



Molecular changes in appearance of a cancer cell among normal HEK293T cells

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Received 1 July 2016; accepted 24 September 2016
Available online xxx

In very early stages of cancer development, one or a few cells expressing cancer-associated genes appear among a much larger number of surrounding normal cells. To analyze the molecular changes induced by this co-existence, we artificially prepared transformed cells with complete loss of tumor suppressor gene, *SCRIB*, among normal human embryonic kidney (HEK293T) cells. A cell strain with *SCRIB*-knockout was successfully constructed by using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 nuclease system and co-cultured with normal cells. By measuring the time-course changes in cell numbers when *SCRIB*-knockout cells (cancer model) or cells with normal level of *SCRIB* expression were respectively co-cultured with wild-type normal HEK293T cells, it was shown that the *SCRIB*-knockout strain was beneficial for proliferation when mixed together with normal cells. Moreover, as a result of proteome analysis on wild-type cells separated from co-culture with *SCRIB*-knockout cells, a total of 843 proteins were identified, among which 139 proteins were specific. Among the specifically identified proteins, 22 proteins were annotated to be involved in cytoskeletons including microtubule motor activity-associated proteins. It was implied that molecular changes in cytoskeletons occurred in normal cells when co-cultured with *SCRIB* knockout cells, but the *SCRIB* knockout might affect proliferation of the transformed cells with *SCRIB* knockout by defensive or offensive mechanism of surrounding normal cells.

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[**Key words:** Cellular competition; Co-culture; HEK293T; Proteome analysis; Tumor suppressor gene]

In the very early stages of cancer development, cancer first occurs at the single-cell level. For survival, cells with the cancer-associated gene must compete with the surrounding normal cells which exist in much larger numbers. Recently, several studies using *Drosophila melanogaster* or dog-derived epithelial cells showed that when cells with certain cancer-associated gene are co-cultured with normal cells, an interaction occurs between these cancer and normal cells, resulting in elimination from the cell layer or apoptosis of one of the cell types that has been outcompeted by the other (1–3). There is an interesting example of the genetic transformations that induce intercellular interactions between cancer cells by the loss of the *SCRIB* gene and normal cells. *SCRIB* is a well-known tumor suppressor gene that is involved in epithelial cell polarity and proliferative control (4). The homolog of *SCRIB* in mouse has been shown to behave as a tumor suppressor (5); in addition, the expression of this gene has been shown to decrease in human cancers, such as colon and breast cancers (6,7). In experiments using *D. melanogaster* or a dog-derived epithelial cell line, cells with decreased expression of *SCRIB* homologs in these organisms have been shown to be eliminated from the epithelial cell layer when surrounded by a larger number of normal cells (3,8).

However, to date, the cellular changes that occur when ‘human’-derived cells with human *SCRIB* mutation are co-cultured with normal cells have not yet been elucidated. Moreover, to understand

the underlying processes and to effectively take advantage of the molecules that play critical roles, the molecular mechanism of mutual recognition and interaction between early cancer cells and normal cells must be clarified in detail.

In the present study, a co-culture system of *SCRIB*-knockout cancer cells and normal cells was constructed using the HEK293T cell line, and the cellular changes were analyzed by time-course microscopic images and proteome analysis of the whole proteins extracted from the co-cultured cells. This co-culture system might represent the interaction between cancer cells and normal cells surrounding them, in the very early stages of cancer development. Because cancers most frequently occur in epithelial tissues, HEK293T cells, which are generally acknowledged to represent characteristics of kidney epithelial cells (9,10) were used for the construction of the model for early cancer development by co-culture of normal cells and cancer model cells. In order to better represent the very early stages of cancer development where only few single cells obtain a cancer-associated genetic mutation in a single gene, HEK293T cells with *SCRIB*-knockout was used instead of established cancer cell lines. By analyzing the constructed co-culture system, the molecular changes that occur in normal cells as a response to the appearance of a cancer-associated cell among them were elucidated.

MATERIALS AND METHODS

Plasmids and antibodies The target sequence of the guide RNA of the CRISPR-Cas9 system for *SCRIB*-knockout was designed using the on-line tool for designing guide RNA sequence (<https://chopchop.rc.fas.harvard.edu/>). Among the candidate sequences, the sequence with the highest score and the lowest possibility of off-

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target effect was selected for plasmid construction. The target sequence used in the present research is nucleotide sequence from 28th to 47th of the open reading frame of human *SCRIB* gene. To construct the CRISPR-Cas9 system for *SCRIB*-knockout in HEK293T cells, pCas-Guide plasmid (Origene, USA) was amplified by polymerase chain reaction (PCR) using oligonucleotides 5'-GTTTATAGAGCTAGAAATAGCAAGTTAAATAAGGC-3' and 5'-CGATCCCGCTCTTCCAC-3'. Annealed oligonucleotide including the guide RNA sequence to target the *SCRIB* gene in HEK293T cells (Forward: 5'-AAGGACGCGGATCGGTGGCGCTGCAACCGGCACGGTTTATAGAGCTAGAA-3' and Reverse: 5'-TTCTAGCTCTAAACCGTGCCGGTTGCAGCGCCACCGATCCCGCTCCTT-3') was cloned into the amplified pCas-Guide using the In-Fusion HD Cloning Kit (Clontech, USA), resulting in pCas-Guide/hSCRIB (Fig. 1). Left (LHR) and right homologous regions (RHR) to induce homologous recombination following the double-strand break of *SCRIB* were cloned from the genomic DNA of HEK293T cells and inserted into HR410PA-1 plasmid (System Biosciences, USA). Genomic DNA was extracted from wild-type HEK293T cells using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, USA), according to the manufacturer's protocol. The LHR was amplified from the extracted genomic DNA by PCR using oligonucleotides 5'-GACGGCCAGTGAATGAGACCTGGGCAGGGGGTTG-3' and 5'-TTGAGTGGAAAGATCCTTGAGCATGGTGGCGGTGG-3' and cloned into *EcoRI* and *BglII* sites of HR410PA-1. The RHR was amplified using oligonucleotides 5'-TCGGATCCCCGTCGAGAGTCGGTGACAAGCGGCAC-3' and 5'-ATTACGCCAAGCTTGCAAGAACTCAGTAAACGCTCCCAACA-3' and cloned into *Sall* and *SphI* sites of HR410PA-1 into which the LHR has already been cloned. The resulting donor plasmid was designated HR410PA-1/hSCRIB (Fig. 1).

A mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), clone 6C5 (MAB374, Millipore, USA) was used at a dilution of 1:500 for Western blot analysis. Horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) (NA931, GE Healthcare, USA) was used at a dilution of 1:5000 as the secondary antibody for the detection of GAPDH. A goat polyclonal antibody against *SCRIB* protein, clone K-21 (sc-11048, Santa Cruz Biotechnology, USA) was used at a dilution of 1:200 for the detection of *SCRIB* protein production by Western blot analysis. As the secondary antibody for the detection of *SCRIB* protein, HRP-conjugated anti-goat IgG (sc-2020, Santa Cruz Biotechnology) was used at a dilution of 1:5000.

Cell culture and establishment of strains HEK293T cells (RCB2202, RIKEN Cell Bank, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM, Nacalai Tesque, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, USA). For maintenance, 1.0×10^6 cells were seeded on 100-mm dishes and incubated at 37°C in a humidified atmosphere with 95% air and 5% CO₂. Culture medium was replaced every 2–3 days and the cells were passaged every 4–5 days.

To establish the *SCRIB*-knockout cell line, HEK293T cells were transfected with both pCas-Guide/hSCRIB and HR410PA-1/hSCRIB plasmids using X-tremeGENE HP DNA Transfection Reagent (Roche, Switzerland), according to the manufacturer's protocol. To establish the negative control cell line that is GFP-positive but has a normal level of the *SCRIB* expression, HEK293T cells were transfected with only HR410PA-1/hSCRIB plasmid. In both cases, 2 µg of total plasmid and 6 µL of the transfection reagent were used for each well of 6-well cell culture plates. Transfection of the cells was followed by selection in culture medium containing puromycin at a final concentration of 1 µg/mL. All selected cells were further maintained in culture medium containing puromycin at 1 µg/mL.

To analyze the growth of the cells of each constructed strain at single culture, 5.0×10^5 cells were seeded on each well of 6-well cell culture plates, and the number of cells was counted at indicated time points using a hemocytometer.

Sample preparation for Western blot analysis Whole proteins were extracted from normal or *SCRIB*-knockout HEK293T cells for the detection of target proteins by Western blot analysis. Cells were seeded onto 6-well plates at 5.0×10^5 cells per well and cultured for 48 h before protein extraction. Cell culture supernatant was removed from each well and the cells were washed twice with ice-cold 1× phosphate buffered saline (PBS; 137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, 1.47 mM KH₂PO₄, pH 7.4, NIPPON GENE, Japan). Following the addition of 150 µL RIPA buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 1.0% (w/v) NP-40 substitute, Wako, Japan] containing 1% (v/v) protease inhibitor cocktail for mammalian cells (Sigma) to each well, the cells were scraped off from the dish surface using cell scrapers (Iwaki, Japan). After homogenization of the cell suspension by thoroughly pipetting the solution 30 times, it was moved to a new 1.5-mL tube and incubated on ice for 5 min. Cell lysates were centrifuged at 14,000 ×g at 4°C for 20 min and the supernatant was moved to a clean 1.5-mL tube as the whole protein extract. Protein concentration was measured using the Bicinchoninate Protein Assay kit (Nacalai Tesque) and the same amount of protein from each sample was applied to SDS-PAGE and Western blot analysis.

Time-course observation of the co-cultures Wild-type and *SCRIB*-knockout HEK293T cells (KO no. 18 strain), or wild-type and negative control cells transfected only with the donor plasmid (D no. 1 strain) were respectively mixed in a ratio of 9:1 (4.5×10^5 : 5.0×10^4) and seeded on 35-mm glass base dishes with cover glasses of thickness 0.12–0.17 mm (Iwaki) (Fig. 2). Microscopic observation of a fixed area on each co-culture dish was performed at the indicated time points, using the microscope that is attached to the microinjector CI-2500 (Fujitsu Ltd., Japan) (11). To detect the green fluorescence of GFP, the fluorescence filter set with a 460–495 nm excitation filter, a 510 IF emission filter

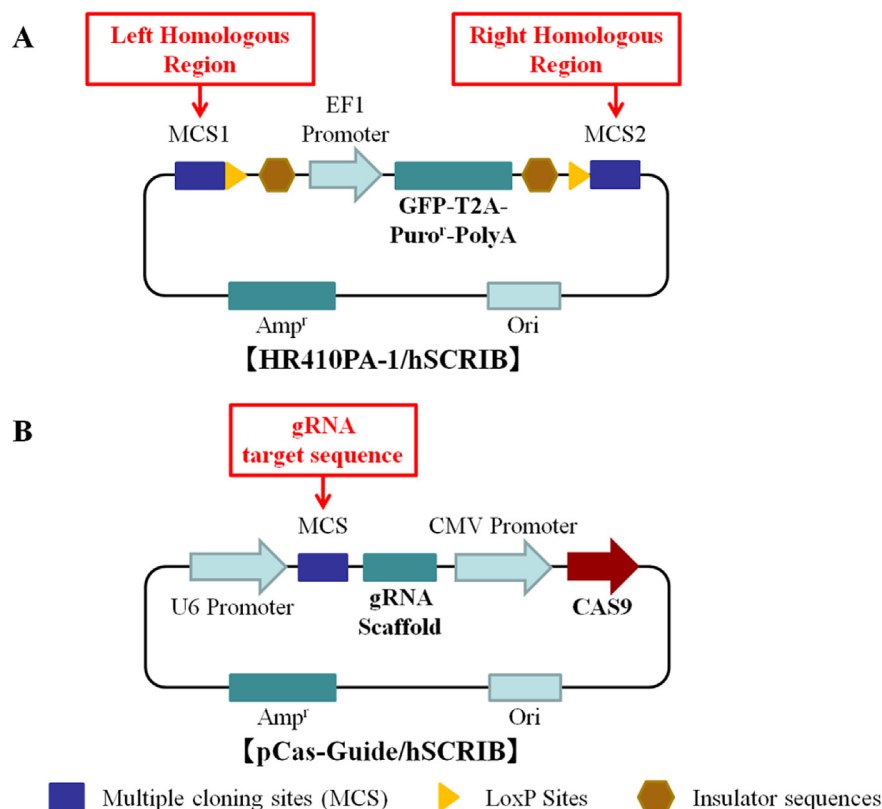


FIG. 1. Plasmids used for *SCRIB* knockout by CRISPR/Cas9 genome editing system. Construct of HR410PA-1/hSCRIB (A); that of pCas-Guide/hSCRIB (B) discussed in Materials and methods.

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