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A Sendai virus-based expression system directs efficient induction of chondrocytes by transcription factor-mediated reprogramming

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Cartilage rarely heals spontaneously once damaged. Osteoarthritis (OA) is the most common degenerative joint disease among the elderly; however, effective treatment for OA is currently lacking. Autologous chondrocyte implantation (ACI), an innovative regenerative technology involving the implantation of healthy chondrocytes, may restore damaged lesions. Chondrocytes for ACI may potentially be induced from differentiated somatic cells using retrovirus (RV)-mediated transduction of three reprogramming factors (SOX9, KLF4, and c-MYC). However, the efficiency of the current induction system needs to be improved and the safety issues arising from the genomic integration of the vector DNA have to be addressed. To solve these problems, we used an RNA vector, termed the replication-defective and persistent Sendai virus vector (SeVdp), to express reprogramming factors for chondrocyte induction. Our results showed that the SeVdp-based vector induced chondrocytes more efficiently than the RV vector, probably because of robust and rapid expression of the transgenes, without any apparent integration of the SeVdp vector. The induced chondrocytes formed cartilage-like tissues when injected subcutaneously into mice. Thus, the SeVdp-based system for inducing chondrocytes may act as a foundation for developing safer and more effective treatments for damaged cartilage.

Keywords Chondrocyte, Direct reprogramming, Sendai virus vector, Osteoarthritis

Osteoarthritis (OA) is a degenerative joint disease that damages the articular hyaline cartilage, predominantly in the elderly, and causes chronic pain that limits mobility in patients with OA. Joint cartilage plays a crucial role in limb movements by absorbing impacts and lubricating the surfaces of joint bones. Owing to its poor vascularity, the capacity of joint cartilage to regenerate when damaged or injured is limited. Although various therapies are available to manage pain and improve mobility, an effective cure for OA is currently lacking. Autologous chondrocyte implantation (ACI) is an innovative technology for repairing cartilage lesions using chondrocytes that have been isolated from patients and expanded ex vivo[1,2]. However, ACI has not been widely used in treating OA, partially because of the limited availability of healthy cartilage for treating patients with OA. Therefore, an alternative source for obtaining chondrocytes in large amounts needs to be developed for enhancing the applicability of ACI in treating OA.

Chondrocyte generation by direct reprogramming is a promising method for obtaining sufficient numbers of chondrocytes for ACI. Chondrocytes can be directly induced from differentiated somatic cells by transducing three reprogramming factors (SOX9, KLF4, and c-MYC)[3,4]. The method depends on the forced expression of the reprogramming factors, KLF4 and c-MYC, together with the chondrocyte-specific transcription factor SOX9, generating chondrocytes directly from somatic cells, such as dermal fibroblasts, without going through the iPSC stage. This reprogramming system enables the generation of large numbers of chondrocytes from cells readily available in patient tissues. Despite these potential advantages, improvements in the efficiency of chondrocyte induction and safety of gene transduction are required before applying direct reprogramming of chondrocytes to ACI.

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In a previous study, we performed alanine scanning mutagenesis of SOX9 and identified a SOX9 variant ($SOX9_{H131A/K398A}$) with potent chondrogenic activity[5]. The use of $SOX9_{H131A/K398A}$ improves the efficiency of chondrocyte induction compared with the original wild-type SOX9. Nevertheless, the induction efficiency remains low, possibly because of the heterogeneous expression of reprogramming factors from retroviral (RV) vectors. Moreover, the use of RV vectors involves genomic integration[6,7], which ensures long-term expression of transduced genes, but also entails a potential risk of accumulating undesired mutations and inappropriate activation of genes, which may eventually lead to malignancy after transplantation[8]. For instance, tumor formation in transplanted iPSCs, possibly caused by multiple RV integrations,[9] and leukemia development after RV-based gene therapy for X-linked severe combined immunodeficiency have been reported [10]. Thus, gene transduction without genomic integration is imperative for the clinical application of chondrocytes generated via direct reprogramming.

RNA viruses that do not integrate into the host genome, including negative-sense single-stranded RNA viruses, provide a safe platform for vectors to transduce exogenous genes into cells. Sendai virus (SeV)-based vectors are attractive options because of their broad cell tropism and low pathogenicity in human cells. SeV vectors have been used for transcription factor-mediated reprogramming to generate iPS cells[11] or cardiomyocyte-like cells[12] free of genomic integration. One of the Sev-based vectors, the replication-defective and persistent SeV vector (SeVdp), originally developed from the non-cytopathic and persistent SeV Cl.151 strain[11,13], remains stable in cells at a non-permissive temperature (38°C) without chromosomal integration. SeVdp vectors not only have excellent transduction efficiency and low cytopathic effects but also express multiple genes stably at high levels from a single vector.

In this study, we used the SeVdp vector to develop a reprogramming system that directly induces chondrocytes from mouse embryonic fibroblasts (MEFs). This SeVdp-based vector expressed reprogramming factors ($SOX9_{H131A/K398A}$, KLF4, and c-MYC) more rapidly and at higher levels than the RV vectors, and induced chondrocytes more efficiently. Genomic polymerase chain reaction (PCR) analysis did not detect integration of the SeVdp vector in the genome of the induced chondrocytes. Cartilage-like tissues were formed in vivo when SeV-induced chondrocytes were subcutaneously transplanted into mice. Thus, the SeVdp-based vector for direct reprogramming will boost further improvements in the development of a safe and efficient chondrocyte induction system.

Results

Sendai virus-based vector directs reprogramming of fibroblasts to chondrocytes

To prepare an SeV vector that expresses the reprogramming factors, SOX9, KLF4, and c-MYC, to induce chondrocytes, we inserted cognate genes and the blasticidin resistance gene (*Bsr*) into an SeVdp vector[11] (Fig. 1a). The SeVdp vector, originally developed from the non-cytopathic strain Cl.151, is capable of stable and long-term expression of multiple transgenes with minimal cytopathic effects[11]. Moreover, instead of wild-type SOX9, we used $SOX9_{H131A/K398A}$ (SOX9m), which has a mutated DNA-binding domain and SUMOylation site, to improve the efficiency of chondrocyte induction[5] (Supplementary Fig. S1). The SeV-based vector was used to infect MEFs (Fig. 1b) and uninfected cells were removed via blasticidin treatment before chondrocyte induction. The SeV vector also induced chondrocytes in mouse dermal fibroblasts (MDFs) and adipose tissue-derived mesenchymal stem cells, which are readily available for regenerative medicine (Supplementary Fig. S2). For comparison, chondrocyte induction from MEFs using RV-based vectors was performed as described previously[5] (Fig. 1b). When MEFs were infected with RV vectors, polygonal or round chondrocytes appeared in clusters on day 5 and expanded further on day 10 (Fig. 1c, *RV S9mKM*). No such morphological changes were observed in cells infected with the empty RV vector (Fig. 1c, *RV EV*). In contrast, MEFs infected with the SeV vector generated similar chondrocyte clusters in greater numbers than those infected with the RV vector (Fig. 1c, *SeV S9mKM*). The cell clusters observed in SeV- or RV vector-infected cells were positively stained with Alcian blue, with more intense staining observed in SeVdp-infected cells, indicating that SeV vector-induced chondrocytes secreted extracellular matrix more abundantly (Fig. 1d). These data indicated that the SeV vector induced chondrocytes more efficiently than the RV vectors used in our previous study.

SeV-based reprogramming vector induces chondrocytes rapidly and efficiently

To quantitatively assess the molecular mechanism via which the SeV vector efficiently induced chondrocytes, we analyzed the levels of reprogramming factors expressed from each vector during chondrocyte induction. We first performed immunofluorescence staining of RV- and SeV-infected cells using antibodies against the FLAG tag (FLAG-tagged SOX9m), KLF4, and c-MYC. These reprogramming factors were expressed at low levels in a small fraction of RV-infected cells on day 2 but showed higher expression levels in a large number of cells on days 5 and 10 (Fig. 2a and Supplementary Fig. S3a, *RV S9mKM*). As compared to RV-infected cells, SeV-infected cells expressed SOX9m, KLF4, and c-MYC on day 2 at a higher level in a larger fraction of cells, and high expression levels of the reprogramming factors were maintained on days 5 and 10 (Fig. 2a and Supplementary Fig. S3a, *SeV S9mKM*). Importantly, SOX9m and KLF4 expression was more homogenous in SeV-infected cells than in RV-infected cells (Fig. 2a, *merge*). Western blot analysis also showed that the level of SOX9m expression from the SeV vector was high on days 2 and 5 and decreased gradually after day 5. In contrast, SOX9m expression from the RV vector was detectable on day 5, but was lower than that from the SeV vector and decreased slightly on days 10 and 15 (Fig. 2b). Similar expression patterns were observed for KLF4 (Supplementary Fig. S3b). These data suggest that stronger and more rapid expression of reprogramming factors by the SeV vector than by the RV vectors may be critical for the efficient induction of chondrocytes.

Consistent with the strong and rapid expression of SOX9m, KLF4, and c-MYC (Fig. 2a, b, and Supplementary Fig. S3), SeV infection upregulated the expression of chondrogenic genes (*Col2a1*, *Col11a2*, *Acan*, *Sox5*, and *Sox6*) on day 5 to levels comparable with those upregulated in RV-infected cells on day 10 (Fig. 2c). The expression

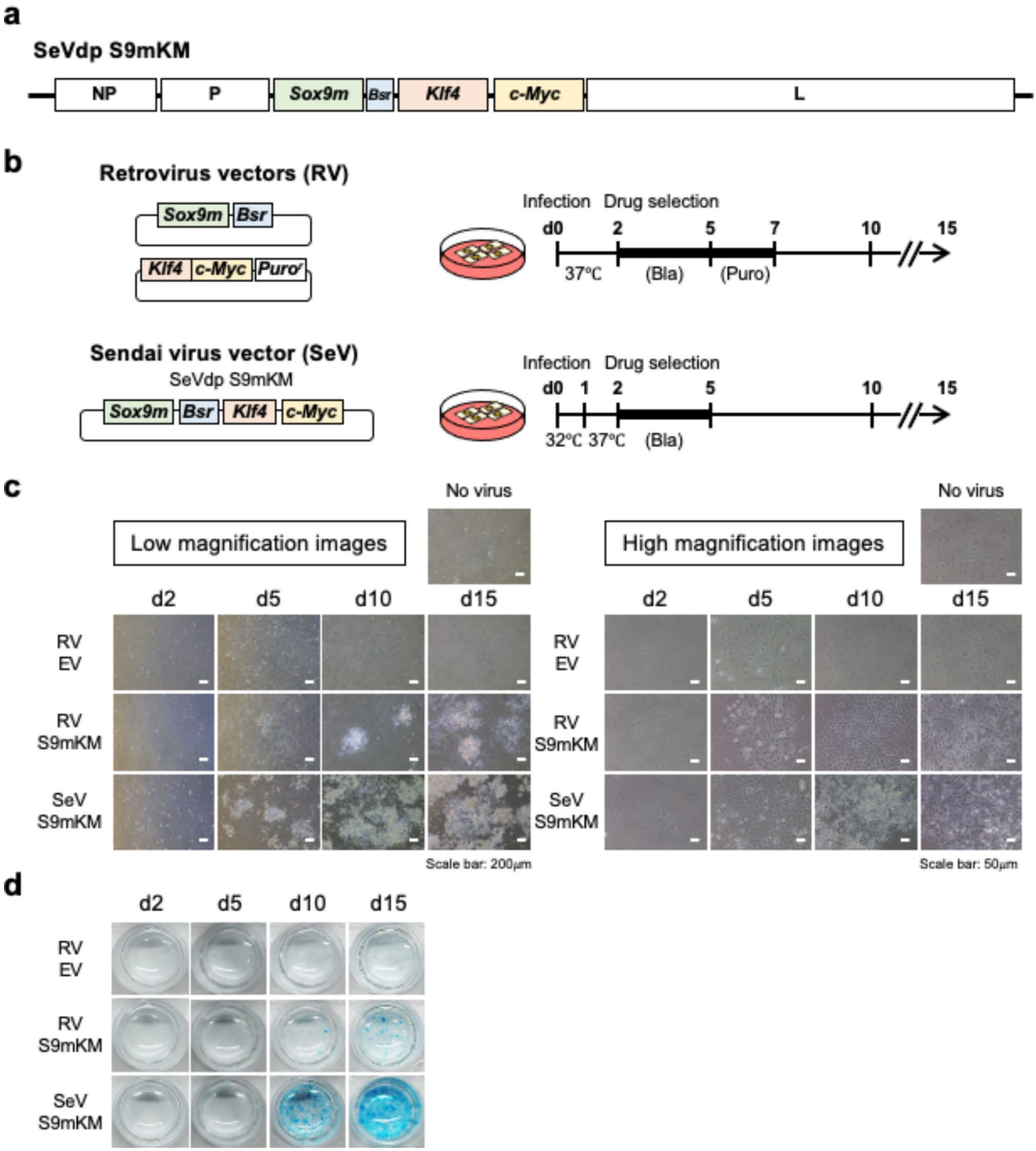

Fig. 1. Sendai-virus-based direct reprogramming system for chondrocytes. (**a**) Structure of the Sendai virus (SeV) vector for inducing chondrocytes. Complementary DNAs encoding $SOX9_{H131A/K398A}$ (SOX9m), blasticidin-resistant gene (Bsr), KLF4, and c-MYC were inserted into the replication-deficient persistent SeVdp vector as shown. (**b**) Structures of the retrovirus vectors and Sendai virus vector used in this study. Experimental schemes for inducing chondrocytes using the retorivirus vectors or the Sendai virus vector are shown. (**c**) Morphological changes of virus-infected MEFs 2, 5, 10, and 15 d after infection with the empty retrovirus vector (RV EV), retrovirus vectors with the direct reprogramming factors (RV S9mKM), or Sendai virus vector with the direct reprogramming factors (SeV S9mKM). (**d**) Alcian blue staining of the differentiated chondrocytes.

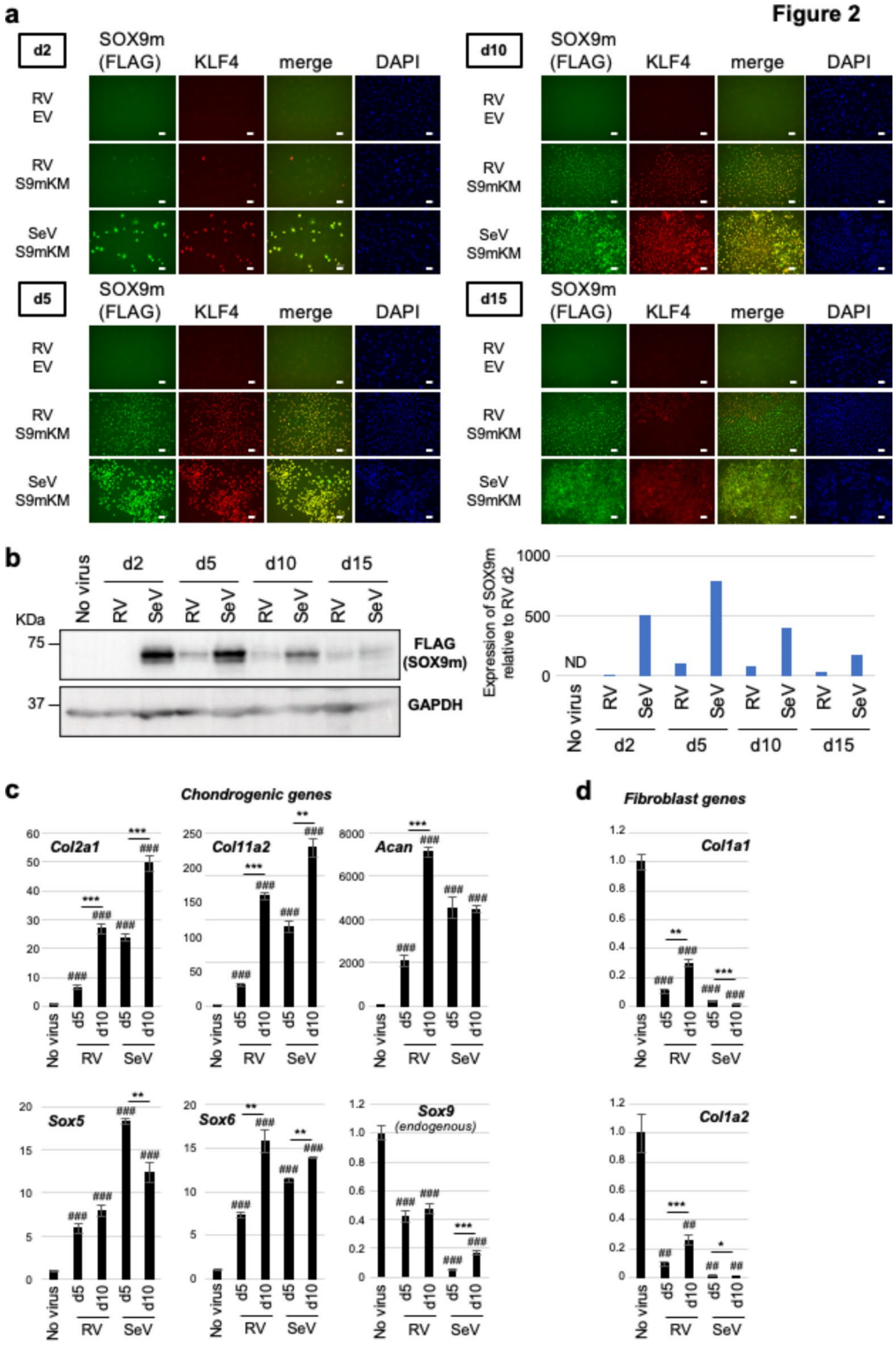

Fig. 2. Rapid and strong expression of the direct reprogramming factors by the Sendai virus vector. (**a**) Immunofluorescence staining of the retrovirus (RV)- or Sendai virus (SeV)-infected cells using an anti-FLAG tag ($Sox9_{H131A/K398A}$) antibody and an anti-KLF4 antibody. (**b**) Western blotting analysis of $Sox9_{H131A/K398A}$ expression in RV- or SeV-infected cells. Quantified data were normalized to GAPDH levels and are shown in the graph on the right. **(c)** Expression levels of the chondrogenic genes in RV- or SeV-infected cells. $n = 3$. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}$ represents a significant difference versus uninfected cells (No virus) ($^{\#\#\#}p < 0.001$). (**d**) Expression levels of the fibroblast genes in RV- or SeV-infected cells. $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}$ represents a significant difference versus uninfected cells ($^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$).

of endogenous Sox9 correlated inversely with the levels of vector-derived SOX9m (Fig. 2b, c). In addition, fibroblast-related genes (*Col1a1* and *Col1a2*) were significantly repressed 5 days after viral infection in both RV- and SeV-infected cells; however, these fibroblast genes were derepressed on day 10 in RV-infected cells, possibly indicating the dedifferentiation of chondrocytes in 2D culture conditions[14]. In contrast, the derepression of fibroblast genes was not observed in SeV-infected cells (Fig. 2d). Together, these results suggest that the strong and rapid expression of reprogramming factors from the SeV vector enables efficient induction of chondrocytes and may prevent dedifferentiation of the induced chondrocytes.

SeV-based reprogramming vector induces chondrocytes in three-dimensional (3D) pellet culture

We then tested SeV-mediated chondrocyte induction in 3D culture, a more physiologically relevant culture system that allows extensive cell-cell interactions that mimic the native in vivo environment of cartilage. SeV-infected cells were selected with blasticidin and then cultured as a pellet, either in standard cell culture medium (Dulbecco's modified Eagle medium [DMEM] plus 10% fetal bovine serum) or chondrogenic medium that included TGFβ and GDF5 (Fig. 3a). Regardless of the medium used (Fig. 3b), the SeV-infected cells expressed chondrogenic genes (*Col2a1*, *Col11a2*, and *Acan*) at significantly higher levels than the uninfected control cells, and repressed fibroblast gene expression (*Col1a1* and *Col1a2*) to almost undetectable levels (Fig. 3c). In addition, there was no significant increase in the expression of the hypertrophic chondrocyte gene, *Col10a1*, in SeV-infected cells (Fig. 3d). Moreover, the expression of *Mmp13* was significantly downregulated in SeV-infected cells (Fig. 3d). These data show that the 3D pellet culture of the SeV vector-infected cells efficiently induce chondrocytes and may minimize the requirement for growth factors, such as TGFβ and GDF5.

SeV-based reprogramming vector does not integrate into the genome of induced chondrocytes

RV-based vectors are integrated into the host genome and may cause insertional mutations or malignant transformation of host cells, posing a potentially serious risk to cell-based therapies. In contrast, the SeV vector genome is a negative-sense single-stranded RNA that resides in the cytoplasm and replicates via an RNA intermediate (positive-stranded anti-genomic RNA). Theoretically, because of this unique replication mechanism, SeVs and their derived vectors are not believed to integrate into the host genome. To confirm that the SeV vector-derived sequence was not present in the genome of the induced chondrocytes, we performed PCR analysis of the genomic DNA of the induced chondrocytes (Fig. 4a). As shown in Fig. 4b, most SeV-infected cells expressed high levels of the viral NP protein, and the SeV RNA was detected at the expected level ($\sim 4 \times 10^4$ copies per cell)[13]. Genomic PCR using a primer set specific for the virus-specific FLAG-tagged *Sox9* detected PCR products of the expected size in the RV-infected cells but not in the SeV-infected or uninfected cells. As a control, the cellular gene encoding glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), was detected in the genomes of both RV- and SeV-infected cells, and the RV and SeV vector plasmids generated PCR products of the same size (Fig. 4c). These data indicated that the SeV vector-derived sequence was not integrated into the genome of the induced chondrocytes.

Chondrocytes induced by the SeV-based reprogramming vector form cartilage-like tissuesin vivo

To validate the ability of the SeV-induced chondrocytes to form cartilage in vivo, we injected SeV-infected cells into the subcutaneous space on the back of immunodeficient mice. Three weeks after the injection, the mice were euthanized and the transplants were isolated (Fig. 5a). Alcian blue staining of the tissue sections revealed chondrocytes in the lacunae surrounded by abundant extracellular matrix, which was consistent with the histological features observed in the cartilage (Fig. 5b). Notably, some areas of the cartilaginous tissue were populated with an array of cells that formed a zone-like structure reminiscent of chondrocytes undergoing endochondral ossification (Fig. 5b, *right lower panel*). As expected, the transplants showed significant induction of chondrogenic genes (Fig. 5d) and type II collagen, but not type I collagen (Fig. 5c). The expression levels of chondrogenic genes were higher than those in the mouse cartilage (Fig. 5d), probably because of the strong transcriptional induction by SeV-derived reprogramming factors. These results demonstrated that SeV-induced chondrocytes retained their ability to form cartilage-like tissues in vivo

Discussion

In this study, we developed an SeV vector that directly induces chondrocytes from MEFs by simultaneously expressing three reprogramming factors ($SOX9_{H131A/K398A}$, KLF4, and c-MYC) and showed that the newly developed SeV vector was superior to our previous RV vector in inducing chondrocytes. First, the SeV-based vector induced chondrocytes more efficiently than the RV vectors (Figs. 1 and 2), which is consistent with the high efficiency of gene delivery and robust expression of reprogramming factors by SeV-based vectors reported for reprogramming somatic cells into iPSCs[11] and fibroblasts directly into cardiomyocytes[11,12]. The high expression levels of reprogramming factors from SeV vectors are possibly due to their high copy number (10^4–10^5 copies per cell)[13] compared to only ~ 10 copies per cell of RVs[15]. Second, the SeV vector achieved more immediate expression of reprogramming factors than our previous RV vectors. This is presumably because SeVs possess an RNA-dependent RNA polymerase that instantaneously initiates transcription and replication in the cytoplasm[16]. In contrast, RVs undergo reverse transcription and integration into the host genome[7], where viral gene expression typically occurs after host cell division[17], which is entirely dependent on the cellular transcriptional machinery. Third, the SeV vector showed a relatively uniform expression of transgenes[11], which ensured the production of a more homogenous population of induced chondrocytes. SeV vectors incorporate multiple exogenous genes into a single vector, from which the genes are expressed in a relatively constant stoichiometry. In addition, the

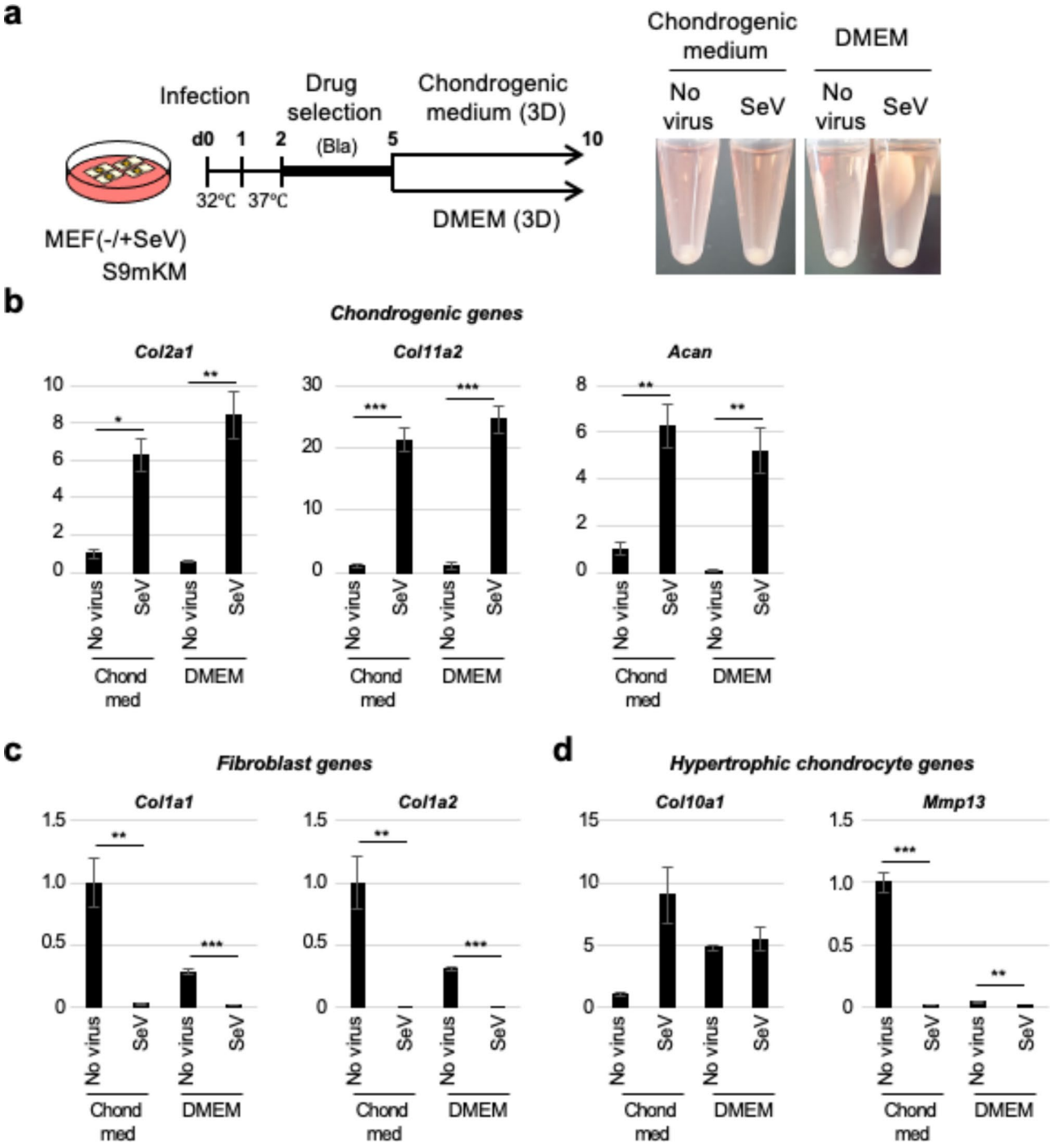

Fig. 3. Three-dimensional pellet culture of SeV-induced chondrocytes. (**a**) Experimental scheme for chondrocyte induction from SeV-infected cells in three-dimensional (3D) pellet culture. The right panels show macroscopic observations of the SeV-infected cells on day 10. (**b**) Expression of chondrogenic genes in the 3D pellet cultured cells. $n = 2 \sim 4$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (**c**) Expression of fibroblast genes in the 3D pellet cultured cells. $n = 3 \sim 4$. $^{**}p < 0.01$; $^{***}p < 0.001$ (**d**) Expression of the hypertrophic chondrocyte genes in the 3D pellet cultured cells. $n = 2 \sim 4$. $^{**}p < 0.01$; $^{***}p < 0.001$.

levels of transgene expression among the infected cells varied to a lesser extent than those expressed by the RV vectors, for which the expression varied widely with the integrated genomic sites. Overall, the newly developed SeV vector enables the efficient induction of a more homogenous population of chondrocytes in a shorter period of cell culture and is therefore better suited for the scalable production of chondrocytes.

The presence of integrated transgenes in the genome is an obvious cause of concern when transcription factor-induced chondrocytes are used in regenerative medicine, largely because of their potential to form tumors

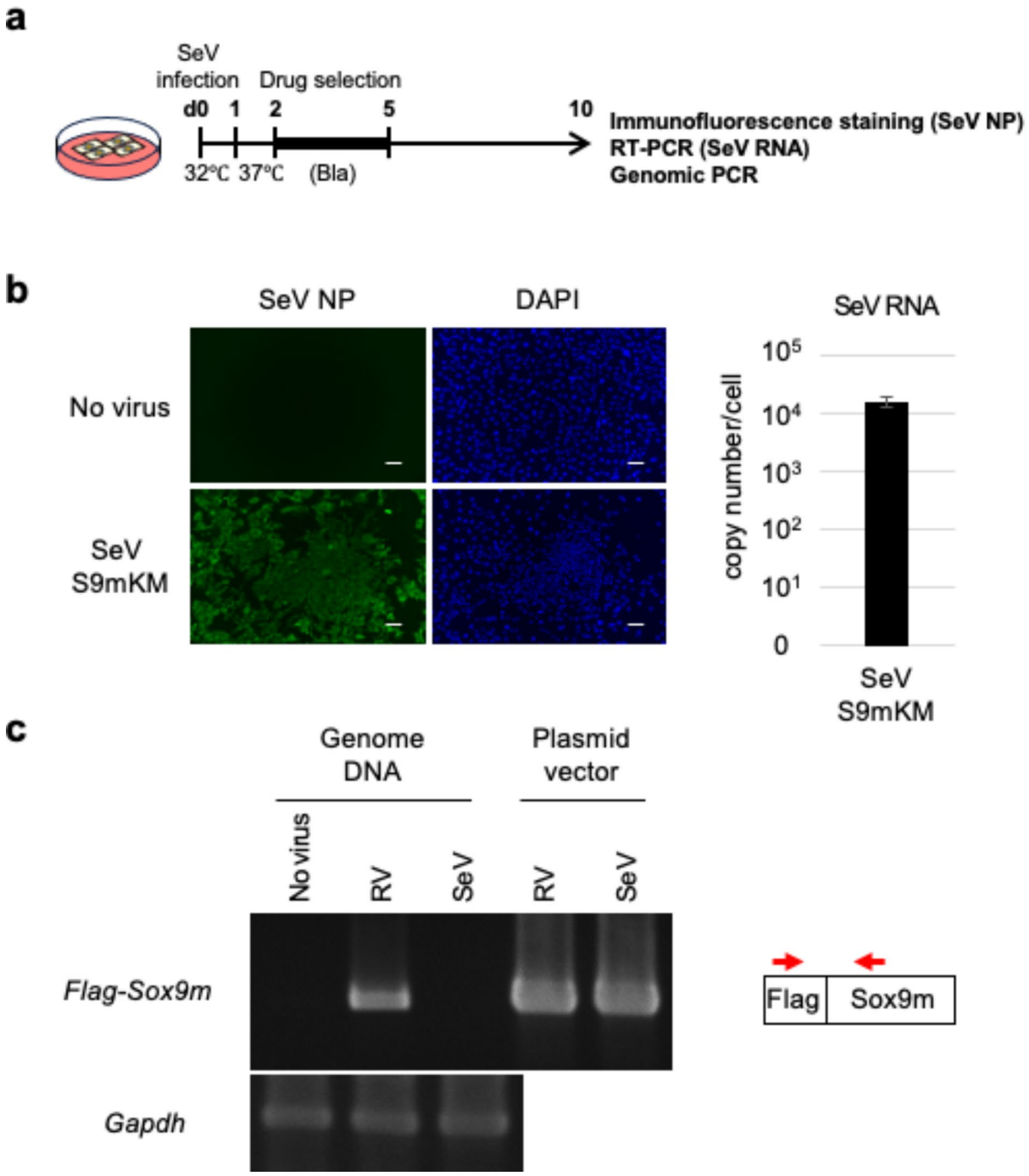

Fig. 4. No integration of the SeV vector in induced chondrocytes. (**a**) Experimental scheme for chondrocyte induction and genomic PCR. (**b**) Detection of the SeV NP protein using immunofluorescence staining (left panel) and SeV RNA using RT-qPCR (right panel) in SeV-infected cells. Scale bar: 100 μm. (**c**) PCR analysis of the virus vector-derived sequence (FLAG-tagged Sox9m) in genomic DNA prepared from noninfected, RV-infected, or SeV-infected cells. The position of the primers used for genomic PCR is shown in red arrows.

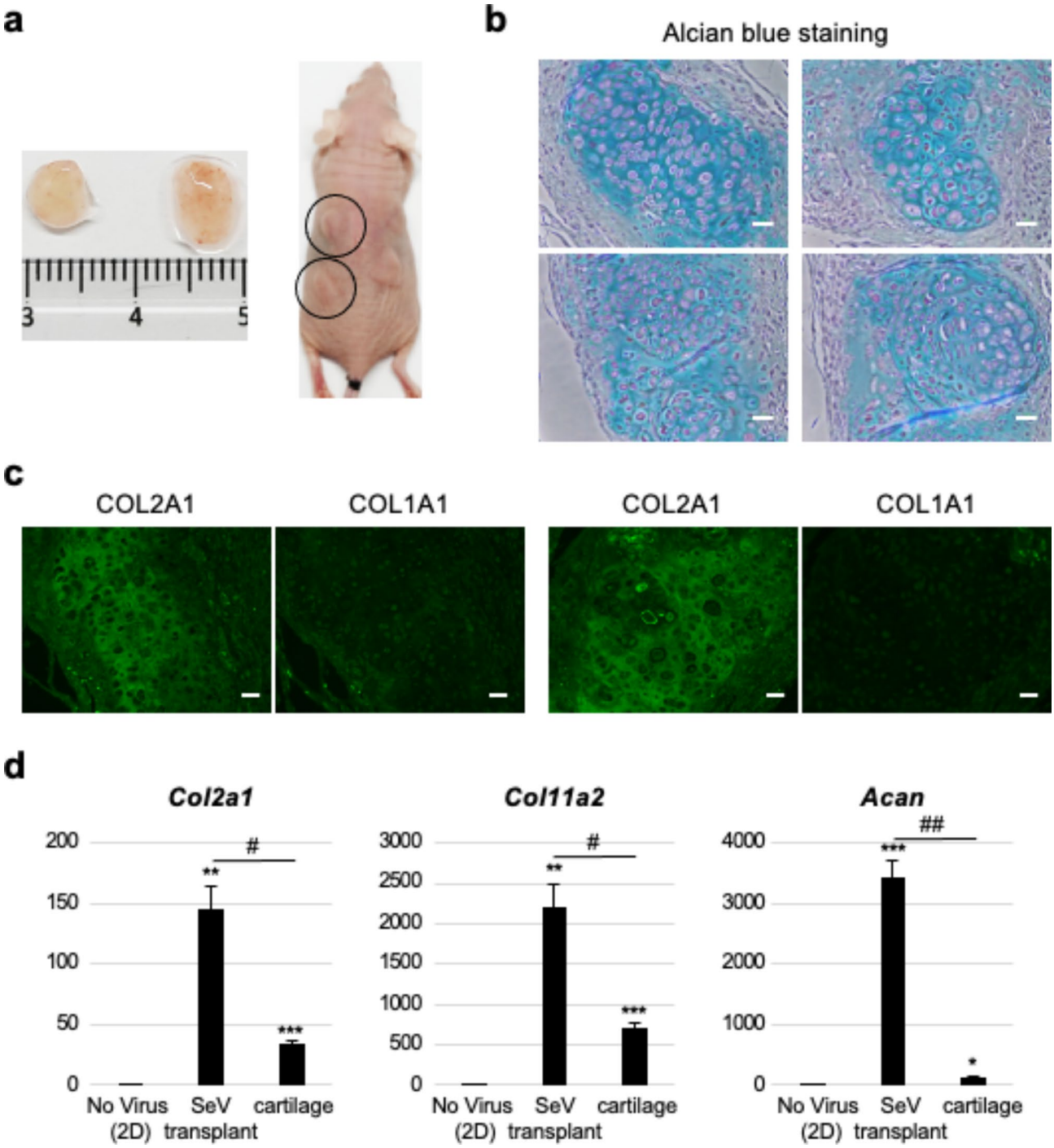

Fig. 5. In vivo cartilage formation from SeV-induced chondrocytes. (**a**) Formation of cartilaginous tissues from the transplanted SeV-induced chondrocytes in mice. (**b**) Alcian blue staining of the tissue sections. Scale bar: 25 μm (**c**) Expression of chondrogenic genes in the cartilaginous tissues generated from SeV-induced chondrocytes. $n = 2$. $^{**}p < 0.01$; $^{***}p < 0.001$.

after transplantation. This is particularly true when the reprogramming system requires *c-Myc* and *Klf4*, which act as oncogenes[18,19]. Indeed, chondrocytes induced by RV vector-directed reprogramming have been reported to generate tumors after transplantation[3]. Considering that SeV is a negative-sense single-stranded RNA virus (NSRV), it is generally believed that SeV does not integrate into the host genome. However, some reports have suggested that RNA viruses that do not generate DNA intermediates for replication can accidentally integrate into the host genome[20–22]. As shown in Fig. 4c, we did not observe integration of the current SeV vector into the genome of induced chondrocytes, at least during the period required for cell culture, indicating that the current SeV vector poses little concern for integration into the host genome.

Despite these substantial advantages of SeV vectors over RV vectors, certain limitations must be overcome to further improve the transcription factor-mediated induction of chondrocytes. As shown in Fig. 5b, some chondrocytes appeared enlarged, possibly indicating hypertrophic changes due to differentiation via the endochondral ossification pathway. Articular chondrocytes in osteoarthritic lesions often display a hypertrophic phenotype that is believed to play a causative role in OA progression. Although gene expression analyses indicated that SeV-induced chondrocytes did not display any evident gene expression signature of hypertrophic chondrocytes, they may easily respond to external cues to become hypertrophic. If these chondrocytes are implanted into an osteoarthritic lesion, they can exacerbate rather than ameliorate OA. To prevent this untoward hypertrophic differentiation, an SeV vector carrying a dominant negative form of a transcription factor (such as *Runx* or *Osterix*) that plays crucial roles in chondrocyte hypertrophy and subsequent differentiation into osteocytes may be helpful[23,24].

Another limitation of current SeV vectors is their immunogenicity. Although the SeV vector used in this study lacks envelope-related genes and largely escapes the innate immune response, it still elicits an acquired immune response[25] that could eliminate transplanted cells in vivo. For instance, in immunodeficient mice, transplanted SeV-infected cells remain intact for at least 60 days; however, they disappear after only a few days in immunocompetent mice, even when immunosuppressants are administered[26]. Therefore, removal of the SeV vector from induced chondrocytes before implantation may be beneficial for the long-term preservation of implanted cells. Possible methods for removing SeV vectors from reprogrammed cells include the use of a vector backbone derived from temperature-sensitive mutations[27], treatment of reprogrammed cells with an siRNA targeting the SeV L gene[11], inclusion of a target sequence of cell-type-specific miRNAs,[28] or installation of a Csy4 endoribonuclease-based system in the vector[29,30]. The incorporation of these technologies into the current vector may lead to the development of an SeV vector that generates chondrocytes free of the SeV vector initially used to induce them.

In summary, the SeV vector-based direct reprogramming system reported in this study not only represents a considerable advancement in generating induced chondrocytes for cell therapy but also provides a solid foundation for further improvements in the scalable production of vector-free chondrocytes.

Methods

Preparation of viral vectors

The recombinant DNA experiments performed in this study were approved by the Recombinant DNA Experiment Committee of the University of Tsukuba (approval numbers: 190121, 210260, and 220144). Complementary DNAs encoding human $SOX9_{H131A/K398A}$, *KLF4*, *c-MYC*, and blasticidin resistance genes were inserted into the SeVdp vector[11] (Fig. 1a). Using the SeVdp vector and expression vectors for SeV proteins, a virus-containing culture medium was prepared as described previously[11], except that NIH3T3 cells were used to determine the titer. The retroviral vectors were prepared as described previously[5].

Induction of chondrocytes using the SeV-based direct reprogramming system

MEFs were prepared from C57BL/6J mouse embryos as described previously[31]. MEFs were cultured at 37℃ in an atmosphere of 5% O_2 and 5% CO_2 in DMEM containing 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (hereafter referred to as standard medium). The MEFs were seeded in a 24-well plate at a density of 4×10^4 cells/well and were infected with SeV on the next day at a multiplicity of infection (MOI) of approximately 1.0 for 24 h at 32℃ in an atmosphere of 5% CO_2. The virus-infected cells were then cultured at 37℃ in an atmosphere of 5% O_2 and 5% CO_2 for 24 h. The uninfected cells were removed by culturing in standard medium containing 8 μg/mL blasticidin for 3 d. The surviving cells were further cultured at 37℃ in an atmosphere of 5% O_2 and 5% CO_2 in standard medium without blasticidin, and the medium was changed every 2 d. The chondrogenic medium was DMEM containing 1% (v/v) fetal bovine serum, 10 ng/mL GDF5 (Biolegend, San Diego, CA, USA; 779506), 10 ng/mL TGF-β1 (Biolegend, 580702), 50 μg/mL L-ascorbic acid 2-phosphate (Sigma, St Louis, MO, USA; A8960), 0.1 μM dexamethasone (Nacalai Tesque, Inc., Tokyo, Japan; 11107-64), 1 mM sodium pyruvate (Nacalai Tesque, Inc.; 06977-34), and ITS (Thermo Fisher Scientific Inc., Waltham, MA, USA; 41400045). Phase-contrast images of the cells were acquired using a Nikon ECLIPS TS100 microscope.

Preparation of stromal vascular fraction (SVF) cells derived from inguinal white adipose tissue (iWAT) and mouse dermal fibroblasts (MDFs)

iWAT was isolated from a 5-month-old female mouse and was minced. The minced iWAT was dispersed using collagenase (Roche, ~ 1.5 units/g tissue) and dispase II (Roche, ~ 2.4 units/g tissue) in phosphate buffered saline (PBS) containing 10 mM $CaCl_2$ (~ 1 ml/g tissue) at 37℃ for 1 h. The dispersed cells were centrifuged at 700 × *g* for 10 min, and the pellet was isolated as SVF cells. The SVF cells suspended in standard medium were filtered through a 70 μm strainer and cultured at 37℃ in an atmosphere of 20% O_2 and 5% CO_2. To isolate MDFs, the hair around the chest of the same female mouse was shaved, and the exposed skin was removed and minced. The skin tissue was dispersed using collagenase and dispase II, and the dispersed cells were filtered and cultured as described for the SVF cells. Chondrocytes were induced using the same method as used for MEFs, except that the cells were cultured in the presence of 20% O_2 instead of 5% O_2.

Alcian blue staining

The induced chondrocytes were washed with PBS and fixed in methanol at room temperature for 2 min. After removing methanol, the cells were washed using 0.1 M HCl and stained with Alcian blue staining solution (pH 2.5, Nacalai Tesque, Inc.) at room temperature for approximately 60 min. The cells were washed with 0.1 M HCl.

Immunofluorescence staining

The virus-infected cells were washed with PBS and fixed using 10% formalin (v/v) at room temperature for 10 min. Then, the cells were washed with PBS, treated with 50 mM NH_4Cl at room temperature for 10 min, treated with 0.1% (w/v) Triton X-100 at room temperature for 5 min, and washed with PBS. The cells were then incubated with the primary antibody in 0.1% (w/v) saponin/PBS at room temperature for 60 min. The cells were washed with PBS and incubated with fluorescent-labeled secondary antibodies in 0.1% (w/v) saponin/PBS at room temperature for 60 min. After washing with 0.1% (w/v) saponin/PBS, the cells were treated with one drop of VECTASHIELD containing DAPI (Nacalai Tesque, Inc.). Fluorescent images were acquired using an All-in-One Fluorescence Microscope BZ-710 (Keyence corp.). The antibodies and dilutions used for immunofluorescence staining are listed in Supplementary Table S1.

Immunoblotting

MEFs were seeded in 60 mm plate at a density of 4×10^5 cells/plate and infected with RVs or SeV. The virus-infected cells were collected 2, 5, 10, and 15 days after virus infection and suspended in radioimmunoprecipitation assay buffer containing protease inhibitor cocktail, 1 μM MG132, and 0.5 mM phenylmethylsulfonyl fluoride (approximately 5×10^5 cells/50 μL). After brief sonication using Bioruptor II (30 s ON-30 s OFF, two cycles, power: high; BM Equipment Co., Ltd., Tokyo, Japan), the insoluble fraction was removed via centrifugation at 20,000 × *g* at 4℃ for 5 min. The cell extracts (approximately 28 μg protein each) were used for immunoblotting with an anti-DYKDDDDK (Flag-SOX9) antibody (1:1,500 dilution). Chemiluminescent images were acquired using a Fusion FX7. EDGE (M&S Instruments Inc.) and quantification were performed using the Evolution Capt software, and the original images of the blots are shown in Supplementary Fig. S1.

Reverse transcription and quantitative PCR (RT-qPCR)

Total RNA was extracted from the induced chondrocytes and RT-qPCR was performed as described previously[5]. The primer sets used for qPCR are listed in Supplementary Table S2. *P*-values were calculated using the Student's t-test. To quantify the SeV genomic RNA, total RNA was purified from virus-infected cells and used for RT-qPCR. The amount of SeV genomic RNA was calculated based on a standard curve constructed using the plasmid DNA vector used for the SeV preparation.

Genomic PCR

Chondrocytes were induced by RV or SeV in a 12-well plate (approximately 10^5 cells/well) for 10 d and lysed using 100 μL of lysis buffer (50 mM Tris-HCl, pH 7.5, 1% SDS, 20 mM EDTA, 0.1 M NaCl) containing 50 μg/mL RNase A at 37℃ for 15 min. Proteinase K (2 μg) was added and the cells were incubated at 55℃ for 5 h. Genomic DNA was extracted using 100 μL of phenol/chloroform and precipitated by adding 100 μL of 2-propanol. After centrifugation at 15,500 × *g* at 4℃ for 7 min, the pellet was rinsed with 70% ethanol. The pellet was dissolved in 10 μL of TE (pH 8.0). For genomic PCR, 10 ng of genomic DNA or 1 ng of the plasmid vector was used as the template. To detect the virus vector-derived sequence (Flag-tagged Sox9m-coding gene), PCR was performed using KOD-plus-Neo (TOYOBO) with a two-step cycle (94℃, 2 min > [98℃, 10 s; 68℃, 30 s] × 35 cycles > 4℃). To detect *Gapdh*, PCR was performed using a three-step cycle (94℃, 2 min > [98℃, 10 s; 58℃, 30 s; 68℃, 20 s] × 35 cycles > 4℃). The primers used for PCR are listed in Supplementary Table S3. The PCR products were analyzed by electrophoresing on a 1% agarose gel. Original images of the gels are shown in Supplementary Fig. S2.

Transplantation of SeV-induced chondrocytes in mice

The animal experiments performed in this study were approved by the Animal Experimental Committee of the University of Tsukuba (approval numbers: 22–488 and 23–312). All experiments were performed in accordance with the relevant guidelines, regulations, and ARRIVE guidelines. MEFs were infected with SeV at an MOI approximately 1.0 in 100 mm plates, and uninfected cells were removed by treating with blasticidin (8 μg/mL) for 3 d. The virus-infected cells were cultured without blasticidin for 1 d and transplanted subcutaneously into the backs of immunodeficient mice (BALB/cAJc1-nu/nu, 6 weeks old, female). The mice were euthanized via cervical dislocation under isoflurane anesthesia 3 weeks after transplantation and the grafts were isolated. Paraffin sections were prepared from the grafts and stained using Alcian Blue. Immunofluorescence staining of paraffin-embedded sections was performed according to standard protocols using anti-COL2A1 or anti-COL1A1 antibody (Santa Cruz Biotechnology). Total RNA was extracted from the grafts and gene expression was analyzed using RT-qPCR.

Data availability

The data underlying this article are available from the corresponding author on reasonable request.

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Author contributions

J.Z., A.F., and Y.S. performed the experiments and analyzed the data. M.S. and K.N. provided experimental resources. A.F. managed the project and wrote the first manuscript draft. K.H. supervised the study and edited the manuscript. All the authors have reviewed and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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