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Reprogramming of retinoblastoma cancer cells into cancer stem cells

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ABSTRACT

Retinoblastoma is the most common intraocular malignancy in pediatric patients. It develops rapidly in the retina and can be fatal if not treated promptly. It has been proposed that a small population of cancer cells, termed cancer stem cells (CSCs), initiate tumorigenesis from immature tissue stem cells or progenitor cells. Reprogramming technology, which can convert mature cells into pluripotent stem cells (iPS), provides the possibility of transducing malignant cancer cells back to CSCs, a type of early stage of cancer. We herein took advantage of reprogramming technology to induce CSCs from retinoblastoma cancer cells. In the present study, the 4 Yamanaka transcription factors, Oct4, Sox2, Klf4 and c-myc, were transduced into retinoblastoma cells (Rbc51). iPS-like colonies were observed 15 days after transduction and showed significantly enhanced CSC properties. The gene and protein expression levels of pluripotent stem cell markers (Tra-1-60, Oct4, Nanog) and cancer stem cell markers (CD133, CD44) were up-regulated in transduced Rbc51 cells compared to control cells. Moreover, iPS-like CSCs could be sorted using the Magnetic-activated cell sorting (MACS) method. A sphere formation assay demonstrated spheroid formation in transduced Rbc51 cells cultured in serum free media, and these spheroids could be differentiated into Pax6-, Nestin-positive neural progenitors and rhodopsin- and recoverin-positive mature retinal cells. The cell viability after 5-Fu exposure was higher in transduced Rbc51 cells. In conclusion, CSCs were generated from retinoblastoma cancer cells using reprogramming technology. Our novel method can generate CSCs, the study of which can lead to better understanding of cancer-specific initiation, cancer epigenetics, and the overlapping mechanisms of cancer development and pluripotent stem cell behavior.

1. Introduction

Retinoblastoma is the most common intraocular malignancy in pediatric patients. Though most children survive this cancer, they may lose their vision in the affected eye(s) or need to have the eye removed. This cancer develops rapidly in the retina and can be fatal if not treated promptly. The mechanism involved in resistance and recurrence is still not clear, and improved targeted therapies are essential for alleviating this devastating malignancy [1].

In recent years, a small population of cancer cells with both self-renewal potential and multi-potential properties, termed cancer stem cells (CSCs), have been identified as a sufficient population to form tumors [2,3]. Moreover, these CSCs are associated with poor prognosis of patients, higher rates of therapeutic resistance and higher rates of recurrence [4–6]. Therefore, CSCs provide a potential therapeutic target. However, it is difficult to identify and collect CSCs because of their rarity in cancer tissues. Generating CSCs from cancer cells or tissues to investigate their characteristics is a potential method to overcome this problem. Reprogramming reverts terminally differentiated somatic cells into embryonic stem (ES)-like pluripotent stem cells (iPS) with the ectopic expression of only three or four transcription factors (OCT3/4, SOX2 and KLF4 with or without C-MYC) [7,8]. The process of iPSC derivation shares similar characteristics with cancer development. Therefore, generation of iPS-like CSCs through reprogramming cancer cells could offer a tool for understanding the origin of cancer stem cells, tumor initiation-progression in vitro, and tumor heterogeneity, which could lead to advancements in cancer type-specific drug discovery.

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In this study, we reprogrammed retinoblastoma cancer cells into iPS-like CSCs with ectopic expression of four transcription factors. This work will contribute to the understanding of CSC initiation and to the development of new therapies targeting CSCs.

2. Materials and methods

2.1. Cell line and cell culture

The human retinoblastoma cancer cell line (JCRB1182, NCC-RbC-51, RIKEN Japan) was cultured in RPMI1640 (Wako, Japan) containing 20% FBS (Gibco, BRL, USA) and used at an early passage for the following reprogramming experiments.

2.2. CytoTune Sendai virus infection

A total of 2×10^5 retinoblastoma cells were infected with CytoTune Sendai virus containing the transcription factors Oct3/4, Sox2, Klf4 and C-myc (CytoTune®-iPS 2.0. MBL, USA). The next day, the cells were harvested and cultured with fresh MEF medium. At day 3, transduced cells were plated on MEF culture dishes. Starting on day 7, the medium was changed to a complete iPSC medium and cultured until the appearance of the iPSC-like colonies was observed.

2.3. RNA isolation and quantitative reverse-transcription polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Quantitative PCR analysis was performed as described previously [9] using a Thermal Cycler Dice Real-Time System (Takara Bio, Otsu, Japan).

2.4. DNA microarray

Total RNA was 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).

2.5. Data analysis of microarrays

Intensity values of each scanned feature were quantified using Agilent feature extraction software version 10.7.3.1. The data were normalized using Agilent GeneSpring GX version 13.1.1 (per chip: normalization to 75th percentile shift; per gene: normalization to median of all samples).

2.6. Immunocytochemistry

The attached cells were fixed in 4% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.4, for 20 min at room temperature and immunostained as described previously [10]. Antibodies against Oct-4, SSEA-4, Nanog, CD133, Pax6, Nestin, Rhodopsin and Recoverin were used in this study.

2.7. Purification of Tra-1-60 positive cells by the MACS method

Tra-1-60 positive cells were sorted using the EasySep selection kit (StemCell, Tech. USA) according to the manufacturer's instructions. After being detached with Accutase and washed with PBS, transduced RBC51 cells were labeled with Tra-1-60 antibodies and magnetic particles and separated using an EasySep magnet.

2.8. Sphere formation assay

A total of 5×10^3 cells were transferred to Ultra Low Attachment plates (Corning Incorporated, Corning, New York, USA) in serum-free DMEM/F12 supplemented with 20 ng/ml of EGF, 10 ng/ml bFGF, 2% B27 supplement without vitamin A, and 1% N2 supplement (Gibco, BRL, USA) and incubated at 37 °C in a 5% CO_2 incubator for 10 days.

2.9. 5-FU-chemoresistance analysis

The cell viability after 5-fluorouracil (5-FU, KYOWA KIRIN, Tokyo, Japan) treatment was measured by colorimetric MTT assay (cell growth assay kit, Millipore, USA). A total of 5×10^4 cells were seeded in 96-well plates containing 1 or 50 μg/ml of 5-FU. After incubation for 48 h, the absorbance at 570 nm was measured using a microtiter plate reader.

3. Results

3.1. Induction of iPS-like colonies with 4 transcription factors

We transduced the transcription factors Oct3/4, Sox2, Klf4 and C-myc into retinoblastoma RBC51 cells (Fig. 1a). From day 2, the small and round RBC51 cells grown in suspension culture attached to the plate and grew as flat, fibroblast-like cells. From day 15, iPS-like colonies were observed on MEF culture dishes (Fig. 1b and c). To examine the stem cell status of the transduced retinoblastoma, we evaluated the protein expression of pluripotent stem cell markers Tra-1-60 in living cells, and Nanog/SSEA4, Oct4/CD133 in fixed iPS-like colonies. A small population of iPS-like colonies was positive for Tra-1-60 (Fig. 1d). Double-positive staining of both pluripotent stem cell markers, Nanog/SSEA4 and Oct4/CD133, was observed in some iPS-like colonies (Fig. 1e). Next, we evaluated the gene expression levels of the stem cell markers. The qPCR analysis showed that the transduced retinoblastoma cells had significantly increased gene expression of the stem cell markers Oct4 and Nanog (Fig. 3). The results suggested that retinoblastoma RBC51 cells were reprogrammed using ectopic transcription factor expression into stem cells with pluripotent properties.

3.2. Sorting iPS-like CSCs using the MACS method

To isolate highly purified Tra-1-60-positive cells from transduced retinoblastoma RBC51 cells, we used Magnetic-activated cell sorting (MACS) technology. RBC51 cells isolated by an EasySep kit (StemCell Technologies) were cultured for 3 passages and then analyzed for expression of Tra-1-60 and double staining of SSEA4/Nanog. The results showed that many more iPS-like colonies were positive for Tra-1-60 staining after sorting (Fig. 1f). Double staining for SSEA4/Nanog (Fig. 1g) suggested that the sorted tra-1-60-positive iPS-like colonies maintained pluripotent stem cell properties.

3.3. Identification of CSC properties

3.3.1. Protein and gene expression of CSC markers

The neural cancer stem cell marker CD133, which had been reported previously [11], was co-expressed with stem cell marker Oct4 in transduced retinoblastoma cells (Fig. 2a), and more iPS-like colonies expressed Oct4/CD133 proteins after sorting with the MACS method (Fig. 2b). The gene expression levels of CD133 and CD44 were significantly up-regulated after reprogramming (Fig. 3). These results indicated that transcription factors had the ability to evoke CSC signatures in retinoblastoma RBC51 cells.

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