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Simple and effective generation of transgene-free induced pluripotent stem cells using an auto-erasable Sendai virus vector responding to microRNA-302

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ABSTRACT

Transgene-free induced pluripotent stem cells (iPSCs) are valuable for both basic research and potential clinical applications. We previously reported that a replication-defective and persistent Sendai virus (SeVdp) vector harboring four reprogramming factors (SeVdp-iPS) can efficiently induce generation of transgene-free iPSCs. This vector can express all four factors stably and simultaneously without chromosomal integration and can be eliminated completely from reprogrammed cells by suppressing vector-derived RNA-dependent RNA polymerase. Here, we describe an improved SeVdp-iPS vector (SeVdp(KOSM)302L) that is automatically erased in response to microRNA-302 (miR-302), uniquely expressed in pluripotent stem cells (PSCs). Gene expression and genome replication of the SeVdp-302L vector, which contains miRNA-302a target sequences at the 3′ untranslated region of *L* mRNA, are strongly suppressed in PSCs. Consequently, SeVdp(KOSM)302L induces expression of reprogramming factors in somatic cells, while it is automatically erased from cells successfully reprogrammed to express miR-302. As this vector can reprogram somatic cells into transgene-free iPSCs without the aid of exogenous short interfering RNA (siRNA), the results we present here demonstrate that this vector may become an invaluable tool for the generation of human iPSCs for future clinical applications.

1. Introduction

Induced pluripotent stem cells (iPSCs) are stem cells generated by artificial reprogramming of somatic cells with defined factors, such as Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006), which are supplied to target cells by exogenous gene expression. iPSC generation comprises simultaneous and continuous expression of multiple exogenous reprogramming factors until the establishment of endogenous pluripotency circuitry, followed by suppression of exogenous factor expression to negligible levels. Cell reprogramming is a relatively slow process and the first step usually takes 10 to 30 days. Most of the gene delivery vectors capable of stable gene expression can induce the generation of iPSCs to some extent, while transient gene delivery systems (e.g., transfection of cells with synthetic mRNA) require repetitive delivery.

Compared with the first step in this process, the significance of the latter is often undervalued, although it is essential for the generation of high-quality iPSCs. Continuous expression of exogenous factors affects pluripotency and causes undesired side effects, such as tumorigenesis. Nevertheless, few of the vectors that are capable of stable gene expression have mechanisms for its active and irreversible suppression. The expression of genes carried by the integrative vectors (retroviral and lentiviral vectors) is often suppressed by epigenetic modifications, but this is a reversible process: the complete suppression involves the excision of vector DNA from the host genome, which requires special conditions. Non-integrative vectors, including episomal plasmids and Sendai virus vectors, are used for iPSC generation, but their removal from iPSCs relies on passive and inefficient omission, and sometimes several months are needed until transgene-free iPSCs are obtained (Fusaki et al., 2009; Yu et al., 2009).

Previously, we developed a replication-defective and persistent Sendai virus (SeVdp) vector for highly efficient transgene delivery into mammalian cells (Nishimura et al., 2011). SeVdp vector lacks all

Abbreviations: SeV, Sendai virus; SeVdp, replication-defective and persistent Sendai virus; PSC, pluripotent stem cell; RdRp, RNA-dependent RNA polymerase; MEF, mouse embryonic fibroblast; UTR, untranslated region; Cluc, *Cypridina noctiluca* luciferase; LNA, locked nucleic acid; Bs, blasticidin S; *Bsr*, blasticidin-resistance gene; KO, Kusabira-Orange; NP, nucleocapsid protein; L, large protein.

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structural genes necessary for viral particle production (viral replication-deficient). However, it encodes a viral RNA-dependent RNA polymerase (RdRp) responsible for the replication of SeVdp RNA genome, allowing persistent transgene expression. We reported that the SeVdp vector carrying *OCT4*, *SOX2*, *KLF4*, and *c-MYC* genes (SeVdp-iPS) efficiently reprogrammed mouse embryonic fibroblasts (MEFs), human dermal fibroblasts, and human hepatocytes into transgene-free iPSCs (Kawagoe et al., 2013; Nishimura et al., 2011,2014; Takayama et al., 2014). SeVdp-iPS can express reprogramming factors stably in various somatic cells. Moreover, this vector can be removed from the iPSCs by targeting viral RdRp with short interfering RNA (siRNA) (Nishimura et al., 2011), which makes this vector a unique tool among other reprogramming strategies.

Although removal of the SeVdp vector with siRNA is very efficient, siRNA transduction to iPSCs is less efficient than to standard cell cultures. Furthermore, transfection of synthetic siRNA is a relatively time- and cost-consuming process, and additional factors should be validated during iPSC production under Good Manufacturing Practice guidelines (Barry et al., 2015). To overcome this, we developed an SeVdp-iPS vector, which can be auto-erased in response to microRNA-302 (miR-302).

MicroRNAs (miRNAs) represent a class of non-coding RNAs that can regulate gene expression primary through mRNA interactions. Brown et al. (2007) reported that the expression of genes inserted in lentiviral vectors can be downregulated by specific miRNAs when miRNA target sequences are incorporated into the 3′ untranslated region (UTR) of these genes. The miR-302 family members are conserved in vertebrates and highly enriched in pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and iPSCs, but not in the differentiated cells (Gao et al., 2015; Landgraf et al., 2007). Previously, miR-302 was shown to specifically inhibit lentiviral vector-mediated transgene expression in PSCs (Brown et al., 2007; Kamata et al., 2010). Therefore, we investigated whether miR-302 may replace the siRNAs, inhibiting RdRp encoded by vector-derived *L* gene.

Here, we describe the characteristics of a novel SeVdp-iPS vector (SeVdp(KOSM)302L) containing miR-302a target sequences at the 3′-UTR of the *L* gene. This vector can be used for the efficient reprogramming of MEFs into transgene-free iPSCs without the aid of siRNAs, observed using human embryonic fibroblasts as well.

2. Materials and methods

2.1. Production of SeVdp vectors

The SeVdp genomic cDNA was constructed as described previously (Nishimura et al., 2011). For the construction of the SeVdp(BG302C) cDNA, four copies of miR-302a target sequence (5′-TCACCAAA ATATGGAAGCACTTACGATTCACCAAAACATGGAAGCACTTAGGTACCTC-ACCAAAAACATGGAAGCACTTACGATTCACCAAAACATGGAAGCACTTA-3′) were inserted into the 3′-UTR of *Cypridina noctiluca* luciferase (Cluc) gene of the SeVdp(BGC). For the construction of the SeVdp(BO)302L and SeVdp(KOSM)302L, the miR-302a target sequences were inserted into the 3′-UTR of *L* gene of SeVdp(BO) (Nishimura et al., 2011) and SeVdp(KOSM) (Nishimura et al., 2014), respectively. Preparation of vector-packaging cells and the production of SeVdp vectors were previously described elsewhere (Nishimura et al., 2011).

2.2. Cell culture and transfection

MEFs and human embryonic fibroblasts (TIG-3) (Matsuo et al., 1982) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) and Minimum Essential Medium Eagle (MEM; Sigma-Aldrich), respectively, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep; Wako, Osaka, Japan). Nanog-green fluorescent protein (GFP) MEFs were isolated from transgenic mice, carrying the Nanog-GFP-IRES-Puro^r reporter construct (Okita et al., 2007), and they were obtained from the RIKEN BioResource Center (Tsukuba, Japan). Mouse (m)ESCs (EB5; RIKEN BioResource Center) and miPSCs (Nishimura et al., 2011) were cultured in mESC medium [DMEM (Nacalai Tesque, Kyoto, Japan) supplemented with 15% FBS (Hyclone, Logan, UT, USA), 0.1 mM non-essential amino acids (NEAA; Thermo Fisher Scientific, Waltham, MA, USA), 55 μM 2-mercaptoethanol (2-ME; Thermo Fisher Scientific), 1% Pen-Strep, and 1000 U/mL leukemia inhibitory factor (LIF; Wako)]. Human (h)iPSCs (454E2; RIKEN BioResource Center) were cultured in the Primate ES cell culture medium (ReproCell, Yokohama, Japan).

To block the activity of miR-302, cells were transfected with 50 nM of the miR-302 seed-targeting 8-mer locked nucleic acid (LNA) oligonucleotide (antimiR-302) or the LNA scramble (Obad et al., 2011) using Lipofectamine RNAi MAX reagent (Thermo Fisher Scientific) a day prior to the infection. The LNA oligonucleotides were synthesized by GeneDesign (Osaka, Japan).

2.3. Luciferase assay

MEFs, mESCs, and miPSCs were infected with the SeVdp(BGC) or SeVdp(BG302C), and treated with blasticidin S (Bs) for 5 days (days 2–6). At day 6, 5×10^5 cells were passaged, and the supernatants were collected 1 day after passage. Cluc activity was determined using Cluc Reporter Assay Kit (ATTO, Tokyo, Japan), according to the manufacturer's instructions.

2.4. Characterization of iPSCs

Detailed methods for iPSC generation, quantitative reverse transcription (RT)-PCR, immunofluorescence staining, and teratoma formation are described in Supplementary Materials and Methods. Animal experiments were carried out in accordance with the protocols approved by University of Tsukuba Ethics Committee for Animal Experiments.

2.5. Statistical analysis

All statistic data presented are representative of at least three independent experiments. Statistical analyses were performed using the Student's *t*-test.

3. Results and discussion

3.1. miR-302 can regulate SeVdp-mediated gene expression in PSCs

To examine whether miR-302 specifically inhibits SeVdp vector-mediated transgene expression in PSCs, we constructed the SeVdp(BG302C) vector containing Cluc and four copies of miR-302a target sequences at its 3′-UTR (Fig. 1). This vector also contains blasticidin-resistance gene (*Bsr*), and a gene for enhanced green fluorescent protein (*EGFP*). We prepared SeVdp(BGC) vector, with the same genomic structure as the SeVdp(BG302C) but without miR-302a target sequences, as a control. MEFs, mESCs, and miPSCs were infected with each of the vectors, and Cluc activities were determined after Bs selection. As shown in Fig. 2A, Cluc activity in mESCs and miPSCs infected with the SeVdp(BG302C) was significantly decreased compared with that in the cells infected with SeVdp(BGC). In contrast to this, both vectors induced comparable Cluc activity in MEFs. EGFP expression was shown to be similar when SeVdp(BG302C) was used to that of SeVdp(BGC) in MEFs and mPSCs (Fig. 2B), indicating that the incorporation of miR-302a target sequences into the SeVdp vector leads to a significant reduction in the expression of desired transgenes in mPSCs but not in MEFs.

We previously demonstrated that the transfection of cells with siRNA targeting the *L* gene inhibits the replication of SeVdp RNA genome in HeLa cells (Nishimura et al., 2011). To examine whether miR-302 blocks the replication of SeVdp genome by suppressing RdRp