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Generation of *SMURF2* knockout human cells using the CRISPR/Cas9 system

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ABSTRACT

The HECT domain E3 ubiquitin ligase SMURF2 regulates stability of several key protein targets involved in tumorigenesis, cell proliferation, migration, differentiation, and senescence. While altered levels and aberrant cellular distribution of SMURF2 were reported in different types of cancer, its role in tumorigenesis is far from understood. To elucidate the role of SMURF2 in cancer, appropriate human cancer cell models are needed. Here, we describe approaches that can be used to generate human normal and cancer cell strains knocked-out for *SMURF2* using the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) gene-editing technology.

Introduction

Smad Ubiquitination Regulatory Factor 2 (SMURF2) is an E3 ubiquitin ligase that functions in a cascade of reactions leading to protein ubiquitination. Ubiquitination is a major mechanism for posttranslational protein modification, and governs nearly every biological process in the cell. This evolutionary conserved cascade consists of three sequential steps: (1) activation of ubiquitin by an activating enzyme (E1) using energy from ATP hydrolysis, (2) transthiolation of ubiquitin from E1 to a ubiquitin-conjugating enzyme (E2), and (3) conjugation of ubiquitin to target proteins by a ubiquitin ligase (E3). In this process, SMURF2 as a HECT-type (homologous to E6-AP COOH-terminus) E3 ligase, accepts ubiquitin from E2 to its active-site cysteine 716 (Cys 716) and then transfers it to the target protein, thereby controlling its stability, localization and/or functions [1]. By providing substrate specificity, SMURF2 represents a potentially attractive target for cancer treatment [2].

SMURF2 is considered to play a dual role in carcinogenesis, as both a tumor suppressor and an oncogene. Aberrant cellular distribution and altered levels of SMURF2 were reported in patients with several types of cancer. On the one hand, strong evidence on the tumor suppressor functions of SMURF2 was provided by Blank et al., and subsequently by other studies [1,3]. In particular, *Smurf2*-deficient mice are prone to development of a wide spectrum of tumors. On the other hand, some studies suggested that SMURF2 could exert pro-oncogenic functions, for example by stabilizing the epidermal growth factor receptor (EGFR) [4], cancer-associated mutant K-RAS, or by promoting Wnt/β-catenin signaling [1]. Despite this progress, our understanding of the biological functions of SMURF2 and its dual behavior in cancer is limited by the lack of appropriate human cell models (i.e. *SMURF2* knockout human cells).

The introduction of targeted genome editing in living cells and organisms has become a powerful tool for biological research and is a potential avenue for therapy of genetic diseases [5]. A general strategy to induce targeted deletions and insertions in a range of cell types and organisms consists of creating DNA double-strand breaks (DBSs) using nucleases [6]. Early methods for gene editing relied on protein-based systems, for instance zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). However, these methods have not been widely adopted due to their complexity and cost [7]. More recently, a new method based on the bacterial nuclease CRISPR-associated protein 9 (Cas9) from

Abbreviations used: SMURF2, Smad Ubiquitination Regulatory Factor 2; HECT, homologous to E6-AP COOH-terminus; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9; ZFNs, zinc finger nucleases; TALENs, transcription activator-like effector nucleases; BCA, bicinchoninic acid; PBS, phosphate-buffered saline.

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Streptococcus pyogenes has been developed. This system has the unique characteristic of dependence on RNA, as opposed to protein-DNA interactions as in ZFNs and TALENs, as a moiety that targets the nuclease to a desired DNA sequence [5].

The hallmark of the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system consists of CRISPR arrays that are composed of spacers interspaced with direct repeats and *cas* genes present in operons [8,9]. CRISPR, together with Cas protein, provide adaptive immunity against viruses and plasmids in many bacterial species and most archaea [10]. Therefore, organisms can have long-term genomic records of infections resulting from the integration of novel short DNA sequences (spacers) into CRISPR arrays [11]. Processed transcripts derived from arrays can base pair with a complementary strand from the incoming invading foreign nucleic acid, thereby recruiting Cas proteins to bind and cleave pathogenic DNA [12]. This mechanistic understanding led to development of CRISPR/Cas9 genome engineering by triggering the repair of double strand DNA breaks at desired sites. Thus, the CRISPR/Cas9 technology provides a precise and simplistic molecular mechanism for editing DNA in cells, tissues and whole organisms, with widespread uses in experimental and applied systems [9].

Here, we describe different approaches for generation of *SMURF2* knockout human cells by using the latest CRISPR/Cas9 genome-editing tool. Importantly, while we demonstrated that these approaches could be applied to efficiently knockout the *SMURF2* gene expression in different human normal and cancer cell strains, the data suggest that the efficiency of *SMURF2* knockout depends on particular cell strain.

Materials

To establish the CRISPR knockout cell lines, the following materials were used:

- Human osteosarcoma (U2OS), breast adenocarcinoma (MDA-MB-231) and prostate cancer (DU145) cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS) (Biological Industries), 100 mg/ml streptomycin and 100 U/ml penicillin (GIBCO).
- Human mammary epithelial (MCF10A) cells were cultured in DMEM/F12 (Biological Industries) supplemented with 5% donor horse serum (Sigma), 20 μg/ml epidermal growth factor (EGF; Peprotech), 10 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma), 100 mg/ml streptomycin and 100 U/ml penicillin (GIBCO).
- Plasmids-bearing *E. coli* strain were grown in 200 ml of LB medium (tryptone 10.0 g/L, yeast extract 5.0 g/L and NaCl 10.0 g/L) supplemented with ampicillin (100 μg/ml) at 37 °C on orbital shaker for 12 h. DNA was extracted using midiprep according to manufacturer's instruction (NuceloBond Xtra Midi Plus, Macherey-Nagel).
- Different transfection reagents were used depending on the type of cell line according to manufacturer's instructions, as follows: FuGENE6 (cat. no. E2691, Promega) for U2OS, Lipofectamine 2000 (P/N 100022050, Invitrogen) for MDA-MB-231, and Lipofectamine 3000 (P/N 52758, Invitrogen) for MCF10A and DU145 cells.
- Opti-MEM (cat. no. 31985-047, GIBCO).
- Genome-wide knockout kit (cat. no. KN210866, Origene).
- Antibiotic: Puromycin (cat. no. P-600-500, Gold Biotechnology).
- RIPA lysis buffer (50 mM Tris-Cl [pH 7.8], 1% NP-40, 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS) and 0.5% sodium deoxycholate) supplemented with protease inhibitors (Complete Protease Inhibitor cocktail, Roche) and phosphatase inhibitors (cat. no. P5726 and P0044, Sigma-Aldrich).
- Sorting buffer (2 mM HEPES, 2 mM EDTA and 2% FBS in phosphate buffer saline (PBS)) was filter sterilized with 0.22 μm pore filter membrane.
- Pierce BCA (bicinchoninic acid) Protein Assay Kit (cat. no. 23225).
- Primary antibodies: rabbit anti-Smurf2 (cat. no. 12024S, 1:1500, Cell Signaling), and rabbit anti-β-actin (cat no. 600401886, 1:3000, Rockland).
- Secondary antibody: horseradish peroxidase-conjugated donkey anti-rabbit (code no. 711-036-152, 1:10,000, Jackson Immuno Research Laboratories).

A genome-wide knockout kit was used to establish SMURF2 CRISPR cells according to manufacturer's instructions. Briefly, the kit contained two pCas-Guide vectors and a donor vector. Sequences targeting the *SMURF2* gene (guide RNA, gRNA) and Cas9 endonuclease were encoded in pCas-Guide vectors, whereas a functional cassette containing the green fluorescent protein (GFP) and the puromycin resistance gene was encoded in the donor vector. Of note, the GFP-puro functional cassette is flanked by left and right homologous arms (LHA and RHA), each 600 bp long, which are complementary to sequences upstream (64,661,881–64,662,480 from *Homo sapiens* chromosome 17, GRCh38. p2 Primary Assembly) and downstream (64,661,191–64,661,790 from the same Assembly) of the *SMURF2* target sequence. Cas9 protein, guided by gRNA, recognizes and precisely cleaves double-stranded DNA at the 5′ end of SMURF2 gene loci; concomitantly, the functional cassette is integrated in the genome at the site of cleavage during homology-directed repair. Therefore, GFP will be under the control of the native *SMURF2* promoter and the puromycin resistant gene will be under the phosphoglycerate kinase (PGK) promoter. In the current study, the following target sequences were used: Sequence 1 (gRNA 1), 5′–CCCGTCAAGCTGCGCCTGAC-3'; and, Sequence 2 (gRNA 2), 5′-GGGCCCGTTCCTCCGGCCTC-3'.

Methods

1. U2OS, MDA-MB-231, DU145 and MCF10A cells were seeded 24 h before transfection in 10 cm dishes, in order to obtain 70–80% confluence on the following day.
2. Cell lines were co-transfected with one or the other pCas-Guide vector (5 μg) and donor vector (5 μg), according to manufacturer's instructions.
3. Medium was replaced 24 h post-transfection, and the cells were split 2–7 days post-transfection at ratio 1:5 to 1:8 (passage 1, P1), depending on the growth rate of the specific cell line.
4. The same splitting ratios were used each 2–6 days throughout the next 6 passages (total 7 passages) for around 3 weeks without antibiotic selection.[2]

Four different methods (A-D) were used to evaluate which approach is suitable for a particular cell line and which approach would more efficiently generate knockouts (Fig. 1).

Method A

U2OS, MDA-MB-231 and DU145 cells were split (P7) to obtain low density by diluting them at ratio 1:10, 1:50, 1:100 and 1:500. The antibiotic puromycin (2.5 μg/ml for U2OS, 10 μg/ml for MDA-

[2] The reason for this is that the donor vector before genomic integration will also provide puromycin resistance to cells. By passaging cells 7 times before adding the antibiotic, the cells containing donor vector in episomal form will be diluted.