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Antioxidant Supplementation Reduces Genomic Aberrations in Human Induced Pluripotent Stem Cells

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SUMMARY

Somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) using oncogenic transcription factors. However, this method leads to genetic aberrations in iPSCs via unknown mechanisms, which may limit their clinical use. Here, we demonstrate that the supplementation of growth media with antioxidants reduces the genome instability of cells transduced with the reprogramming factors. Antioxidant supplementation did not affect transgene expression level or silencing kinetics. Importantly, iPSCs made with antioxidants had significantly fewer de novo copy number variations, but not fewer coding point mutations, than iPSCs made without antioxidants. Our results suggest that the quality and safety of human iPSCs might be enhanced by using antioxidants in the growth media during the generation and maintenance of iPSCs.

INTRODUCTION

A safe and efficient method for generating patient-specific pluripotent cells from easily accessible tissues via expression of the transcription factors *OCT4*, *SOX2*, *KLF4*, and *c-MYC* (collectively referred to as the four factors or 4F) (Takahashi et al., 2007), or some other combination of defined factors (Yu et al., 2007), would have important applications in cell-replacement therapy and disease modeling. A key concern with reprogramming somatic cells into induced pluripotent stem cells (iPSCs) is the increased load of genomic aberrations. We and others have shown that human iPSCs harbor genomic aberrations and point coding mutations that are absent in the parental cells (Gore et al., 2011; Hussein et al., 2011; Ji et al., 2012; Martins-Taylor et al., 2011; Pasi et al., 2011; Taapken et al., 2011). These mutations occur despite the exclusion of *c-MYC* as the reprogramming factor and the use of non-integrating methods for transgene delivery (Gore et al., 2011; Young et al., 2012). The causes of these mutations remain largely unknown.

Evidence indicates that preferential reprogramming of mutated cells in the starting somatic cell population (Gore et al., 2011; Ji et al., 2012) and adaptation to growth in culture (Hussein et al., 2011; Laurent et al., 2011) may contribute to the somatic mutations found in iPSCs. However, a significant proportion of the mutations in iPSCs cannot be exclusively attributed to preexisting rare mutations in the parental cells or to acquisition of mutations during the passaging of iPSCs (Ji et al., 2012), suggesting that the mutation rate might be elevated during reprogramming. This finding is consistent with the oncogenic potential of at least some of the reprogramming factors, and with the fact that reprogramming-factor-transduced mouse cells develop genome instability (González et al., 2013; Marión et al., 2009). It is known that the P53 pathway is activated in cells transduced with reprogramming factors and results in apoptosis, cell-cycle arrest, and senescence (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Krizhanovsky and Lowe, 2009; Marión et al., 2009; Utikal et al., 2009). However, in a previous study, we found that the TP53 gene was not mutated in any of the iPSCs that harbored mutations (Ji et al., 2012). Thus, an important challenge now is to identify the aspects of reprogramming that cause mutations to optimize reprogramming, in order to minimize genome instability during the derivation of iPSCs.

The acceleration of growth rate following the induction of reprogramming factors (Ruiz et al., 2011) is expected to impose greater metabolic demands for energy and precursors for biosynthesis on the cells. During reprogramming, mitochondria get progressively smaller and less active (Prigione et al., 2010; Suhr et al., 2010), and metabolism shifts from oxidative respiration to oxidative glycolysis (Varum et al., 2011). Such a metabolic shift can lead to a buildup of electrons in the electron transport chain, increasing their

leakage into the cytoplasm as reactive oxygen species (ROS) that will cause oxidative stress if the radical-scavenging systems in the cell are not sufficiently upregulated. High ROS levels can result in the modification of individual nucleotide bases (such as the mutagenic 7,8 dihydro-8-oxoguanine), single- and double-strand breaks (Vafa et al., 2002), and telomere attrition (von Zglinicki, 2002). Indeed, reprogramming-factor-transduced fibroblasts have elevated levels of oxidative DNA damage and ROS (Banito et al., 2009; Esteban et al., 2010). One way to prevent this damage is to supplement the reprogramming cells with ROS scavengers such as N-acetyl-cysteine (NAC) or vitamin C (Vc). Here, we show that supplementation of NAC and Vc during reprogramming and early passaging of iPSCs generated from human fibroblasts results in a significant reduction in de novo copy number variations (CNVs).

RESULTS

Antioxidant Reduces ROS and DNA Damage to Promote Cell Survival during Reprogramming

For reprogramming, we used primary human fibroblasts that we had previously subjected to whole-exome sequencing and confirmed that they had no obvious genetic defects in DNA repair genes (Ji et al., 2012). Furthermore, in another study, we found no evidence of an increased incidence of de novo genomic aberrations near viral integration sites (Hussein et al., 2011). Here, we used retroviruses encoding human *OCT4*, *SOX2*, *KLF4*, and *c-MYC* (hereafter referred to as 4F) for reprogramming. To confirm that reprogramming-factor-transduced human fibroblasts experience oxidative stress, we measured ROS levels 6 days postinfection. We found that 4F-transduced human fibroblasts had significantly higher levels of ROS (Figure 1A). Supplementation of growth media with NAC reduced ROS levels in 4F- and *c-MYC*-, but not *OCT4*, *SOX2*, and *KLF4* (hereafter referred to as 3F)-transduced cells (Figure 1A; Figures S1A and S1B available online). In agreement with the previously reported role of *c-MYC* in increasing ROS levels (Vafa et al., 2002), these results suggested that *c-MYC* is the major factor underlying elevation of ROS levels during reprogramming. Oxidative damage has been reported to induce double-strand breaks (Vafa et al., 2002), and the phosphorylated histone variant H2AX (γH2AX) is a widely used marker for monitoring the levels of DNA double-strand breaks. To assess the extent of DNA double-strand breaks, we performed immunostaining for γH2AX in reprogramming-factor-infected fibroblasts. Treatment with NAC resulted in a significant reduction in the proportion of 4F-infected cells with γH2AX foci ($p = 0.029$, Fisher's exact test; Figure 1B). However, γH2AX levels in 3F-infected cells were not affected by NAC treatment (Figures S1C and S1D). Collectively, our results suggest that a significant proportion of DNA double-strand breaks arise due to elevated ROS levels during reprogramming of human fibroblasts with 4F.

DNA damage caused by forced expression of 4F has been shown to trigger the DNA damage response and increase apoptosis and senescence during reprogramming (Marión et al., 2009; Banito et al., 2009). To determine whether NAC influences the cell survival during reprogramming, we performed a flow-cytometry-based assay to measure apoptosis. We found that NAC treatment during reprogramming promoted the survival of 4F-infected cells (Figure 1C). Taken together, our results suggest that antioxidant treatment reduces ROS levels and genome instability, leading to the increased survival of cells during reprogramming with 4F.

Antioxidant Does Not Alter Transgene Expression and Silencing during Reprogramming

We used a common viral titer for all of the reprogramming experiments in antioxidant-treated and -untreated conditions to avoid genetic variability potentially caused by different levels of expression of the 4F. However, it is possible that the increased survival observed during reprogramming in the presence of NAC is not due to reduced ROS levels, but rather reflects the reduced dosage of transgene expression. To examine whether antioxidants alter the viral transduction efficiency and transgene expression level, we used the GFP retrovirus to infect the cells and treated them with or without NAC. GFP viral infection reached almost 100% transduction efficiency as revealed by flow-cytometry analysis. NAC treatment did not significantly alter the proportion of GFP^+ cells or the expression level of GFP (Figures S2A–S2C), demonstrating that NAC treatment does not affect viral transduction efficiency and transgene expression. We conclude that the reduction in genome instability and apoptosis that occurs during reprogramming is unlikely to be due to reduced transgene load in NAC-treated cells.

Another possible explanation for the reduction in genotoxicity and apoptosis observed during reprogramming in the presence of NAC may be the shorter duration of transgene expression. To test whether antioxidant treatment affects the kinetics of exogenous 4F silencing, we performed quantitative PCR (qPCR) analysis at different time points of reprogramming (Figure 2). We found that the exogenous expression levels of 4F declined with the progression of reprogramming in both control and NAC-treated conditions (Figures 2A–2D), indicative of transgene silencing. The transgene expression levels of the 4F did not differ significantly between the untreated and NAC-treated cells at all tested time points after viral infection (Figures 2A–2D). Taken together, these results suggest

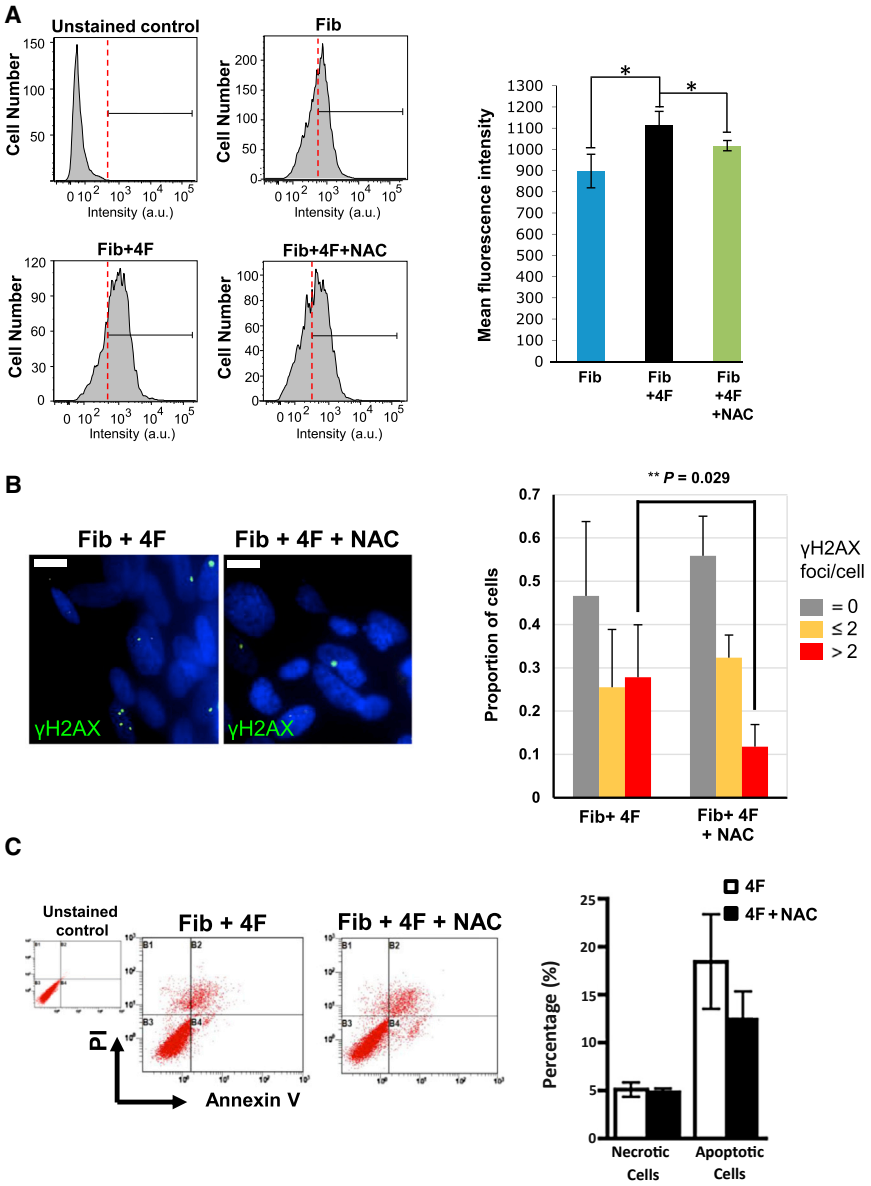

Figure 1. Antioxidant Reduces ROS and DNA Damage to Promote Cell Survival during Reprogramming

(A) Flow-cytometry analysis of ROS levels during reprogramming. Cells at day 6 of infection with 4F were analyzed and the mean ± SEM is represented (n = 3 independent experiments, Student's t test, p = 0.01 for the difference between fibroblasts (Fib) and Fib+4F, and p = 0.035 for the difference between Fib+4F and Fib+4F+Nac).

(B) Immunostaining of γH2AX during reprogramming. Cells at day 5 of infection with 4F were stained and 100 cells of each condition were counted for gH2AX-positive foci. Mean ± SEM is represented (n = 3 independent experiments, Student's t test); scale bar: 5 μm.

(C) Flow-cytometry analysis of cell death during reprogramming. Cells at day 7 of reprogramming were analyzed and the mean ± SEM is represented (n = 3 independent experiments, Student's t test).

that antioxidant treatment does not influence transduction efficiency, transgene expression, and silencing during reprogramming, and thus this variable is unlikely to underlie the observed reduction in stress and increased cell survival during reprogramming.

Antioxidants Reduce Genomic Aberrations in iPSCs

To determine whether mitigation of oxidative stress via antioxidant supplementation during reprogramming leads to the generation of iPSCs with better genome integrity, we generated iPSCs with and without antioxidant supplementation during reprogramming using the same viral titer and a common batch of parental cells. Growth-media supplementation with NAC did not significantly change the reprogramming efficiency (Figures 3A and 3B). Characterization of iPSCs generated with or without antioxidant treatment showed that they expressed the pluripotency markers TRA-1-60, SSEA4, and NANOG, and were able to differentiate into lineages representative of three germ layers (Figure 3C), suggesting that they are bona fide iPSCs. The iPSCs made with antioxidant treatment were passaged in the presence of antioxidants until CNVs were assessed. Antioxidant treatment during the passaging of iPSCs did not influence their proliferation or survival (Figures S2D–S2G). Furthermore, we confirmed that iPSCs made with or without antioxidants did not have significant differences in the number of transgene integrations (Figures S3A and S3B).

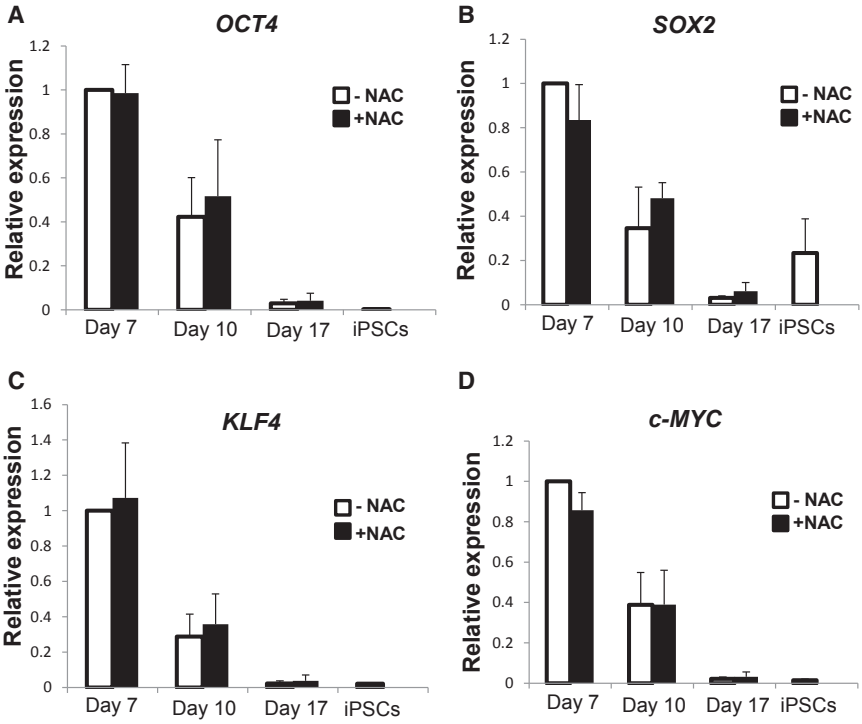

Figure 2. Antioxidant Does Not Change Transgene Expression and Silencing during Reprogramming

(A–D) qPCR analysis of exogenous *OCT4* (A), *SOX2* (B), *KLF4* (C), and *c-MYC* (D) expression at days 7, 10, and 17 of reprogramming with or without NAC treatment. Mean ± SEM is represented (n = 3 independent experiments, Student's t test). An iPSC line generated with 4F without NAC treatment was used to assess transgene silencing after reprogramming.

We assessed the levels of point mutations in iPSCs prepared with or without antioxidant supplementation via high-throughput, whole-exome sequencing (Gore et al., 2011; Ji et al., 2012). DNA from parental fibroblasts, control iPSCs, and iPSCs made with NAC or Vc supplementation was subjected to exome capture using the TruSeq exome capture kit, which targets ~62 Mb of coding and conserved noncoding regions of the human genome, and the enriched DNA was sequenced on the Illumina HiSeq platform. We aligned the sequence data to the human reference (hg19) and identified single-nucleotide variants (Table 1). Mutations in iPSCs were defined as variants that are not found in the Database of Single Nucleotide Polymorphisms (dbSNP v129) and parental fibroblasts in the genomic regions (33.9 Mb) that had at least 8-fold sequencing coverage in the data for each exome. The numbers of coding point mutations in untreated iPSCs and iPSCs made with NAC or Vc were highly variable and consequently we were unable to detect differences with the number of samples that were sequenced (Table 1).

We then examined CNVs of iPSCs using the Affymetrix SNP 6.0 copy number platform. Only arrays that met stringent quality-control criteria (Figure S4A) were retained, and Genotype Console (v 4.0) was used to identify CNVs as previously described (Hussein et al., 2011), requiring a minimum of ten consecutive concordant probes with at least 10 kb in length. The number of parental CNVs in each line was similar (Figure 3D), suggesting that the data from each sample have similar false-negative rates. We discarded CNVs that overlapped with CNVs in the parental fibroblasts or those that are documented in the Database of Genome Variation (http://projects.tcag.ca/variation/) because these might represent common polymorphisms that may preexist in the parental fibroblasts. The median number of novel CNVs in iPSCs (passage 6) treated with antioxidants (NAC and Vc) was significantly lower than the number of CNVs in the untreated iPSCs (passage 6; median number of de novo CNVs in untreated iPSCs = 47, median number of de novo CNVs in antioxidant-treated iPSCs = 12; $p < 0.02$, Mann-Whitney U test; Figure 3E). The length distribution of the novel CNVs was similar among different lines (Figure S4B). We also used an alternative CNV calling method called PennCNV (Wang et al., 2007) to verify the results. As before, the antioxidant-treated iPSCs had fewer CNVs than the control iPSCs (Figure S4C). Taken together, our results suggest that antioxidant treatment during the generation and maintenance of iPSCs reduces their genomic aberrations.

DISCUSSION

One way to obtain good-quality iPSCs is to screen many iPSCs lines derived from the same patient tissue for genomic aberrations. However, given the high number of

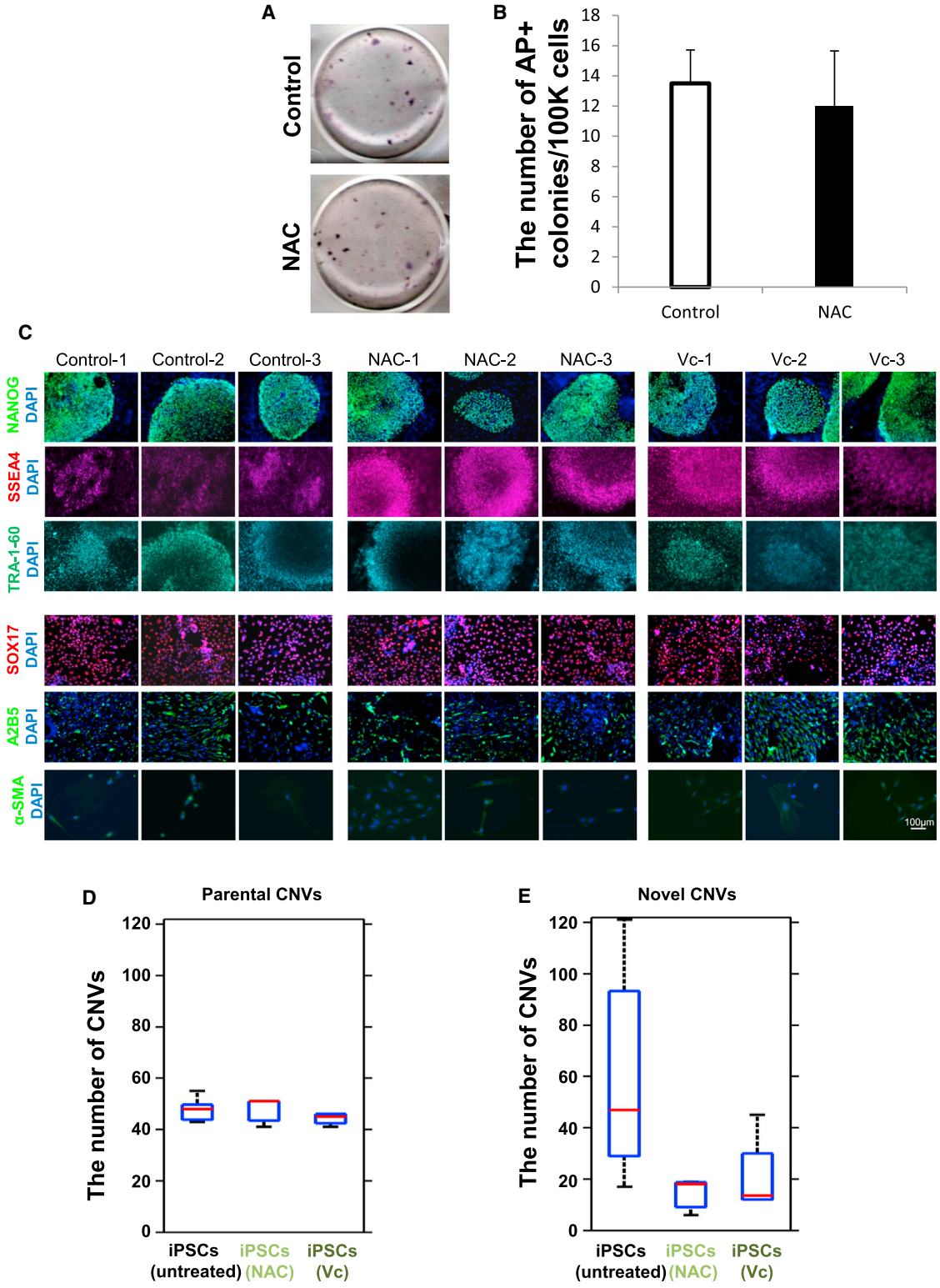

Figure 3. Antioxidants Reduce Genomic Aberrations in iPSCs

(A and B) Reprogramming efficiency with or without NAC. Cells were stained with alkaline phosphatase (AP) at day 27 of reprogramming with 4F. The number of AP^+ colonies per 100,000 cells was quantified. Mean ± SEM was represented (n = 4 independent experiments, Student's t test).

(legend continued on next page)

Table 1. Coding Variants Identified in iPSCs via Exome Sequencing

Sample Name	Number of Variants	% Known (dbSNP)	Nonparental Variants	Exonic Nonparental Variants
Fibroblasts	14,590	91	NA	NA
CTRL-1	14,754	91	54	19
CTRL-2	14,843	91	49	15
CTRL-3	14,697	91	48	15
CTRL-4	14,920	90	53	17
CTRL-5	14,724	91	40	14
NAC-1	14,752	91	36	11
NAC-2	14,954	90	57	11
NAC-3	14,725	91	43	16
NAC-4	14,814	90	53	18
NAC-5	14,714	91	41	14
VC-1	14,800	91	49	19
VC-2	14,656	91	49	16
VC-3	14,472	90	60	11
VC-4	14,894	90	63	21

The variants shown are in the genomic regions targeted by Truseq exome capture (including noncoding targeted regions) in all of the iPSCs and fibroblasts with a minimum of 8-fold coverage (33.9 Mb for total, 18.5 Mb for exonic). dbSNP (v.129) is the database of known SNPs.

cell divisions, compounded by the increased rate of genotoxicity during reprogramming (Banito et al., 2009; González et al., 2013; Marión et al., 2009; Figure 1B), it is likely that very few iPSCs can be generated without de novo mutations using current protocols. Thus, the identification of mutation-free iPSCs would require genome-wide screening of many colonies, which is labor intensive and costly. Given the challenges in determining the functional consequences of genomic aberrations in genes not obviously implicated in cancer or other diseases, it would be more desirable to identify the aspects of reprogramming that cause mutations, and use this knowledge to optimize reprogramming methods to preserve genetic integrity during the derivation and maintenance of iPSCs.

Using two independent platforms and different algorithms to process CNV data, we observed that antioxidant supplementation significantly reduced CNVs in iPSCs. By directly modulating the levels of CNVs in iPSCs with antioxidant supplementation, we were able to demonstrate that a significant proportion of genomic aberrations in iPSCs are incurred after delivery of the reprogramming factors. If all the mutations in iPSCs were due to preexisting parental mutations, then the CNVs in iPSCs derived from a common batch of parental cells with and without antioxidant supplementation during reprogramming would not be different. Theoretically, it is possible that the reprogramming-associated CNVs arise after picking the iPSC colonies and during early passaging rather than during the reprogramming phase. However, our results do not support this view. Differential genome instability would be expected to correlate with increased apoptosis, but we failed to observe such a difference (Figure S2G).

Vc was shown to improve the quality of mouse iPSCs based on the tetraploid complementation assay, and this improvement was attributed to the reduction in DNA methylation aberrations during reprogramming (Stadtfeld et al., 2012). As Vc is already present in the standard media used for the derivation of iPSCs, it was suggested that further Vc supplementation may not reduce mutations in iPSCs (Esteban and Pei, 2012). However, our findings indicate that the amount of Vc that is present in commonly used reprogramming media, such as knockout serum replacement, may be insufficient to protect 4F-driven reprogramming cells from the elevated oxidative stress that is incurred during iPS derivation. Our results also suggest that the improvements observed in tetraploid complementation upon Vc supplementation (Stadtfeld et al., 2012) might also be due to a reduction of genomic aberrations acquired during the derivation of iPSCs.

Our study suggests that a proportion of the somatic CNVs observed in iPSCs are due to elevated oxidative stress; however, oxidative stress does not appear to underlie the point mutations observed in iPSCs (Table 1). The reason for the specificity of antioxidants for CNVs is unclear, but it might reflect the fact that the repair of DNA double-strand breaks is more error prone than the repair of

(C) Characterization of iPSCs. iPSCs of different conditions were stained with NANOG, SSEA4, and TRA-160, or SOX17, A2B5, and alpha-smooth muscle actin (alpha-SMA) before or after differentiation, respectively. Scale bar: 5 μm.
(D) CNV analysis of iPSCs. CNV profiling was performed using Affymetrix SNP 6.0 and copy number regions were called using Affymetrix Genotype Console (v 4.0). The numbers of parental CNVs present in control (n = 5), NAC (n = 3), or Vc (n = 4) are not significantly different from each other (Mann-Whitney U test, $p > 0.05$).
(E) CNV analysis of iPSCs. Data were analyzed as in (D) except that the numbers of nonparental and novel (i.e., not documented in the Database of Genomic Variants) CNVs present are displayed. The numbers of nonparental CNVs are significantly greater in control (n = 5) than in NAC (n = 3) and Vc (n = 4) combined (Mann-Whitney U test, $p < 0.02$).

oxidative DNA lesions. Previous studies have reported that Vc increases the efficiency of reprogramming human cells in media devoid of antioxidants (Esteban et al., 2010), and Vc seems to increase reprogramming efficiency by acting as a cofactor for the JHDM1a/b histone demethylase (Wang et al., 2011). The reduced number of CNVs we observed in iPSCs generated in standard reprogramming conditions with Vc supplementation is unlikely due to Vc's nonantioxidant function, since NAC, which is thought to function exclusively as an antioxidant, leads to a similar reduction in CNVs in iPSCs (Esteban et al., 2010).

In conclusion, mitigation of oxidative stress during reprogramming and early passaging by antioxidant supplementation protects the genome of reprogramming cells from DNA damage and results in iPSCs with fewer overall genomic aberrations. Although the mutation load is reduced, it remains to be determined whether the use of antioxidants during reprogramming endows iPSCs with an improved differentiation potential and/or a reduced risk of tumorigenicity.

EXPERIMENTAL PROCEDURES

Cell Culture

Human neonatal foreskin fibroblasts (ATCC) were cultured in growth medium consisting of Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone Laboratories) and 1 mM L-glutamine (Invitrogen). See the Supplemental Experimental Procedures for additional details.

Generation of iPSCs

iPSCs were generated as previously described (Ji et al., 2012). See the Supplemental Experimental Procedures for additional details.

Measurement of ROS

The ROS level of each sample was measured using 2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen) according to the manufacturer's instructions with some modifications. See the Supplemental Experimental Procedures for additional details.

Immunocytochemistry

Cells were fixed with PBS containing 4% paraformaldehyde for 20 min at room temperature and washed with PBS. See the Supplemental Experimental Procedures for additional details.

Alkaline Phosphatase Staining

Staining was performed with an alkaline phosphatase detection kit (Millipore) according to the manufacturer's manual. See the Supplemental Experimental Procedures for additional details.

Preparation of Illumina Sequencing Libraries

Genomic DNA was extracted from iPSCs using the ZYMO DNA extraction kit, and 1 μg of DNA was used to create Truseq paired-end libraries according to the manufacturer's protocol. See the Supplemental Experimental Procedures for additional details.

Affymetrix SNP 6.0 CNV Profiling

CNVs were measured using Affy SNP 6.0 arrays at the Center for Genomic Analysis facility at Sickkids Hospital (Toronto, Canada). See the Supplemental Experimental Procedures for additional details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2013.11.004.

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