Isolation of human iPS cells using EOS lentiviral vectors to select for pluripotency

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Induced pluripotent stem (iPS) cells may be of use in regenerative medicine. However, the low efficiency of reprogramming is a major impediment to the generation of patient-specific iPS cell lines. Here we report the first selection system for the isolation of human iPS cells. We developed the EOS (Early Transposon promoter and *Oct-4* (*Pou5f1*) and *Sox2* enhancers) lentiviral vector to specifically express in mouse and human embryonic stem cells but not in primary fibroblasts. The bicistronic EOS vector marked emerging mouse and human iPS cell colonies with EGFP, and we used puromycin selection to aid the isolation of iPS cell lines that expressed endogenous pluripotency markers. These lines differentiated into cell types from all three germ layers. Reporter expression was extinguished upon differentiation and therefore monitored the residual pluripotent cells that form teratomas. Finally, we used EOS selection to establish Rett syndrome–specific mouse and human iPS cell lines with known mutations in *MECP2*.

The initial identification of mouse iPS cell colonies used transgenic mice bearing reporter genes inserted into pluripotency genes such as *Fbxo15* (ref. 1), *Nanog* (ref. 2) or *Oct-4* (also known as *Pou5f1*)[3]. Isolation of iPS cells is possible without pluripotency reporters; however, further optimization of the reprogramming process in mouse cells continues to use stably integrated reporters. At present, isolation[4–6] and optimization of methods to reprogram human iPS cells relies on morphological criteria and skilled stem cell culture expertise. To facilitate these efforts by less experienced or novice laboratories we developed an accurate pluripotency reporter system which can be transduced efficiently into human primary somatic cells.

The most efficient systems for stable reporter gene transfer into primary human cells are retroviral or lentiviral vectors, but these suffer from frequent transcriptional silencing in pluripotent stem cells[7], including in iPS cells[2,3]. Here we use the strong LTR (long terminal repeat) promoter from an Early Transposon (ETn) that is highly transcribed in embryonic stem (ES) cells[8] and combine it with Oct-4 and Sox2 binding motifs in ES cell–specific enhancers to overcome vector silencing and specifically mark pluripotent stem cells. These EOS lentiviral vector reporters robustly mark human iPS cells with EGFP during reprogramming and aid the isolation of mouse and human iPS cell lines under puromycin selection. As EOS vector expression is extinguished during differentiation, they monitor the presence of undifferentiated pluripotent stem cells that form teratomas. Our selection method facilitates isolation of mouse iPS cells lines produced by three-factor retroviral-mediated reprogramming, and we provide proof-of-principle that EOS selection can be used to generate disease-specific mouse and human iPS cell lines to model Rett syndrome.

RESULTS

EOS lentiviral vectors mark mouse and human ES cells

To identify the two most effective EOS cassettes, regulated by the multimerized *Oct-4* core enhancer element CR4 (conserved region 4)[9] or the *Sox2* core enhancer element SRR2 (Sox2 regulatory region 2)[10] (**Supplementary Fig. 1** online), we performed preliminary experiments to evaluate expression of several cassettes after retroviral vector transfer into mouse ES cells (data not shown). To test EOS expression in a vector suited for high-efficiency transduction of primary somatic cells, we generated self-inactivating lentiviral vectors bearing the EOS-C(3+) and EOS-S(4+) cassettes identified in the preliminary screen (**Fig. 1a**). These vectors were infected into mixed cultures of J1 mouse ES cells and mouse embryonic fibroblasts (MEFs). Control vectors, in which *EGFP* was driven by the ubiquitous *EF1α (EEF1A1)* and *PGK (PGK1)* promoters, showed EGFP expression in MEFs and, at a lower level, in mouse ES cells. In contrast, lenti-EOS-C(3+) and EOS-S(4+) vectors directed specific EGFP expression in mouse ES cell colonies, and EGFP expression was higher than with the *Oct-4*

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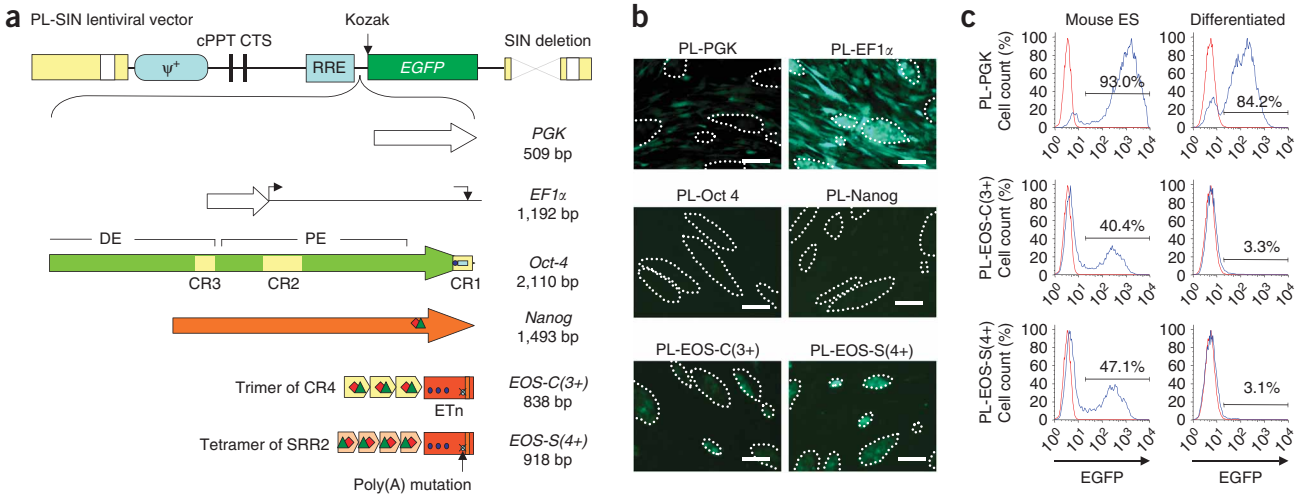

Figure 1 | EOS lentiviral vectors in mouse ES cells. (**a**) Schematic of lentiviral vectors with several different promoters. The vector backbone has a self-inactivating (SIN) deletion in U3 of the 3′ LTR so that EGFP is expressed from the internal promoter. The start codon of EGFP contains a Kozak consensus sequence. Ψ^+, enhanced packaging signal; cPPT, central polypurine tract; CTS, central termination signal; RRE, Rev response element. EOS, ETn promoter with poly(A)-site mutation, plus Oct-4 (red diamond) and Sox2 (green triangle) binding sites. CR1–CR4, Oct-4 enhancer conserved regions 1–4; DE, distal enhancer; PE, proximal enhancer; C(3+), trimer of CR4 enhancer; S(4+), tetramer of SRR2 enhancer; bp, base pairs. (**b**) EGFP expression in mixed cultures of J1 mouse ES cells and MEFs (without mitomycin C treatment) infected with the indicated lentiviral constructs. Images were taken 2 d after infection. Dotted lines outline colonies. Scale bars, 100 μm. (**c**) Flow cytometry analysis of EGFP expression from the indicated vectors in mouse ES cells upon *in vitro* differentiation. Representative result from three independent differentiation experiments. Flow cytometry histograms show percentage of the maximum cell count. Blue, histograms of each infected sample; red, uninfected controls.

or *Nanog* promoter vectors (**Fig. 1b**). We obtained similar results when infecting into each cell type individually (**Supplementary Fig. 2** online). After differentiation of EOS-infected mouse ES cells, EOS-EGFP expression was extinguished and the cells were almost indistinguishable from mock-infected cells by flow cytometry (**Fig. 1c**; **Supplementary Fig. 3a** online). We observed occasional residual EGFP-positive cells only in ES-like colonies after differentiation (**Supplementary Fig. 3b**). These data show that EOS cassettes are live-cell markers specific for undifferentiated mouse ES cells.

Because little is known about *Oct-4* regulatory elements in human ES cells and the ETn promoter has not been studied in this context, we next examined EOS lentivirus expression in the CA-1 human ES (hES) cell line[11]. Similarly to mouse ES cells, the ubiquitous *PGK* and *EF1α* promoters drove EGFP expression in hES cells and in surrounding MEF feeders (**Fig. 2a**). Expression from *Oct-4* and *Nanog* promoter vectors was difficult to detect, whereas the EOS cassettes showed specific expression in hES cells (**Fig. 2a**). Differentiation with retinoic acid for 9 d showed that EGFP expression from the EOS vectors was extinguished, whereas the control *PGK* and *EF1α* promoter vectors maintained expression (**Fig. 2b**; **Supplementary Fig. 4** online). Also, experiments in primary human dermal fibroblasts and well established human cell lines demonstrated that the ubiquitous *PGK* and *EF1α* promoter vectors expressed EGFP, whereas the EOS vectors did not (**Supplementary Fig. 5a**,**b** online).

We also infected the H1 hES cell line with the EOS lentiviral vector. We isolated by cell sorting the subset of cells that expressed both EGFP from the EOS cassette and the endogenous IGF-1 receptor (IGF-1R), which is specific for undifferentiated hES cells

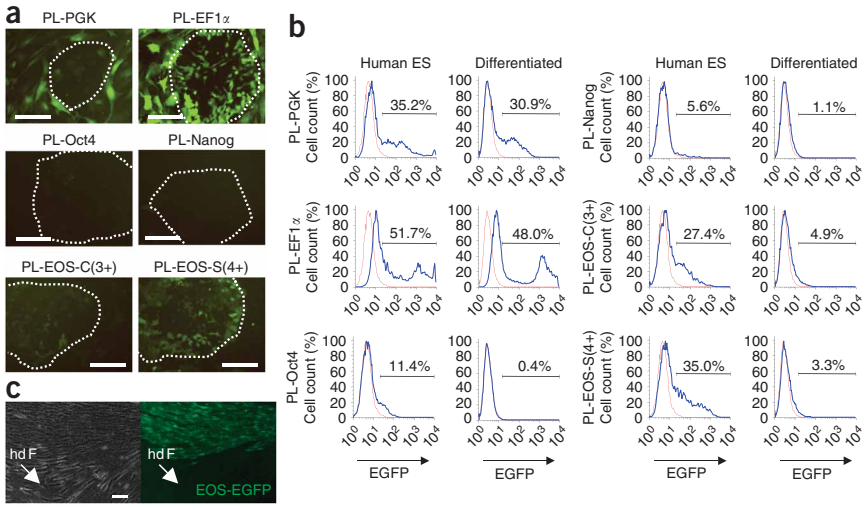

Figure 2 | EOS lentiviral vectors in human ES cells. (**a**) EGFP expression from the indicated EOS lentiviral vectors in CA-1 human ES cell colonies cultured on MEF feeder cells (mitomycin C treated). Images were taken 3 d after infection. Scale bars, 200 μm. (**b**) Flow cytometry analysis of EGFP expression from the indicated vectors in human ES cells upon *in vitro* differentiation, as described in **Figure 1c**. (**c**) Phase contrast (left) and EGFP fluorescence (right) micrographs of EOS-EGFP expression in a representative H1 hES cell colony. Arrow: nonexpressing, hES cell–derived, fibroblast-like (hdF) cells that surround the colony and represent the supportive ES cell niche. Scale bars, 200 μm.

(**Supplementary Fig. 6a** online). We subjected the cells to clonogenic analysis for hES-colony initiating cells (CIC) as previously reported[12]. The frequency of CICs in EOS and IGF-1R positive populations was 1 in 1,000 cells, demonstrating enrichment for the most primitive self-renewing hES cells (normal CIC frequency: 1 in 5,000). EGFP expression was restricted to the CIC colony, with no expression in surrounding hES-derived fibroblast-like cells that represent the supportive hES cell niche[13] (**Fig. 2c**). No distinction of EOS expression could be made between sorted cells positive and negative for the endogenous pluripotency marker SSEA-3. However, cells gated for IGF-1R expression versus cells devoid of IGF-1R expression showed bifurcation of EOS expression (**Supplementary Fig. 6b**). These data indicate that EOS lentivirus expression correlates strongly with the functional clonogenicity of hES cells, and with the IGF-1R marker of the most primitive hES cell subset.

Improved isolation of mouse iPS cells using EOS selection

To use the EOS vector for selection and maintenance of iPS cells, we constructed an EOS-C(3+) lentiviral vector containing an *EGFP*-IRES-*Puro*^{R} cassette to mark iPS cells generated in a reprogramming experiment. As a proof-of-principle, we infected MEFs isolated from genetically unmodified CD-1 mice with concentrated EOS lentiviral vector at a multiplicity of infection (MOI) of 4 (**Fig. 3a**). Next, we reprogrammed wild-type or EOS-infected MEFs into iPS cell colonies by infection with Moloney murine leukemia virus (MoMLV)-based retroviral vectors (pMXs) encoding the four Yamanaka factors (*Oct-4*, *Sox2*, *Klf4*, *c-Myc*) or the three factors without *c-Myc*. EGFP expression from EOS was first detected by day 6, when iPS cell colonies were first visible (**Fig. 3b**). Previous selection using *Oct4-neo* and *Nanog-puro* markers was successful when applied from days 3 to 6 (refs. 2,3). Therefore, at day 7 after induction, we subjected the EOS-infected MEFs to puromycin selection. Surviving colonies maintained EGFP expression coincident with alkaline phosphatase staining (**Fig. 3b**). Colonies resulting from four-factor induction (EOS-4F) assumed an ES cell–like colony morphology in the first week, whereas this was delayed in the three-factor inductions (EOS-3F), as previously reported[14,15].

To examine the reprogramming frequencies, we stained the remaining colonies in the dish for alkaline phosphatase. Puromycin selection of EOS-infected cells increased the number of alkaline phosphatase–positive ES-like colonies approximately threefold with four-factor induction and 1.6-fold with three-factor induction (**Fig. 3c**). Puromycin selection provided a growth advantage and space for smaller iPS cell colonies to expand, resulting in increase of colony numbers. We picked 12 to 33 putative colonies from each plate between days 17 and 22 to establish into lines. The four-factor colonies generated in the presence and absence of puromycin selection expanded into lines at equal efficiency (50%, 6/12). In contrast, the use of EOS and puromycin selection increased by eightfold the efficiency with which the relatively unstable[16] three-factor colonies could be expanded into lines. For the three-factor

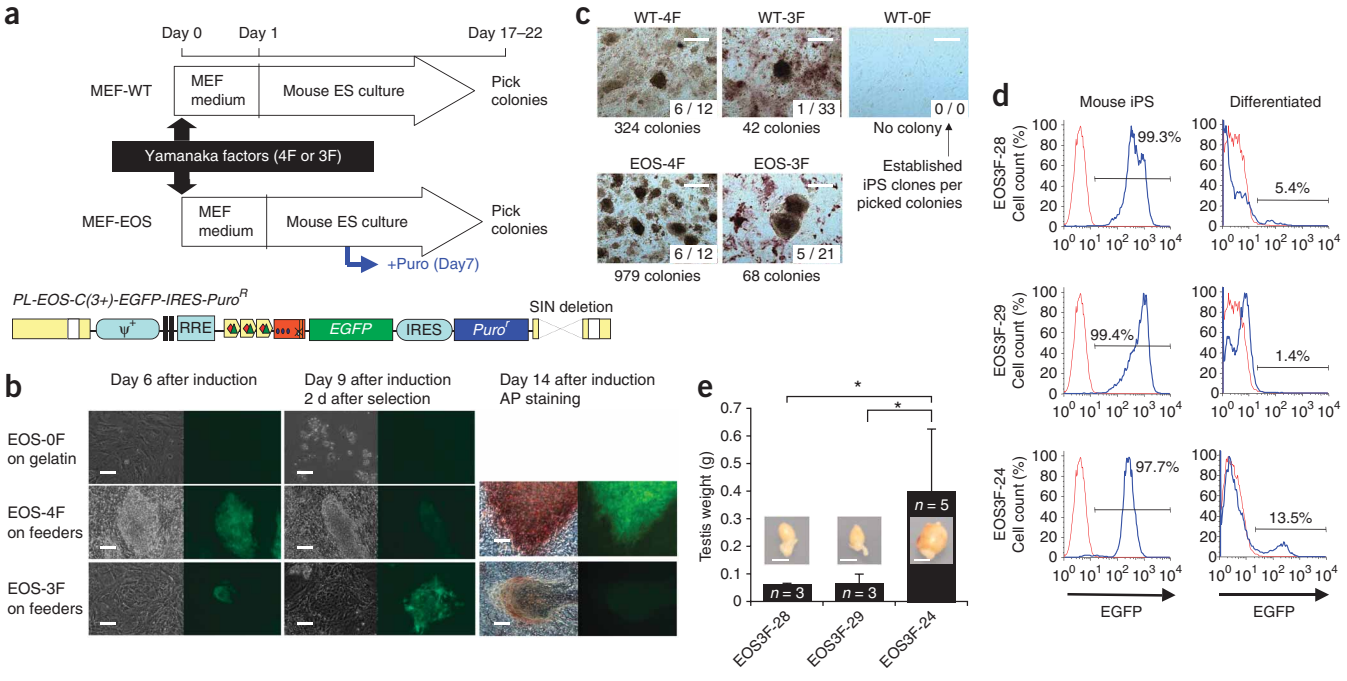

Figure 3 | EOS lentiviral vector in reprogrammed mouse iPS cells. (**a**) Experimental outline of mouse iPS cell induction. MEFs were infected with the indicated bicistronic EOS lentiviral vector, to yield MEF-EOS cells. iPS cells were induced 24 h later with either four factors (4F: *Oct-4*, *Sox2*, *Klf4*, *c-Myc*), three factors (3F: without *c-Myc*) or no factors (0F). Puromycin (puro) selection was applied at 7 d and emerging ES-like colonies were picked at 17–22 d. (**b**) Emerging colony morphology, alkaline phosphatase staining and activated EGFP fluorescence from the EOS cassette for cells reprogrammed with four or three factors at the indicated times after induction. Scale bars, 100 μm. (**c**) Alkaline phosphatase (AP) staining of cells with (EOS-4F, EOS-3F) and without (WT-4F, WT-3F) EOS vector infection and puromycin selection. WT-0F is the no-induction control. The number of colonies per representative plate is indicated; the ratio within the panel shows established iPS cell clones relative to total picked iPS-cell colonies. Scale bars, 500 μm. (**d**) Flow cytometry analysis of EGFP expression (blue) in three mouse iPS cell lines upon *in vitro* differentiation. Representative result from three independent differentiation experiments. Red, simultaneously differentiated WT4F-1 iPS clone (negative control). (**e**) Comparison of tumors formed by EGFP$^+$ EOS3F-24 iPS cells with those formed by EGFP$^-$ EOS3F-28 and EOS3F-29 iPS cells, 5 weeks after injection. Error bars, s.d.; $^*P < 0.05$. Scale bars, 5 mm.

lines, the expansion efficiency was 3% (1/33) without selection and 24% (5/21) with puromycin selection (**Fig. 3c**). The iPS cell lines showed ES-like morphology (**Supplementary Fig. 7** online), and EGFP expression coincided with the endogenous pluripotent markers SSEA-1 and Nanog (**Supplementary Fig. 8** online). Copy number of integrated EOS lentiviral vector ranged from 2 to 4 in the lines examined, as expected from the MOI used (**Supplementary Fig. 9** online). We conclude that EOS is an effective marker of reprogrammed colonies and enriches for the isolation and maintenance of iPS cell lines.

Pluripotency of the established mouse iPS cell lines was confirmed by staining for markers of the three germ layers (βIII-tubulin, ectoderm; α-actinin, mesoderm; and α-fetoprotein, endoderm) after embryoid body–mediated *in vitro* differentiation (**Supplementary Fig. 10** online) and teratoma formation (**Supplementary Fig. 11** online). As expected from ES cell differentiation experiments, EOS-EGFP expression was extinguished upon *in vitro* differentiation of mouse iPS cell lines EOS3F-28 and EOS3F-29 (**Fig. 3d**). Of note, one iPS cell line (EOS3F-24) did not readily differentiate but instead retained some cells with ES-like morphology, even after growth in normal fibroblast medium without leukemia inhibitory factor (LIF) and feeders. As expected, these ES cell-like colonies continued to express EGFP from the EOS cassette (**Fig. 3d** and **Supplementary Figs. 3c** and **10**). To test whether residual EOS-EGFP expression marked persisting undifferentiated cells, we injected the differentiated cells into testes of immunodeficient mice for teratoma formation. Five weeks after injection, the differentiated EOS3F-24 cells (EGFP positive) formed

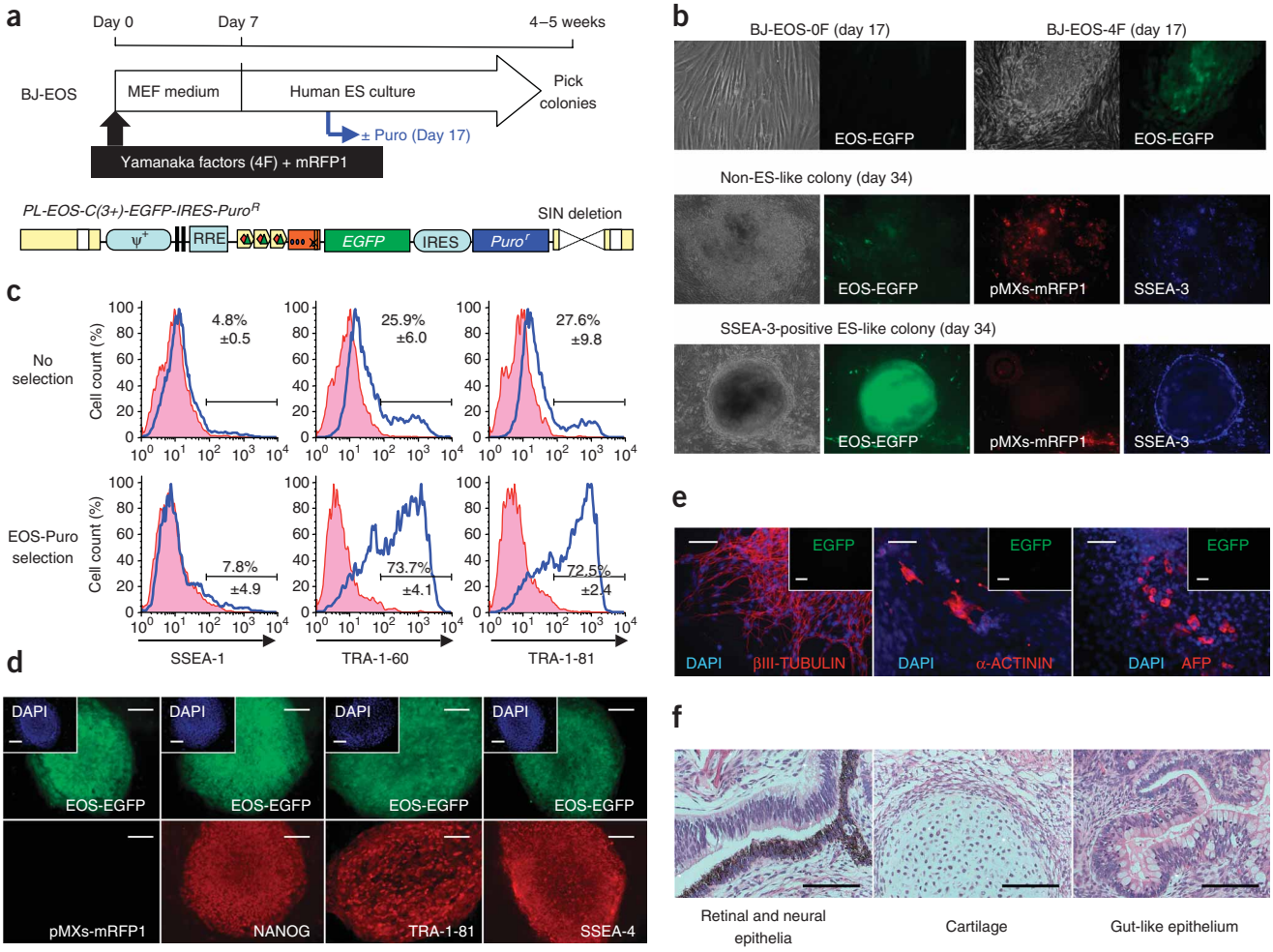

Figure 4 | EOS lentiviral vector in reprogrammed human iPS cells. (**a**) Experimental outline of human iPS cell induction. BJ cell line fibroblasts were infected with the indicated EOS lentiviral vector and with a lentivirus encoding the ecotropic gammaretrovirus receptor, to yield BJ-EOS cells. The cells were then infected with gammaretroviral vectors encoding the four human Yamanaka factors (4F: *OCT4*, *SOX2*, *KLF4*, *c-MYC*) and a pMXs-mRFP1 reporter. Puromycin (puro) selection was applied at 17 d and colonies picked 4 to 5 weeks after induction. (**b**) Emerging colony morphology, EGFP expression and marker expression at the indicated times after induction. Top row, emerging EGFP expression at day 17. Middle and bottom rows, representative colonies with poor (middle) or good (bottom) morphology at day 34. Scale bars, 200 μm. (**c**) Flow cytometry analysis showing the percentage (mean ± s.d. from three independent induction plates) of TRA-1-60$^+$ and TRA-1-81$^+$ cells with and without puromycin selection. SSEA-1 is a differentiation marker. Blue, histograms of each stained sample; red, secondary-antibody controls. (**d**) Expression of EGFP, TRA-1-60, SSEA-3 and mRFP1 in human iPS cell lines (clone 4YA). Scale bars, 100 μm. (**e**) Expression of lineage markers in a human iPS cell line (clone 4YA) after *in vitro* differentiation. Markers: βIII-tubulin (ectoderm), α-actinin (mesoderm) and α-fetoprotein (endoderm). Scale bars, 200 μm. (**f**) Sections, stained with hematoxylin and eosin, of mature teratomas formed from human iPS cells (clone 4YA), containing structures typical of each of the three germ layers: neural tissue (ectoderm), cartilage (mesoderm) and ciliated epithelium (endoderm). Scale bars, 200 μm.

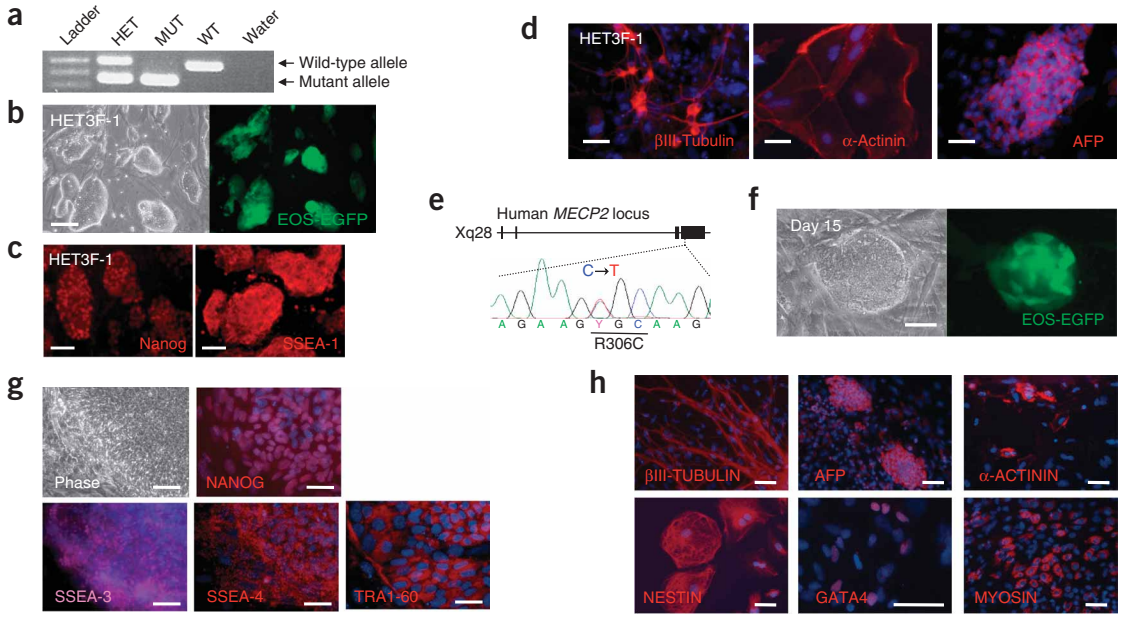

Figure 5 | EOS lentiviral vector in Rett syndrome–specific mouse and human iPS cell lines. (**a**) PCR genotyping of MEFs from the *Mecp2*^{308} mouse model to identify a heterozygous *Mecp2*$^{+/308}$ (HET) embryo. WT, wild-type; MUT, homozygous *Mecp2*$^{308/308}$ embryos. (**b**) Phase-contrast (left) and fluorescence micrographs (right) of *Mecp2*^{308} HET 3-factor iPS cell line (HET3F-1). Scale bars, 250 μm. (**c**) The HET3F-1 mouse iPS cell line stained for pluripotency markers. Scale bars, 250 μm. (**d**) Expression of lineage markers in HET3F-1 mouse iPS cells after *in vitro* differentiation. Markers are βIII-tubulin (ectoderm), α-actinin (mesoderm) and α-fetoprotein (endoderm). Scale bars, 50 μm. (**e**) Sequencing chromatogram of genomic DNA derived from an individual with Rett syndrome. (**f**) Phase contrast (left) and fluorescence (right) micrographs of a representative colony during reprogramming of human cells at 15 d after induction. Scale bar, 50 μm. (**g**) Colony morphology and immunostaining for pluripotency markers of the R306C human iPS cell line. Scale bars, 50 μm. (**h**) Expression of lineage markers in the R306C human iPS cell line upon *in vitro* differentiation. Markers: βIII-tubulin and Nestin (ectoderm), α-actinin and smooth-muscle myosin (mesoderm), and α-Fetoprotein and GATA4 (endoderm). Blue, DAPI staining, in all cases. Scale bars, 50 μm.

significantly larger teratomas, with a wide variety of tissue types, whereas the differentiated EOS3F-28 and EOS3F-29 (EGFP negative) cells did not form teratomas (**Fig. 3e**). We conclude that the EOS vector is an effective live-cell marker to monitor the differentiation state. These experiments also suggest that the EOS vector could be used to purge residual undifferentiated cells after *in vitro* differentiation and thus to prevent teratoma formation, by sorting EGFP-negative cells or by expressing a suicide gene.

Improved isolation of human iPS cells using EOS selection

To assess the use of EOS vector and puromycin selection on reprogramming of human somatic cells, human fibroblasts expressing *mSlc7a1* (ecotropic gammaretrovirus receptor) were infected with EOS lentiviral vectors containing an *EGFP*-IRES-*Puro*^{R} sequence, before infection with MoMLV-based retroviral vectors (pMXs) encoding the four human Yamanaka factors[4] (**Fig. 4a**). At the same time, pMXs-mRFP1 vector, encoding monomeric red fluorescent protein 1 (mRFP1), was infected along with the four factors to assess the gene transfer efficiency and to monitor retroviral vector silencing in the reprogrammed iPS cells[17–19]. EGFP expression and colony formation was detected 2 weeks after induction and puromycin selection was applied at day 17 (**Fig. 4b**). By 4 weeks of induction, colonies with non-hES-like morphology continued to express mRFP1 with occasional cells expressing EGFP and SSEA-3, whereas those with hES-like morphology expressed EGFP and SSEA-3 but not pMXs-mRFP1 (**Fig. 4b**). Puromycin selection increased the frequency of SSEA-3 positive iPS cell colonies with good hES-like morphology ninefold, from 4.8% to 46%, and raised the overall number of colonies with good morphology 12-fold, from 11 to 135. Puromycin selection also increased the percentage of human cells positive for the endogenous pluripotency markers TRA-1-60 and TRA-1-81 by threefold, to approximately 72% after 5 weeks, in three independent reprogramming experiments (**Fig. 4c**).

These selected iPS cell lines were isolated (**Supplementary Fig. 12a** online) and continued to express EGFP coincident with the endogenous pluripotent markers NANOG, TRA-1-81, SSEA-4, TRA-1-60 and SSEA-3 (**Fig. 4d** and **Supplementary Fig. 12b,c**). After embryoid body–mediated differentiation, the established human iPS cells extinguished EOS-EGFP expression as expected, and spontaneously formed several different cell types corresponding to the three germ layers indicating functional pluripotency (**Fig. 4e**). As the most stringent test of pluripotency for human cells, we injected the cells into immunodeficient mice for teratoma formation. Injected mice developed tumors that contained several mature tissue types corresponding to the three germ layers (**Fig. 4f**). These data demonstrate that the EOS lentiviral vector marks human iPS cells despite silencing of the MoMLV-based retroviral vector (pMXs-mRFP1). Puromycin selection of EOS-infected somatic cells enriches for iPS cell lines that express pluripotent stem cell markers.

Isolation of mouse and human Rett syndrome iPS cell lines

Finally, as a proof-of-principle for reproducibility of selection based on the EOS system in a disease context, we generated Rett

syndrome–specific iPS cell lines. Rett syndrome is an X-linked, postnatal neurodevelopmental disorder caused by a heterozygous mutation in the methyl CpG-binding protein 2 (*MECP2*) gene that affects neuronal maturation[20]. Genotyped heterozygous MEFs from *Mecp2*^{308} mice[21] (**Fig. 5a**) were reprogrammed by three-factor retroviral infection, and EOS puromycin selection allowed isolation of EGFP positive colonies that were established into iPS cell lines (**Fig. 5b**). The lines expressed pluripotency markers (**Fig. 5c**) and differentiated *in vitro* into the three germ layers, including neurons (**Fig. 5d**). Similarly, we reprogrammed fibroblasts from an 8-year-old girl with the common heterozygous R306C missense mutation in *MECP2* (**Fig. 5e**) by four-factor retroviral infection. Puromycin selection identified EGFP positive colonies (**Fig. 5f**), to produce subject-specific R306C-iPS cell lines that expressed pluripotency markers (**Fig. 5g**) and differentiated *in vitro* into several cell types, corresponding to the three germ layers, including neurons (**Fig. 5h**). Genotypes of the final mouse and human iPS cell lines were confirmed by PCR and sequencing respectively (data not shown). These results demonstrate that EOS selection can be used to establish disease-specific iPS cell lines from knockout mice and from patients.

DISCUSSION

To our knowledge, the EOS lentiviral vectors presented here are the first reporters to robustly mark human iPS cells during reprogramming. They direct pluripotent stem cell–specific expression and resist vector silencing while under puromycin selection. The previously unexplored approach of combining an endogenous retrotransposon promoter with pluripotent state–specific core enhancers holds promise for further vector improvements using new variants of embryonic-specific retrotransposon elements. The EOS vectors mark, enrich and maintain high-quality ES and iPS cell lines. This makes them useful reporters to more efficiently isolate accurately reprogrammed iPS cell lines from knockout or transgenic mice, which may be difficult to cross with *Nanog*- or *Oct4*-reporter mice, and from human biopsies to model disease *in vitro*[22–24]. Plasmids described in the paper will be available from Addgene (http://www.addgene.org/).

The Rett syndrome iPS cell lines described here may be used to validate that the known neuronal maturation defects in the mouse knockout model of the disease are recapitulated in neurons derived by directed differentiation of the iPS cells. Once the specificity of the *in vitro* assays is established for the mouse iPS-cell Rett syndrome model, we foresee a similar approach being applied to characterize neurons derived from the patient-specific human iPS cells. Ultimately, the human iPS cells will provide a renewable source of neurons that may be applied toward screens for small molecules or neuroactive drugs that rescue neuronal function in Rett syndrome.

In comparison to live-cell staining for the TRA-1-81 surface marker to identify reprogrammed human iPS colonies[19], EOS vectors increased the frequency of obtaining colonies with good morphology and permit constant selection to maintain and expand iPS cells in the pluripotent state. In the context of *in vitro* iPS cell applications, such as the study of disease-specific iPS cell lines, retroviral or lentiviral integrations do not hinder disease-specific iPS cell line generation[22] nor do they necessarily influence phenotyping of affected cell types[23,24]. More recent reprogramming methods that use adenoviral vectors[25,26] or plasmid transfection[25] have promising future applications. The use of EOS vectors to allow imaging of cells for EGFP expression or selection for puromycin resistance may be valuable attributes for optimizing new reprogramming technologies using transient-factor delivery methods or for high-throughput screens of small molecules that enhance reprogramming[27]. Furthermore, directed differentiation procedures may need to be optimized for each disease-specific iPS cell line generated, and EOS vector expression could be used to monitor nonresponding pluripotent stem cells in this context. Finally, the addition of an EOS-regulated suicide gene may hold the potential to purge undifferentiated pluripotent stem cells *ex vivo* to prevent teratomas[28] in future clinical trials aiming for regenerative medicine therapies, or to eliminate them *in vivo* after transplantation.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

A.H., D.L.M. and J.E. conceived the project; I.A.M. provided reagents; A.H., M.B., J.R. and J.E. designed experiments; A.H. developed the EOS vectors and performed the iPS cell reprogramming experiments; A.Y.L.C. and N.F. performed the Rett syndrome iPS cell experiments; K.V. and J.S.D. performed the hES cell experiments; C.A.S. and P.P. performed the teratoma experiments; A.H., J.R., M.B. and J.E. wrote the manuscript.

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ONLINE METHODS

Plasmid construction. The PL (self-inactivating) lentiviral vector backbones have been previously described[29]. The ETnII LTR#6 promoter has been described previously[8], but we introduced a single nucleotide mutation in the poly(A) signal by a two-step PCR method using primers ETn-pA-Mu-s, ETn-pA-Mu-a, RVP3 and GLP2 (primer sequences used in this study are listed in **Supplementary Table 1** online). The human *NANOG* promoter was PCR amplified from bacterial artificial chromosome (BAC) RP11-277J24 (AC006517), containing human chromosome 12, using primers 'Nanog-fwd (*Nco*I)' and 'Nanog-rev (*Bam*HI)'. Mouse *Oct-4* promoter was derived from GOF-18 (ref. 30). Mouse *Oct-4* enhancer CR4 (ref. 9) and *Sox2* enhancer SRR2 (ref. 10) were PCR-amplified from genomic DNA of J1 ES cells (strain 129S4/Jae) using primers 'mOct4-CR4-s (*Eco*RI)', 'mOct4-CR4-a (*Xho*I)', 'mSox2-SRR2-s (*Eco*RI)' and 'mSox2-SRR2-a (*Xho*I)'. All promoters and enhancers were verified by DNA sequencing. The cloning strategy and sequence of the constructs is available upon request. Plasmids described in this paper will be available from Addgene (http://www.addgene.org/).

Cell culture. J1 mouse ES cells and mouse iPS cells were cultured in mouse ES medium: Dulbecco's Modified Eagle's Medium (DMEM) with 15% fetal bovine serum (FBS) supplemented with 4 mM L-glutamine, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 0.55 mM 2-mercaptoethanol (all from Invitrogen) and purified recombinant LIF. Plat-E retrovirus packaging cells were maintained in DMEM with 10% FBS containing blasticidin (10 μg ml^{-1}) and puromycin (1 μg ml^{-1}). NIH3T3, 293T and MEF cells were cultured in DMEM with 10% FBS supplemented with 4 mM L-glutamine. MEFs were isolated from embryonic days 15.5–17.5 CD-1 mouse embryos. CA-1 human ES cell line was maintained on feeders in Knockout DMEM (Invitrogen) supplemented with 15% Serum Replacement (Invitrogen), 2 mM Glutamax (Invitrogen), penicillin, streptomycin, 0.1 mM nonessential amino acids, 0.5 mM 2-mercaptoethanol, and 10 ng ml^{-1} recombinant human basic fibroblast growth factor (bFGF; PeproTech). H1 human embryonic stem cells were grown on Matrigel (BD Biosciences) in the presence of MEF-conditioned medium as previously reported[13]. Human dermal fibroblasts were isolated from a skin biopsy of an 8-year-old male by distal humerus osteotomy under Research Ethics Board approval from the Hospital for Sick Children. Feeders for iPS cell derivation were isolated from day 15.5 embryos of Tg(DR4)1Jae/J mice (Jackson Laboratory) for puromycin resistance.

Viral vector production and infection. Plat-E (retrovirus packaging cells) or 293T cells for lentivirus were plated at a density of 1×10^5 cells cm^{-2}. The following day, the cells were transfected using Lipofectamine 2000 (Invitrogen) with appropriate plasmids. The supernatant containing virus was collected 48 h after transfection and passed through a 0.45-μm filter to remove cellular debris. Lentiviruses were concentrated by ultracentrifugation at 4 °C, 2 h, 30,000 r.p.m. with a T-865 rotor (Sorvall). The viral pellet was resuspended in 40 μl Hank's balanced salt solution (Invitrogen) overnight at 4 °C. One day before infection, target cells were seeded at 5×10^4 cells (for NIH3T3 and MEFs) or 1×10^4 cells (for J1) per well of a 24-well plate. For infection, virus was added to the target cells in the presence of 8 μg ml^{-1} Polybrene (hexadimethrine bromide, Sigma). Titer for PL-EOS-C(3+)-EGFP-IRES-Puro lentiviral vectors was approximately 1×10^7 IU ml^{-1} assayed on J1 mouse ES cells, and the titer was used to estimate the MOI of fibroblast infections.

Immunocytochemistry. Cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 20 min, permeabilized with 0.2% NP-40 for 5 min, blocked with 0.5% BSA and 6% normal goat serum for 1–2 h and incubated with primary antibodies with 0.25% BSA and 3% normal goat serum in PBS overnight. After washing three times with PBS, cells were incubated with secondary antibodies for 45 min. Immunostaining images were taken with a Zeiss Axiovert 200M microscope equipped with AxioCam HRm camera and AxioVision software. Antibodies used in this study are listed in **Supplementary Table 2** online.

Flow cytometry. Trypsinized cells were suspended in PBS with 5% FBS. Single-cell suspensions were filtered through 70-μm-pore nylon membranes and analyzed by FACScan (Becton Dickinson) flow cytometry using CellQuest software (Becton Dickinson). Before each experiment, the machine was calibrated using calibration beads (Spherotech). Cell debris was excluded from analysis by using forward- and side-scatter gating. For each cell type, mock-infected or uninfected cells were used as a negative control to adjust fluorescence channel 1 gain to detect EGFP fluorescence. The data obtained were analyzed with FlowJo software (Tree Star).

Microscopy. Live cell images were captured using a Leica DM IL microscope equipped with Leica DC500 digital color camera and OpenLab software (Improvision) or a Leica DMI4000B microscope equipped with Leica DFC340FX camera and Leica Application Suite software. For EGFP fluorescence, a band-pass 450–490 nm filter was used for excitation and low-pass 520 nm filter was used for detection.

Mouse ES or iPS cell differentiation. Mouse ES or iPS cell colonies cultured on gelatin-coated dishes were loosely detached by trypsin-EDTA treatment and suspended in mouse ES medium without LIF. The ES colonies were cultured in suspension in untreated Petri dishes for 4 d to make embryoid bodies. The cells were induced to differentiate with 5 μM all-*trans* retinoic acid (Sigma) for 24 h and cultured further as embryoid bodies for 3 d. Then embryoid bodies were trypsinized into single cells and plated onto tissue culture-grade dishes for 3–5 d more.

Human ES or iPS cell differentiation. Human iPS cell colonies were loosely detached by collagenase treatment and suspended in human ES medium without bFGF. The ES colonies were cultured in Ultra Low Attachment six-well plates (Costar) for 8 d to make embryoid bodies. Then, embryoid bodies were loosely dissociated by trypsin and plated onto tissue culture-grade dishes for another 2 weeks. For CA-1 differentiation (**Fig. 2b**), we added 5 μM all-*trans* retinoic acid to human ES cell culture medium (without bFGF) for 9 d.

Alkaline phosphatase staining. Cells were fixed with 4% formaldehyde and stained with 1 mg ml^{-1} Fast Red TR, hemi(zinc chloride) salt (Sigma) and 0.4 mg ml^{-1} naphthol phosphate,

disodium salt (Sigma) in 0.1 M Tris-HCl, pH 8.6, for 5–10 min. Wild-type J1 ES cells were used as a staining control and NIH3T3 or MEF cells were used as negative controls.

Mouse iPS cell induction. The induction of iPS cells was based on the published protocol[14,16]. In brief, retroviral vectors encoding *Oct-4*, *Sox2*, *Klf4* and *c-Myc* were produced using Plat-E cells by plasmid transfection of either pMXs-Oct4, pMXs-Sox2, pMXs-Klf4 or pMXs-c-Myc (Addgene plasmids 13366, 13367, 13370 and 13375, respectively). One million cells per 10-cm dish of MEFs (isolated from wild-type CD-1 strain or *Mecp2* heterozygous mutant mice[21] (Jackson Laboratory)) were infected with 2.5 ml each of unconcentrated retroviral vector in the presence of 8 μg ml^{-1} Polybrene. One day after infection, the cells were trypsinized, and 6 × 10^5 cells were transferred onto feeders in a 10-cm dish in mouse ES cell medium. Puromycin selection (1 μg ml^{-1}) was applied at 7 d and emerging ES-like colonies were picked from 17–22 d and dissociated by trypsinization. All EOS-infected iPS cell lines were maintained in mouse ES medium containing 1 μg ml^{-1} puromycin on drug-resistant feeders. iPS cell lines will be available through the Ontario Human iPS Cell Facility (http://www.ontarioips.ca/).

Human iPS cell induction. Human BJ fibroblasts (American Type Culture Collection, CRL-2522) or Rett syndrome fibroblasts (Coriell, GM11270) were infected with pLenti6/UbC/mSlc7a1 lentiviral vector (Addgene, 17224)[4] expressing the mouse *Slc7a1* gene (encoding the receptor for ecotropic gammaretrovirus) and selected with blasticidin before reprogramming experiments. Cells were seeded at 8 × 10^5 cells per 10-cm dish and transduced twice with pMXs retroviral vectors encoding human *OCT4* (*POU5F1*), *SOX2*, *KLF4* and *c-MYC* (*MYC*) (Addgene 17217, 17218, 17219 and 17220, respectively)[4], together with pMXs-mRFP1 retrovirus for monitoring infectivity and viral silencing. One week after transduction, cells were trypsinized and seeded onto a 10-cm feeder dish in human ES cell medium. We added puromycin (1 μg ml^{-1}) at day 17 after transduction. After 4 to 5 weeks of induction, emerged colonies were picked and mechanically dissociated at initial passages until the cells became confluent on the six-well plate, then adapted to collagenase treatment. All EOS-infected iPS cell lines were maintained in human ES medium containing 1 μg ml^{-1} puromycin on feeders.

Teratoma formation. Mouse iPS cells were suspended in PBS with 5% FBS and injected into the testes of NOD/SCID (nonobese diabetic, severe combined immunodeficiency) mice. Four to five weeks after injection, testes were weighed. Human iPS cells were suspended in a mixture of Knockout DMEM, Matrigel and collagen (StemCell Technologies) to inject intramuscularly into NOD/SCID mice, as previously described[6]. Tumors were harvested 9 weeks after injection. Fixed tumors were embedded in paraffin, sectioned and stained with hematoxylin and eosin for pathological analysis. Mouse and human ES cells were used as positive controls for teratoma formation. Parental fibroblasts for iPS derivation did not form teratomas (data not shown). All procedures using animals were approved by the SickKids Animal Care Committee under the auspices of The Canadian Council on Animal Care.

Genotyping of MeCP2 mutation. For Rett syndrome mouse iPS cells, PCR on genomic DNA yielded an amplicon of 396 bp for wild-type *Mecp2* and 318 bp for the truncated *Mecp2*^{308} allele using primers oIMR3912, oIMR3913 and oIMR3914. For Rett syndrome human iPS cells, genomic DNA was extracted from R306C iPS cells and PCR was performed using primers RTT-fwd and RTT-rev (**Supplementary Table 1**). The PCR amplicon was isolated and DNA sequencing was performed using the RTT-fwd primer.

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