

Vitamin C Enhances the Generation of Mouse and Human Induced Pluripotent Stem Cells

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SUMMARY

Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by defined factors. However, the low efficiency and slow kinetics of the reprogramming process have hampered progress with this technology. Here we report that a natural compound, vitamin C (Vc), enhances iPSC generation from both mouse and human somatic cells. Vc acts at least in part by alleviating cell senescence, a recently identified roadblock for reprogramming. In addition, Vc accelerates gene expression changes and promotes the transition of pre-iPSC colonies to a fully reprogrammed state. Our results therefore highlight a straightforward method for improving the speed and efficiency of iPSC generation and provide additional insights into the mechanistic basis of the reprogramming process.

INTRODUCTION

Animal development starts with the fertilized egg undergoing a programmed process of cell proliferation and differentiation that generates all cell types of an individual. This process was thought to be irreversible in mammals, but the cloning of Dolly proved that fully differentiated somatic cell nuclei can be reprogrammed back to an embryonic-like state by factors present in oocytes (Wilmut et al., 1997). More recently, Yamanaka and colleagues demonstrated that mouse somatic cells can also acquire a pluripotent state in vitro after the introduction of a defined combination of transcription factors that are highly enriched in embryonic stem cells (ESCs) (Takahashi and Yamanaka, 2006). Mouse iPSCs are similar to ESCs in most aspects and can generate entire individuals after tetraploid complementation (Kang et al., 2009; Okita et al., 2007; Wernig et al., 2007; Zhao et al., 2009). iPSCs have also been produced from other species including human and pig (Esteban et al., 2009; Takahashi et al., 2007; Yu et al., 2007), raising the possibility of clinical

application of personalized stem cell-based therapies without immune rejection or ethical concerns. Human iPSCs also provide a unique platform for studying genetic diseases in vitro (Park et al., 2008). However, the low efficiency of iPSC generation is a significant handicap for mechanistic studies and high throughput screening, and also makes bona fide colony isolation time consuming and costly. The efficiency of alkaline phosphatase-positive (AP⁺) colony formation with the four Yamanaka's factors (Sox2, Klf4, Oct4, c-Myc; SKOM) in mouse fibroblasts is about 1% of the starting population, but only around 1 in 10 of those colonies is sufficiently reprogrammed to be chimera competent (Silva et al., 2008). In human fibroblasts, only about 0.01% of cells transduced with SKOM form AP⁺ iPSC colonies (Takahashi et al., 2007; Yu et al., 2007). In our search for compounds that improve the efficiency of somatic cell reprogramming, we have found that a vitamin (Vc) that is highly abundant in our diet significantly increases mouse and human iPSC colony formation, at least in part by alleviating cell senescence.

RESULTS

Vitamin C Can Improve iPSC Generation

Somatic cells including fibroblasts quickly undergo senescence in culture, in part as a result of accumulation of reactive oxygen species (ROS) produced by cell metabolism (Parrinello et al., 2003). We studied ROS generation during the reprogramming of mouse embryonic fibroblasts (MEFs) and noticed a significant early increase (2.5- to 3-fold) in cells transduced with Sox2/Klf4/Oct4 (SKO) compared to SKOM and the control (Figure 1A). This is consistent with SKO being less efficient than SKOM in generating iPSCs, and led us to hypothesize that antioxidants might improve the efficiency of SKO-based reprogramming by suppressing ROS. We found that a combination of vitamin B1 (Vb1), reduced glutathione (GSH monoethyl ester, GMEE), sodium selenite (Sel), and ascorbic acid (vitamin C, or Vc) (Arrighoni and De Tullio, 2002) significantly accelerated the appearance of GFP⁺ cells in MEFs carrying a transgenic Oct4-GFP promoter and bypassed the need to split on feeders (Figure 1B, left). We then measured the contribution of each antioxidant by

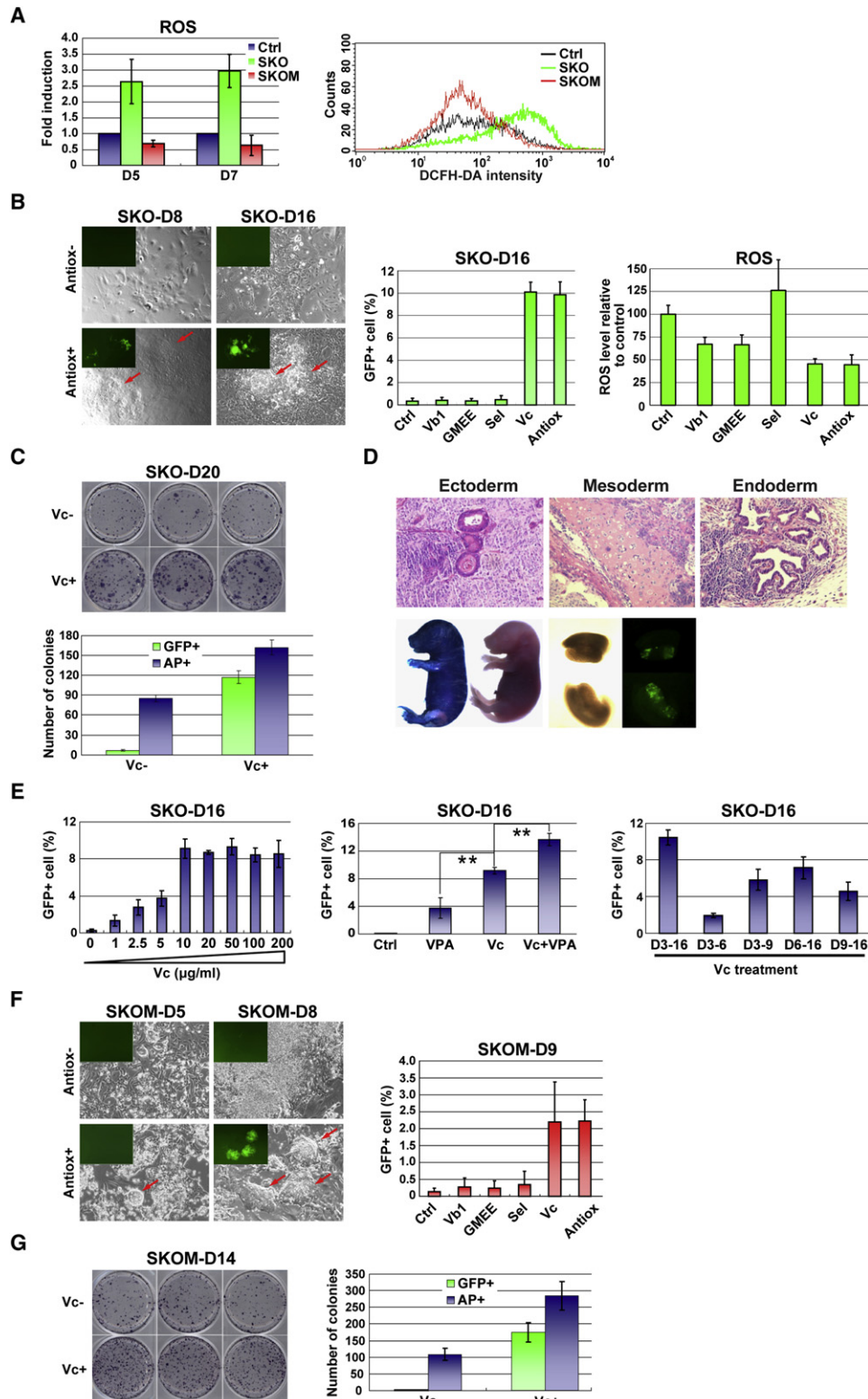


Figure 1. Vitamin C Enhances the Reprogramming of Mouse Fibroblasts

(A) Left: Measurement of ROS in SKO- and SKOM-infected MEFs (from ICR mice) compared to empty vector. Mean values + standard deviation (SD) of three independent experiments are shown. D indicates day hereafter. Right: Scatter plot histogram of a similar representative experiment, only 1 time point is shown. (B) Left: Phase contrast and fluorescence photographs of SKO-infected MEFs untreated or treated with mixed antioxidants (Antiox). Arrows point to emerging colonies, a representative experiment is shown. Middle: FACS quantification of GFP⁺ cells in SKO-infected MEFs treated with mixed antioxidants or each

using FACS and found that Vc accounted for the entire increase in reprogramming efficiency (Figure 1B, middle), achieving a remarkable 10% of cells being GFP⁺ at day 16. Vb1, GMEE, Sel, and other compounds with compelling antioxidant activities including n-acetylcysteine, resveratrol, α -lipoic acid, vitamin E, and L-carnitine hydrochloride (not shown) did not have any noticeable effect. Vb1, GMEE, and Vc all reduced the steady-state ROS level in SKO-infected MEFs (Figure 1B, right), suggesting that the activity of Vc in this context may in fact be independent of its antioxidant properties. Next, we evaluated the effect of splitting SKO-infected MEFs (3×10^3 cells) on feeders at day 7 postinfection, because under these circumstances GFP⁺ colonies originate from single cells. At day 20, most alkaline phosphatase-positive (AP⁺) colonies in wells treated with Vc were GFP⁺ compared to very few in the control (Figure 1C), and the overall efficiency (number of colonies divided by number of starting cells) was $\sim 3.8\%$. iPSC clones generated with mixed antioxidants or Vc under these conditions were pluripotent as demonstrated by standard characterization procedures and the formation of teratomas and chimeric mice with contribution to the germline (Figure 1D and Figures S1A–S1H available online). Addition of Vc to SKO-infected adult mammary gland fibroblasts (MaFs) showed a similar increase in GFP⁺ cells compared to MEFs (Figure S1I), demonstrating that the effect of Vc is not restricted to a specific cell type. A dose response experiment in SKO-infected MEFs also showed that a low dose of Vc (10 $\mu\text{g/ml}$) can achieve a maximum effect (Figure 1E, left), suggesting that the enhanced reprogramming is not due to cell death or selection of resistant populations. Notably, Vc had a more potent effect in increasing GFP⁺ cells in SKO-infected MEFs than the widely used histone deacetylase inhibitor valproic acid (VPA) (Huangfu et al., 2008), and the combination of both was additive (Figure 1E, middle), suggesting that they act through different mechanisms. Vc had to be added for the duration of the experiment to achieve its full potential (Figure 1E, right). Consistently, DNA microarray analysis of an SKO time course experiment showed sustained acceleration of transcriptional changes in the presence of Vc: Vc-treated cells at days 6, 8, and 10 clustered with day 10 untreated cells (Figure S1J).

We then tested whether Vc can enhance reprogramming efficiency with SKOM. Mixed antioxidants promoted the appear-

ance of GFP⁺ colonies by day 8 without splitting on feeders (Figure 1F, left), compared to none in the control. FACS analysis was performed at day 9 to prevent cell overgrowth and detected an average 2% of GFP⁺ cells in Vc-treated cells while the other antioxidants had no effect (Figure 1F, right). When SKOM-infected MEFs (2×10^3 cells) were split on feeders and allowed to grow until day 14, the corrected efficiency of AP⁺/GFP⁺ colony formation was $\sim 8.75\%$ (Figure 2G). Thus, Vc improves reprogramming efficiency with both SKO and SKOM, and the effect is not mediated, at least not exclusively, by a reduction in ROS.

Vitamin C Converts Pre-iPSCs into iPSCs

Our observation that Vc increases the ratio of GFP⁺/AP⁺ colonies suggested that it may promote the transition from pre-iPSCs to iPSCs as described previously (Silva et al., 2008). To test this idea, we added mixed antioxidants or Vc to pre-iPSC clones derived from MEFs or MaFs and observed highly homogeneous acquisition of ESC-like characteristics and GFP fluorescence within the course of a few passages (Figures 2A and 2C). Newly reprogrammed iPSCs derived from pre-iPSCs were stable in continuous culture as evaluated by qPCR of pluripotent markers, demethylation of the *Nanog* proximal promoter, and formation of chimeric mice (Figures 2B and 2D–2F). Consistent with the rapid conversion to a pluripotent state, DNA microarray analysis showed quick prominent changes in gene expression in MaF pre-iPSCs treated with Vc (Figures S2A–S2D). A summary of the iPSC cell lines generated from pre-iPSCs via Vc is included in Figure S2E.

We also compared the ability of Vc and the two inhibitor cocktail 2i (ERK and GSK3 β inhibitors) described by Silva et al. (2008) to transform pre-iPSCs into iPSCs. In standard mouse ESCs medium the percentage of GFP⁺ cells was higher in Vc-treated cells than in 2i-treated ones (Figure 2G). In N2B27 plus LIF, the medium used in the original study, the percentage of GFP⁺ cells was similar for Vc and 2i but the proliferation potential was superior in Vc-treated cells (Figure 2H). Moreover, western blot of lysates from pre-iPSCs treated with Vc did not show a reduction in total ERK or active ERK (pERK), although 2i eliminated the pERK signal as expected (Figure 2I). Therefore, Vc efficiently transforms pre-iPSC clones into pluripotent iPSCs via a mechanism that seems different from 2i.

antioxidant separately. Mean values of five independent experiments (performed in triplicate) + SD are shown. Right: ROS measurement at day 5 postinfection of SKO-infected MEFs (from ICR mice) treated as indicated; mean values + SD of three independent experiments are shown.

(C) Top: AP staining of SKO-infected MEFs untreated or treated with Vc and split in triplicate on 6-well plates coated with feeders. A representative experiment is shown. Bottom: Counting of AP⁺ and GFP⁺ colonies in the same experiment, mean values + SD are shown.

(D) Top: Hematoxylin/eosin-stained sections of teratomas formed after 3 weeks with a MEF iPSC clone generated with SKO and mixed antioxidants (SKOAntiox-1). Bottom left: Beta galactosidase staining of a nonchimeric and a chimeric embryo (blue) produced with a MEF iPSC clone generated with SKO and Vc (SKOVc-1). Bottom middle: phase contrast and fluorescence photographs of Oct4-GFP⁺ germline-progenitor cells in the genital ridge of another embryo produced with the same clone.

(E) Left: Dose-response experiment to determine the optimal concentration of Vc that increases reprogramming in SKO-infected MEFs. GFP⁺ cells were measured by FACS, mean values + SD of a representative experiment measured in triplicate are shown. Middle: SKO-infected MEFs were treated with VPA, Vc, or a combination of both. GFP⁺ cells were measured by FACS, mean values + SD of a representative experiment measured in triplicate are shown. Asterisks indicate p value < 0.01. Right: SKO-infected MEFs were untreated or treated with Vc for selected periods of time during iPSCs generation. GFP⁺ cells were measured by FACS; mean values + SD of a representative experiment measured in triplicate are shown.

(F) Left: Phase contrast and fluorescence photographs of SKOM-infected MEFs untreated or treated with mixed antioxidants presented as in (B). Right: GFP⁺ cells measured by FACS in SKOM-infected MEFs treated as indicated, mean values + SD of three independent experiments (each measured in triplicate) are shown.

(G) Left: AP staining of SKOM-infected MEFs untreated or treated with Vc and split in triplicate on 6-well plates coated with feeders. A representative experiment is shown. Right: counting of AP⁺ and GFP⁺ colonies in the same experiment, mean values + SD are shown.

Vitamin C Alleviates the Senescence Roadblock during iPSC Generation

We did not observe a significant change in apoptosis after Vc treatment of SKO- or SKOM-infected MEFs at any of the time points evaluated (Figure 3A). However, we detected increased proliferation in the middle phase of reprogramming (Figure 3B), suggesting a bypass of cell senescence. Supporting this idea, we had seen that nontransduced MEFs treated with Vc have a prolonged lifespan (up to 12 passages in this study) compared to control MEFs untreated or those treated with other antioxidants (Figures S3A and S3B). We then performed a western blot with lysates from an SKO time course experiment and observed a significant reduction in p53 and p21 levels in Vc-treated cells (Figure 3C). The cells retained basal levels of p53, so the recruitment of Tp53BP1 to nuclear foci (indicative of functional DNA repair machinery) was unaffected by Vc in both SKO- and SKOM-infected MEFs (Figure 3D). Next, we studied the effect of exogenous p53 activation or knockdown on Vc-mediated reprogramming. p53 adenoviruses or the p53-activating compound nutlin-3a inhibited the formation of GFP⁺ colonies in SKO-infected MEFs in a dose-dependent manner (Figure 3E; Figure S3C). On the other hand, p53 shRNA increased GFP⁺ colonies by ~100-fold without Vc and 2- to 3-fold with Vc (Figure 3F). The latter modest increase might reflect the fact that Vc treatment alone reduces but does not abolish p53 expression, allowing the shRNA to reduce it further. Our data therefore suggest that Vc improves iPSC generation by reducing p53 levels and alleviating cell senescence while still maintaining an intact DNA repair machinery.

Vitamin C Improves the Generation of Human iPSCs

Reprogramming is more challenging in human cells, raising a barrier for producing iPSCs and concerns about the quality and homogeneity of clones that do arise (Yamanaka, 2009). While our mouse experiments were in progress, we tested whether the antioxidant mix can also enhance reprogramming of human somatic cells. We used skin fibroblasts from a fetus with beta thalassemia, placental corionic mesenchymal cells (CMCs), and cells from the periosteal membrane. Addition of mixed antioxidants to KSR medium did not increase the basal low efficiency of reprogramming for any of these cell types (not shown), and we noticed that KSR already contains antioxidants including Vc (Garcia-Gonzalo and Izpisua Belmonte, 2008). We then switched to a full serum protocol (Figure 4A), previously thought to be ineffective for human cells, to mimic the culture conditions of our mouse experiments. Mixed antioxidants alone or in combination with VPA potently increased the number of AP⁺ ESC-like colonies in SKOM-infected cells (Figure 4B). No increase was observed with SKO (not shown), possibly because of low efficacy of this combination in the human context (Naka-

gawa et al., 2008; Wernig et al., 2008). Once we had identified that Vc is the key compound in the antioxidant mix, we added Vc + VPA to a different set of human fibroblasts transduced with SKOM and observed very high reprogramming efficiency, up to 6.2% of the cells with fibroblasts from a patient with ornithine transcarbamylase deficiency (OTCD) (Figure 4C). Vc or Vc + VPA could reprogram adipose stem cells (ASCs) with even higher efficiency (up to 7.06%), in agreement with a recent report describing superior susceptibility of these cells (Figure 4D; Sun et al., 2009). Selected iPSC colonies from these experiments were expanded in KSR medium on feeder layers or in mTeSR medium on Matrigel. They homogeneously displayed features of human ESCs (hESCs) through multiple passages, as evaluated by morphology, number of chromosomes, activation of the endogenous ESC program, silencing of the transgenes, and demethylation of *Oct4* and *Nanog* proximal promoters (Figures 4E–4H). Our iPSCs also acquired markers of all three germ layers after differentiation into embryonic bodies (EBs) and developed complex teratomas (Figures 4I and 4J); a summary of human iPSCs characterization is shown in Figure 4K.

DISCUSSION

We show here that vitamin C, a common nutrient vital to human health, enhances the reprogramming of somatic cells to pluripotent stem cells. By adding Vc to the culture medium, we can now obtain high-quality iPSCs from mouse and human cells routinely. While our work was in progress, six independent laboratories identified cell senescence as a roadblock for reprogramming (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009; Zhao et al., 2008), creating significant interest in finding compounds that alleviate cell senescence without increasing the risk of mutations. Vc reduces p53 levels during reprogramming and this may raise concerns regarding safety, but at least based on the parameters tested, our cell lines are devoid of noticeable side effects.

We uncovered this new role of Vc by trying to correct increased ROS production in SKO-infected MEFs. Vc function in this context seems to be unrelated to its antioxidant activity, but it nevertheless seems likely that ROS contribute to the lower reprogramming efficiency of SKO compared to SKOM. The mechanism underlying this increase in ROS merits further investigation and suggests that metabolic changes are triggered differentially by SKO and SKOM. Besides reducing p53, Vc accelerates transcriptome changes during reprogramming and allows the conversion of pre-iPSCs to iPSCs. The extent to which these observations relate to cell senescence is unclear, and it is possible that Vc is acting in other ways as well. For example, it could accelerate stochastic events during reprogramming,

(E) DNA methylation profile of the *Nanog* proximal promoter in selected pre-iPSC clones before and after treatment with mixed antioxidants or Vc.

(F) Chimeric mice produced with a MEF pre-iPSC clone (MEF C2) treated with mixed antioxidants. Asterisks indicate control mice.

(G) Left: Phase contrast and fluorescence photographs of a representative MaF pre-iPSC clone (MaF 4FC9) cultured for 1 passage on feeders in either mouse ESCs medium or N2B27 + LIF, in both cases untreated or treated with Vc or 2i. Similar results were observed with a different clone (MaF 4FC10). Right: FACS quantification of GFP⁺ cells with a pre-iPSC clone (MaF 4FC9) cultured for 1 passage with mouse ESCs medium + Vc or 2i on feeders or without them on gelatin.

(H) FACS quantification of GFP⁺ cells and proliferation of two pre-iPSC clones (MaF 4FC9 and 4FC10) cultured for 1 passage with N2B27 + LIF and untreated or treated with Vc or 2i.

(I) Western blot for ERK and pERK of lysates from MaF 4FC9 cultured on gelatin and untreated or treated with Vc or 2i; tubulin is the loading control.

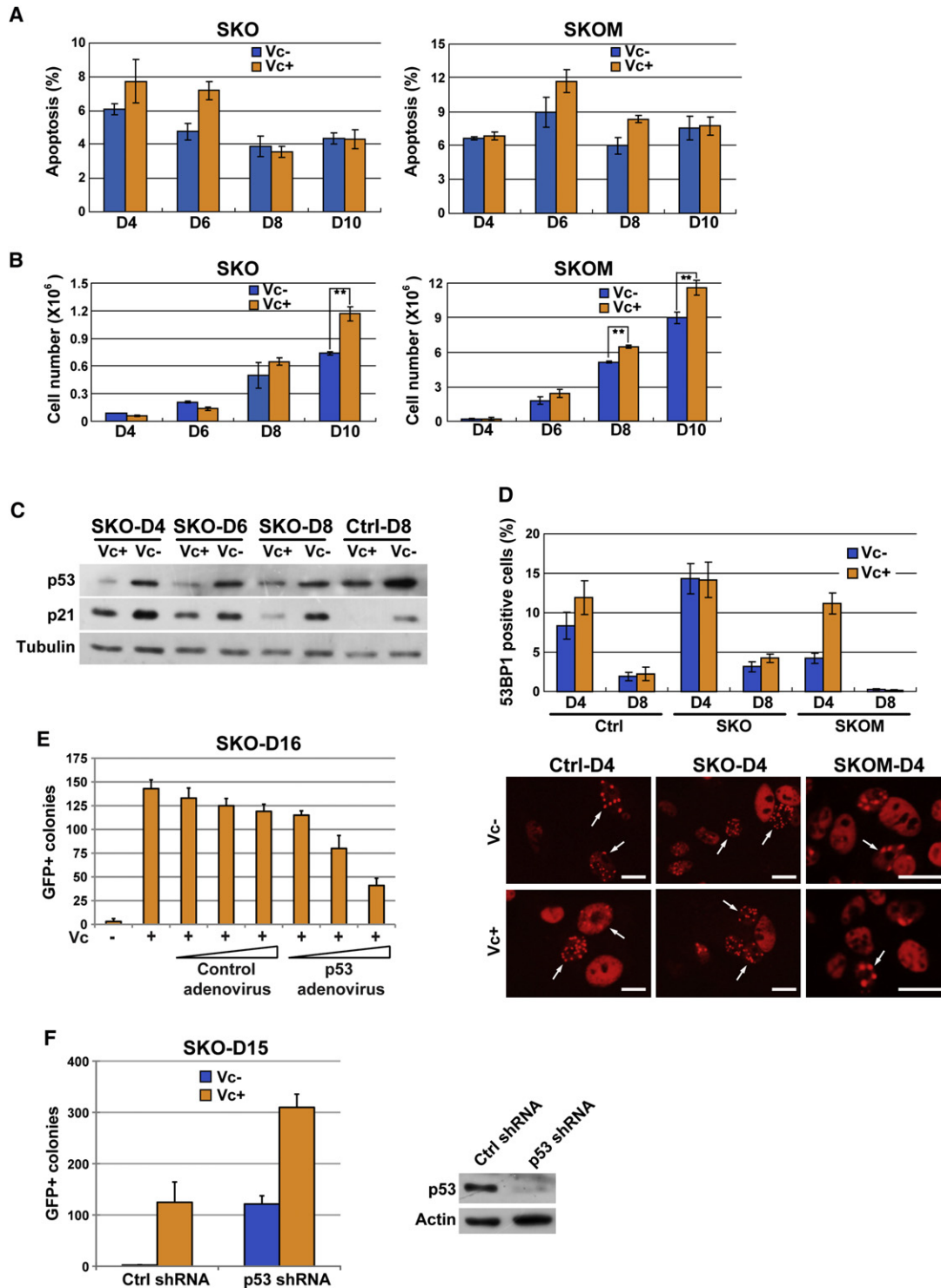


Figure 3. Vitamin C Alleviates the Senescence Roadblock in Reprogramming Fibroblasts

(A) Measurement of apoptosis during iPSCs generation in SKO- and SKOM-infected MEFs untreated or treated with Vc, a representative time course experiment performed in triplicate (mean values + SD) is shown.

(B) Measurement of proliferation by FACS with the same samples. Mean values + SD is shown, asterisks indicate p value < 0.01.

(C) Representative western blot for p53, p21, and tubulin in MEFs infected with SKO or empty vector untreated or treated with Vc.

(D) Top: Bar graph showing the quantification of Tp53BP1-positive cells assessed by immunofluorescence microscopy in duplicate coverslips (at least 500 nuclei were counted in total and mean values + SD are shown) of a representative experiment with SKO- and SKOM-infected MEFs untreated or treated with Vc; two time points are shown. Bottom: immunofluorescence captures for Tp53BP1 at day 4 postinfection of a similar representative experiment; scale bars represent 25 μ M.

perhaps by promoting epigenetic modifications that allow further changes to proceed. In this regard, Vc is a cofactor in reactions driven by dioxygenases including collagen prolyl hydroxylases, HIF (hypoxia-inducible factor) prolyl hydroxylases, and histone demethylases (Shi, 2007), and it is interesting to consider that Vc might influence reprogramming by increasing the activity of these enzymes. Histone demethylases are important for development and modulate the expression of the ESC master transcription factor Nanog (Cloos et al., 2008), so it is possible that Vc allows the reprogramming to run more smoothly by facilitating histone demethylation. While additional mechanistic studies are done, Vc may prove useful for high-throughput screening of compounds and siRNA oligos or for enhancing the efficiency of virus-free delivery systems (Kim et al., 2009; Yu et al., 2009). It is also an interesting concept that a vitamin with long-debated anti-aging effect (Harman, 1956; Massip et al., 2009) has such a potent effect on reprogramming, and our work may stimulate further research in this area as well.

EXPERIMENTAL PROCEDURES

Cell Culture

Mouse fibroblasts were transduced (40,000 cells) at passage 2 or 3 with pMX-based retroviruses in 6-well culture dishes as in our previous reports (Qin et al., 2008). MEFs and MaFs from OG2 mice, which carry the *Rosa26-lacZ* allele and a transgenic *Oct4* promoter driving GFP expression (Huangfu et al., 2008; Silva et al., 2008), were used in all experiments unless otherwise indicated. For human iPSCs generation, 50,000 cells at early passages were transduced in 6-well dishes with pMX-based vectors that encode human factors (Adgene). At day 6, 5,000 to 10,000 cells were split on feeders. ROS and apoptosis were detected with 2,7-dichlorofluorescein diacetate (DCFH-DA, Beyotime) and the PE Annexin V Apoptosis Detection Kit (BD PharMingen) according to the manufacturers' manuals. Vc and the other antioxidants and ERK (PD0325901) and GSK3b inhibitors (CHIR99021) were purchased from Sigma; VPA was purchased from Merck. The working concentrations were: Vc 25 or 50 $\mu\text{g}/\text{ml}$ (unless otherwise indicated), Vb1 9 mg/L, Sel 20 nM, GMEE 1.5 mg/L, n-acetylcysteine 1 mM, resveratrol 10 μM , α -lipoic acid 5 $\mu\text{g}/\text{ml}$, vitamin E 25 μM , L-carnitine hydrochloride 15 $\mu\text{g}/\text{ml}$, PD0325901 1 μM , CHIR99021 3 μM , and VPA 1 mM. Antioxidants including Vc were added from day 2 till the end of each experiment unless otherwise indicated and maintained for the continuous culture of picked mouse iPSC colonies. VPA was added from day 3 to 8 and day 8 to 16 of mouse and human iPSCs generation, respectively. p53 and control (empty) adenoviruses were purchased from Saibainuo Gene Technology. pRetroSuper vectors containing shRNA sequences for p53 (GTACATGTGTAATAGCTCC) (Kawamura et al., 2009) or the control firefly luciferase gene were made by us; after infection, cells were selected in puromycin for 4 days.

Cell Line Characterization

AP staining, immunofluorescence microscopy, karyotyping, bisulfate sequencing, and chimeric mice generation were performed as described (Esteban et al., 2009; Qin et al., 2008). A Zeiss SteREO Lumar V12 fluorescence microscope was used to observe GFP⁺ germ cells. For teratoma formation, 1,000,000 cells were injected subcutaneously into nude mice for mouse iPSCs and 3,000,000 into SCID mice for human iPSCs. EBs from human iPSCs were produced by detaching cells growing on feeders with trypsin and seeding in nonadherent plates for 7–9 days; medium (human ESCs maintenance

medium without bFGF) was replaced every other day. Western blotting detection was performed with ECL⁺ (Amersham). qPCR was done with SYBR Green (Takara), samples were analyzed in duplicate or triplicate, and beta actin values were used for normalization. Primers for genomic qPCR, semiquantitative RT-PCR, and bisulfate sequencing are available upon request. DNA microarrays were performed with Affymetrix MoGene 1.0 ST chip.

ACCESSION NUMBERS

The array data described in this study have been deposited in the GEO database with the accession numbers GSE19377 and GSE19378.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at doi:10.1016/j.stem.2009.12.001.

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(E) FACS quantification of GFP⁺ cells in SKO-infected MEFs treated with Vc, increasing concentrations of p53-expressing or control adenoviruses (250, 1000, and 4000 viral particles/cell in this order) were added at day 4 postinfection. A representative experiment (measured in triplicate, mean values +SD are shown) is presented (also in F).

(F) Left: Effect of p53 shRNA knockdown on the number of GFP⁺ colonies induced by Vc treatment in SKO-infected MEFs. Right: Western blot demonstrates specificity of our shRNA vectors; lysates were extracted at day 8.

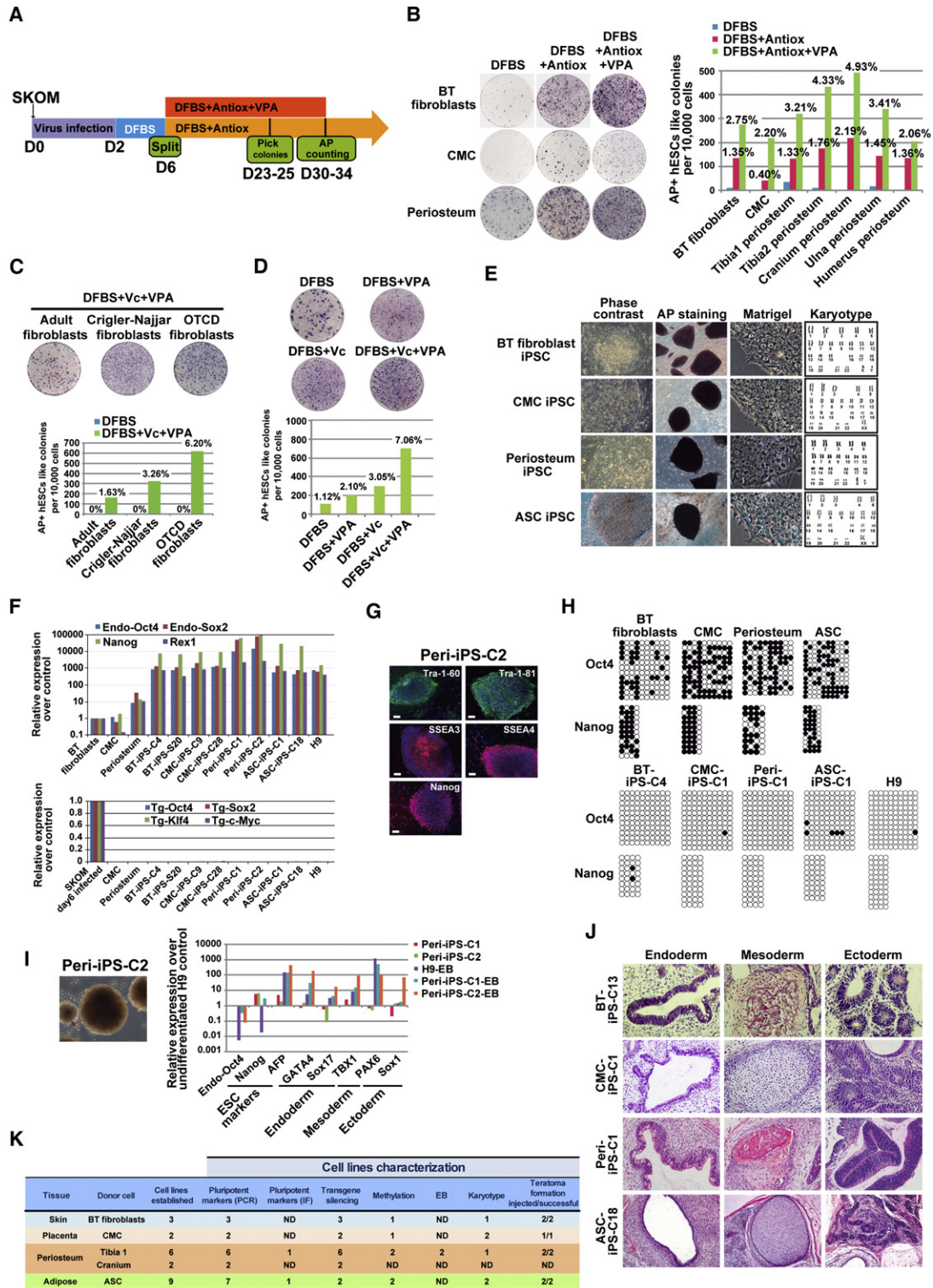


Figure 4. Vitamin C Enhances the Generation of Human iPSCs

(A) Schematic representation of our human iPSC generation protocol.

(B) Left: AP staining of selected iPSCs generation experiments in 10 cm dishes with the indicated cell types and treatments. DFBS, DFBS-based medium; BT, beta thalassaemia. Right: quantification of the same dishes and additional experiments, efficiency is labeled (when convenient) on the top of each bar.

(C) Top: AP staining in 10 cm dishes of additional experiments with fibroblasts treated with Vc + VPA. Bottom: bar graph showing quantification of the same experiment.

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(D) Top: AP staining in 10 cm dishes of a representative iPSCs generation experiment with ASCs. Bottom: bar graph showing quantification of the same experiment.

(E) Phase contrast photographs of established iPSC clones grown on feeder or Matrigel, AP staining on feeders, and karyotype analysis.

(F) qPCR for the indicated endogenous genes and also the transgenes in selected iPSC clones (Peri indicates periosteum-derived iPSCs); H9 hESCs and the starting population before infection or extracted at day 6 postinfection are the controls where appropriate.

(G) Immunofluorescence microscopy for the indicated markers of a representative periosteum iPSC clone, similar results were observed with iPSCs from BT fibroblasts, CMCs, and ASCs (not shown). Scale bars represent 50 μ m.

(H) DNA methylation profile of the *Oct4* and *Nanog* proximal promoters in the indicated cell types.

(I) Left: phase contrast captures of EBs formed by a representative periosteum iPSC clone. Right: qPCR analysis of endogenous *Oct4* and *Nanog* and also markers for the three germ layers in EBs from two periosteum cell lines; EBs from H9 hESCs and undifferentiated H9 cells were the control.

(J) Hematoxylin/eosin-stained sections of teratomas formed after 7–8 weeks with iPSCs produced from BT fibroblasts (C-13), CMCs (C-1), periosteum (C-1), and ASCs (C-18).

(K) Summary of human iPSC cell lines generated in this study and their characterization.