved to iPS cells after reprogramming, they were not completely identical to iPS cells. Significantly elevated gene expression of Decorin and CD90 was observed in CSC-like cells. Together, these results show that reprogramming of cancer cells produced not pluripotent stem cells but CSC-like cells, and these findings will provide biological information about genuine CSCs and help establish new CSC-targeted therapies.

Keywords Cancer stem cells (CSCs) · Reprogramming · Colorectal cancer · Induced pluripotent stem (iPS) cells

Introduction

Cancer stem cells (CSCs) retain a stem cell-like phenotype, e.g., their quiescence, multipotency, and self-renewal capacity, and exhibit resistance against chemotherapeutic agents or radiation therapy. Since they play crucial roles in metastasis and tumor relapse, CSCs are a promising therapeutic target for curative anti-cancer remedies. However, their properties and molecular mechanisms are still unclear because of their low abundance in tumor tissues. To eliminate the need to isolate CSCs from cancer cells or tissues, we aimed to design a method for generating CSC-like cells from original cancer cells.

Although there have been many reports on CSCs, most studies have concentrated on CSCs isolated from cancerous tissue and focused on CSC markers. CD133 and CD24 are putative CSC markers and are associated with aggressive colon tumors that have poor prognoses (Chu et al. 2009). The precise function of CD133 is unclear, but CD133 is believed to play roles in the tumorigenicity and progression of colon

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cancer, and its upregulation is associated with poor prognoses in liver cancer and liver metastases (Horst et al. 2009). CD24 is anchored to the cell surface, and in colon cancer, CD24-positive subpopulations show CSC-like properties (Ke et al. 2012). CD24 may play roles in differentiation, but its distribution in colon cancer is debatable. High expression or high activity of the acetaldehyde dehydrogenase (ALDH) family, especially ALDH1A or ALDH3A1, has also been used as a CSC marker in various cancer types (Chen et al. 2015; Huang et al. 2009; Pan et al. 2017; Tanei et al. 2009). ALDHs protect cells from oxidative stress, which can contribute to chemoresistance in various cancer types including colon cancer (Liu et al. 2013; Moreb et al. 2006; Raha et al. 2014; Xu et al. 2015; Yan et al. 2014).

Takahashi and Yamanaka discovered that differentiated adult human fibroblast cells could be dedifferentiated or induced into undifferentiated stem-like cells by transducing four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc (Takahashi et al. 2007). This reprogramming technology brought about a distinct paradigm shift in regenerative medicine. In cancer research, Carette et al. first succeeded in establishing CSC-like cells by transducing the four transcription factors into chronic myelogenous leukemia cells (Carette et al. 2010). Other studies have also demonstrated that CSC-like cells can be established from cancer cell lines by the transduction of these four transcription factors (Kim et al. 2013; Chen et al. 2012; Miyoshi et al. 2010; Oshima et al. 2014).

Despite the importance of understanding CSC biology and the potential clinical applications, the rarity of CSCs within tumors makes the isolation of CSCs difficult. To address this obstacle, previous studies have successfully demonstrated the use of reprogramming factors to induce CSC-like cells. However, these studies used retroviruses or lentiviruses for expression of these factors, and thus these strategies run the risk of genomic insertion of the reprogramming factors into the host genome by the viruses. To overcome these problems, we generated CSCs in vitro using a Sendai virus vector kit and the established reprogramming techniques. The Sendai viral vector system avoids the risk of undesirable genomic insertions into the host genome since Sendai virus is an RNA virus. Our method enabled transduced cells to maintain their CSC-like properties in vitro with repeated subculture (passage numbers > 10), which allowed for biological profiling of the CSCs.

Materials and methods

Cell lines

The colon cancer cell lines LoVo (IFO50067; adenocarcinoma, carcinoembryonic antigen producing) and OUMS-23 (JCRB1022; metastatic colon cancer cells from pericardial fluid) were supplied from the JCRB cell bank (National Institute of Biomedical Innovation, Health and Nutrition, Osaka, Japan). Both lines were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Wako) in a 37 °C humidified incubator with 5% CO_2.

Reprogramming

A total of 2×10^5 colorectal cancer cells were treated with Sendai virus vectors (CytoTune-iPS 2.0L; Medical & Biological Laboratories, Aichi, Japan) containing the transcription factors Oct3/4, Sox2, Klf4, and L-myc. The cells were trypsinized and suspended in reprogramming virus cocktails with fibroblast medium (DMEM high glucose medium with 10% FBS, 1% penicillin-streptomycin). According to a recommended protocol for floating cells, the cells were then centrifuged at 500×*g* for 90 min at room temperature to improve the transduction efficiency with mechanical stress. After 24 h of transduction, the medium was replaced with fresh fibroblast medium. This study followed the Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms and was reviewed and approved by the Gene Recombination Experiment Safety Committee, Shinshu University (permit number 12-044).

Culture of reprogrammed cells

At an early stage (days 1–6), reprogrammed cells were harvested in fibroblast medium as control cells. After confirming the formation of iPS cell-like colonies (day > 30), a Life Technologies Tra-1-60 Alexa Fluor™ 488 Conjugate Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for live cell imaging to pick Tra-1-60-positive colonies. Tra-1-60-positive colonies were mechanically selected using pipette tips. Tra-1-60-positive colonies were cultured in embryonic stem (ES) medium, which consisted of 80% DMEM/F12 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 20% Gibco KnockOut™ Serum Replacement (Thermo Fisher Scientific), 100 μM non-essential amino acids (Wako), 2 mM L-glutamine (Wako), 100 μM 2-mercaptorthanol (Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin-streptomycin, and 4 ng/ml basic fibroblast growth factor (Wako) on a feeder cell layer of mouse embryonic fibroblasts (MEFs; Oriental Yeast Co, Tokyo, Japan) inactivated with mitomycin C (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan).

Magnetically activated cell sorting of Tra-1-60-positive cells

To isolate Tra-1-60-positive CSC-like cells, the EasySep™ selection kit (Stem Cell Technologies, Vancouver, CA) was used according to the manufacturer's instructions. Briefly, cells were detached with 0.1% trypsin on passage 3 and labeled with anti-human Tra-1-60 antibodies. Labeled cells were separated by magnetic beads and the EasySep magnet system.

Quantitative real-time PCR

Total RNA was extracted from cancer cells using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. First-strand complementary DNAs were synthesized from RNAs using the PrimeScript™ RT-PCR (reverse transcription polymerase chain reaction) Kit (Takara Bio, Otsu, Japan). Quantitative real-time PCR (qPCR) analysis was performed as described previously using a Thermal Cycler Dice Real-Time PCR System and SYBR Premix Ex Taq™ II (Takara Bio). The qPCR primers used for detecting CSC markers and undifferentiated markers are shown in Table 1. Each PCR cycle consisted of a denaturation step for 1 min at 94 °C, an annealing step for 1 min at 60 °C, and an extension step for 2 min at 72 °C.

Alkaline phosphatase staining

At day 7, reprogrammed cells were cultured in Matrigel-coated 6-well plates. After 96 h of incubation at 37 °C with 5% CO_2, the cells were stained with an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions.

Immunofluorescence staining

Original cells were plated on gelatin-coated coverslips; reprogrammed cells were seeded on MEF-coated coverslips. Cells were fixed with 4% paraformaldehyde, permeabilized and incubated overnight at 4 °C with antibodies against CD133 (orb99113; rabbit IgG, Biorbyt, Cambridge, UK), Tra-1-60 (mouse mAb Alexa Fluor 488 Conjugate Kit, Life Technologies, Thermo Fisher Scientific), Nanog (hNanog2, rabbit IgG, eBioscience, Thermo Fisher Scientific), and Oct3/4 (SC-5279; mouse mAb, Santa Cruz Biotechnology, Dallas, TX, USA). Cells were then labeled with secondary antibodies (goat anti-mouse/rabbit/rat Alexa Fluor™ 488/568, Thermo Fisher Scientific). Nuclei staining was performed using DAPI (4′,6-diamino-2-phenylindole). Cells without primary antibody incubation were used as negative controls. Fluorescence microscopy (BZ-X700; Keyence, Osaka, Japan) with digital photographic capability was used to visualize cells at several magnifications.

DNA microarray and data analysis

To evaluate stem cell-like phenotypes in reprogrammed cells, we compared the gene expression profiles of original and reprogrammed colon cancer cells with 253G1 standard induced iPS cells. Total RNAs were amplified and labeled with Cy3 using an Agilent Low Input Quick Amp Labeling kit, Agilent Human 8 × 60 K Ver.3 (ID 72363). Microarrays were scanned using an Agilent DNA microarray scanner (DNA Chip Research Inc., Tokyo, Japan). Intensity values of each scanned profile were quantified using Agilent profiling extraction software, version 10.7.3.1. Data were normalized using Agilent GeneSpring GX version 3.1.1 (per chip: normalization to 75th percentile shift; per gene: normalization to median of all samples).

MTT assays

Original and reprogrammed cells (5×10^3 cells/well) were exposed to 1 or 50 μg/ml 5-fluorouracil (5-FU) (Kyowa Hakko Kirin Co., Ltd.) in 96-well plates. After 48-h incubation, the MTT assay was performed using a cell growth assay kit (MSD Millipore, Billerica, MA, USA) and the absorbance at 570 nm was measured using a microtiter plate reader.

Tumorigenicity assay and histochemistry

Original and reprogrammed cells in D-PBS (−) were injected into the fibrous capsule of both kidneys ($1.6–2.5 \times 10^3$ cells/kidney) of 8-week-old immune-deficient female mice (SCID/beige, Charles River Laboratories International Inc.,

Table 1 Real-time PCR primer sequences

	Forward	Reverse
CD133	5′-TGG GGC TGC TGT TTA TTA TTC T-3′	5′-TGC CAC AAA ACC ATA GAA GAT G-3′
CD24	5′-TGC TCC TAC CCA CGC AGA TT-3′	5′-GCT CAA CCC AGA GTT GGA A-3′
Nanog	5′-GGT GGT AGG AGG AGT AAA GG-3′	5′-AGC TAC AAA CAG GTG AAG AC-3′
Oct3/4	5′-TCT ATT TGG GGT ATT CAG C-3′	5′-ATT GTT GTC AGC TTC CTC AC-3′
ALDH1	5′-TCC TGG TTA TGG GCC TAC AG-3′	5′-CTG GCC CTG GTA GAA TA-3′
β-actin	5′-TGG CAC CCA GCA CAA TGA A-3′	5′-CTA AGT CAT AGT CCG CCT AGA AGC A-3′

Yokohama, Japan) for the low-dose transplantation model (LoVo_i, two mice; LoVo_ORG, two mice; OUMS-23_i, four mice; OUMS-23_ORG, two mice). In another experiment, a high dose of the original cells (2.5×10^4 cells/kidney) was transplanted in another cohort of mice (LoVo_ORG, two mice; OUMS-23_ORG, two mice). All experiments were reviewed and approved by the Animal Ethics and Research Committee, Shinshu University (permit number 260037) and conducted in accordance with their guidelines. The mice were bred and observed for palpable tumor formation up to 10 weeks after cell transplantation to assess tumor formation, and mice were then euthanized. After tumors were collected, they were photographed, cut into small fragments with a scalpel, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 5 days at 4 °C. Samples were stained using HE (hematoxylin and eosin), PAS (periodic acid-Schiff), and AB (Alcian Blue) stains.

Statistical analysis

All in vitro experiments were performed in triplicate unless specified. The significance of differences among mean values was evaluated by Student's *t* test using GraphPad Prism7 (GraphPad Software). $P < 0.01$ was considered statistically significant.

Results and discussion

Results

Induction of colonies similar to iPS cells after reprogramming

The schematic for the generation of CSCs in vitro is shown in Fig. 1(a). We transduced the four reprogramming transcription factors into colon cancer cell lines using CytoTune Sendai virus vectors at a multiplicity of infection of 3 on day 0. The transgenes were detected by RT-PCR after day 7 (first passage, iP1), but gradually became undetectable due to evanescence of the vectors (Fig. 1(d, d′)). Colonies similar to induced iPS cells appeared 2 weeks after transduction; both reprogrammed colon cancer cell lines formed iPS cell-like colonies (Fig. 1(b″″, b″″′, c″″, c″″′)). According to our reprograming protocol, the colonies were then cultured on MEFs. Isolated cells showed strong alkaline phosphatase (ALP) activity on passage 3 and had a larger fraction of ALP-positive undifferentiated cells compared with the original cancer cell lines (Fig. 1(e, e′, f, f′)). Far more ALP-positive undifferentiated cells were observed among the reprogrammed cells. The changes in c-myc expression during the reprogramming procedure (Fig. 1(d, d′)) had no impact on the induction of cells.

Isolating CSC-like cells among the reprogrammed cells using the MACS method

Colonies similar to iPS cells were labeled by FITC-conjugated anti-Tra-1-60 IgG and selected under a live imaging microscope (Fig. 1(h, h′)). To isolate Tra-1-60-positive cells among the reprogrammed cells in the colonies, we used the EasySep magnetically activated cell sorting (MACS) method to purify Tra-1-60-positive cells. The purified cells formed colonies similar to iPS cells, but the proliferation rates were remarkably lower than those of standard ES/iPS cells (data not shown). The Tra-1-60-positive colonies could be maintained at least 10 weeks under ES/iPS culture conditions. These results suggested that the morphology and proliferation potential of reprogrammed Tra-1-60-positive colon cancer cells were drastically changed compared with the original cells. When the number of proliferating cells increased, it was necessary to re-pick iPS-like colonies and re-sort via MACS to concentrate the Tra-1-60-positive cells and maintain high-quality iPS-like colonies.

Estimation of CSC properties

The expression of cell surface markers was evaluated by qPCR to estimate the stemness of the reprogrammed cells (Fig. 2). Although previous studies have established that various CSCs express stemness genes such as Nanog, Oct3/4, and Sox2 (Li et al. 2011; Lonardo et al. 2011), we also observed upregulated CD133 in OUMS-23 cells at an early stage (Fig. 2(b)), in addition to Nanog (Fig. 2(b″″)) and Oct3/4 (Fig. 2(b″″′)). CD133 and ALDH1 expressions were somewhat high in untreated LoVo cells (Fig. 2(a, a‴)), while reprogramming induced CD24 (Fig. 2(a″)), Nanog (Fig. 2(a″″)), and Oct3/4 (Fig. 2(a″″′)) downregulation. The variability of CD24 expression did not appear in either cell line before or after reprogramming (Fig. 2(a″, b″)). After several rounds of passage, CSC markers were downregulated.

Next, we evaluated the levels of CD133, Nanog, Tra-1-60, and Oct3/4 protein expression by immunofluorescent staining (Fig. 3). CSC marker protein expression was upregulated immediately after reprogramming. We did not detect an upregulation of CSC marker and undifferentiated marker expression between original and reprogrammed LoVo cells in later passages (passage 17). However, CD133 expression was significantly upregulated in reprogrammed OUMS-23 cells compared with original cells.

DNA microarray and data analysis

To analyze gene expression alterations before and after reprogramming, a DNA microarray analysis was performed with 253G1 iPS cells serving as the control. The heat map showed that the gene expression profiles of the colon CSC-

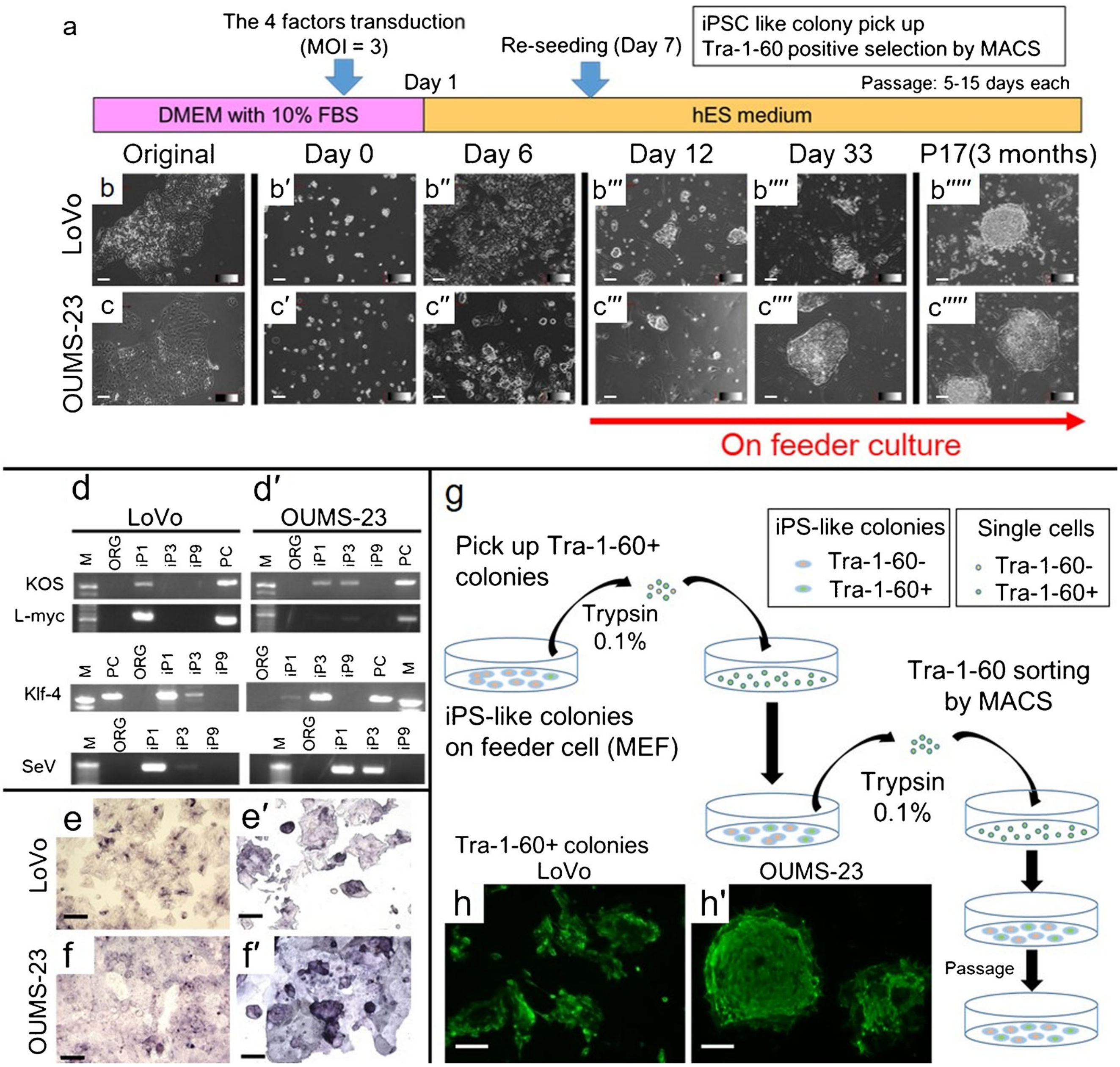


Fig. 1 Induction of CSC-like colonies from colon cancer cells after reprogramming. Schematic representation of the reprogramming protocol (a) and microscopy images of LoVo (b, original cells; b′, day 0; b″, day 6; b‴, day 12; b⁗, day 33, b′′′′′, passage 17) and OUMS-23 (c, original cells; c′, day 0; c″, day 6; c‴, day 12; c⁗, day 33; c′′′′′, passage 17) colon cancer cells at various stages. Original cell lines are b (LoVo) and c (OUMS-23). Scale bars = 100 μm. RT-PCR analysis of the four transgenes used for reprogramming in colon cancer cells at various stages of the reprogramming protocol (d, LoVo; d′, OUMS-23). M, marker; ORG, original cells; iP1, reprogrammed cells on passage 1 (day 7); iP3, reprogrammed cells on passage 3 (day > 20); iP9, reprogrammed cells on passage 9 (day > 40); PC, positive control (reprogrammed retinoblastoma cells on day 1). Alkaline phosphatase staining. Cells were stained with an alkaline phosphatase kit before reprogramming (e, LoVo_ORG; f, OUMS-23_ORG) and at 11 days after transduction (e′, LoVo_i; f′, OUMS-23_i). Scale bars = 100 μm. Schematic showing identification of CSC/multipotent stem cells after reprogramming using MACS (g). Tra-1-60-positive colonies were selected from the reprogrammed induced pluripotent iPS cells in the colonies (left), cultured (middle), and then purified using MACS (right) as described in the "Materials and methods" section. Representative images of Tra-1-60-positive LoVo_i (h) and OUMS-23_i (h′) colonies established by MACS

like cells were similar to the original cancer cells (Fig. 4(a)). Notably, the gene expression profiles of the colon CSC-like cells were different from 253G1 iPS cells. These findings suggested that the colon cancer cells were transformed to CSC-like cells with some characteristics of the original cells.

Principal component analysis (PCA) of the total microarray data showed that the three-dimensional positions of the colon cancer cells closely approached 253G1 iPS cells upon reprogramming (Fig. 4(b)). This revealed that epigenetic changes occurred in the reprogrammed colon cancer cells, and these cells are similar to iPS cells in their gene expression profiles. Interestingly, CD90 and Decorin were uniquely upregulated in reprogrammed colon cancer cells compared with original cells, with statistical significance (Fig. 4(c)). PCA also showed different lengths in the data from the original cancer cells compared with the reprogrammed cells in both cell lines; the plot length from OUMS-23 cells was longer than that of LoVo cells, indicating that OUMS-23 cells were more sensitive to transduction than LoVo cells.

Sensitivity to 5-FU

We next examined chemoresistance of the reprogrammed cells to 5-FU by measuring cell viability after 48-h treatment with 1 or 50 μg/ml 5-FU (Fig. 4). The survival rate of the reprogrammed LoVo cells (85.3%) was nearly two times as high as that of the original cell line (45.7%) in response to 50 μg/ml 5-FU (Fig. 4(d); $P < 0.01$). In OUMS-23 cells, the viability of 50 μg/ml 5-FU-treated reprogrammed OUMS-23 cells (101.5%) was significantly higher than that of original cells (90.0%) (Fig. 4(d′); $P < 0.01$). These results suggested that reprogrammed LoVo and OUMS-23 cells acquired 5-FU chemoresistance.

Fig. 3 Immunofluorescence staining of pluripotent stem cell markers and CSC markers in reprogrammed and control cells. Original cells were plated on gelatin-coated coverslips; reprogrammed cells were seeded on MEF-coated coverslips. (a) LoVo_ORG, original LoVo cells, passage number = 32; LoVo_i, reprogrammed cells picked from iPS-like colonies, passage number = 25 after reprogramming. (b) OUMS-23_ORG, original OUMS-23 cells, passage number = unknown (U) + 17; OUMS-23_i, reprogrammed OUMS-23 cells picked from iPS-like colonies, passage number = 15 after reprogramming. Scale bars = 100 μm ▶

Tumor formation in immune-deficient mice

To evaluate the tumorigenicity of the reprogrammed cells, we transplanted original or reprogrammed cancer cells into the sub-renal capsule of immune-deficient mice. Both the original colon cancer cell lines were transplanted at 1.6–2.5 × 10^3 cells/kidney but did not result in tumor formation (no tumors/four kidneys for both cell lines). In contrast, tumorigenesis was observed in immune-deficient mice after transplanting reprogrammed cancer cells (LoVo_i, two tumors/two kidneys; OUMS-23_i, five tumors/seven kidneys). We performed transplantations with higher concentrations of the original cancer cell lines (2.5 × 10^4 cells/kidney) and the tumorigenicity acquired under this high-dose condition produced 1/15–1/10 times more cells.

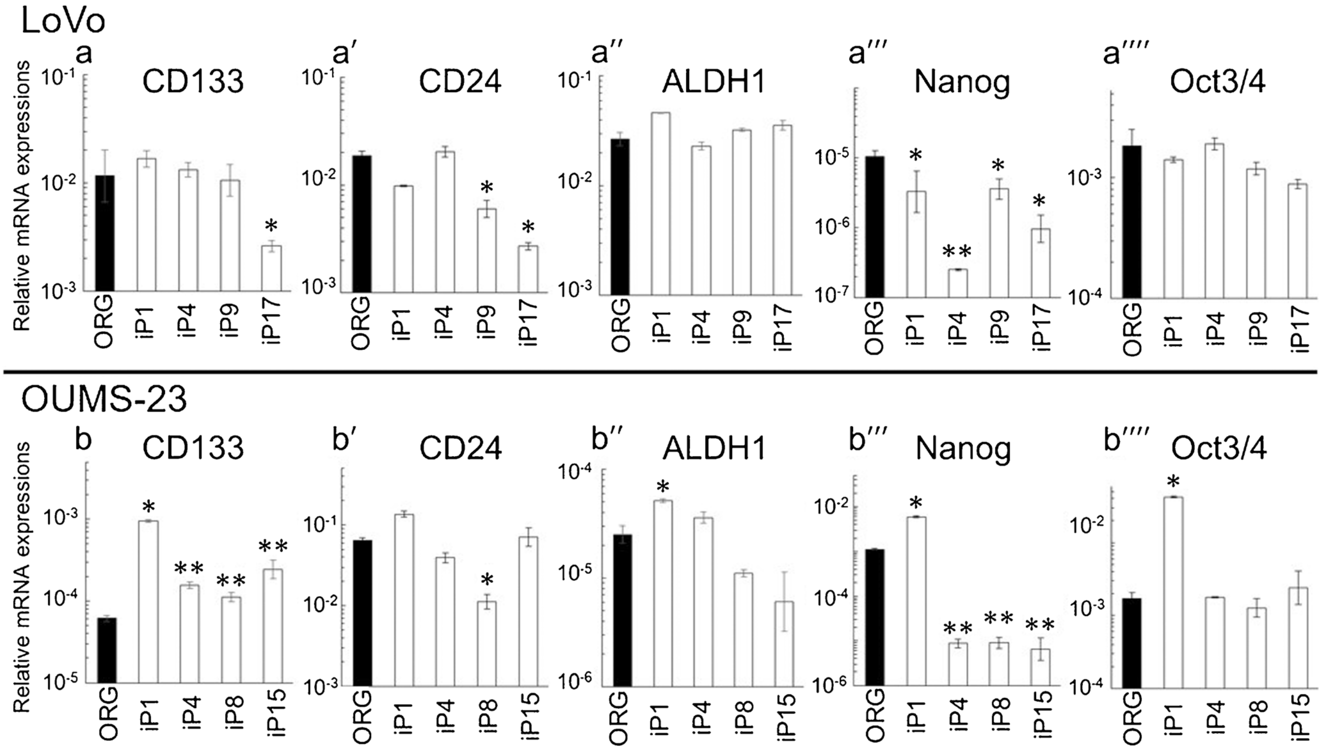
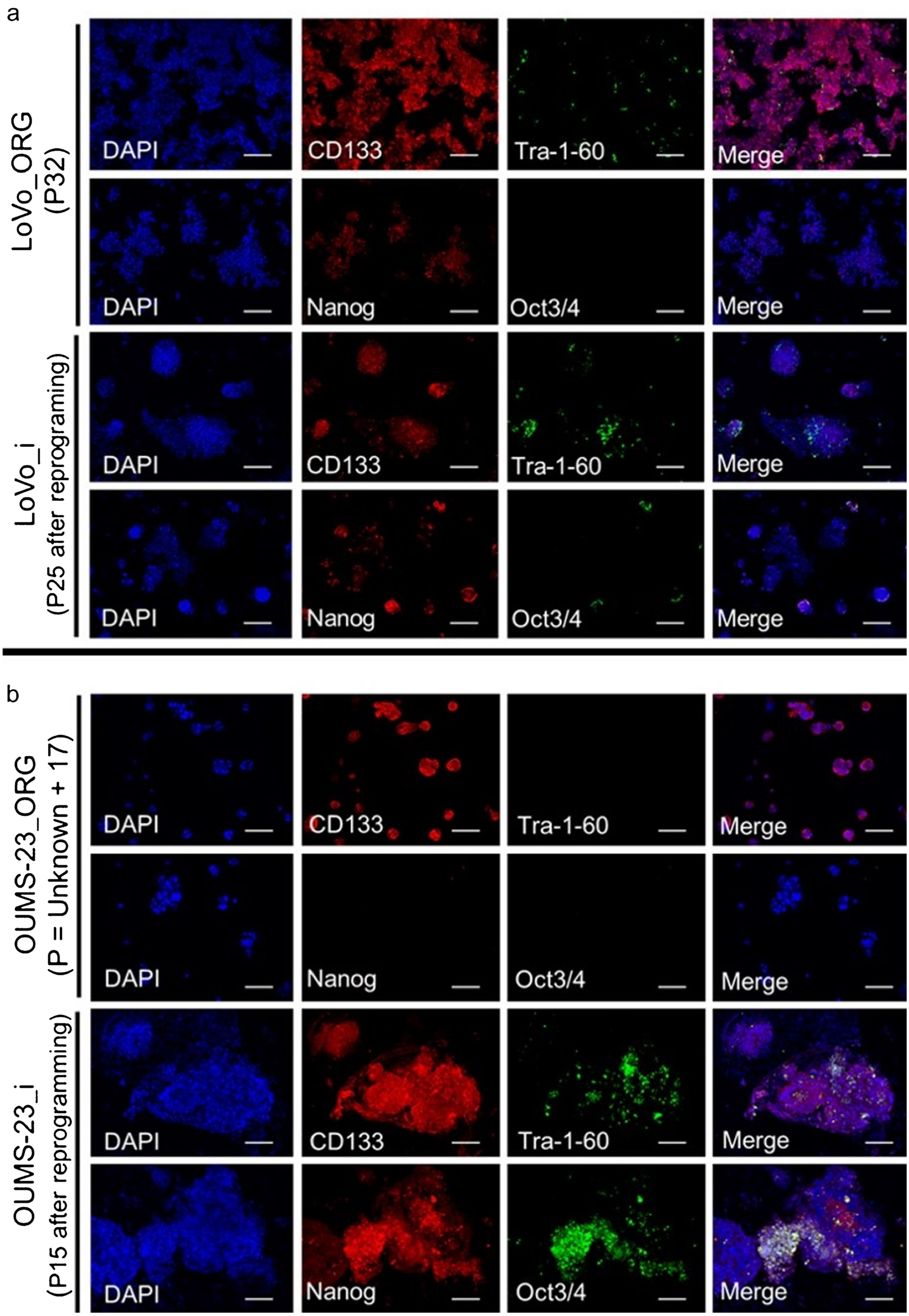


Fig. 2 mRNA expression of pluripotent stem cell markers and CSC markers in reprogrammed and control cells. mRNA expressions of CSC markers CD133 (a, b), CD24 (a′,b′), and ALDH1 (a‴, b‴) and the pluripotent stem cell markers Nanog (a‴, b‴) and Oct3/4 (a⁗, b⁗) in original and reprogrammed LoVo (a–a⁗) and OUMS-23 (b–b⁗) cells. Data from original cell lines are shown in closed bars, and data from reprogrammed cells are shown in open bars. ORG, original colon cancer cells; iPx, × times passaged reprogrammed cells. mRNA expression levels were normalized to β-actin. Data are expressed as means ± standard deviation. *$P < 0.01$, compared with ORG; **$P < 0.01$, compared with ORG and *


a
LoVo_ORG
(P32)
DAPI
CD133
Tra-1-60
Merge
DAPI
Nanog
Oct3/4
Merge
LoVo_i
(P25 after reprograming)
DAPI
CD133
Tra-1-60
Merge
DAPI
Nanog
Oct3/4
Merge
b
OUMS-23_ORG
(P = Unknown + 17)
DAPI
CD133
Tra-1-60
Merge
DAPI
Nanog
Oct3/4
Merge
OUMS-23_i
(P15 after reprogramming)
DAPI
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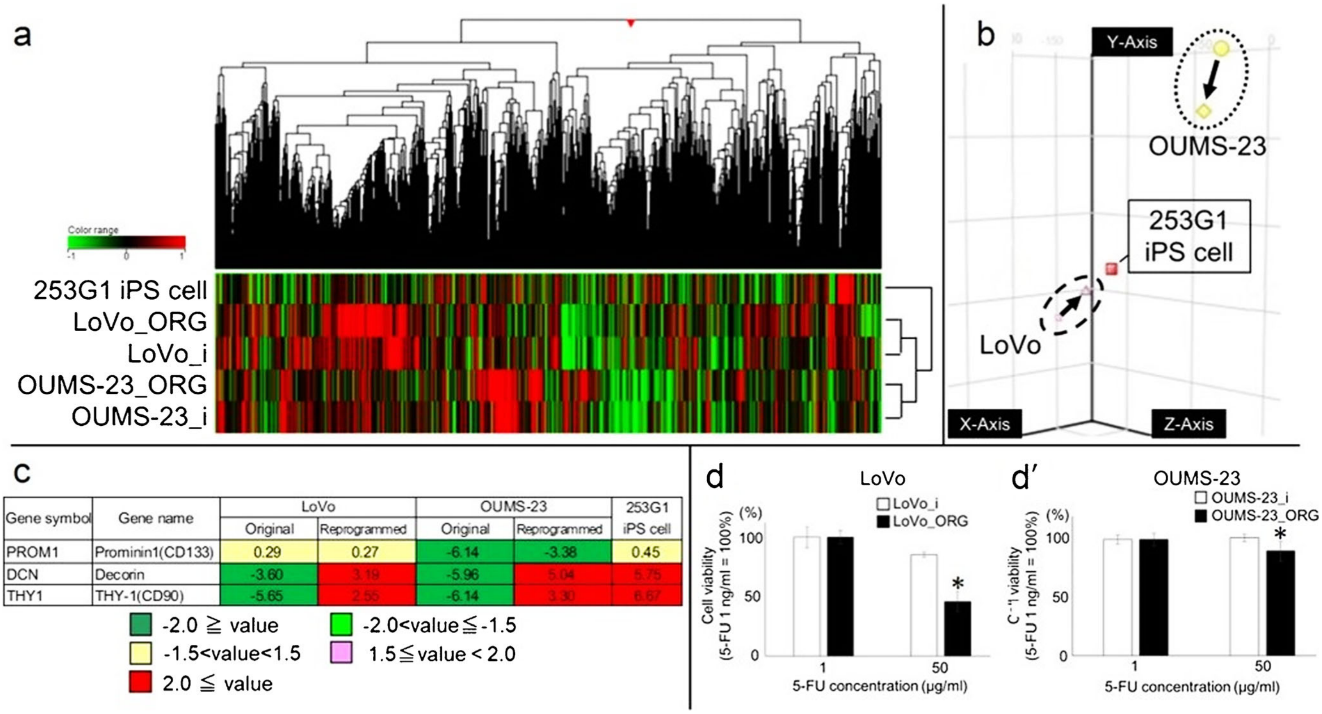

Gene symbol	Gene name	LoVo		OUMS-23		253G1 iPS cell
		Original	Reprogrammed	Original	Reprogrammed	
PROM1	Prominin1(CD133)	0.29	0.27	-6.14	-3.38	0.45
DCN	Decorin	-3.60	3.19	-5.96	5.04	5.75
THY1	THY-1(CD90)	-5.65	2.55	-6.14	3.30	6.67

🟩 -2.0 ≧ value
🟩 -2.0<value≦-1.5
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🟥 2.0 ≦ value

Fig. 4 Changes in gene profiling in reprogrammed colon cancer cells by DNA microarray. Heat map representation of the gene profiles of original and reprogrammed colorectal cancer cells and 253G1 iPS cells (a). LoVo_ORG, original LoVo cells; LoVo_i, reprogrammed LoVo cells picked from iPS-like colonies; OUMS-23_ORG, original OUMS-23 cells; OUMS-23_i, reprogrammed OUMS-23 cells picked from iPS-like colonies. Principal component analysis plots of the transcriptome data sets (b). Clipped representation from the normalized microarray data of CD133 (prominin 1), Decorin, and THY-1 (CD90) (c). 5-FU chemoresistance assay. Original (d) and reprogrammed (d′) colon cancer cells were treated with 1 μg/ml or 50 μg/ml 5-FU for 48 h, and MTT assays were performed. The survival rates of 1 μg/ml 5-FU-treated cells were set as 100%. Data are expressed as means ± standard deviation. *$P < 0.01$

The microscopy findings of tumors derived from high-dose original carcinoma cell transplantation and low-dose reprogrammed carcinoma cell transplantation are shown in Fig. 5. Tissue specimens of tumors derived from high-dose original carcinoma cell transplantation exhibited the morphology of tubular adenocarcinoma, in which the original carcinoma cells formed glandular structures (Fig. 5(a)), and the morphology of poorly differentiated adenocarcinoma, in which original carcinoma cells formed solid structures with cytoplasmic mucin (Fig. 5(b)). In contrast, both tissue specimens of tumors derived from low-dose reprogrammed carcinoma cells transplantation revealed, for the most part, the morphology of undifferentiated carcinoma in which the reprogrammed carcinoma cells formed solid structures without cytoplasmic mucin (Fig. 5(c, d, d′)). Both reprogrammed carcinoma cells seemed to be dedifferentiated. However, both tissue specimens showed focal papillary structures indicating remnant of adenocarcinoma differentiation (Fig. 5(e, e′, f, f′)).

Discussion

In this study, we produced CSC-like reprogrammed colorectal cancer cells using a set of defined factors (Oct3/4, Sox2, Klf4, and L-myc) and demonstrated that these cells could be repeatedly passaged and maintained the CSC properties in vitro. iPS cell-like colonies appeared 2 weeks after reprogramming and were Tra-1-60-positive, which enabled colony selection. Reprogrammed cancer cells demonstrated CSC properties compared with control cells. Original LoVo and OUMS-23 cells did not form iPS cell-like or Tra-1-60-positive colonies under the same condition as reprogrammed cells. These results are partially consistent with those of earlier studies that showed CSC-like cells could be established by reprogramming (Carette et al. 2010; Kim et al. 2013; L. Chen et al. 2012; Miyoshi et al. 2010; Oshima et al. 2014). However, these studies used retroviruses or lentiviruses for transduction and thus ran the risk of genomic insertion into the host genome by the viruses. To avoid the risk of genomic insertion by viral vectors, we used Sendai virus (RNA virus) vectors and succeeded in establishing CSC-like cells. In addition, Oshima et al. reported the induction of colorectal CSC-like cells by transduction of Oct3/4, Sox2, and Klf4 into SW480 cells, but the colorectal CSC-like cells had to be subcultured in vivo. Our CSC-like cells established in this study could be subcultured in vitro for a long time (passage number > 10), which allowed for easy cell biological analysis. In fibroblast medium, reprogrammed cells differentiated and lost their CSC-like features immediately. These results suggest

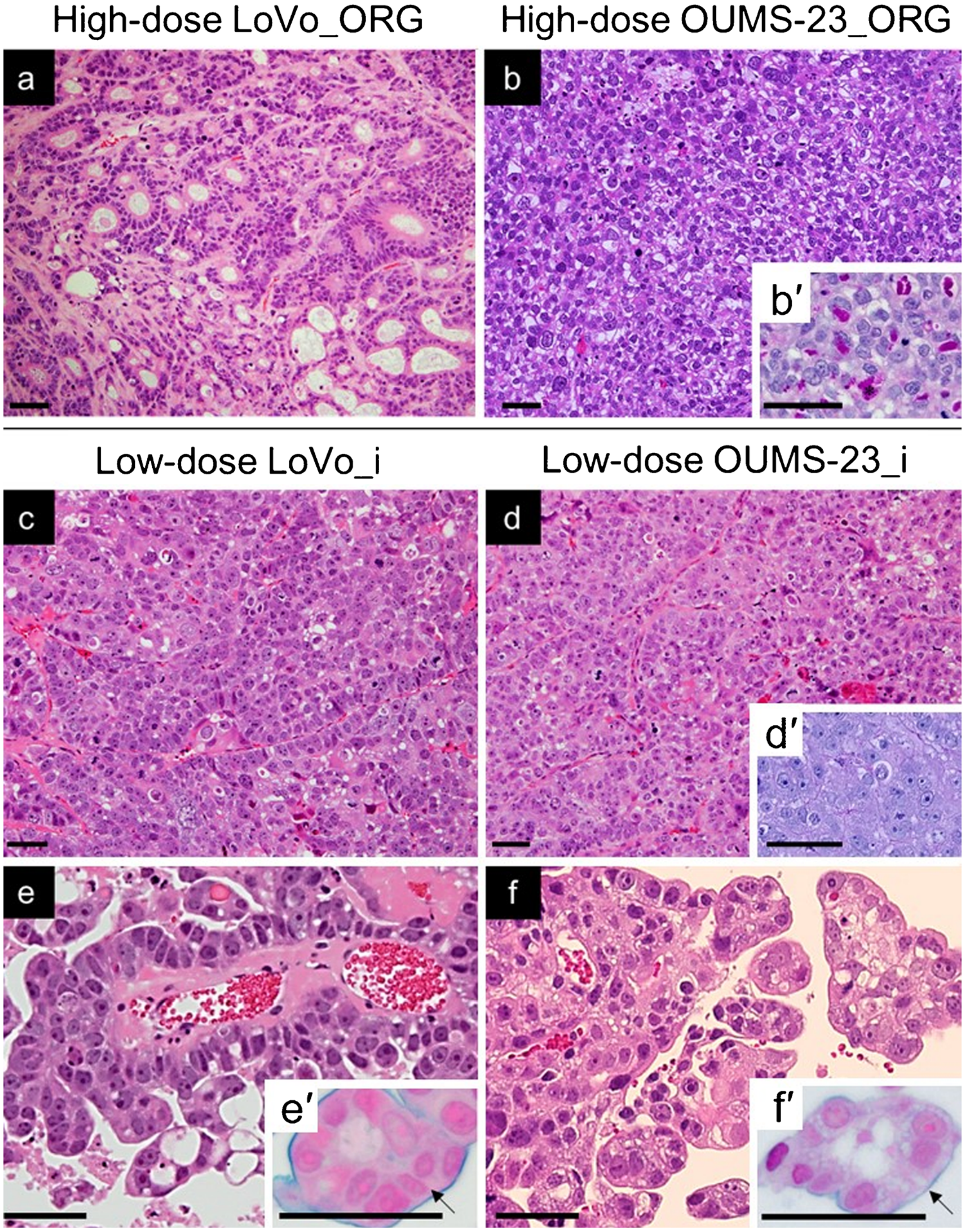

Fig. 5 Staining analysis of xenograft tumors in SCID/beige mice. Hematoxylin and eosin (HE) staining of the tumor tissue derived from high-dose LoVo_ORG cell transplantation (a). The original carcinoma cells form glandular structures and exhibit findings of tubular adenocarcinoma. HE staining (b) and diastase-treated periodic acid-Schiff (D-PAS) staining (b′; purplish-red dyed area) section of the tumor tissue from high-dose OUMS-23_ORG transplantation. The original carcinoma cells form solid structures with D-PAS-positive cytoplasmic mucin (b′) and show findings of poorly differentiated adenocarcinoma. HE staining of the tumor tissue derived from low-dose LoVo_i transplantation (c). The reprogrammed carcinoma cells form solid structures. HE staining (d) and D-PAS staining (d′) of the tumor tissue derived from low-dose OUMS-23_i transplantation. The reprogrammed carcinoma cells form solid structures without cytoplasmic mucin (d′). HE staining and Alcian Blue (AB) staining (e′, f′) section of the tumor tissue derived from low-dose LoVo_i (e) and OUMS-23_i (f) transplantation. Both of the reprogrammed carcinoma cells form papillary structures in part with AB-positive apical surface (e, f, arrow). Original magnification × 200 (a–d), × 400 (b′, d′, e, e′, f, f′). Scale bars are 50 μm

that microenvironment has a great influence for reprogrammed cells to keep their CSC-like features during post reprogram cultivation.

The PCA results in DNA microarray are helpful in explaining the unique characteristics of both the cancer cell lines and the reprogrammed CSC-like cells. After reprogramming, the plots showed that the data from both cancer cell lines moved toward that of iPS cells and our results showed that the cells acquired iPS cell-like characteristics, i.e., upregulation of undifferentiated markers, but were not completely identical to true iPS cells. This transition of cancer cell lines in acquiring iPS cell-like characteristics may be perceived as a vector with direction and length. A discussion of our findings in the context of this vector may provide new insights.

First, how are our findings reflected in “direction”? The purified cells from iPS cell-like colonies showed tumorigenicity in immune-deficient mice after transplantation of a small number of cells. The tumor sections from original LoVo cancer cells showed typical tubular formation and were relatively differentiated; however, the sections from reprogrammed LoVo CSC-like cells changed into a solid structure type and transformed into poorly differentiated type. Moreover, the tumor sections from original OUMS-23 cancer cells appeared to initially be solid type and contained much mucin in the cytoplasm, which exhibited features of glandular structures. However, the reprogrammed OUMS-23 CSC-like cells maintained solid type, like the original cells, but lost cytoplasmic mucin, which was one sign of dedifferentiation. Although the reprogrammed cancer cells showed dedifferentiation, transformed into a more poorly differentiated state, and showed the morphology of undifferentiated carcinoma, a focal adenocarcinoma component remained among tissues as focal papillary components, confirmed with AB-positive apical surface, which means that they retain original characteristics. This unique change, i.e., dedifferentiation as a transition from one point to another, can be perceived as "direction" of the vector in PCA.

Next, how are our findings illustrated in "length"? Our results suggested that CSC and undifferentiated markers (CD133, CD24, Oct3/4, and ALDH1) were upregulated in OUMS-23 cells immediately after reprogramming but were not drastically changed in LoVo cells. The vector length of LoVo is shorter than that of OUMS-23 as seen in PCA, indicating a smaller change in LoVo cells. The vector length quantifies the amount of change, in which specific factors associated with reprogramming are produced. Decorin and CD90 mRNA levels were markedly increased in reprogrammed colon cancer cells. CD90 is a 25–37-kDa glycophosphatidylinositol-anchored glycoprotein that is mainly expressed on leukocytes, where it contributes to cell-cell/cell-matrix adhesion (Rege and Hagood 2006). Several papers have shown that CD90-positive cells have CSC features, and there is evidence that CD90 is a prognostic marker in many cancers (Kumar et al. 2016; Yang et al. 2008). Decorin expression was reported to be a key factor in chemoresistance and promoting invasiveness by affecting signaling molecules involved in cell growth, survival, metastasis, and angiogenesis in bladder cancer (Mohamed El Behi et al. 2013) and oral cancer (Kasamatsu et al. 2015). Interestingly, we observed that reprogrammed LoVo and OUMS-23 CSC-like cells acquired 5-FU chemoresistance. These results suggested that acquiring chemoresistance may be related with Decorin overexpression in reprogrammed cells.

In addition, as the PCA shows, the reprogrammed cancer cell data shifted toward, but did not fully reach, normal pluripotent cells, i.e., iPS cells, but could come back only to the CSC phase without complete loss of genetic lesions of the original cancer cells and with newly gained CSC genes. It thus appears difficult to generate true iPS cells from cancer cells beyond the CSC phase, which suggests that reprogrammed cancer cells do not completely lose their identity as cancer cells. They come back only to the CSC stage and are more malignant, but do not move toward normal pluripotent stem cells. In our previous study on the reprogramming of retinoblastoma (Yue et al. 2017), it was very easy to establish CSC-like cells, but we could not return these cells to non-cancerous cells. Although the tip of the vector showed direction from each cancer to reprogrammed cancer cells, i.e., CSC-like conditions, it was very difficult to shorten the distance further from the tip of each cancer cell to the location of iPS cells despite repeated gene transduction. Based on these results, as each length of the vector shows, there are genetic targets to differentiate reprogrammed cancer cells (CSCs) from the cancer cells (originals). The length is shorter in LoVo cells than that of OUMS-23 cells, which may suggest OUMS-23 cells have more unique targets than LoVo cells. Moreover, there are genetic targets unique to each reprogrammed cell, which are different from normal iPS cells, because the direction of each vector deviates from the location of normal pluripotent stem cells and the distance between the tip of each arrow, and its location does not coincide with it. Therefore, this experimental system will be useful to investigate the process of malignancy from the original cancer cell to a CSC and to identify CSCs in tumor tissues; these results will also contribute to establishing new therapies, e.g., immunotherapy targeting CSC-like cells and molecularly targeted therapy. Moreover, the PCA plots from the LoVo and OUMS-23 data show clearly that they have different characteristics and/or target factors that may identify these populations as CSCs. Thus, PCA is a simple and useful tool to evaluate reprogramming conditions and estimate cell biological dynamics.

Together, our findings show that reprogramming of cancer cells by transduction of four established reprogramming factors did not produce pluripotent stem cells but instead produced CSC-like cells. These findings help provide biological information and expand our understanding on genuine CSCs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All experiments were reviewed and approved by the Animal Ethics and Research Committee, Shinshu University (permit number 260037), and were conducted in accordance with their guidelines.

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