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Site-specific recombinase strategy to create iPS cells efficiently with plasmid DNA

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Abstract

Induced pluripotent stem cells (iPSC) have revolutionized the stem cell field. iPSC are most often produced by using retroviruses. However, the resulting cells may be ill-suited for clinical applications. Many alternative strategies to make iPSC have been developed, but the non-integrating strategies tend to be inefficient, while the integrating strategies involve random integration. Here we report a facile strategy to create murine iPSC that utilizes plasmid DNA and single transfection with sequence-specific recombinases. PhiC31 integrase was used to insert the reprogramming cassette into the genome, producing iPSC. Cre recombinase was then employed for excision of the reprogramming genes. The iPSC were demonstrated to be pluripotent by *in vitro* and *in vivo* criteria, both before and after excision of the reprogramming cassette. This strategy is comparable to retroviral approaches in efficiency, but is non-hazardous for the user, simple to perform, and results in non-random integration of a reprogramming cassette that can be readily deleted. We demonstrated the efficiency of this reprogramming and excision strategy in two accessible cell types, fibroblasts and adipose stem cells. This simple strategy produces pluripotent stem cells that have the potential to be used in a clinical setting.

Keywords

induced pluripotent stem cells (iPSC); non-viral reprogramming strategy; phage integrase; adipose-derived mesenchymal stem cells (ASC)

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST
MPC is an inventor on Stanford-owned patents covering phiC31 integrase.

INTRODUCTION

Site-specific integrases are recombinases encoded in bacteriophage genomes that facilitate their stable integration into the host genome. Through recognition and recombination of specific sequences called attachment (*att*) sites residing in phage and bacterial genomes, phage integrases are able to direct stable insertion of large sequences into the genome. The demonstration that the serine recombinase fC31 integrase could function efficiently in mammalian cells initiated the use of phage integrases for engineering eukaryotic genomes [1, 2]. In particular, treatments for genetic diseases could potentially be achieved by integrase-mediated insertion and sustained expression of the wild-type version of the defective gene. For example, ϕC31 integrase was shown to direct integration of a plasmid encoding human factor IX in hepatocytes, providing long-term treatment of hemophilia B [3, 4].

As an alternative to *in vivo* delivery of plasmid DNA, it is often desirable to carry out gene therapy on cells isolated *in vitro*, followed by transplantation of the corrected cells to the patient. This *ex vivo* strategy thus combines gene therapy and cell therapy. In the optimal scenario, autologous stem cells from a patient with a genetic disease are isolated, gene corrected, expanded *in vitro,* and reimplanted into the patient, where they contribute to tissue/organ regeneration and disease treatment. However, the limited availability and *in vitro* expansion potential of patient-derived adult stem cells are major obstacles to this strategy. The successful reprogramming of somatic cells into a state of pluripotency by ectoptic expression of the transcription factors Oct4, Sox2, Klf4, and cMyc[5] led to a substantial expansion in possibilities for the stem cell field. The generation of such induced pluripotent stem cells (iPSC), closely resembling embryonic stem cells (ESC), offered not only a tool to study developmental processes, but also a potential therapeutic for use in gene and cell replacement strategies.

It has been demonstrated over the past few years that the original retrovirally-based methods of generating iPSC, which may lead to insertional mutagenesis and oncogene activation, can be replaced by alternative approaches, each with its own advantages and limitations [6, 7]. However, to date, such lentiviral [8], episomal plasmid [9–11], transposon [12, 13], protein [14], mRNA [15], or microRNA-based [16, 17] methods have not displaced the original retroviral approaches, because the alternative approaches tend to be less efficient or more difficult to perform, offering insufficient advantages. We report here a strategy for generating iPSC that provides significant advantages in terms of ease, accessibility, and efficiency. We have also given consideration to the availability and reprogramming efficiency of the cell type used as a starting population, to facilitate translation of the reprogramming approach to the clinic. In this regard, we have demonstrated these methods in fibroblasts, a commonly-used, accessible cell type [5] and also adipose-derived mesenchymal stem cells (ASC), an abundant and accessible cell type that has recently been shown to reprogram more easily than fibroblasts [18–20].

MATERIALS AND METHODS

Cell culture

Mouse embryonic fibroblasts (MEF) were prepared from E13.5 embryos (C57Bl/6) as described elsewhere [21] and cultivated in DMEM high glucose supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Carlsbad, CA). Adipose-derived mesenchymal stem cells (ASC) were isolated from the inguinal fat pads of 8–10 weeks old mice (C57Bl/6). Briefly, dissected fat pads were minced and subsequently digested in 0.1% collagenase type IV (Worthington, Lakewood, NJ) at 37°C for 1 h. After separation of

adipocytes by centrifugation at 400 × g for 10 min and filtration through a 100 μm filter mesh, cells were plated onto 10 cm dishes in the same medium used for MEF. After 24 h, cells were moved into incubators providing physiological oxygen conditions (5% O_2; Sanyo, Wood Dale, IL). Medium was changed daily until the first passage of the cells. By using flow cytometry, ASC were confirmed to be >90% $CD29^+$ and $Sca\text{-}1^+$ and >95% $CD34^-$ (Fig. S1a). To validate the isolation of bona fide ASC, differentiation ability along mesodermal lineages was assessed. ASC were differentiated into the osteogenic and adipogenic lineages, as shown by Alizarin Red and Oil Red O staining, respectively (Fig. S1b). All iPSC lines were maintained on a mitomycin C-treated MEF feeder layer plated on 0.1% gelatin in ES medium containing 20% ES-qualified FBS (Invitrogen, Carlsbad, CA), 1 × non-essential amino acids, 55 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml leukemia inhibitory factor [19] (Millipore, Billerica, MA). Murine ES cells from strains C57BL/6 and 129 constitutively expressing emerald green fluorescent protein (EmGFP) from Invitrogen were used as positive controls.

Plasmid construction

The reprogramming plasmid p4FLR (Fig. 1a), containing the four Yamanaka factor genes *cMyc, Klf4, Oct4*, and *Sox2* and the *EGFP* gene, all expressed from a CAG promoter, and a series of recombinase recognition sites, was cloned by using adaptor ligation and a series of PCR reactions. A 415 bp fragment carrying the ϕC31 *attB* site and R4 *attP* site, flanked by *loxP* sites, was synthesized and utilized in the construction. The reprogramming genes were derived from PB-TET-MKOS [12]. The EGFP sequence and plasmid backbone were derived from pEGFP-1 (Clontech, Palo Alto, CA), which carries a neomycin/kanamycin resistance gene under the control of the SV40 early promoter. The sequence of p4FLR includes 11,884 bp and will be made available upon request. Plasmid pVI, expressing wild-type ϕC31 integrase, and pVmI, expressing non-functional ϕC31 integrase, have been described elsewhere [4]. pCAG-CRE, expressing the Cre recombinase gene, was purchased from Addgene (www.addgene.org).

Nucleofection and reprogramming

A total of 1×10^6 each of MEF or ASC were nucleofected (Lonza, Walkersville, MD) according to the manufacturer's instructions using MEF nucleofector kit I (program T-20) or human MSC nucleofector kit (program U-23), respectively. One nucleofection was sufficient; multiple nucleofections were not required. Upon nucleofection with 3 μg total DNA (pVI : p4FLR ratio 1 : 1 by mass), ASC were cultivated under low oxygen conditions (5% O_2) for 48 h. On day 2, $1–3 \times 10^5$ MEF or ASC were transferred from uncoated plastic 6-well plates onto a mitomycin C-treated MEF feeder layer plated on 0.1% gelatin on 10 cm dishes. Medium was changed every other day. For MEF reprogramming, cells were maintained in an atmospheric oxygen incubator for 10 days after nucleofection, then transferred to a low oxygen incubator (5% O_2) for 2 weeks. Colonies were visible starting from day 8–12 and picked between days 20–26.

Introduction of Cre in iPSC

Lipofection of iPSC with pCAG-Cre was performed by using Effectene (Qiagen, Valencia, CA). For this purpose, 1 μg DNA was diluted in 100 μl EC buffer and mixed with 3.2 μl enhancer solution provided in the kit. Upon 10 min incubation at room temperature, 8 μl Effectene reagent was added and incubated for a further 15 min. This transfection mix was added to 2×10^5 cells plated on 0.1% gelatin. Medium was changed after 48 h.

Immunofluorescence and cell staining

Cells grown on 4-well glass chamber slides (Millipore) were fixed with 4% paraformaldehyde and immunostained with anti-Oct4 (all Abcam, Cambridge, MA, 1:200 dilution), anti-SSEA-1 (Scbt, Santa Cruz, CA, 1:100 dilution), anti-Nanog, anti-Sox2, or anti-GFP (Rockland, Gilbertsville, PA) and the respective secondary antibodies labeled with Alexa594 or Alexa488 (Invitrogen) in PBS-BT buffer (PBS, 3% BSA, 1% Triton X-100). For counterstaining of the nuclei, DAPI was included in the mounting medium (ProLong Gold; Molecular Probes, Carlsbad, CA). Alkaline phosphatase staining was performed according to the manufacturer's instructions (Stemgent, Cambridge, MA). Images of stained sections were taken on an Axioshop 2 Plus microscope with an AxioCam MRc camera (Zeiss, Thornwood, NY).

In vitro differentiation

For *in vitro* differentiation of iPSC, embryoid bodies were formed within 3–6 days by transfer into suspension culture on non-tissue culture-treated 10 cm plates. To allow spontaneous differentiation, cells were grown in ES cell medium in the absence of LIF. After transfer from suspension culture onto 0.1% gelatin-coated 60 mm dishes, day 10–14 embryoid bodies were stained for the respective markers of the three germ layers. Anti-smooth muscle actin (SMA; Sigma, St. Louis, MO), anti-alpha-fetoprotein (AFP, Scbt), and antibeta III tubulin (Tuj1, Scbt) were used. Nuclei were counterstained with Hoechst 33342 (Invitrogen).

Teratoma and chimera formation

Teratoma formation and chimera formation were carried out at the Transgenic Service Center of the Comprehensive Cancer Center at Stanford University School of Medicine. To generate teratomas, $1–2\times10^6$ iPSC generated from a C57BL/6 background were mixed 1:1 with Matrigel (BD Biosciences, San Diego, CA) and injected into the kidney capsules of 8 week-old immunodeficient SCID beige mice. After 4 weeks, tumors were subjected to histological analyses. To form chimeric mice, iPSC were injected into the blastocysts of albino B6 mice and implanted into the uteri of pseudopregnant foster mothers using routine techniques. Chimerism was revealed by the development of black coat color on the host white coat color background. Mice were housed and maintained in the Research Animal Facility at Stanford University in accordance with the guidelines of the Administrative Panel on Laboratory Animal Care (APLAC) of Stanford University.

Quantitative RT-PCR analyses

Total RNA was prepared using the RNeasy Mini Plus kit (Qiagen), and subsequently 1 μg of the RNA was used for reverse transcription using the iScript cDNA synthesis kit (BioRad, Hercules, CA), following the manufacturer's instructions. mRNA expression levels were analyzed using iQ SYBR green supermix (BioRad) and the real time PCR detection system CFX96 (BioRad). Expression levels of individual transcripts (*Klf4, cMyc, GFP, Oct4, Sox2, Rex1, and Nanog*) were normalized to GAPDH expression and compared to the expression levels in mES cells. Primers (Table S1) and PCR conditions are listed in the Supplementary Materials and Methods Section.

Bisulfite mutagenesis and analysis

Primers developed by EpigenDx (Worcester, MA) were used to analyze CpG sites within the proximal promoter regions of the murine Oct4 and Nanog promoters. One μg of genomic DNA extracted using the DNeasy Blood and Tissue kit (Qiagen) was sent to Epigendx for bisulfite treatment, PCR, and pyrosequencing.

Southern blotting

Ten μg of genomic DNA from iPSC lines were digested overnight with *Hind*III and resolved by agarose gel electrophoresis. After transfer and UV crosslinking onto Hybond N+ nylon membrane (GE Healthcare, Piscataway, NJ), the DNA was hybridized with an *EGFP* probe generated by DIG High Prime Labeling and Detection Starter Kit II (Roche, Indianapolis, IN).

LM-PCR

1 μg of genomic DNA was digested with *Mse*I overnight (10 μl total reaction), followed by heat inactivation of the enzyme at 65°C for 20 min. The linker (antisense 5'-/5Phos/TAG TCC CTT AAG CGG AG/3AmMO/-3'; sense 5'-GTA ATA CGA CTC ACT ATA GGG CTC CGC TTA AGG GAC-3'; Integrated DNA Technologies, San Diego, CA) was ligated with T4 ligase to the entire digest at a final concentration of 0.7 μM at 16°C overnight. The first round of the nested PCR used linker primer-1 (5'-GTA ATA CGA CTC ACT ATA GG*G*C-3') and either attB-F2 (5'-ATG TAG GTC ACG GTC TCG AA*G*C-3') or attB-R1 (5'-TCC CGT GCT CAC CGT GAC C*A*C-3'). The second round of the nested PCR used 2 μl of the product from the first round plus linker primer-2 (5'-AGG GCT CCG CTT AAG GG*A*C-3') and either attB-F3 (5'- CGA AGC CGC GGT G*C*G-3') or attB-R2 (5'-ACT ACC GCC ACC TCG*A*C-3') to amplify the integration junctions. The asterisk is used to denote a phosphorothioate bond. PCR conditions used were 98°C for 2 min, 10 cycles of 98°C for 15 sec, 60–55°C for 30 sec with 0.5°C/cycle decrements, 72°C for 30 sec, and 30 cycles of 98°C for 15 sec, 55°C for 30 sec with 72°C for 30 sec, and a final elongation at 72°C for 2 min. Upon column purification (Zymoclean Gel Recovery Kit, Zymo Research, Irvine, CA) fragments were cloned into the blunt-end vector pJET (Fermentas, Glen Burnie, MD) according to the manufacturer's instructions. DNA sequencing was performed by Elimbiopharm (Hayward, CA) using standard techniques.

RESULTS

Generation of iPSC by using φC31 integrase

The delivery of the reprogramming factors into either MEF or ASC was performed by co-nucleofection of plasmid pVI carrying the ϕC31 integrase gene and the reprogramming plasmid p4FLR (Fig.1a). Plasmid p4FLR included cDNA sequences for the murine *cMyc, Klf4, Oct4*, and *Sox2* genes under the control of the CAG promoter and connected via 2A peptides, facilitating polycistronic mRNA expression. In order to screen for stable integrants, the reporter gene *EGFP* was included in the reprogramming plasmid, linked via an internal ribosomal entry site or IRES at the 3'-end of the polycistronic mRNA gene product. Downstream of the *EGFP* gene was placed a cassette carrying recognition sites for three site-specific recombinases. The ϕC31 *attB* site was utilized for primary integration, while the R4 *attP* site provided for potential secondary integration. These *att* sites were flanked by two *loxP* sites to facilitate Cre-mediated removal of the reprogramming cassette (Fig.1a). Nucleofection efficiencies, as judged by scoring of GFP+ cells by FACS analysis performed 48 – 72 hours after nucleofection, were in the range of 35 – 64% (Fig. S2).

Forty-eight hours after nucleofection, cells were plated onto mitomycin C-treated MEF feeder layers and switched to ES medium. The reprogramming efficiency was calculated by dividing the number of iPSC colonies on each plate that stained positive for alkaline phosphatase or SSEA1 (Fig.1b upper panel) by the number of cells plated on the respective plate. iPS colonies were obtained from MEF at an efficiency of 0.01% ± 0.006, while iPS colonies from ASC occurred at 0.014% ± 0.009. Factoring in the transfection efficiency, reprogramming efficiencies of ~0.03% were typically observed. After picking individual colonies 18 – 24 days after nucleofection, iPS cell lines were established. These cell lines

stained positive for alkaline phosphatase (Fig. 1b lower panel) and were subsequently evaluated for the number of integration events via Southern blot analysis by using a probe directed against the *EGFP* reporter gene on the reprogramming plasmid (Fig.1c).

Among 19 MEF-derived iPSC clones tested, 37% exhibited a single integration event, while of 13 ASC-derived iPSC clones, 31% exhibited single-copy integration of the plasmid. Overall, ~50% of the analyzed genomic DNA samples obtained from iPSC clones exhibited a double integration of the reprogramming plasmid, while the remaining 16% showed a triple integration. An overview of the different integration events among MEF-iPSC and ASC-iPSC is given in Fig. S3. For simplicity and as a proof of concept, we focused on two clones with a single integration site, one derived from MEF and one from ASC. To determine the chromosomal location of each integration site, the single integrants were subjected to linker-mediated (LM)-PCR. By using this method, the MEF-iPSC line was shown to possess a single integration into an intronic region of the *Ptpn1* gene on chromosome 2. The ASC-iPSC line was found to have a single integration in an intergenic region on chromosome 1. The locations of both integration sites were verified via PCR of the genomic locus by using a combination of genomic and plasmid-binding primers, as depicted schematically in Fig. 1d. Chromosome spreads of metaphase cells of the selected clones were analyzed and revealed the correct chromosome number and no major differences from mouse ESC (Fig. S4). However, more refined cytogenetic techniques would be required to reveal more subtle chromosomal rearrangements that may occur in IPSC. Such analyses will be carried out in the next study.

Of the 14 integration sites we evaluated by LM-PCR, six clones were found in intergenic regions, six were located within an intron, and two sites were in an exon (Tab S2). These results are similar to those obtained in a previous report and largely reflect the proportions of these elements in the genome, with some skewing toward genes [22]. We evaluated the integration sites obtained in our study according to the criteria articulated in a recently published study from Papapetrou et al. [23], which defined so-called genomic safe harbors, Of the six intergenic sites, two met the criteria proposed by this work, which represented 14% of all integration sites analyzed. The context of the integration sites is summarized in supplementary Table S2, in which the genomic safe sites are highlighted in gray. As discussed in the Discussion section, 23% of ϕC31 integration sites in the human genome are in safe locations.

Deletion of reprogramming genes from iPSC by using Cre recombinase

To remove the reprogramming cassette, the iPSC clones carrying one copy of the reprogramming plasmid were transiently exposed to Cre recombinase (Fig. 1a). Cre was introduced by lipofection with Effectene of a plasmid expressing Cre. By visually tracking the loss of EGFP expression, transgene-free iPSC clones were easily detected and picked for clonal expansion. Typically, 50% or more of the clones exhibited loss of EGFP expression. Excision of the reprogramming plasmid was verified by Southern blot (Fig. 1c). The Cre-mediated removal of the reprogramming cassette from the respective genomic loci was further demonstrated by PCR of the genomic locus using a combination of genomic and plasmid-binding primers (Fig. 1d). Moreover, the absence of the integrase-encoding plasmid pVI, which was used to integrate p4FLR, could be shown by PCR (Fig. 1d, lower panel). The excised clones were designated MEF-iPSC-X and ASC-iPSC-X.

Pluripotency of iPSC before and after Cre-mediated excision

To evaluate the pluripotency of the iPSCs generated by using ϕC31 integrase, both before and after Cre-mediated excision of the reprogramming cassette, the following assays were carried out. The mRNA expression profiles of the pluripotency-associated genes *Oct4, Klf4,*

Sox2, Nanog, and Rex1, as well as *EGFP,* were determined via qRT-PCR and compared to the respective transcript levels in mouse ESC. By comparing the transcript levels before and after removal of the ectopically expressed genes, reactivation of the endogenous gene transcripts was verified. As depicted in Fig. 2a, the expression levels in the iPSC lines were similar to those in ESC.

In order to assess epigenetic changes in the DNA methylation status of the Oct4 and Nanog promoter regions, bisulfite sequencing was performed. Pyrosequencing revealed the full reactivation of the respective promoters, showing low methylation levels that were comparable to those of ESC. In contrast, analysis of the promoter methylation in the parental MEF and ASC showed a high rate of methylation. Fig. 2b schematically depicts the results of the bisulfite pyrosequencing. The quantification can be seen in supplementary Fig. S5. Immunofluorescence staining for Oct4, SSEA1, Nanog, and Sox2 (Fig. S6 for the latter two markers) revealed expression of those ESC/iPSC-characteristic proteins (Fig. 2c). The removal of the reprogramming cassette, including the reporter gene *EGFP,* allowed validation of transgene-free iPSC by the absence of EGFP staining. In the EGFP-negative MEF-iPSC-X and ASC-iPSC-X clones, sustained expression of the endogenous pluripotency-associated proteins was verified (Fig. 2c).

In order to assess the *in vitro* differentiation potential across all three germ layers of the iPSC clones, we carried out embryoid body formation. By staining for SMA, Tuj1, and AFP, we demonstrated differentiation into cells of mesodermal, ectodermal, and endodermal origin, respectively (Fig. 3a). Furthermore, the pluripotency of the iPSC lines was not altered after removal of the reprogramming cassette, since the differentiation potential of MEF-iPSC-X and ASC-iPSC-X was not reduced (Fig. 3a).

To evaluate pluripotency *in vivo*, the iPSC clones were injected into the kidney capsule of immune-deficient SCID/beige mice, and teratoma formation was evaluated 4 weeks after injection. As shown in Fig. 3b for MEF-iPSC and ASC-iPSC, histological analysis of the teratoma revealed that cell types derived from all three germ layers were included in the tumors. The injection of MEFiPSC-X and ASC-iPSC-X all led to teratoma formation to a similar extent (data not shown).

As a final proof of pluripotency, iPSC were injected into the blastocysts of albino B6 mice and implanted into the uteri of pseudopregnant foster mothers. Contribution to chimeras was observed by patched coat color (Fig. 3c). Thus, our recombinase-generated iPSC were genuinely reprogrammed, fulfilling all criteria of pluripotency.

DISCUSSION

In addition to the immunological compatibility of using patient-derived iPSC, the method of their derivation is a key aspect of their suitability for use in gene/cell therapeutic approaches. Although retroviral approaches to generate iPSC are commonly used, the risks of insertional mutagenesis and transgene reactivation leading to tumor formation are not negligible [24, 25]. Excision of the reprogramming factors after derivation of iPSC has been performed by several groups to circumvent transgene reactivation [13, 26–28]. However, the retroviral approach still requires the expertise and biosafety risks entailed in handling retroviruses. In order to avoid insertional mutagenesis completely, non-integrating strategies such as the use of proteins [14], episomal plasmids [11, 29], mRNA [15], and miRNAs [16, 17] have been described, although often with low efficiencies. In this study, we demonstrated a straightforward non-viral method to generate iPSC, complete with an easy-to-follow protocol to excise the reprogramming cassette. The reprogramming step can be accomplished without any complex or biohazardous techniques, by a single nucleofection with the reprogramming and

ϕC31 integrase plasmids. In previous reports with non-integrating plasmids, a single nucleofection was not sufficient, and multiple rounds of transfection were required [30, 31], whereas with the mRNA method, daily, large doses of modified RNA need to be transfected [15]. Multiple transfections are tedious to perform and can cause significant cell death. Our efficiencies of up to 0.03% were substantially higher than previously described plasmid-based approaches, which ranged between 0.0015% [9], and 0.005% [11].

After derivation of iPSC clones, integration events can easily be analyzed by Southern blot using an *EGFP* probe to identify single integrants, which are used for the subsequent steps. More than 30% of all analyzed iPSC clones showed a single integration of the plasmid p4FLR. This fraction is superior to the fraction of single-copy integrants seen with other non-viral methods such as Piggybac transposon-based (15%) approaches [12, 13, 26]. Our strategy utilizes ϕC31-mediated integration to place the reprogramming cassette at transcriptionally favored locations (Fig. 1a). To determine the genomic locus of integration, we applied the LM-PCR technique, which by a simple protocol reliably elucidates the DNA sequence of the genomic junctions. After determining the genomic integration site, PCR was used to confirm the locus and also to verify excision of the reprogramming cassette after Cre exposure (Fig. 1d). For the MEF-iPSC and the ASC-iPSC clones illustrated throughout the paper, we could verify single integration of the reprogramming plasmid via Southern blot (Fig. 1c), as well as genomic site-precise PCR. By using primers specific for the ϕC31 integrase *attB* site and the genomic site, the detection of the integration and also excision could be accurately determined (Fig. 1d).

The prevalence of intergenic integration sites made it a simple matter to identify a potentially safe integration site. Two out of six of the intergenic sites can be considered safe sites, meeting all the criteria proposed by Papapetrou et al. [23]. To fulfill these criteria, integration sites cannot be within 50 kb of the 5' end of any gene, upstream or downstream, nor within 300 kb of any cancer-related gene 5' or 3' ends, or within 300 kb of miRNA 5' or 3' ends. Moreover, integration sites cannot be inside a gene transcription unit nor within an ultraconserved region (UCR) [23]. To evaluate the translation of our approach in mouse cells to human cells, we re-analyzed previous DNA sequence data of ϕC31 integration sites in human cells [22], according to all of the safe harbor criteria [23]. This analysis revealed that of 107 sequenced sites, 47 were intergenic, representing 44%. Of those 47 intergenic sites, 25 were found to be genomic safe sites, representing 23% of the total integration sites (Table S3), a higher fraction than that described in the lentiviral study (~10%) [23].

While this manuscript was in preparation, a study by Ye et al. [32] was published using ϕC31 integrase to make murine and human iPSCs with an unexcisable vector. A higher intergenic pseudo site frequency was reported (9/10 clones; 90%) [32] than has been previously observed with ϕC31 integrase (~60%) [22, 33, 34]. When we reanalyzed the data of Ye et al. [32] by using the UCSC BLAT/genome browser [22], several integration sites were re-classified, such that the overall intergenic pseudo site frequency dropped to ~60%, which is in closer agreement with the current and previous studies. It was also demonstrated that adjacent gene function was not affected [32]. In that study, all integration events were single-copy, which may be due to features of the reprogramming plasmids that were employed. That study did not demonstrate excision of the reprogramming genes or generation of chimeric mice, both of which were achieved in the present study.

It has been shown that sustained expression of the reprogramming genes can affect the differentiation potential of iPSC [27]. Moreover, genome-wide expression analyses performed in patient-derived iPCSs showed that iPSC exposed to Cre recombinase, thus lacking the reprogramming cassette, were more similar to hES cells than before excision [35]. In our study, we were able to confirm the pluripotency of the iPSC before and after Cre exposure.

We observed that the percentage of chimerism was higher using excised iPSC, although the number of examples was small. Moreover, two out of two chimeric mice derived from non-excised iPSC developed tumours within the first 6 weeks. This finding is consistent with previous reports [36] and may be due to sustained *cMyc* expression in the mice generated with MEF-iPSC carrying the reprogramming cassette. In the other approaches undertaken to prove pluripotency, we did not observe major differences between the iPSC and iPSC-X lacking the reprogramming cassette. For example, the analysis of mRNA expression of pluripotency-associated genes was not altered (Fig. 2a) in iPSC-X, indicating reactivation of the endogenous mRNA expression. Along the same lines, the epigenetic changes documenting the gain of pluripotency were verified by bisulfite modification and sequencing. Among all analyzed iPSC samples, the methylation status of the Oct4 and Nanog promoters was low, whereas this epigenetic mark was high in the parental cells (Fig. 2b and Fig S5). A hallmark of pluripotency is the trilineage differentiation of iPSC, which we assessed *in vitro* via embryoid body formation (Fig. 3a), as well as *in vivo* by evaluating teratoma formation (Fig. 3b). In both cases, the capacity to give rise to cells of all three germ layers was confirmed, independently of the presence or absence of the reprogramming cassette. Collectively, these results showed that iPSC generated via ϕC31 integrase represented *bona fide* pluripotent stem cells. These iPSC expressed genes and markers at comparable levels to mouse ESC and were morphologically indistinguishable from their ESC counterparts (Fig. 1b). Similar observations were made in a recent study in which human iPSCs were generated by a single excisable lentiviral cassette and showed the same *in vitro* behavior before and after excision [37].

In addition to ease of use and safety of resulting iPSC, our approach possesses a further potential advantage. This feature lies in the possibility of using ϕC31 integrase to place simultaneously both the reprogramming genes and a chromosomal target for a second integrase into a safe location in the genome, yielding a valuable approach for gene therapy [38, 39]. For example, the R4 *attP* site present on p4FLR could be retargeted via transient exposure to R4 integrase and a plasmid carrying the R4 *attB* site [38] (Fig. 1a). Recently, this type of strategy has been used in human ESC [40]. However, as we have reported previously [38], R4 integrase is also able to target pseudo *attP* sites. Therefore, it may be preferable to use another of the other integrases we have characterized that does not mediate integration at pseudo *att* sites [41], to minimize the possibility of integration at undesired sites. Such a strategy is part of our current efforts to optimize the recombinase strategy and extend these studies to human cells. This type of protocol to generate iPSC cells and then to integrate a therapeutic gene into a safe site in the genome may offer a strategy well-suited for use in clinical settings.

To summarize the advantages of our approach, it is simpler and safer for the user than retroviral and lentiviral approaches, since no virus needs to be prepared or handled, yet the efficiency is similar. The fraction of single-copy integrations and the fraction of integrations that are in safe sites is higher than with viral or transposon approaches. The approach is more efficient than most non-integrating approaches, and unlike those approaches, carries a built-in capacity to add therapeutic genes to the genome of iPSC at a pre-characterized safe site. In summary, this method may offer a simple, safe solution for generating iPSC, especially when further genetic modification of the cells is desired.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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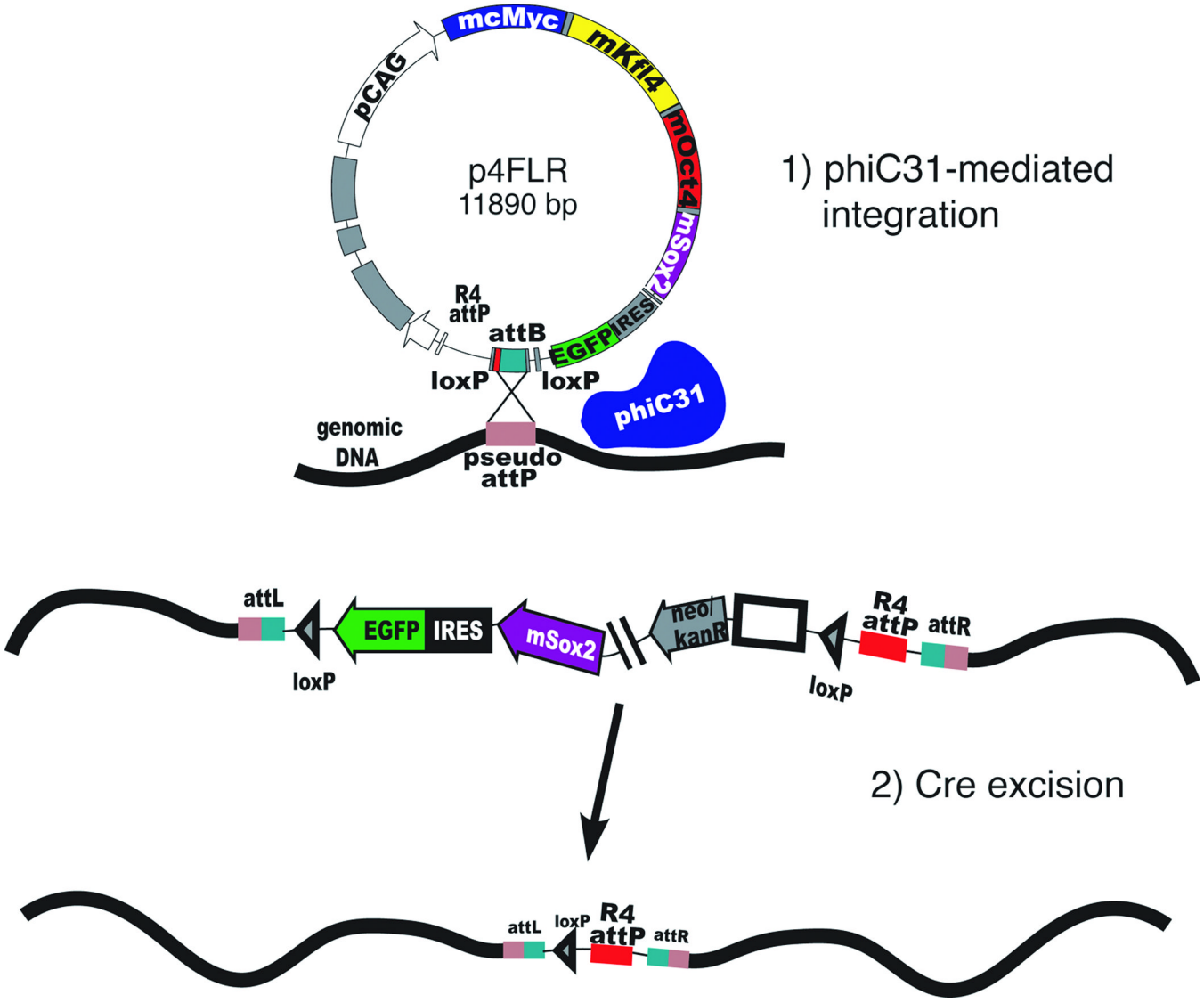
1a



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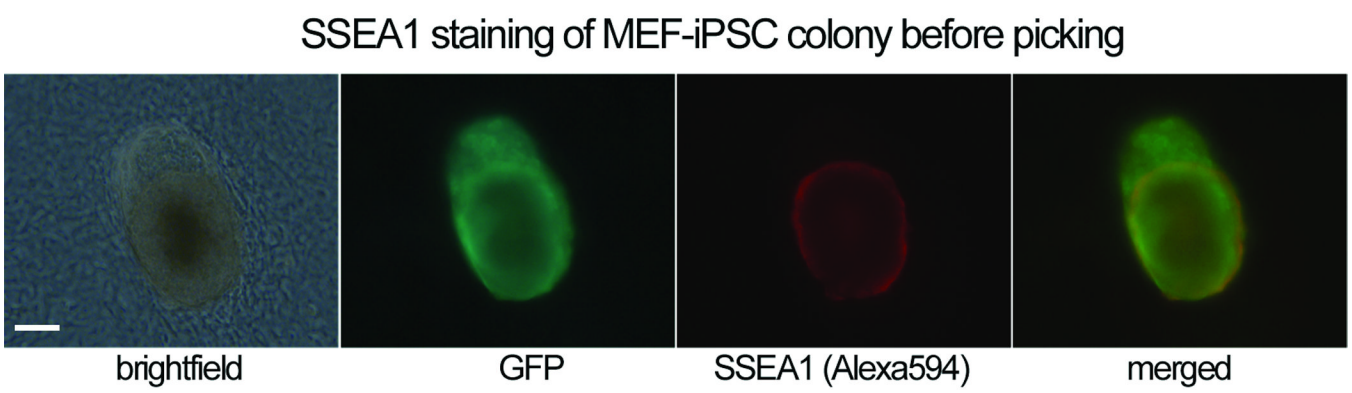
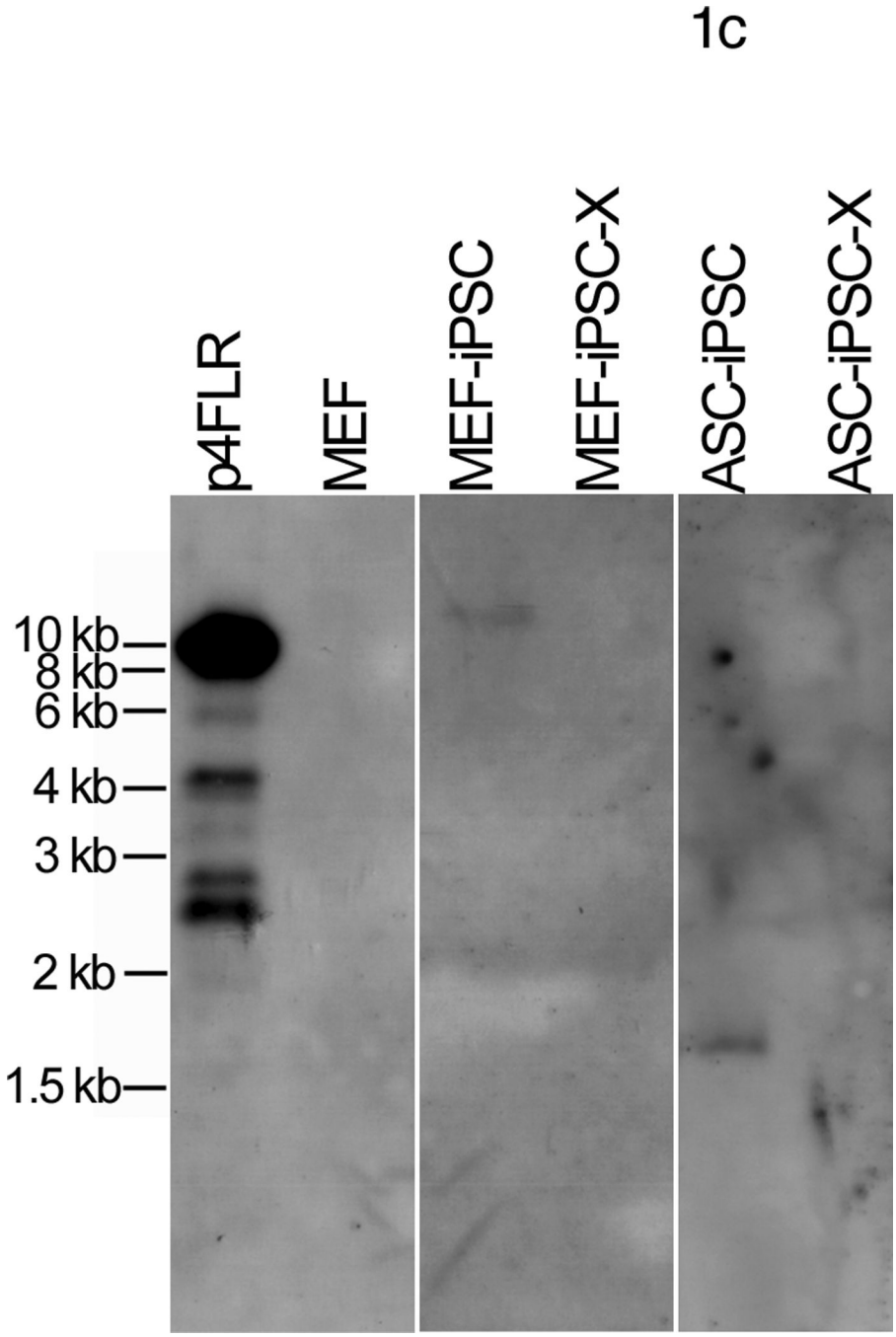




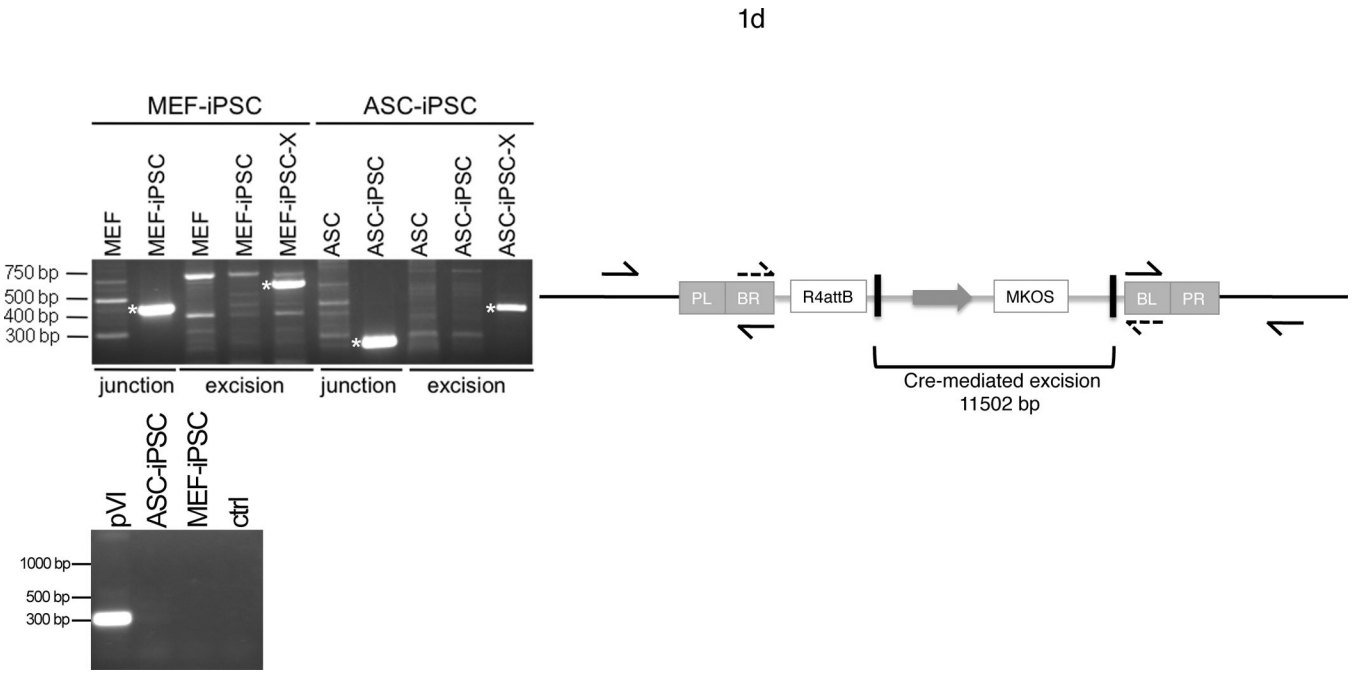
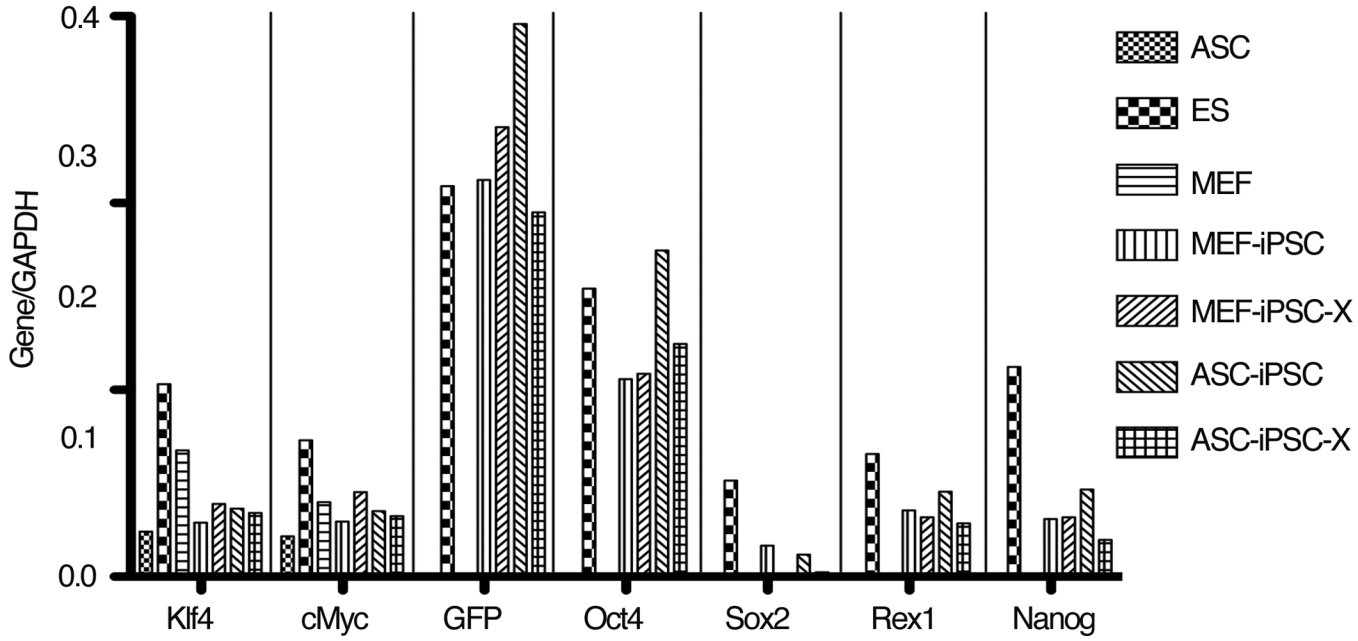


Figure 1. Generation of iPSC and removal of reprogramming factors using site-specific recombinases

(A) Schematic overview of the recombinase strategy3/29/2011. First, ϕC31 integrase is used to integrate the p4FLR plasmid at a preferred location and reprogram somatic cells. A clone with a single copy of the plasmid integrated at a safe, intergenic location is chosen. Second, Cre recombinase mediates precise excision of the reprogramming cassette. **(B)** SSEA1 staining of an ASC-iPSC on day 20 after nucleofection before picking (upper panel) and morphology of established iPSC lines generated from MEF and ASC starting populations shown by alkaline phosphatase staining compared to ESC (lower panel). Scale bars represent 50μm. **(C)** Southern blot analysis of representative MEF- and ASC-iPSC lines before and after Cre-mediated excision of the reprogramming cassette, by using an *EGFP* probe. Both clones carried a single integration of the reprogramming cassette, which was no longer detectable after excision. **(D)** Verification of the genomic integration site determined previously by LM-PCR using pairs of the respective genomic and plasmid-binding primers for both of the iPSC clones as depicted schematically (right panel). Genomic DNA of the parental cells was used as a negative control, proving specific product amplification (left panel). Cre-mediated excision of the reprogramming cassette was verified by amplification of the genomic integration locus (left panel). Genomic DNA of cells bearing the entire cassette served as negative controls, since in those samples the small PCR product could not be detected by using a combination of primers binding adjacent to the genomic integration site and within the recombined sites (dashed lines in right panel). PCR to test for pVI plasmid (lower left panel) showing the absence of the plasmid in established iPSC lines. Ctrl = control.

2a



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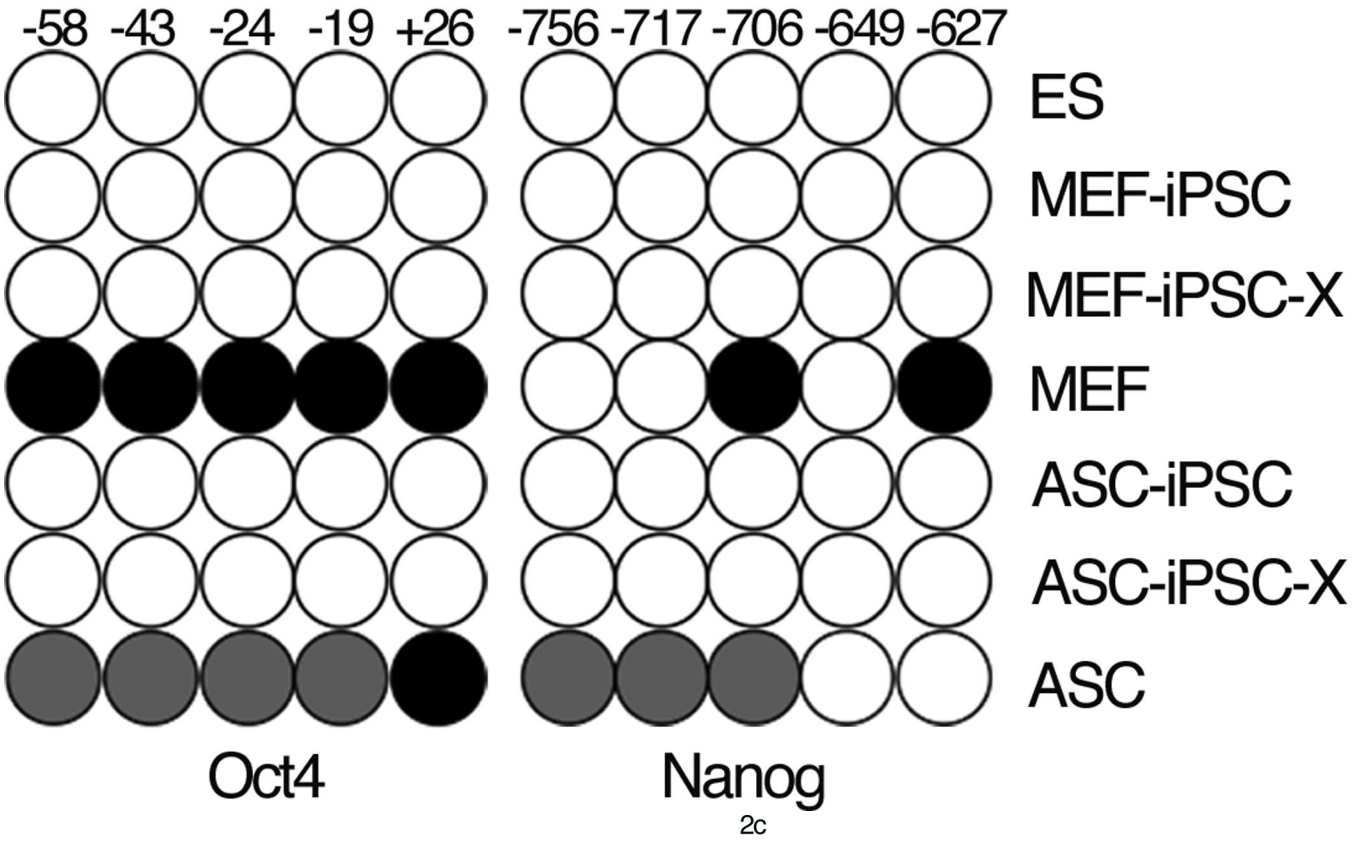

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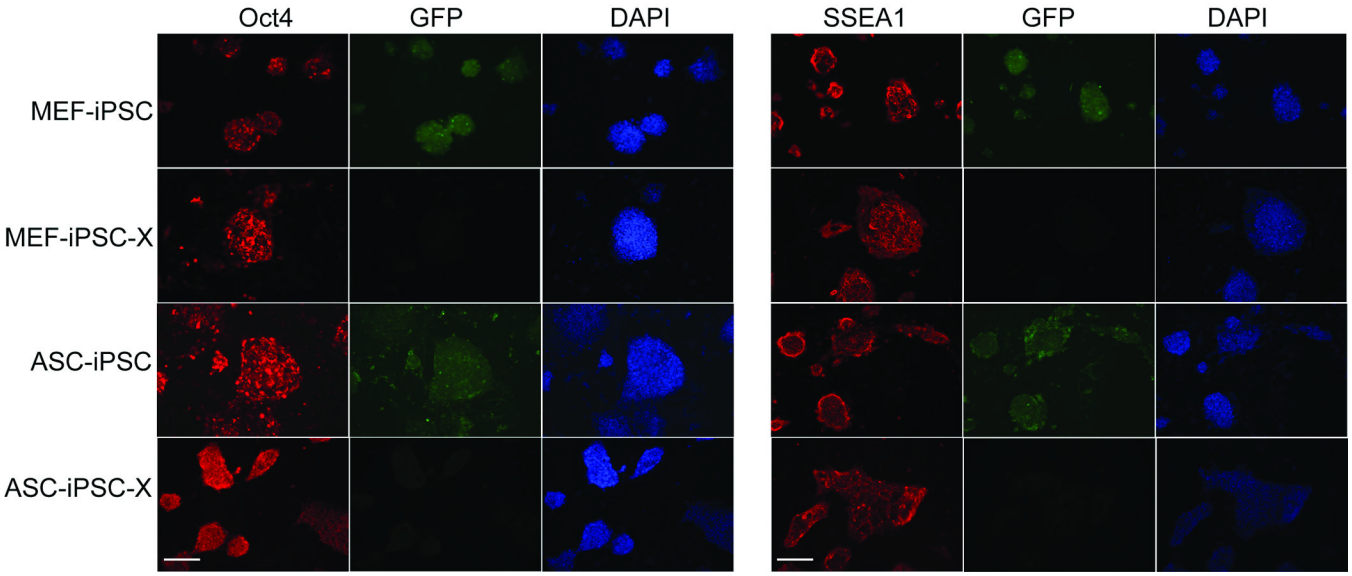
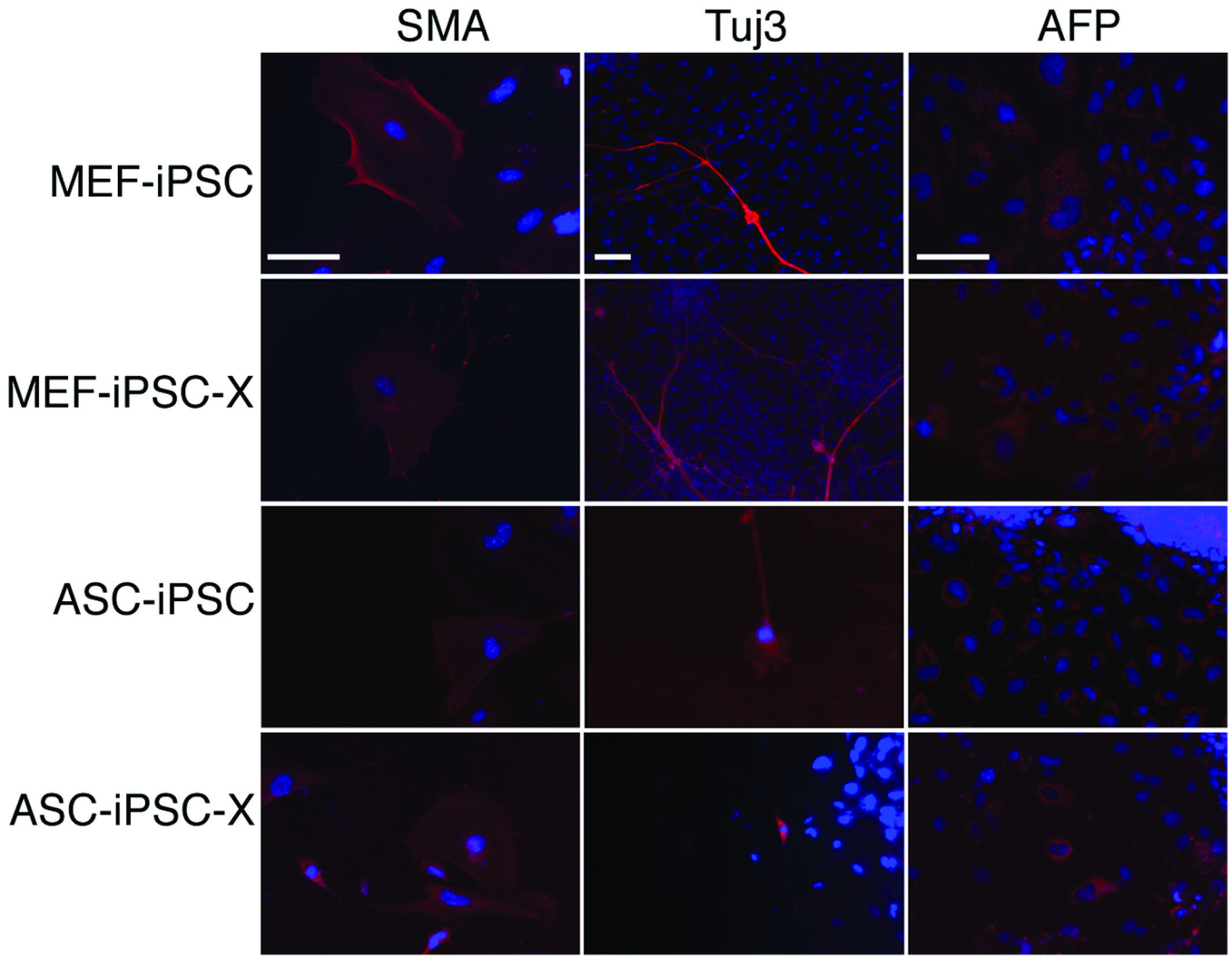


Figure 2. Pluripotency of iPSC before and after Cre-mediated excision

(A) Quantitative RT-PCR data showing expression of *Klf4, cMyc, GFP, Oct4, Sox2, Rex1*, and *Nanog* in MEF- and ASC-iPSC before and after removal of the reprogramming cassette, as well as in the parental MEF and ASC and in ESC controls. **(B)** Promoter methylation status of Oct4 (left panel) and Nanog (right panel) in iPSC and iPSC-X lines. Five different CpG islands for each line were analyzed, indicated by their distance to the respective transcription start site (TSS). Open circles reflect low methylation (0–25%), whereas gray circles represent medium (26–75%) and black circles high (76–100%) methylation. **(C)** Immunofluorescence staining of Oct4, SSEA1, and EGFP in MEF- and ASC-iPSC before and after Cre recombinase treatment. Scale bars represent 50μm.

3a



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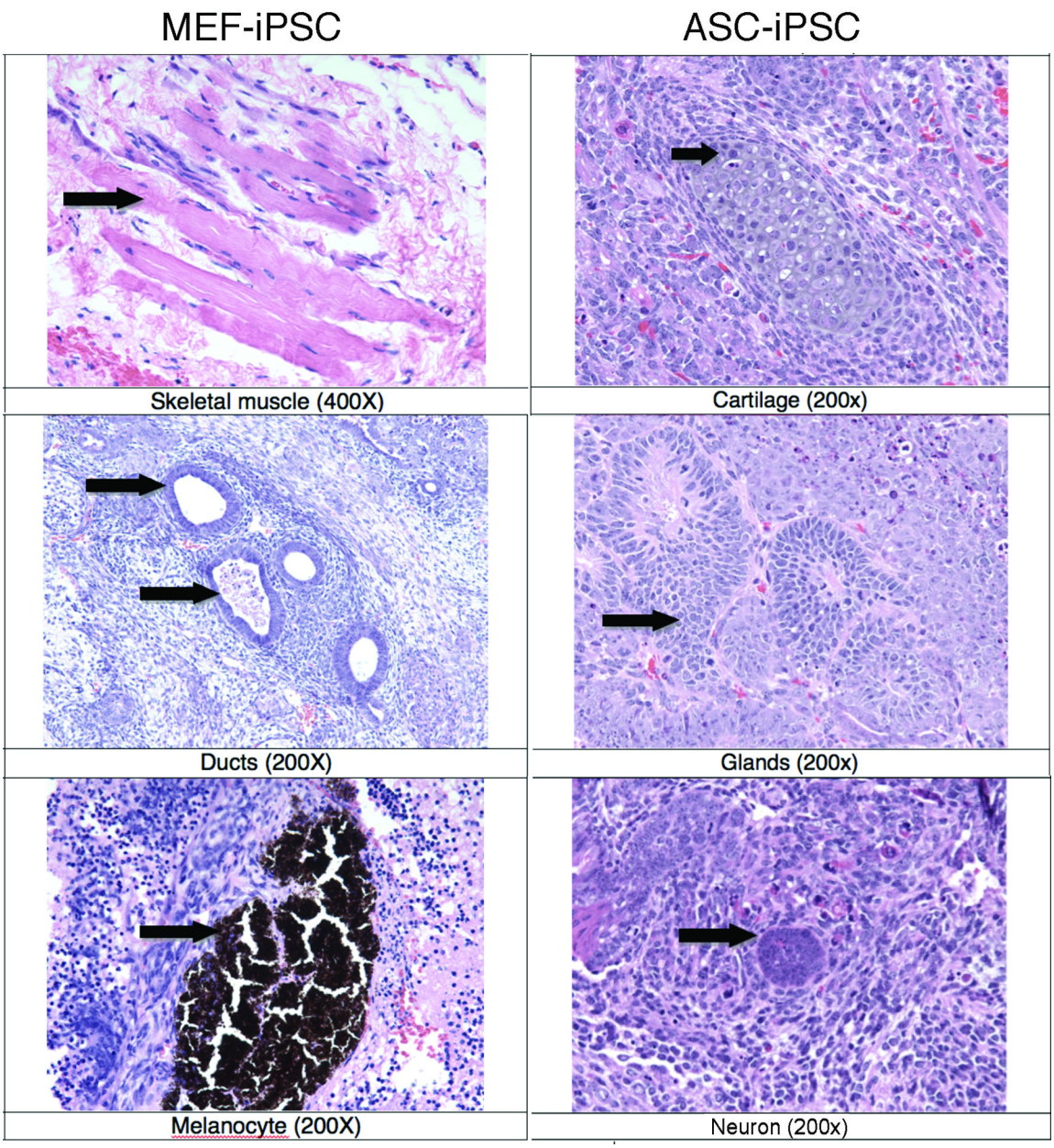

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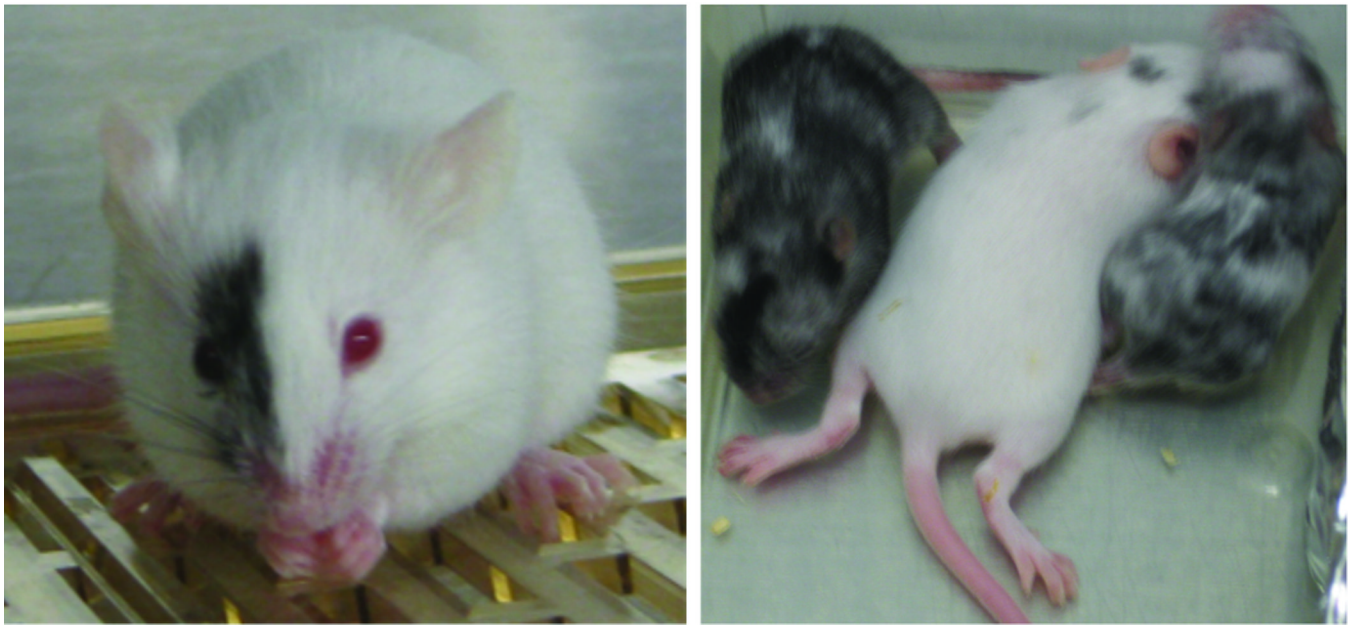

Figure 3. *In vivo* studies of pluripotency of iPSC before and after Cre-mediated excision

(A) Day 14 embryoid bodies were stained with the antibodies anti-SMA, anti-Tuj1, or anti-AFP to show mesodermal, ectodermal, and endodermal differentiation *in vitro*, respectively. Alexa 594-labeled secondary antibodies were used, while Hoechst staining was used to visualize the nuclei. Figure represents merged pictures. Scale bars represent 50 μm. **(B)** Histological samples obtained 4 weeks after injection of MEF-iPSC (left panel) or ASC-iPSC (right panel) into SCID beige mice showing teratomas composed of cells with mesoderm, ectoderm, and endoderm lineages. **(C)** Chimeric pups obtained after injection of MEF-iPSC (left image) and MEF-iPSC-X (right image) into blastocysts of albino B6 mice. iPSC gave rise to black fur.