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The ROSA26-iPSC Mouse: A Conditional, Inducible, and Exchangeable Resource for Studying Cellular (De)Differentiation

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http://dx.doi.org/10.1016/j.celrep.2013.01.016

SUMMARY

Control of cellular (de)differentiation in a temporal, cell-specific, and exchangeable manner is of paramount importance in the field of reprogramming. Here, we have generated and characterized a mouse strain that allows iPSC generation through the Cre/loxP conditional and doxycycline/rtTA-controlled inducible expression of the OSKM reprogramming factors entirely from within the ROSA26 locus. After reprogramming, these factors can be replaced by genes of interest—for example, to enhance lineage-directed differentiation—with the use of a trap-coupled RMCE reaction. We show that, similar to ESCs, Dox-controlled expression of the cardiac transcriptional regulator Mesp1 together with Wnt inhibition enhances the generation of functional cardiomyocytes upon in vitro differentiation of such RMCE-retargeted iPSCs. This ROSA26-iPSC mouse model is therefore an excellent tool for studying both cellular reprogramming and lineage-directed differentiation factors from the same locus and will greatly facilitate the identification and ease of functional characterization of the genetic/epigenetic determinants involved in these complex processes.

INTRODUCTION

Pluripotency can be induced in various cell types by the ectopic expression of four transcription factors Oct4 (also known as Pou5f1), Sox2, Klf4, and c-Myc (hereinafter referred to as OSKM), generating induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Since the initial discovery in 2006, various methods (González et al., 2011) have been developed to generate iPSCs using inducible or excisable transposon-based strategies to control the ectopic expression of the reprogramming factors instead of the more "classical" and uncontrollable retroviral-based systems. The most advanced techniques in mouse iPSC generation are the recently developed double transgenic reprogrammable mouse systems (Carey et al., 2010; Stadtfeld et al., 2010). These doxycycline-inducible Col1a1 locus-targeted transgenic mouse models allow iPSC derivation from different cell populations when crossed with the ubiquitously expressing reverse-tetracycline-dependent-transactivator (rtTA) ROSA26 mouse strain. However, these double transgenic approaches lack tissue/cell-specific OSKM expression, as well as a means of selecting OSKM-expressing cells, and do not allow the exchange of the reprogramming factors by other genes to study iPSC lineage-directed differentiation at a well-defined locus. Here, we report a conditional and inducible (COIN) transgenic mouse model that allows expression of OSKM exclusively from within the ROSA26 locus. Moreover, after reprogramming, the four factors can be replaced in the resulting iPSCs by any cDNA/ORF of interest via a recombinase-mediated cassette exchange (RMCE) strategy.

RESULTS AND DISCUSSION

Generation and Use of the ROSA26-iPSC Mouse Model to Create Reprogrammed Cells

We generated a COIN *OSKM* construct by using similar conditional strategies (Belteki et al., 2005), as well as previously published (Nyabi et al., 2009) Gateway cloning technologies, and subsequently targeted this construct to the ROSA26 locus via homologous recombination in robust hybrid F1 embryonic stem cells (ESCs), i.e., G4 ESCs (George et al., 2007). In this double-locked system (Figure 1A and Figure S1A), a *loxP*-flanked (*floxed*) *neomycin* resistance (Neo^R) transcriptional STOP cassette prevents the expression of the bicistronic *rtTA-IRES-puromycin*-pA mRNA and the reprogramming factors. Through Cre-mediated deletion of this STOP cassette, *rtTA* is expressed from the endogenous ROSA26 promoter. Only after administration of the inducing drug doxycycline (Dox), rtTA will undergo nuclear translocation and activate the Tet-responsive element (TRE; tet(o)-CMV_{min}) that will drive expression of the polycistronic OSKM cassette along with a reporter gene

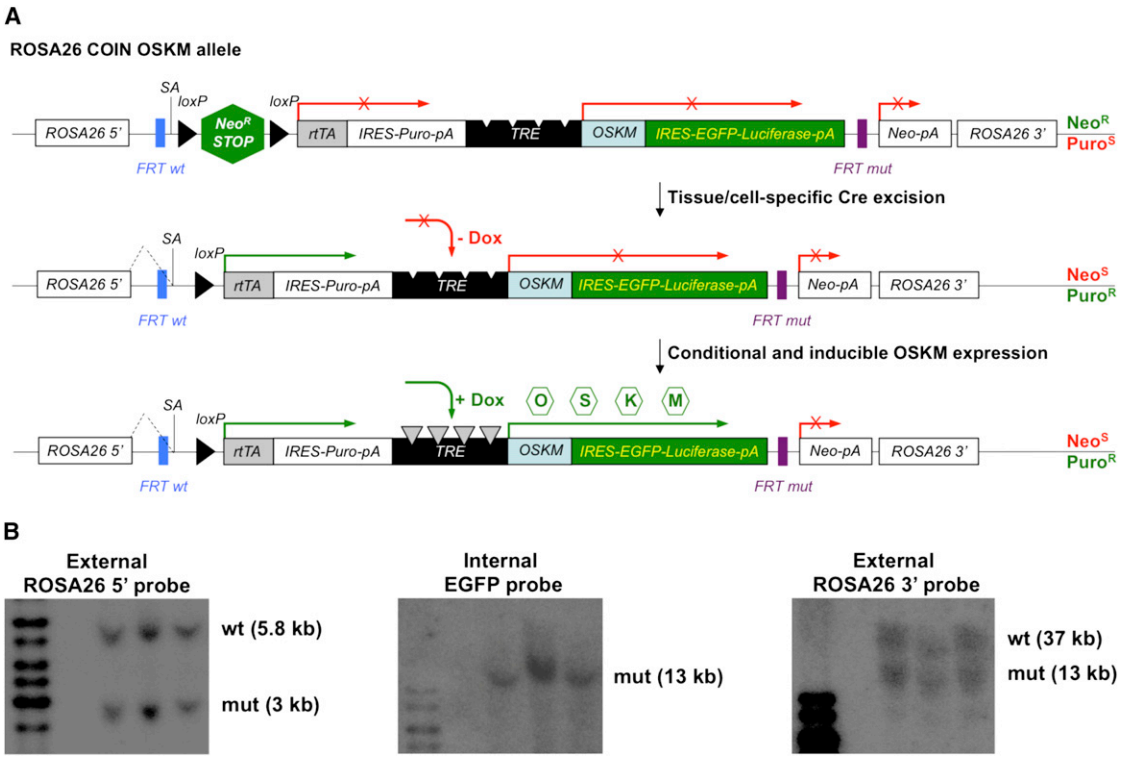

Figure 1. The ROSA26-iPSC Mouse Model

(A) Schematic overview of the conditional and inducible (COIN) OSKM-targeted ROSA26 locus and its use. Cre-mediated removal of the conditional Neo^R STOP cassette leads to ROSA26-based rtTA expression. Inducible expression of the reprogramming factors (OSKM, encoding for the polycistronic *Oct4*, *Sox2*, *Klf4*, and *c-Myc* transgene) will only be achieved upon Dox administration and can be monitored by the inducible EGFP expression. SA, splice acceptor; IRES, internal ribosome entry site; pA, poly(A) sequence.

(B) Southern blot analysis (5′, internal EGFP, and 3′ probe) of three correctly targeted ESC clones. wt, wild-type; mut, mutant.

For details, see Figure S1A.

(*enhanced green fluorescent protein-Luciferase*; *EGFP-Luciferase*). Via diploid embryo aggregation with correctly targeted ESCs (Figure 1B), a stable mouse line was generated from several strong, germline-transmitting chimeras (Figures S1B and S1C). These ROSA26-iPSC mice can be bred to the wide range of tissue/cell-specific Cre mouse lines available. This allows conditional and inducible expression of the reprogramming factors in the derived primary cell cultures isolated either from embryonic or adult offspring for cellular reprogramming experiments (Figure 2A).

To test our ROSA26-iPSC transgenic model functionally, we isolated mouse embryonic fibroblasts (MEFs) and adult tail tip fibroblasts (TTFs) from Sox2 Cre X ROSA26-iPSC mice. We cultured and expanded these cells under puromycin (Puro) selection before inducing the reprogramming factors with Dox. After 3 weeks of continuous Dox induction, several OSKM-EGFP-expressing colonies with an ESC/iPSC-like morphology could be detected (Figure 2B). Individual picking and expansion of these colonies led to the establishment of multiple Dox-independent iPSC lines expressing pluripotency-related markers such as alkaline phosphatase (Alk. Phos.), Oct4, Nanog, and SSEA1 (Figure 2C) and increased *Nanog* mRNA expression levels that are similar to wild-type G4 ESCs (Figure 2D). In vitro differentiation of these iPSCs using embryoid body differentiation assays demonstrated no differences compared to wild-type ESCs in terms of the formation of embryoid bodies, beating cardiomyocyes, sprouting vascular endothelial channels, or their hematopoietic differentiation potential (Figure 2E; data not shown). To further characterize the differentiation potential of our iPSC clones in vivo, cells were subcutaneously injected into immunocompromized nude mice, which led to teratoma formation for all analyzed clones. Histological analysis of the tumors demonstrated their differentiation potential toward cell types of the three different germ layers (Figure 2F). Moreover, adult iPSC-derived chimeric mice could be generated using diploid complementation assays, thereby further underscoring the pluripotent nature of these cells (Figure 2G).

However, as previously reported for other TRE-based transgenic mouse lines (Beard et al., 2006; Premsrirut et al., 2011), we also observed significant mosaic transgene expression in our ROSA26-iPSC model. Indeed, of our starting fibroblast populations, only around 8% of the MEFs (OSKM-heterozygous) and 16% of the TTFs (OSKM-homozygous) appeared to be capable of turning on the *OSKM-EGFP* expression cassette after 2 days of in vitro Dox induction (Figure S2A). As well, when we turned on the reprogramming factors in vivo for 5 days in

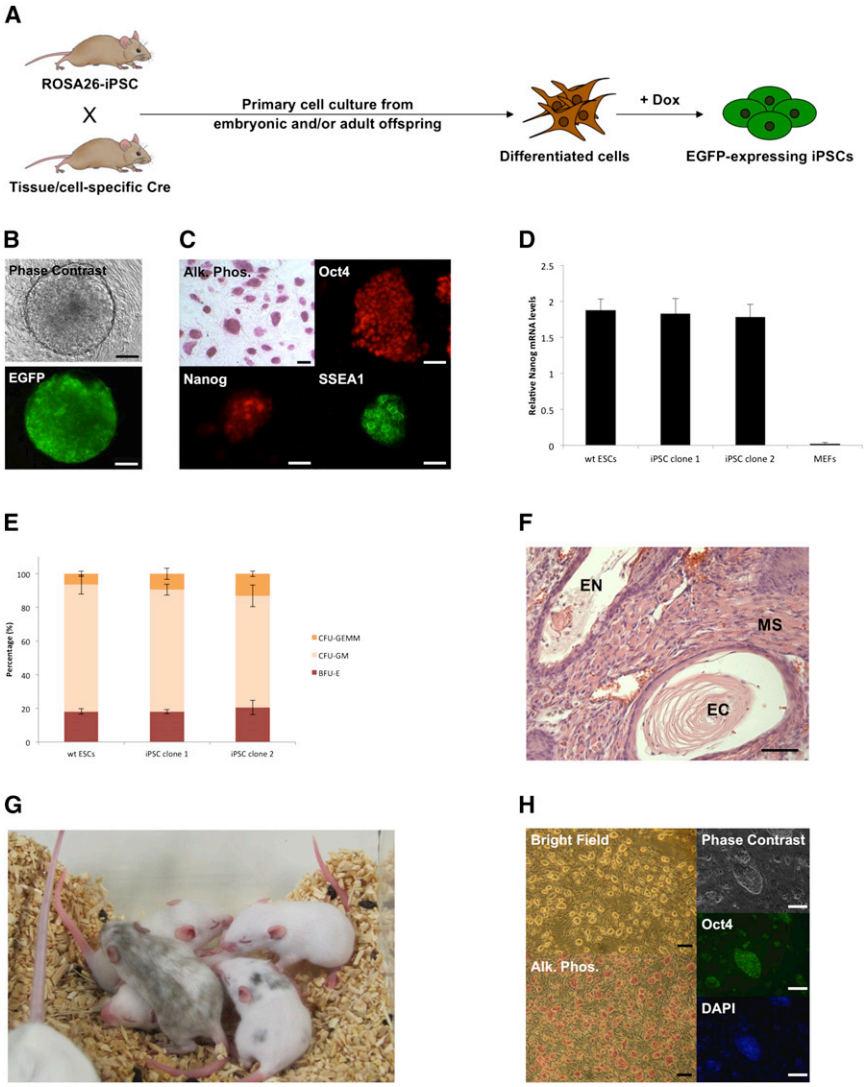

Figure 2. Derivation and Characterization of ROSA26-iPSC Mouse Model-Derived iPSCs

(A) The ROSA26-iPSC mouse can be bred to a tissue/cell-specific Cre mouse line. Isolation of Cre-excised differentiated cells from hereof-derived embryonic/adult offspring and subsequent Dox administration leads to EGFP-expressing iPSCs.

(B) Phase contrast and fluorescent picture of an OSKM-EGFP-expressing MEF-iPSC colony after 3 weeks of continuous Dox induction. Scale bars, 100 μm.

(C) Bright field (Alk. Phos.) and fluorescent pictures (Oct4, Nanog, and SSEA1) of pluripotency markers of a MEF-iPSC clone. Scale bars, 100 μm.

(D) qRT-PCR analysis on pluripotency marker *Nanog* of wild-type G4 ESCs and MEF-iPSC clones compared to MEFs.

(E) Graph representing the methylcellulose assay comparing the hematopoietic cell colonies (BFU-E, CFU-GM, and CFU-GEMM) after hematopoietic-specific in vitro differentiation of wild-type G4 ESCs versus two MEF-iPSC clones.

(F) An H&E-stained section of a MEF-iPSC-derived teratoma (endoderm, [EN], ciliated columnar epithelium with goblet cells; mesoderm [MS], muscle and ectoderm [EC], squamous epithelium). Scale bar, 50 μm.

(G) Picture of TTF-iPSC-derived chimeric mice.

(H) Characterization of EC-iPSCs. Scale bars, 200 μm. Error bars represent the SD of the average of triplicate assays.

See also Figures S2 and S3.

a hematopoietic/endothelial lineage-specific manner using Tie2 Cre X ROSA26 OSKM-homozygous mice, we could sort, based on the EGFP-based transgene expression of our mouse model, EGFP/c-Kit-positive hematopoietic stem/progenitor cells (HS/PCs) (Figure S2B) and reprogram them toward iPSCs with similar characteristics as described before (data not shown). Therefore, using our model and EGFP-based fluorescence-activated cell sorting (FACS), it is possible to isolate cells either from in vivo sources or after in vitro expansion that are still capable of turning on the OSKM reprogramming factors.

Additionally, it should be noted the reprogramming efficiency in our developed mouse model is somewhat less efficient in generating iPSCs (Table S1) than the other two previously published *Col1a1*-based reprogrammable mouse models (Carey et al., 2010; Stadtfeld et al., 2010). To understand the reason for this lower efficiency, we directly compared the OSKM mRNA and protein levels in our ROSA26-iPSC model with those of the model developed by Carey et al., 2010. Upon Dox administration, significantly lower mRNA and protein levels of the reprogramming factors were observed in OSKM-homozygous, EGFP-positive sorted MEFs from our ROSA26-based model compared to OSKM-homozygous MEFs from the Carey et al., 2010 Col1a1-based model (Figures S2C and S2D). The lower frequencies of reprogramming observed in our ROSA26-OSKM-based model can therefore be explained by the overall lower OSKM expression levels. It is interesting that these differences are less pronounced in our ROSA26-based TTF-iPSCs that appear to have basal OSKM levels more similar to wild-type ESCs than in the Col1a1-derived TTF-iPSCs (Figure S2E). As well, unlike the model published by Stadtfeld et al. (2010), we see no evidence of spontaneous tumor formation in adult Sox2 Cre X OSKM-homozygous mice under noninduced conditions up to 18 months of age. This demonstrates the tightness of *OSKM* expression in our system as also shown by quantitative real-time PCR (qRT-PCR) analysis of derived MEFs compared to wild-type MEFs (Figure S2C).

Not only did we obtain fully reprogrammed iPSCs from our ROSA26-iPSC model, but we also generated interesting partially reprogrammed cells (discussed later) derived from EGFP/CD31-positive endothelial cells (ECs) (Figure S3A). Upon Dox removal, these cells have the potential to differentiate spontaneously back into CD31-positive, vascular-like channels in vitro even without

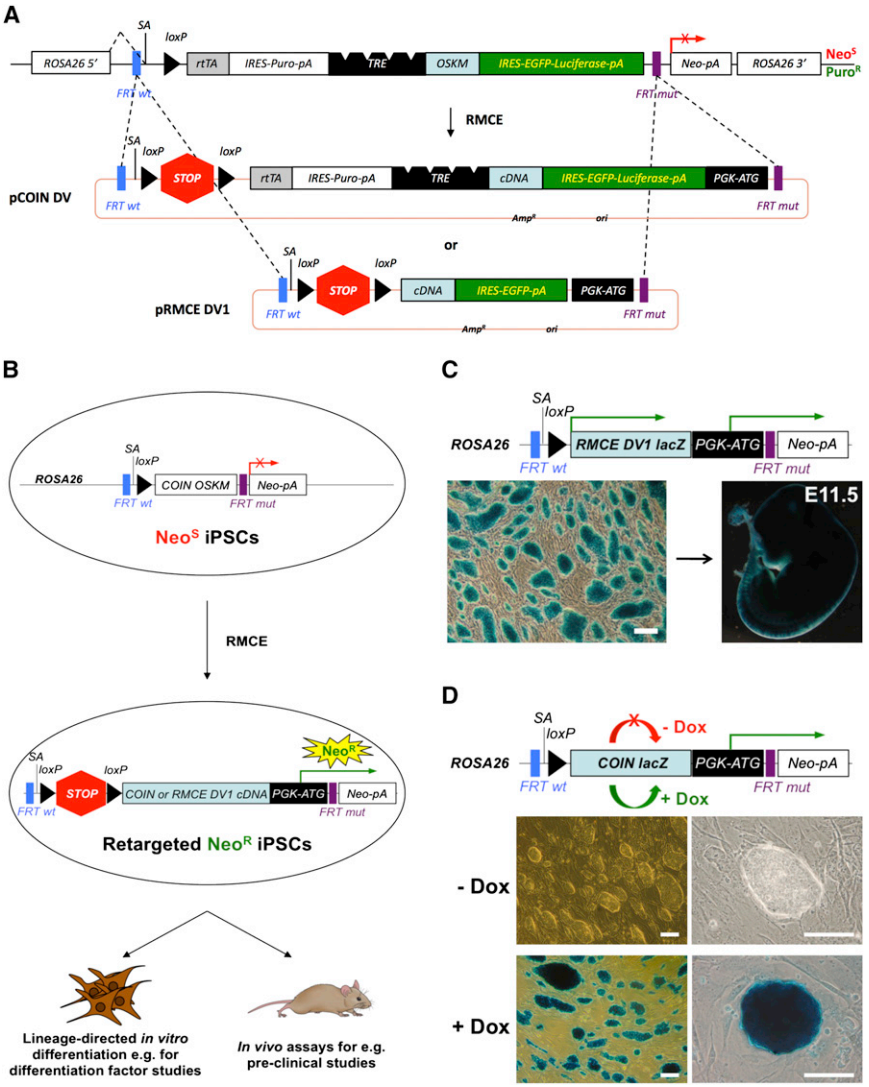

Figure 3. RMCE Removal of the Reprogramming Factors

(A) Schematic overview of the RMCE strategy to replace the reprogramming factors by conditional (pRMCE DV1) or conditional and inducible (pCOIN DV) incoming vectors.

(B) Using a trap-coupled RMCE reaction, neomycin-sensitive (Neo^S) iPSCs can be efficiently retargeted with pRMCE DV1 or pCOIN cDNA constructs replacing the reprogramming factors and restoring Neo^R.

(C) Scheme and bright field pictures from X-gal-stained Cre-excised pRMCE DV1 lacZ-retargeted TTF-iPSCs and derived E11.5 chimeric embryos. Scale bar, 200 μm.

(D) Scheme and bright field pictures of noninduced (−Dox) and induced (+Dox) X-gal-stained Cre-excised, Neo^R COIN lacZ-retargeted MEF-iPSC subclones. Scale bars, 200 μm.

the addition of additional cytokines such as VEGF (Figures S3B and S3C). These cells are somewhat similar to what has recently been described for rat OSKM-induced vascular progenitor cells (iVPCs) (Yin et al., 2012) in their ability to differentiate spontaneously back into CD31-positive cells when Dox was removed from the system even in late passage ($p > 10$) clones. Of the three iVPC clones analyzed in greater detail, all showed discrete populations of Alk. Phos.-positive cells (Figure S3D), but only clone 3 was capable of forming a tumor when injected into immunocompromised mice. It is interesting that the tumor did not contain a variety of different tissues derived from all three different germ layers characteristic for teratomas but instead displayed a rather limited developmental potential with high vascular channel formation capacity as judged by CD31 immunostaining analysis (Figure S3E). By selectively replating clone 3 on to gelatinized plates and subcloning ESC-like colonies, we could derive Dox-independent EC-derived iPSCs (EC-iPSCs) that ubiquitously expressed ESC cell markers such as Alk. Phos. and Oct4 (Figure 2H). These reprogrammed EC-iPSCs have a greater developmental potential than the original iVPC precursor cells and could form teratomas with all three germ layer derivatives along with a more limited angiogenic potential (Figure S3E). Although not "truly" pluripotent, the iVPCs in comparison to EC-iPSCs can be of great interest in preclinical studies as well as in further unraveling the molecular mechanisms of cellular reprogramming and identifying genetic determinants of EC memory. Using this conditional and inducible ROSA26-iPSC model system with other tissue/cell-restricted Cre lines will also enable researchers to identify other stable partially reprogrammed cell lines similar to our mouse iVPCs, partially described here. This is an important issue as several concerns have recently been raised about the genetic stability (Blasco et al., 2011) and immunogenicity (Zhao et al., 2011) of true pluripotent iPSCs.

Characterization and Validation of the RMCE Component of the ROSA26-iPSC Mouse Model in Lineage-Directed Cellular Differentiation Assays

After successful reprogramming, the ectopic expression of OSKM is no longer needed and can be shut off by withdrawal of Dox. However, we designed our construct in such a way that we can actually use the same ROSA26 locus to remove and, more important, exchange the reprogramming factors using a trap-coupled RMCE reaction with high efficiencies as previously reported (Sandhu et al., 2011). Other cDNAs/ORFs of interest can hereby be efficiently cloned into Gateway-compatible vectors and introduced in a conditional (pRMCE DV1) or conditional and inducible (pCOIN DV) manner (Figure 3A). By cotransfecting such RMCE-compatible incoming vectors

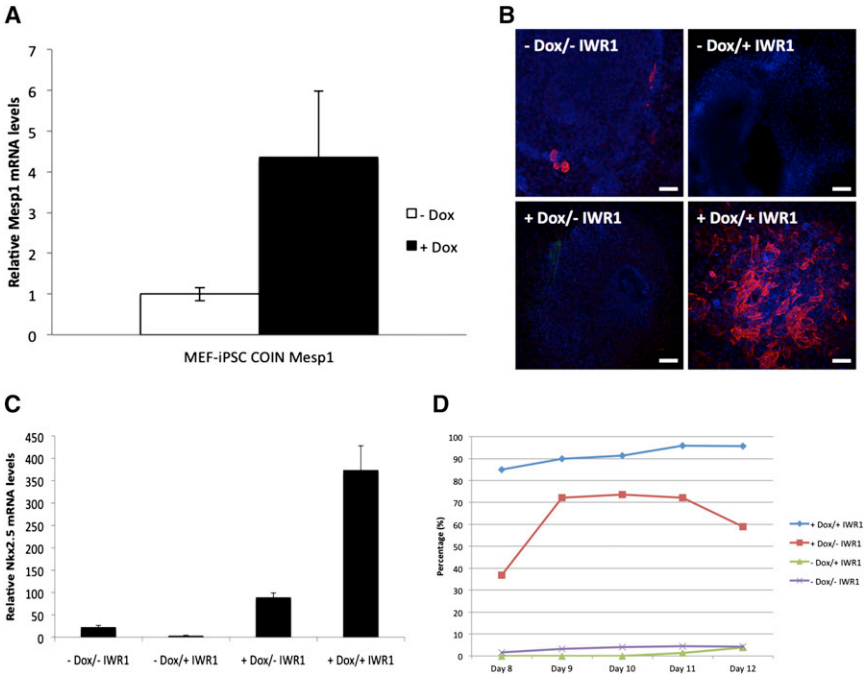

Figure 4. Cardiomyocyte Lineage-Directed Differentiation of COIN Mesp1-Retargeted MEF-iPSCs

(A) qRT-PCR analysis of pulsed *Mesp1* expression at day 4 of in vitro differentiation in induced, COIN *Mesp1*-retargeted MEF-iPSCs compared to non-induced control cells.

(B) Fluorescent immunohistochemistry pictures showing TroponinT-positive structures (in red) in induced and Wnt-inhibited (+Dox/+IWR1) cells at day 8 of in vitro differentiation. Representative images for each of the conditions are shown. Hoechst (blue) DNA staining is monitoring cellularity. Scale bars, 100 μm.

(C) qRT-PCR analysis at day 8 of differentiation on cardiac marker for Nkx2.5 mRNA levels showing the increased levels mainly in induced differentiating iPSCs with Wnt inhibition (+Dox/+IWR1).

(D) Beating cardiomyocyte assay showing the dramatically enhanced differentiation potential of our COIN *Mesp1*-retargeted MEF-iPSC toward beating cardiomyocytes when pulsed with Mesp1 together with (+Dox/+IWR1) (or without, +Dox/−IWR1) Wnt inhibition compared to noninduced differentiating cells (−Dox/−IWR1 and −Dox/+IWR1). Error bars represent the (SD of the average of triplicate assays.

together with a FlpE-expressing plasmid, the resulting Neo^R iPSC clones should be correctly retargeted and can, for example, subsequently be used for lineage-directed differentiation studies (Figure 3B). To demonstrate this, we created two incoming in vitro Cre-excised lacZ-encoding constructs: one pRMCE DV1 lacZ construct and one pCOIN DV lacZ construct. In separate experiments, we successfully replaced the reprogramming factors by these lacZ constructs. For the pRMCE DV1 lacZ, we obtained constitutive ROSA26-based lacZ expression in the retargeted iPSCs (Figure 3C). Using beta-galactosidase staining analysis, we could show that the RMCE approach was working at high efficiencies in MEF-derived (12/12 lacZ-positive/Neo^R clones = 100%), TTF-derived (22/22 lacZ-positive/Neo^R clones = 100%), and EC-derived (18/19 lacZ-positive/Neo^R clones = 95%) iPSC clones. The exchange was further validated using three randomly chosen Neo^R RMCE DV1 lacZ MEF-iPSC clones to demonstrate the complete removal of the COIN OSKM transgene by genomic PCR analysis (Figures S4A and S4B). Moreover, high percentage E11.5 chimeric embryos that constitutively express lacZ could be derived from retargeted TTF-iPSC clones, thereby demonstrating that the pluripotency of these cells is not affected after RMCE with lacZ (Figure 3C). For the pCOIN DV lacZ system, the expression was, as expected, only induced after Dox supplementation, which once again demonstrates the tight regulation of this COIN system (Figure 3D).

To functionally test our system further and specifically differentiate our iPSCs toward beating cardiomyocytes as a promising cell type for preclinical regenerative and tissue repair studies, we successfully exchanged the OSKM factors in MEF-iPSCs by a Cre-excised pCOIN DV construct encoding Mesp1, a known key transcriptional regulator of cardiovascular development (Bondue and Blanpain, 2010). Similar to previously published results in ESCs (Lindsley et al., 2008), a brief 2-day pulse of Mesp1 expression in early differentiating iPSCs (Figure 4A), together with Wnt inhibition (using the Wnt pathway inhibitory compound IWR1; Chen et al., 2009), preferentially generated enhanced numbers of cells positive for the definitive cardiac marker TroponinT (cTnT) (Figure 4B). We also observed significantly increased mRNA expression levels of other cardiac marker genes such as *Nkx2.5* as judged by qRT-PCR mRNA expression analysis (Figure 4C; data not shown) and in the number of beating cardiomyocytes (Figure 4D; Movies S1 and S2).

We believe that the exchange of the reprogramming factors by other genes from within the same ROSA26 locus via an efficient RMCE reaction, together with in vitro validation of the resultant exchanged iPSC clones, will be of great importance to the field of cellular (de)differentiation in efficiently identifying genes that can control cell lineage-directed differentiation. This was demonstrated by the cardiomyocyte-specific differentiation of mouse-derived iPSCs by ectopic Mesp1 expression together with Wnt inhibition. This regulated transgene expression will be particularly useful in preclinical regenerative medicine approaches where continued expression of powerful transcriptional modulators may be detrimental for terminal cellular differentiation necessary for tissue repair.

In summary, we have created an in vivo resource that will add to the toolbox of existing mouse resources that will allow scientists to identify genes more easily and understand their roles in cellular (de)differentiation processes. It should also be noted that, given the discovery of the human counterpart of the ROSA26 locus (Irion et al., 2007) as well as other safe genomic loci (Hockemeyer et al., 2009), our system could be similarly

adapted for human cell-based studies and identifying factors involved in human ESC/iPSC (de)differentiation.

EXPERIMENTAL PROCEDURES

Generation of the ROSA26-iPSC Mice

The construction of the expression vector, subsequent ESC targeting/screening, and final generation of ROSA26-iPSC transgenic mice were performed using a similar strategy as described elsewhere (Nyabi et al., 2009). Briefly, the individual constructs were first created by directional cloning strategies of the specific sequences into Gateway-compatible vectors and subsequently recombined using a multisite Gateway LR reaction. PvuI-linearized DNA of the resulting expression vector was then electroporated into wild-type G4 ESCs (George et al., 2007) and grown under standard ESC conditions with G418 selection drug added to the culture medium. Individual G418-resistant ESC colonies were picked, expanded, and screened by PCR and Southern blot analysis. One of the correctly ROSA26-targeted ESC clones was subsequently aggregated with diploid E2.5 Swiss embryos and generated several high-grade, germline-transmitting chimeric animals.

Cellular Reprogramming

(1) Fibroblasts isolated from Sox2 Cre (Lu and Robertson, 2004) X ROSA26-iPSC E13.5 embryos and tail tips of 6-week-old adult mice were first expanded under Puro selection before being plated on to culture dishes coated with a mitomycin C-inactivated feeder layer and reprogrammed in daily refreshed ESC medium or KSR medium (ESC medium with 15% Knockout Serum Replacement [Invitrogen] instead of fetal bovine serum) supplemented with doxycycline hyclate (Dox, 2 μg/ml, Sigma-Aldrich). (2) OSKM-EGFP-expressing HS/PCs were isolated from 3-week-old and in vivo Dox-induced (5 days of daily refreshed Dox in the drinking water, 1 mg/ml) Tie2 Cre (Kisanuki et al., 2001) X ROSA26-iPSC mice. Cells extracted from the bone marrow or spleen were first stained with a c-Kit-specific antibody (APC-H7 rat antimouse CD117 [c-Kit], PharMingen) before double EGFP/c-Kit-positive HS/PCs were sorted on a FACSAria II machine (BD Biosciences) and allowed to reprogram similarly as described for the fibroblasts. (3) Similarly as for the HS/PCs, ECs were isolated from lungs of 3-week-old and in vivo Dox-induced (5 days of daily refreshed Dox in the drinking water, 1 mg/ml) Tie2 Cre (Kisanuki et al., 2001) X ROSA26-iPSC mice. CD31-positive ECs were isolated using magnetic-activated cell sorting (MACS) with incubated CD31 (PECAM, rat antimouse CD31 antibody, BD Biosciences) precoated sheep antirat immunoglobulin G (IgG) magnetic Dynabeads (Dynal, Life Technologies) and reprogrammed similarly as described for the fibroblasts. Initially, only partially reprogrammed individual iVPC colonies could be observed and expanded in the presence of Dox. Subcloning of one particular iVPC clone on gelatinized plates eventually led to Dox-independent EC-iPSCs.

Immunohistochemistry

Immunohistochemistry labeling of various different markers were performed using standard procedures and/or commercially available kits according to the manufacturer's instructions: Alk. Phos. (Mouse ESC/iPSC Characterization Set [Stemgent] or Leukocyte Alkaline Phosphatase Kit [Sigma-Aldrich]), Oct4 (Mouse ESC/iPSC Characterization Set [Stemgent] or mouse anti-Oct4 primary antibody (Santa Cruz Biotechnology]), Nanog (Mouse ESC/iPSC Characterization Set [Stemgent]), SSEA1 (Mouse ESC/iPSC Characterization Set [Stemgent]), CD31 (rat anti-CD31 antibody [BD Biosciences]), cTnT (mouse anti-cTnT antibody [NeoMarkers]) and X-gal (previously described in Novak et al., 2000).

qRT-PCR

Total RNA was isolated using RNeasy Plus Mini Kit (QIAGEN). cDNA was synthesized using the First Strand cDNA Synthesis Kit (Roche) with oligo(dT) primer by starting from equal amounts of RNA as measured by a NanoDrop spectrophotometer (Thermo Scientific). qRT-PCRs were performed using the LightCycler 480 SYBR Green I Master (Roche), monitored on a LightCycler 480 system (Roche), and analyzed using qBase software (Hellemans et al., 2007). Gene expression was standardized against housekeeping genes *hydroxymethylbilane synthase* (*Hmbs*), *β-actin*, and *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*). All primers are listed in Table S2. The error bars in the graphs (Figures 2D, 4A, 4C, and S2C) represent the SDs of the average of triplicate reactions (technical replications).

In Vitro Differentiation

(1) Differentiation of ESCs/iPSCs toward hematopoietic cell lineages was performed first by embryoid body (EB) formation in 1% methylcellulose (R&D Systems) followed by replating and further differentiation in Methocult GF3434 medium (Stem Cell Technologies) according to the manufacturer's instructions. Colonies were blindly scored after 12 days of culture. The error bars in the graph (Figure 2E) represent the SD of the average of triplicate biological assays. (2) iPSC differentiation preferentially toward cardiomyocytes was performed as previously described for ESCs (Lindsley et al., 2008). Early differentiating iPSC EBs were plated (1.5×10^5 cells per milliliter) and grown in bacterial grade Petri dishes in EB differentiation medium and supplemented with Dox (2 μg/ml) and/or IWR1 (10 μM, Sigma-Aldrich).

Teratoma Formation

iPSCs were trypsinized and subcutaneously injected into the flank of immunocompromised nude mice. Forming teratomas were isolated 5 to 8 weeks later and further processed to paraffin sections stained with hematoxylin and eosin (H&E) for histological analysis. All experiments performed on mice were approved by the animal ethical committee of Ghent University.

RMCE Targeting

For the trap-coupled RMCE experiments, Effectene transfection reagent (QIAGEN) was used according to the manufacturer's instructions to cotransfect an incoming vector, created via directional cloning steps of individual Gateway-compatible constructs and subsequently recombined using Gateway LR reactions as previously described (Nyabi et al., 2009), together with a FlpE-expressing plasmid (pCAGGS-FlpE-IRES-puromycin-pA) (Schaft et al., 2001) in a 1:1 ratio into RMCE-compatible iPSCs. G418 selection (200 μg/ml) was started 48 hr after transfection. After 7 days, individual Neo^R RMCE-retargeted iPSC colonies could be observed and further expanded.

Beating Cardiomyocyte Assay

For each condition, 96 individually plated embryoid bodies were blindly scored from day 8 until day 12 of differentiation for the presence of beating cardiomyocytes.

For further details, please see the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, two tables, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.01.016.

LICENSING INFORMATION

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ACKNOWLEDGMENTS

We thank A. Bradley from the Wellcome Trust Sanger Institute for providing us with the polycistronic OSKM construct, G. Berx from the VIB Department for Molecular Biomedical Research (VIB-DMBR), University of Ghent, for the EGFP-Luciferase reporter construct, and C. Blanpain and A. Bondue from the Free University of Brussels for the Mesp1 construct. We also thank our VIB-DMBR microscopy core facility for their help with the microscopy analysis, K. Deswarte from B. Lambrecht's group for his help with the sorting experiments, and T. Pieters from Professor Frans Van Roy's group (VIB-DMBR) for providing us the Oct4 antibody. L.H. was funded by a Ph.D. grant of the

Agency for Innovation by Science and Technology. S.G. and P.D. were supported by the Fonds voor Wetenschappelijk Onderzoek–Vlaanderen.

L.H. and J.J.H. designed the research; L.H. performed the research with help from S.G., P.D., S.B., and J.J.H.; J.D., K.H., and T.H. performed aggregations; L.H. and J.J.H. analyzed the data; A.N. and D.W. provided essential reagents and ideas; L.H. wrote the manuscript together with S.G. and J.J.H.

Received: June 12, 2012
Revised: December 3, 2012
Accepted: January 14, 2013
Published: February 7, 2013

REFERENCES

Beard, C., Hochedlinger, K., Plath, K., Wutz, A., and Jaenisch, R. (2006). Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. Genesis *44*, 23–28.

Belteki, G., Haigh, J., Kabacs, N., Haigh, K., Sison, K., Costantini, F., Whitsett, J., Quaggin, S.E., and Nagy, A. (2005). Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. Nucleic Acids Res. *33*, e51.

Blasco, M.A., Serrano, M., and Fernandez-Capetillo, O. (2011). Genomic instability in iPS: time for a break. EMBO J. *30*, 991–993.

Bondue, A., and Blanpain, C. (2010). Mesp1: a key regulator of cardiovascular lineage commitment. Circ. Res. *107*, 1414–1427.

Carey, B.W., Markoulaki, S., Beard, C., Hanna, J., and Jaenisch, R. (2010). Single-gene transgenic mouse strains for reprogramming adult somatic cells. Nat. Methods *7*, 56–59.

Chen, B., Dodge, M.E., Tang, W., Lu, J., Ma, Z., Fan, C.W., Wei, S., Hao, W., Kilgore, J., Williams, N.S., et al. (2009). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nat. Chem. Biol. *5*, 100–107.

George, S.H., Gertsenstein, M., Vintersten, K., Korets-Smith, E., Murphy, J., Stevens, M.E., Haigh, J.J., and Nagy, A. (2007). Developmental and adult phenotyping directly from mutant embryonic stem cells. Proc. Natl. Acad. Sci. USA *104*, 4455–4460.

González, F., Boué, S., and Izpisúa Belmonte, J.C. (2011). Methods for making induced pluripotent stem cells: reprogramming à la carte. Nat. Rev. Genet. *12*, 231–242.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. *8*, R19.

Hockemeyer, D., Soldner, F., Beard, C., Gao, Q., Mitalipova, M., DeKelver, R.C., Katibah, G.E., Amora, R., Boydston, E.A., Zeitler, B., et al. (2009). Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nat. Biotechnol. *27*, 851–857.

Irion, S., Luche, H., Gadue, P., Fehling, H.J., Kennedy, M., and Keller, G. (2007). Identification and targeting of the ROSA26 locus in human embryonic stem cells. Nat. Biotechnol. *25*, 1477–1482.

Kisanuki, Y.Y., Hammer, R.E., Miyazaki, J., Williams, S.C., Richardson, J.A., and Yanagisawa, M. (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. Dev. Biol. *230*, 230–242.

Lindsley, R.C., Gill, J.G., Murphy, T.L., Langer, E.M., Cai, M., Mashayekhi, M., Wang, W., Niwa, N., Nerbonne, J.M., Kyba, M., and Murphy, K.M. (2008). Mesp1 coordinately regulates cardiovascular fate restriction and epithelial-mesenchymal transition in differentiating ESCs. Cell Stem Cell *3*, 55–68.

Lu, C.C., and Robertson, E.J. (2004). Multiple roles for Nodal in the epiblast of the mouse embryo in the establishment of anterior-posterior patterning. Dev. Biol. *273*, 149–159.

Novak, A., Guo, C., Yang, W., Nagy, A., and Lobe, C.G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis *28*, 147–155.

Nyabi, O., Naessens, M., Haigh, K., Gembarska, A., Goossens, S., Maetens, M., De Clercq, S., Drogat, B., Haenebalcke, L., Bartunkova, S., et al. (2009). Efficient mouse transgenesis using Gateway-compatible ROSA26 locus targeting vectors and F1 hybrid ES cells. Nucleic Acids Res. *37*, e55.

Premsrirut, P.K., Dow, L.E., Kim, S.Y., Camiolo, M., Malone, C.D., Miething, C., Scuoppo, C., Zuber, J., Dickins, R.A., Kogan, S.C., et al. (2011). A rapid and scalable system for studying gene function in mice using conditional RNA interference. Cell *145*, 145–158.

Sandhu, U., Cebula, M., Behme, S., Riemer, P., Wodarczyk, C., Metzger, D., Reimann, J., Schirmbeck, R., Hauser, H., and Wirth, D. (2011). Strict control of transgene expression in a mouse model for sensitive biological applications based on RMCE compatible ES cells. Nucleic Acids Res. *39*, e1.

Schaft, J., Ashery-Padan, R., van der Hoeven, F., Gruss, P., and Stewart, A.F. (2001). Efficient FLP recombination in mouse ES cells and oocytes. Genesis *31*, 6–10.

Stadtfeld, M., Maherali, N., Borkent, M., and Hochedlinger, K. (2010). A reprogrammable mouse strain from gene-targeted embryonic stem cells. Nat. Methods *7*, 53–55.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Yin, L., Ohanyan, V., Pung, Y.F., Delucia, A., Bailey, E., Enrick, M., Stevanov, K., Kolz, C.L., Guarini, G., and Chilian, W.M. (2012). Induction of vascular progenitor cells from endothelial cells stimulates coronary collateral growth. Circ. Res. *110*, 241–252.

Zhao, T., Zhang, Z.N., Rong, Z., and Xu, Y. (2011). Immunogenicity of induced pluripotent stem cells. Nature *474*, 212–215.