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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

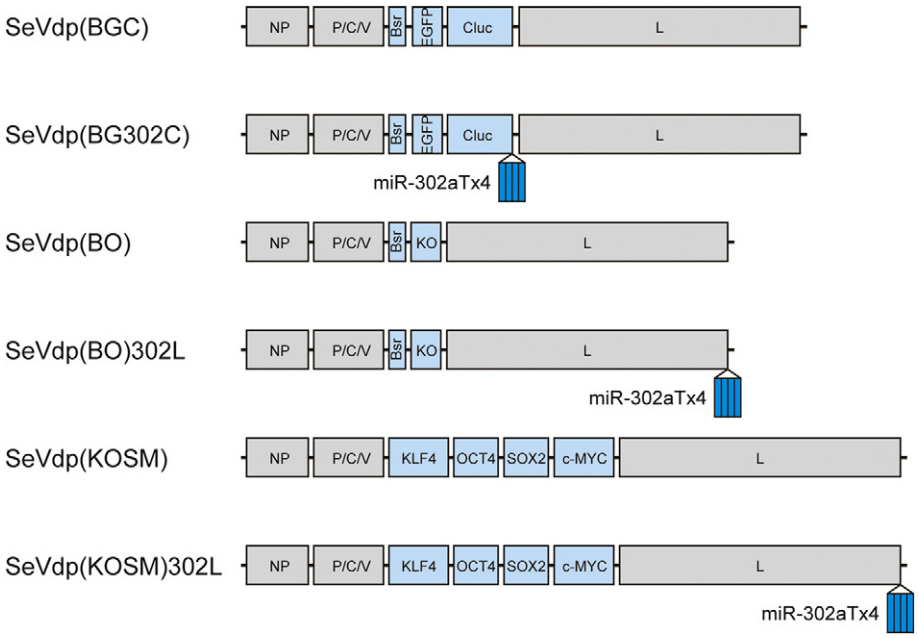

Fig. 1. SeVdp vector structure. The *NP*, *P*, and *L* genes are necessary for the replication of SeVdp RNA genome and transcription. The *P* gene contains multiple open reading frames encoding the P, C, and V proteins. Other genes were inserted into the SeVdp backbone as transgenes.

activity, we prepared SeVdp(BO) and SeVdp(BO)302L, which contained *Bsr* and a Kusabira-Orange (KO) sequence. The SeVdp(BO)302L contained miR-302a target sequences at the 3′-UTR of *L* gene as well (Fig. 1). MEFs, mESCs, and miPSCs were infected with each of the vectors, and KO expression was examined by fluorescence microscopy. Two days after the infection, KO expression was shown to be induced

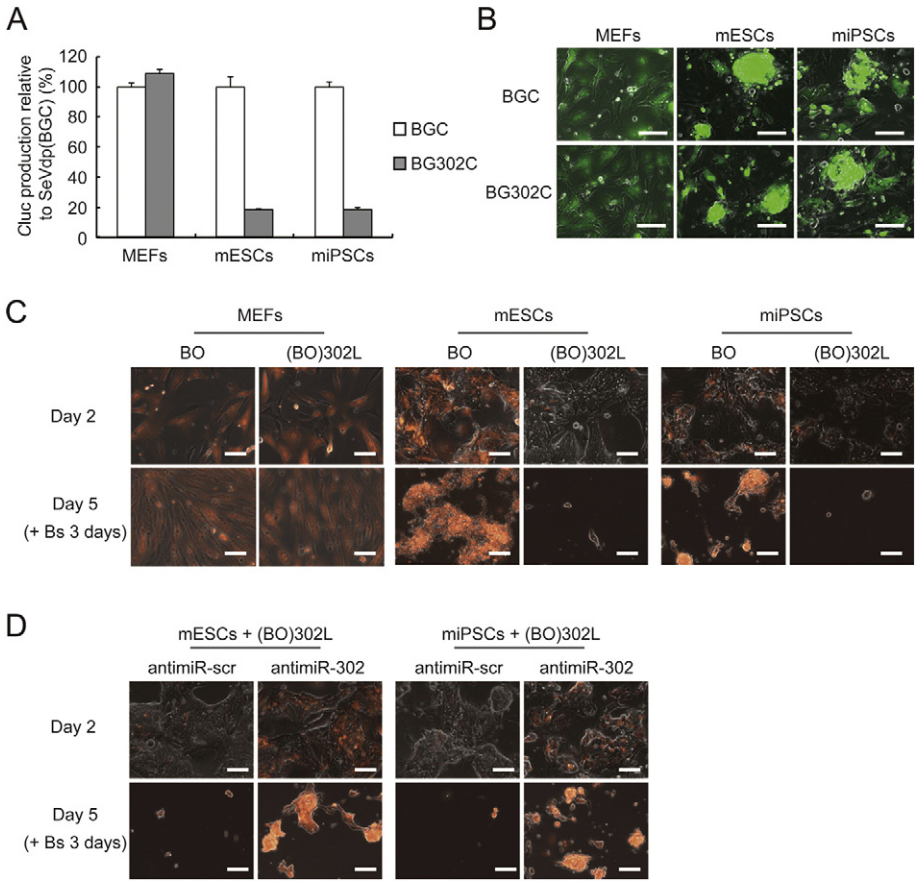

Fig. 2. Regulation of the expression of SeVdp vectors containing miR-302a target sequences. (A) miR-302-mediated inhibition of transgene expression. Cluc activity in the supernatants of the indicated cultures was determined. (B) EGFP expression in SeVdp(BGC)- and SeVdp(BG302C)-infected MEFs, mESCs, and miPSCs was observed 6 days after the infection. Scale bars, 100 μm. (C) miR-302-induced inhibition of the replication of SeVdp genome. The cells were infected with SeVdp(BO) or SeVdp(BO)302L, and treated with Bs at day 2–5. KO expression was observed at day 2 and 5. Scale bars, 100 μm. (D) Recovery of the replication of SeVdp genome by miR-302 inhibition. mESCs or miPSCs were transfected with the antimiR-302 or antimiR-scr prior to the infection with the vectors, and KO expression was observed at day 2 and 5.

in all cells infected with SeVdp(BO) (Fig. 2C). In contrast, SeVdp(BO)302L-mediated KO expression was strongly inhibited in mESCs and miPSCs, but not in MEFs. Additionally, mPSCs infected with the SeVdp(BO)302L were shown to be sensitive to Bs whereas the cells infected with the SeVdp(BO) were resistant to it (Fig. 2C). To examine the specificity of miR-302-mediated suppression of the SeVdp(BO)302L, we transfected mPSCs with a miR-302 inhibitor, antimiR-302, prior to the infection. This significantly restored KO expression and Bs resistance (Fig. 2D), suggesting that miR-302 activity can prevent the replication of SeVdp genome through a specific knockdown of *L* gene in mPSCs.

3.2. An auto-erasable SeVdp vector facilitates the generation of transgene-free miPSCs

We investigated whether miR-302-mediated *L* suppression may facilitate the removal of SeVdp-iPS vector from miPSCs. First, we examined the time course of miR-302a expression after the infection of MEFs with the SeVdp(KOSM), and showed that it can be detected at day 6 after the infection, while its level at day 16 was significantly different from the level determined at day 0 (Supplementary Fig. 1A). Expression analysis of the early-phase reprogramming markers (*Cdh1* and *Fbxo15*) and late-phase markers (*Oct4*, *Esrrb*, and *Nanog*) indicated that miR-302a expression was induced at an early phase of reprogramming and gradually increased with iPSC maturation (Supplementary Fig. 1B).

Afterwards, we prepared SeVdp(KOSM)302L vector containing miR-302a target sequences at the 3′-UTR of *L* gene of the SeVdp(KOSM) (Fig. 1), and examined its characteristics. Nanog-GFP MEFs were infected with the SeVdp(KOSM) or SeVdp(KOSM)302L, and SSEA1-positive (SSEA1(+)) colonies were examined by immunofluorescence staining at day 15 after the infection. In accordance with our previous observations (Nishimura et al., 2014), SeVdp(KOSM) was shown to induce the formation of many SSEA1(+) colonies, however, all colonies expressed SeV NP antigen as well (Fig. 3A). In contrast to this, most colonies infected with SeVdp(KOSM)302L expressed SSEA1, but not NP antigen, indicating that SeVdp(KOSM)302L was efficiently erased from the reprogrammed colonies. Notably, we detected no NP-negative (NP(−)) colonies at day 8 after the infection with SeVdp(KOSM)302L, although the number of the NP(−) colonies considerably increased at day 15 (Supplementary Fig. 2A), suggesting that vector removal depends on miR-302 expression levels (Supplementary Fig. 1A).

We next examined the expression of *Nanog*, a more reliable marker of fully reprogrammed miPSCs, by determining *Nanog* promoter-driven GFP expression levels (Okita et al., 2007). Twenty-five days after the infection with SeVdp(KOSM)302L, a large population of *Nanog*-positive

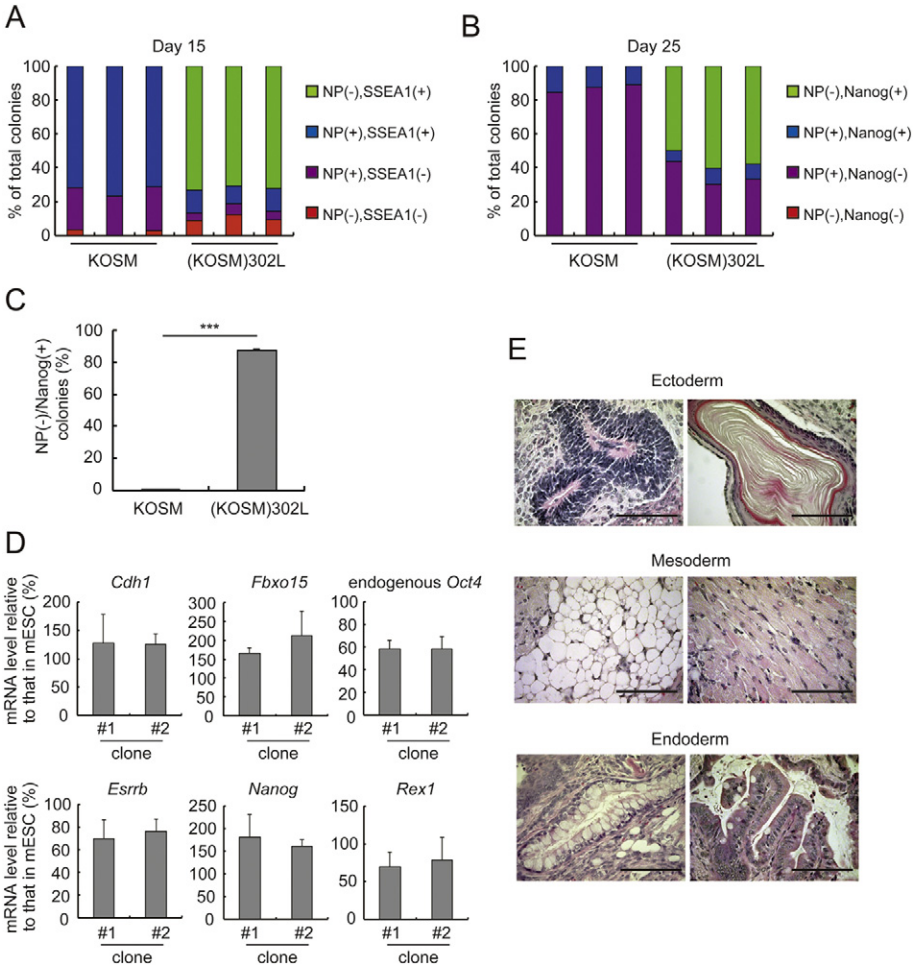

Fig. 3. Generation of vector-free miPSCs using SeVdp(KOSM)302L. (A) Percentage of colonies expressing SeV NP and/or SSEA1. The numbers of NP(−)SSEA1(−), NP(+)SSEA1(−), NP(+)SSEA1(+), and NP(−)SSEA1(+) colonies were counted at day 15, and the percentage of each type of colony in the total number of colonies was obtained in three independent experiments. (B) Percentage of colonies expressing SeV NP and/or Nanog. The numbers of NP(−)Nanog(−), NP(+)Nanog(−), NP(+)Nanog(+), and NP(−)Nanog(+) colonies were counted at day 25, and the results obtained in three independent experiments are presented. (C) Vector-removal efficiency following the reprogramming. Ratio of NP(−) to Nanog(+) colonies at day 25 was calculated. Data are represented as mean ± standard error of the mean (SEM) of three independent experiments. ***$P < 0.005$. (D) Expression of pluripotency markers in miPSC clones generated with the SeVdp(KOSM)302L. miPSC clones (#1 and #2) were isolated, and pluripotency marker expression was determined at day 50. Data are represented as mean ± SEM of three independent experiments. (E) Histological examination of teratomas derived from miPSCs generated using SeVdp(KOSM)302L. Scale bars, 100 μm.

(Nanog(+)) colonies was shown to be generated, unlike in the samples infected with SeVdp(KOSM) (Fig. 3B). Approximately 87% of Nanog(+) colonies were negative for NP antigen when SeVdp(KOSM)302L vector was used (Fig. 3C). However, most cells infected with SeVdp(KOSM) expressed NP antigen and failed to express *Nanog* (Fig. 3B). Interestingly, the number of NP(+)Nanog(+) colonies was considerably lower than that of the NP(+)SSEA1(+) colonies when SeVdp(KOSM) vector was used (Fig. 3A and B), suggesting that the suppression of *trans*-supplying factor expression may be required for the progression to the late phase of reprogramming.

To investigate the characteristics of miPSCs generated using SeVdp(KOSM)302L, Nanog(+) colonies were isolated and cultivated for the additional 20 days. The miPSC clones expressed ESC marker genes at the levels comparable to those determined in the mESCs (Fig. 3D and Supplementary Fig. 2B). Additionally, the clones were shown to differentiate into the derivatives of three germ layers in teratomas (Fig. 3E), indicating that the SeVdp(KOSM)302L-induced miPSCs are as pluripotent as mESCs. We confirmed that the NP-antigen expression was not detected in isolated clones (Supplementary Fig. 2B). Moreover, NP proteins and *NP* and *L* mRNAs were not detected even when the clone was cultured under conditions inducing fibroblast differentiation (Supplementary Fig. 3A, C, and D). Differentiated miPSCs were shown to have a significant reduction in the level of miR-302a (Supplementary Fig. 3B), in comparison with that in the undifferentiated cells, suggesting that elimination of SeV-gene expression was not due to miR-302-mediated suppression of SeV RdRp activity in those cells. These data indicated that SeVdp(KOSM)302L was completely removed from the established miPSCs.

3.3. Generation of transgene-free hiPSCs using the auto-erasable SeVdp vector

We next examined the capacity of the SeVdp(KOSM)302L to reprogram human embryonic fibroblasts (TIG-3 cells) into hiPSCs. The infection of TIG-3 cells with the SeVdp(KOSM) led to the induction of miR-302a expression and the upregulation of ESC marker genes (Fig. 4A and Supplementary Fig. 4A). We infected TIG-3 cells with the SeVdp(KOSM) or SeVdp(KOSM)302L, and the expression of TRA-1-60 and NP antigen was examined by immunofluorescence staining 30 days after the infection. SeVdp(KOSM)302L was shown to induce the formation of NP(−)TRA-1-60(+) colonies, suggesting that this vector can be efficiently removed from hiPSCs after the reprogramming

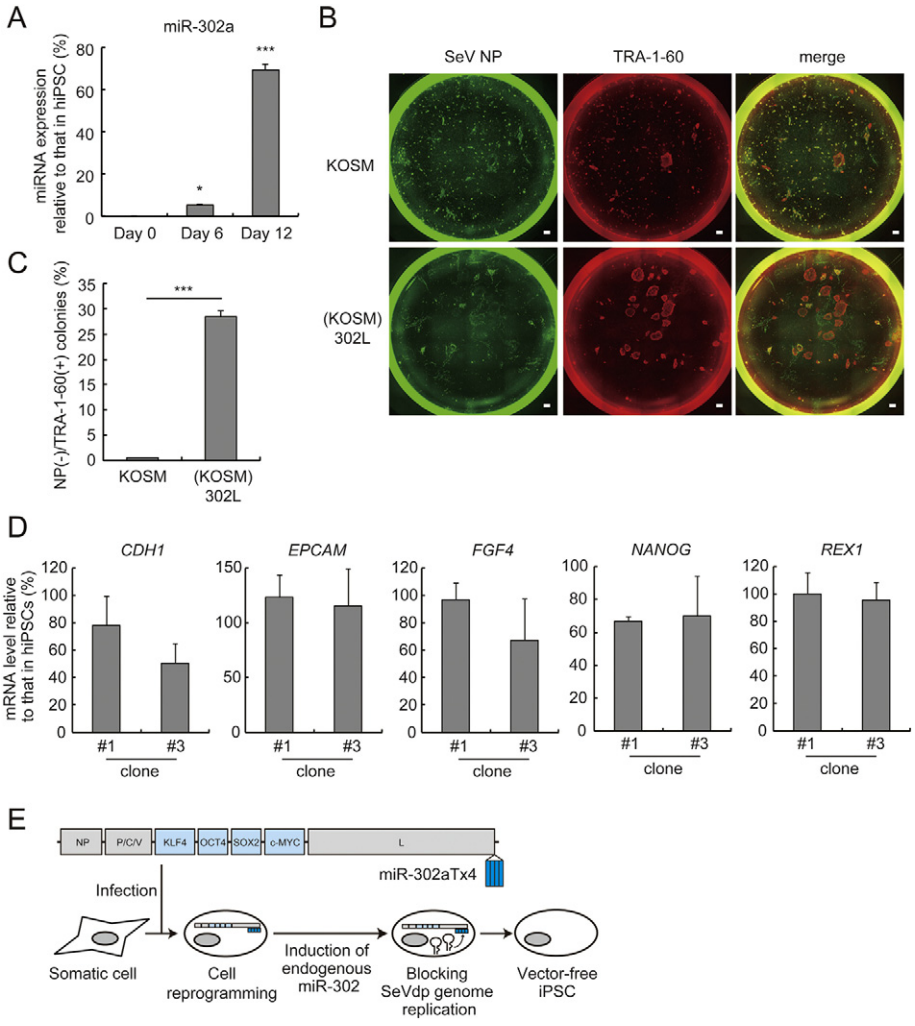

Fig. 4. Generation of vector-free hiPSCs using the SeVdp(KOSM)302L. (A) miR-302a expression in SeVdp(KOSM)-infected TIG-3 cells, at day 0, 6, and 12 after the infection, presented relative to that in the established hiPSCs. Data are presented as mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{***}P < 0.005$, versus day 0. (B) Efficiency of TIG-3 cell reprogramming. SeV NP and TRA-1-60 expression in cells infected with SeVdp(KOSM) or SeVdp(KOSM)302L was determined at day 30 after the infection. Scale bars, 900 μm. (C) Efficiency of vector removal during hiPSC generation. Ratio of NP(−) to TRA-1-60(+) colonies was calculated at day 30. Data are represented as mean ± SEM of three independent experiments. $^{***}P < 0.005$. (D) Expression of pluripotency markers in hiPSCs generated with the SeVdp(KOSM)302L. Gene expression in hiPSC clones was determined after 19 passages (clone #1) or 16 passages (clone #3). Data are represented as mean ± SEM of three independent experiments. (E) Schematic representation of transgene-free iPSC generation using the auto-erasable SeVdp-iPS vector. Introduction of four reprogramming factors induces the expression of miR-302, which binds to the target sequences in the 3′-UTR of *L* gene, suppressing the replication of SeVdp genome, which further leads to the removal of vector from the reprogrammed cells.

(Fig. 4B). In contrast, SeVdp(KOSM) infection significantly induced the formation of TRA-1-60(+) colonies, but most of them remained NP-positive (Fig. 4B). We observed that NP(+) colonies exhibited dome-like shapes (Supplementary Fig. 4B), different from the hESC-like morphology, suggesting that the extended expression of *trans*-supplying reprogramming factors may interfere with hiPSC maturation. Previous reports suggested that transgene silencing is required for the establishment of fully reprogrammed iPSCs in integrative reprogramming systems (Chan et al., 2009). Upon the completion of reprogramming, the expression of transgenes should be replaced with the expression of endogenous counterparts, to acquire pluripotency (Jeanisch and Young, 2008). Therefore, the complete removal of the SeVdp-iPS vector may represent a prerequisite for the generation of fully reprogrammed hiPSCs.

To estimate the efficiency of vector removal, we counted the number of NP(−)TRA-1-60(+) colonies. SeVdp(KOSM)302L led to the generation of more NP(−)TRA-1-60(+) colonies than the SeVdp(KOSM) vector (Fig. 4C). Approximately 28% of SeVdp(KOSM)302L-induced TRA-1-60(+) colonies were shown to be NP(−). Although many SeVdp(KOSM)302L-induced colonies remained NP(+) at day 30, they showed faint and partial staining patterns, and the subsequent passaging and colony selection significantly accelerated the removal of vector. We confirmed that hiPSC clones generated using SeVdp(KOSM)302L expressed hESC marker genes (Fig. 4D and Supplementary Fig. 4C), whereas NP expression was not detected (Supplementary Fig. 4C and D), suggesting that the SeVdp(KOSM)302L vector facilitates the generation of vector-free hiPSCs. Recent reports demonstrated that the SeVdp(KOSM)302L is particularly useful for the generation of transgene-free iPSCs from human peripheral blood cells, including $CD8^+$ T-cells (Nishimura et al., 2013), mucosal-associated invariant T-cells (Wakao et al., 2013), and peripheral blood mononuclear cells (Trokovic et al., 2014).

In this study, we demonstrated that the miR-302-induced inhibition of the replication of SeVdp genome is useful approach for facilitating iPSC generation. Human and mouse iPSCs express a set of unique miRNAs, including miR-290 family (mouse), miR-371-373 (human), and miR-17-92 (mouse and human) (Houbaviy et al., 2003; Suh et al., 2004). These miRNAs are likely to exhibit distinct spatiotemporal expression patterns, indicating a need for the investigation of other ESC-specific miRNAs that may show better efficiency than miR-302 for the rapid removal of the SeVdp-iPS vector during iPSC generation. Further refinement of our strategy should make the SeVdp-iPS vector an easier and more potent tool for the generation of transgene-free iPSCs.

4. Conclusion

Here, we developed a novel SeVdp-iPS vector that can be targeted by miR-302. This vector can efficiently reprogram somatic cells and is automatically removed from the reprogrammed cells when miR-302 expression reaches a level sufficient to block the replication of SeVdp genome (Fig. 4E). Complete inhibition of the SeVdp-iPS vector represents a prerequisite for the promotion of the maturation and proliferation of iPSC colonies. Overall, the auto-erasable SeVdp-iPS vector was shown to allow the effective and easy generation of transgene-free iPSCs, using mouse and human somatic cells. We expect that this approach is applicable in a variety of other cell reprogramming procedures, including direct reprogramming without the initialization to iPSCs, using different sets of reprogramming factors and miRNA target sequences.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2017.06.011.

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Declaration of interest

M.N. is a founder and CTO (Chief Technical Officer) at Tokiwa-bio Inc. The company had no role in study design, data collection and analysis, manuscript preparation, or decision to submit the manuscript for publication.

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