High Glutathione and Glutathione Peroxidase-2 Levels Mediate Cell-Type-Specific DNA Damage Protection in Human Induced Pluripotent Stem Cells

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SUMMARY

Pluripotent stem cells must strictly maintain genomic integrity to prevent transmission of mutations. In human induced pluripotent stem cells (iPSCs), we found that genome surveillance is achieved via two ways, namely, a hypersensitivity to apoptosis and a very low accumulation of DNA lesions. The low apoptosis threshold was mediated by constitutive p53 expression and a marked upregulation of proapoptotic p53 target genes of the BCL-2 family, ensuring the efficient iPSC removal upon genotoxic insults. Intriguingly, despite the elevated apoptosis sensitivity, both mitochondrial and nuclear DNA lesions induced by genotoxins were less frequent in iPSCs compared to fibroblasts. Gene profiling identified that mRNA expression of several antioxidant proteins was considerably upregulated in iPSCs. Knockdown of glutathione peroxidase-2 and depletion of glutathione impaired protection against DNA lesions. Thus, iPSCs ensure genomic integrity through enhanced apoptosis induction and increased antioxidant defense, contributing to protection against DNA damage.

INTRODUCTION

The generation of human induced pluripotent stem cells (iPSCs) from adult somatic cells represents an important advancement in stem cell biology, because of the many potential applications including patient-specific tissue replacement, drug screening, and disease modeling (Okita and Yamanaka, 2011; Robinton and Daley, 2012). In addition, iPSCs derived from patients of diseases caused by known mutations can generate valuable in vitro models for complex disorders, including aging, diabetes, and neurodegeneration. The iPSCs can be generated through forced expression of a set of transcription factors and share with embryonic stem cells (ESCs) the same cardinal features of self-renewal and pluripotency (Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008).

Pluripotent stem cells have the capacity to differentiate into almost any cell type in the adult organism. This pluripotency, however, requires that, unlike differentiated cells, stem cells must be endowed with superior DNA maintenance and repair systems to ensure genomic stability over multiple generations without propagating DNA errors (Liu et al., 2014). The mechanisms required to maintain genomic integrity in response to DNA damage, which could otherwise compromise competency for tissue renewal, are only poorly understood and have been largely investigated in ESCs. Human ESCs and iPSCs, for instance, have an abbreviated cell cycle with a very brief G1 phase, indicating that mechanisms mediating responses to DNA damage may differ from those in somatic cells (Momcilović et al., 2009, 2010; Filion et al., 2009).

Under physiological conditions, reactive oxygen species (ROS) generated as by-product of mitochondrial respiration are the major source of DNA damage (Schieber and Chandel, 2014). DNA lesions in the absence of DNA repair can lead to cell death, genomic instability, and cancer. There are two major ways how ESCs could principally ensure increased genomic integrity. First, mutation frequencies must be suppressed by low levels of DNA damage accumulation and efficient repair systems. Second, ESCs that accumulate mutations or DNA damage must be rapidly eliminated from the stem cell population. Previous studies suggested that mechanisms of genome surveillance, including DNA repair, are indeed superior in ESCs (Saretzki et al., 2008; Maynard et al., 2008). It was shown that murine ESCs possess highly efficient repair mechanisms resulting in a 100-fold lower mutation frequency compared with embryonic fibroblasts (Cervantes et al., 2002). In addition, murine and human ESCs are hypersensitive to several DNA-damaging agents and readily undergo apoptosis (Qin et al., 2007; Roos et al., 2007; Madden et al., 2011; Liu et al., 2013).

Multiple mechanisms have been described that sensitize ESCs to DNA damage-induced apoptosis. First, human ESCs possess unique ROCK-dependent mechanisms in singularized cells that lead to a myosin-mediated form of cell blebbing, which rapidly triggers apoptosis upon cell detachment (Ohgushi et al., 2010). Second, unlike
differentiated cells, certain human ESC lines have been found to express a constitutively pre-activated form of the proapoptotic BCL-2 protein BAX at the Golgi apparatus, which may quickly translocate to the outer mitochondrial membrane and initiate execution of the intrinsic apoptosis pathway upon DNA damage (Dumitru et al., 2012). Notably, the basal level of pre-activated BAX varies among different human ESC lines and, for example, is not detectable in the H1 cell line. Nonetheless, H1 cells show the typical sensitivity to DNA damage, suggesting that additional mechanisms might be involved in priming ESCs for rapid cell death (Liu et al., 2014).

Although the regulation of pluripotency and genomic stability has been mainly studied in ESCs, very little is known regarding the mechanisms controlling their susceptibility to death stimuli. In the present study, we investigated how human iPSCs react to DNA damage induced by several genotoxins and proapoptotic stimuli in comparison to fibroblasts. We found that iPSCs are hypersensitive to agents triggering the mitochondrial death pathway, which is mediated by the increased expression of several proapoptotic BCL-2 proteins. In contrast, iPSCs were largely resistant to death receptor-mediated apoptosis. Interestingly, despite the increased apoptosis sensitivity upon DNA damage, iPSCs displayed very low levels of DNA lesions compared to other cell types under diverse genotoxic conditions. Moreover, we found that iPSCs harbor high glutathione (GSH) levels and strongly express several GSH-dependent antioxidant enzymes. The combined depletion of GSH and glutathione peroxidase-2 (GPX2) levels was able to impair this iPSC-specific resistance to DNA damage. Thus, our results suggest that human iPSCs have a superior DNA maintenance response that is mediated by both an increased antioxidant defense and an elevated mitochondrial priming and apoptosis induction. These data might have profound implications for future iPSC-based therapies that are dependent on the quality of the differentiated cells and their ability to maintain an intact genome.

RESULTS

Human iPSCs Are Highly Susceptible to Mitochondrial Cell Death

To elucidate the relationship between DNA damage acquisition and apoptosis induction in human iPSCs, we first investigated the expression of pluripotency markers. Two iPSC lines that had been generated in our laboratory from adult skin fibroblasts (Lehle et al., 2014) were analyzed in more detail. In vitro analysis and morphological assessment showed that both iPSC lines were very similar to human ESCs and expressed alkaline phosphatase and pluripotency markers including NANOG, SOX2, TRA1-60, TRA1-81, and SSEA-4 (Figure S1). Moreover, visual observation revealed that no significant differentiation had occurred, even on the colony periphery.

As ROS generated during mitochondrial respiration are a major source of DNA damage (Schieber and Chandel, 2014), we first employed hydrogen peroxide to compare the cell death sensitivity of iPSCs and fibroblasts. After treatment with 500 μM H2O2, apoptotic and necrotic cell death was determined by fluorescence-activated cell sorting (FACS) measurement of annexin-V/propidium iodide (PI) staining. As shown Figure 1A, iPSCs were very sensitive to oxidative damage-induced cell death, whereas fibroblasts remained largely viable even after 24 hr of H2O2 treatment. A similar cell-type-specific sensitivity was observed after exposure to UVC light, a condition known to induce cell death mainly by oxidative DNA damage. Whereas iPSCs were already killed after exposure to a low UVC dose of 5 mJ/cm2, their somatic precursor cells remained viable even after a high-dose UVC treatment of 100 mJ/cm2 (Figure 1B). Furthermore, bleomycin, a genotoxic chemotherapeutic drug, efficiently induced cell death in iPSCs, but not in fibroblasts (Figure 1C).

The increased apoptosis sensitivity of human ESCs was reported to be selective to DNA-damaging stimuli (Dumitru et al., 2012). We therefore investigated cell death in response to non-genotoxic ER stress and applied tunicamycin and thapsigargin, which triggered cell death of iPSCs, but only weakly compromised the viability of fibroblasts (Figures 1D and 1E). In addition, brefeldin A, a Golgi complex disassembly agent, induced cell death in iPSCs, but not in fibroblasts (Figure 1F). Finally, we compared death receptor-mediated apoptosis in both cell types. Interestingly and in contrast to the previous stimuli, iPSCs remained largely resistant to Fasl- or TRAIL-induced apoptosis even after prolonged incubation for 72 hr, whereas a considerable fraction of fibroblasts was already killed after a short exposure to both death ligands (Figures 1G and 1H). These results demonstrate that iPSCs display a high and selective apoptosis sensitivity to death stimuli activating the mitochondrial pathway, whereas they are largely resistant to death receptor-mediated apoptosis.

iPSCs Display Increased Mitochondrial Priming

To explore the mechanism underlying the low apoptosis threshold of iPSCs, we investigated the expression of several apoptosis mediators using qRT-PCR analysis (Figure 2A). Transcript levels of members of the inhibitor-of-apoptosis protein family, including XIAP, IAP1, and particularly IAP2, were considerably reduced in iPSCs compared to fibroblasts. In addition, iPSCs displayed lower mRNA expression of several proapoptotic BCL-2 protein members, including BCL2, BCLX, BCLW, and A1. In contrast,
even under basal conditions, TP53 and important proapoptotic BCL-2 members, such as BIM and NOXA, were highly expressed in iPSCs compared to fibroblasts (Figure 2A). Furthermore, in line with previous cell death experiments, iPSCs expressed markedly reduced levels of the death ligand TRAIL as well as several death receptors (Figure 2B).

Since expression of BCL-2 proteins is often regulated by posttranslational events, we further analyzed protein expression using western blot analyses that revealed a high expression of p53 in iPSCs, but not in fibroblasts (Figure 2C). In addition, the p53-regulated proapoptotic proteins BAK and BIM were strongly expressed in iPSCs compared to fibroblasts. No significant differences between both cell types were found for BCL-XL, BID, and BAX, whereas expression of MCL-1, which is regulated at the posttranslational level by Nanog (Noh et al., 2012), was elevated in iPSCs. Altogether, the mRNA and protein expression analyses indicated that iPSCs reveal an increased mitochondrial priming that is presumably mediated by a strong p53 response, resulting in a shift of the balance of antiapoptotic to proapoptotic BCL-2 proteins. In contrast, differentiated cells display low mitochondrial priming, resulting in resistance to DNA-damaging agents and other drugs activating the mitochondrial death pathway. Consistently, boosting the priming with the BH3 mimetic ABT-737 strongly sensitized fibroblasts to DNA damage-induced apoptosis (Figure 2D).

DNA Damage Rates in Genotoxically Stimulated iPSCs Are Lower Than in Fibroblasts and Increase upon Differentiation

Previous studies suggested that mechanisms of genome surveillance, including DNA repair, are superior in ESCs (Saretzki et al., 2008; Maynard et al., 2008). We therefore determined the accumulation of DNA damage in iPSCs and fibroblasts using long-run real-time PCR-based DNA damage quantification (LORD-Q) analysis, a highly sensitive technique for the detection of nuclear and mtDNA damage.
To prevent apoptosis-mediated DNA fragmentation and cellular repair processes, we exposed the cells to UVC light, which induces DNA damage within a few seconds of treatment. Surprisingly and in contrast to the strong apoptotic response induced by UVC, accumulation of both nuclear and mtDNA lesions was significantly lower in iPSCs than in fibroblasts (Figure 3A). To verify these findings, we additionally measured oxidative DNA lesions, such as cyclobutane pyrimidine dimers and pyrimidine(6-4)pyrimidone photoproducts, using an ELISA, which confirmed the low accumulation of DNA damage in iPSCs (Figure 3B). We also analyzed mtDNA damage and the occurrence of 8-hydroxydeoxyguanosine (8-oxo-dG), a mutagenic DNA lesion, in response to H₂O₂ treatment, which also revealed a reduced acquisition of DNA lesions in iPSCs compared to fibroblasts (Figures 3C and 3D).

We next investigated whether this protection against DNA damage was maintained during iPSC differentiation. To induce differentiation, iPSCs were grown in fetal calf serum (FCS)-containing medium in the absence of FGFl. When fibroblasts and undifferentiated and differentiated iPSCs were exposed to different H₂O₂ concentrations, differentiated iPSCs clearly displayed enhanced DNA damage rates compared to their undifferentiated counterparts (Figure 3E). Thus, protection of pluripotent cells against DNA damage is rapidly lost upon differentiation.

Human iPSCs Exhibit Significantly Lower DNA Lesion Rates Than Most Tumor Cell Lines

Since malignant cells exhibit several features of stem cells, we wanted to investigate whether the protection against DNA damage also could be observed in tumor cells. In addition to iPSCs and fibroblasts, we therefore UVC-irradiated 15 different tumor lines that were mostly derived from the NCI60 panel. We found that iPSCs exhibited a significantly lower DNA damage rate than fibroblasts in all three investigated genomic loci, namely mtDNA (Figure 4A), the GAPDH locus (Figure 4B), and the TP53 locus (Figure 4C). In most tumor cell lines, the levels of nuclear and mtDNA damage correlated with each other. Moreover, the damage rate of mtDNA and the two examined nuclear loci was significantly lower in iPSCs than in most tumor cell lines.
Thus, during genotoxic exposure, iPSCs acquire less DNA lesions than parental fibroblasts and cells derived from various tumor entities.

**Human iPSCs Display High GSH Levels and Decreased Oxidative Stress**

Reduced DNA damage could be mediated by increased expression of DNA repair genes, as previously shown in ESCs (Saretzki et al., 2008; Maynard et al., 2008). However, since iPSCs exhibit protection against both nuclear and mtDNA damage, another explanation could be that there is less oxidative damage occurring, possibly due to higher levels of antioxidants. Measurement of the levels of GSH, the most important cellular antioxidant, indeed revealed 3- to 4-fold increased levels of GSH in iPSCs compared to fibroblasts (Figure 5A). To investigate the functional role of increased GSH levels, we depleted cellular GSH using dimethyl fumarate (DMF), which is metabolized in a GSH-dependent manner, and buthionine sulfoximine (BSO), an irreversible γ-glutamylcysteine synthetase inhibitor. Combined treatment of iPSCs and fibroblasts with both agents for 1 hr was sufficient to reduce GSH contents in both cell types by at least 80%, without inducing cell death (Figure 5B). When cells were subsequently treated with H2O2, GSH-depleted fibroblasts exhibited a significant and strong increase in DNA damage that was partially reversed by a cell-permeable glutathione O-ethylester (GSH-OEt) (Figure 5C). Surprisingly, however, only minor effects of GSH depletion and substitution on DNA lesion rates were observed in iPSCs (Figure 5C).

To investigate whether the cells had experienced comparable intracellular stress levels, we measured ROS levels using dihydroorhadamine 123 staining and FACS analysis. As shown in Figure 5D, both cell types displayed considerable ROS level increases after H2O2 exposure, which was significantly boosted by GSH depletion and rescued by GSH O-ethylester, demonstrating that the experimental setup was not responsible for the low effect of GSH depletion on the DNA vulnerability of iPSCs. Notably, the increase of ROS levels in fibroblasts was 5- to 10-fold higher than in iPSCs, suggesting that oxidative stress is efficiently prevented in iPSCs.
High Levels of GPX2 and GSH Protect iPSCs from DNA Damage

The previous results indicated that iPSCs exhibit high GSH levels, but might possess additional safeguard mechanisms. We therefore analyzed mRNA expression of several antioxidant enzymes in fibroblasts and iPSCs (Figure 6). Our qRT-PCR analyses revealed that several glutathione S-transferases (GSTs), which act as antioxidant and detoxifying enzymes, were upregulated in iPSCs compared to their somatic precursor cells. Most striking was GSTA2 that was expressed at more than 80,000-fold higher levels in iPSCs relative to primary fibroblasts. In addition, iPSCs revealed a more than 10,000-fold higher expression of GPX2. Furthermore, several peroxiredoxins, which act as scavengers of H₂O₂ and organic hydroperoxides, as well as glutathione reductase were considerably upregulated in iPSCs, supporting the potent antioxidant status of iPSCs (Figure 6).

In iPSCs the transcript levels of GSTA2 and GPX2 were elevated not only compared to fibroblasts but also compared to most tumor cell lines (Figure 7A). The sole exception was observed in HepG2 hepatocellular carcinoma cells that, as typical for liver cells, expressed not only increased levels of both enzymes (Figure 7A), but were also relatively resistant to DNA damage (Figure 4). To investigate a functional contribution of GSTA2 or GPX2 to DNA damage protection, we reduced mRNA levels of both enzymes in iPSCs by small interfering RNA (siRNA) treatment. However, neither GSTA2 nor GPX2 knockdown alone led to significant increases of detectable DNA damage following H₂O₂ exposure (Figure 7B). We next combined GSH depletion with the knockdown of GPX2 or GSTA2 in iPSCs. In GSH-depleted cells, knockdown of GPX2, but not of GSTA2, significantly rendered the cells more vulnerable to DNA damage following H₂O₂ exposure. Vice versa, the lentiviral overexpression of GPX2 in fibroblasts was sufficient to confer increased resistance to DNA damage (Figure 7C). These results thus indicate that high levels of GSH and GPX2 mediate DNA damage protection of iPSCs. In view of the increased expression of several other antioxidant mediators as well as the reported abundance of DNA repair proteins, it is likely that, in addition, further DNA damage protection mechanisms ensure genomic integrity of iPSCs.

DISCUSSION

Pluripotent stem cells must tightly control the balance between cell survival and death to prevent unfavorable mutations and to ensure genomic integrity. First, maintenance of genomic stability must be particularly stringent, because any genetic alterations in pluripotent stem cells can impair the functionality of their progeny and compromise tissue renewal. Pluripotent stem cells bear an enhanced tumorigenic potential and share several characteristics with cancer cells, such as replicative immortality (Ben-David and Benvenisty, 2011). These properties require a quick and strong apoptotic response in DNA-damaged cells to prevent an accumulation of mutations that could facilitate deregulated proliferation or predispose cells to acquire further mutations associated with cancer development.

It has been shown that ESCs are hypersensitive to DNA damage and readily undergo apoptosis (Qin et al., 2007; Madden et al., 2011; Liu et al., 2013), although much less is known about iPSCs. DNA damage sensitivity in human ESCs was shown to correlate with a property, termed mitochondrial priming, that is determined by the balance between pro- and antiapoptotic BCL-2 proteins (Liu et al., 2013). In the present study, we found that, compared to differentiated fibroblasts, iPSCs exhibit a low apoptosis...
Interestingly, the high apoptosis sensitivity was restricted to stimuli activating the mitochondrial pathway, whereas iPSCs were strongly resistant to the extrinsic apoptosis pathway, presumably by the downregulation of several death receptors. Unlike human ESCs (Dumitru et al., 2012), however, the increased sensitivity of iPSCs was not confined to DNA-damaging stimuli, but also observed after treatment with ER stress- or Golgi disassembly-inducing agents that also trigger the mitochondrial pathway.

Human ESCs have been reported to maintain BAX in an active conformation at the Golgi apparatus under basal conditions (Dumitru et al., 2012), however, the increased sensitivity of iPSCs was not confined to DNA-damaging stimuli, but also observed after treatment with ER stress- or Golgi disassembly-inducing agents that also trigger the mitochondrial pathway.

Human ESCs have been reported to maintain BAX in an active conformation at the Golgi apparatus under basal conditions (Dumitru et al., 2012), but this mechanism is unlikely the sole reason of their increased apoptosis sensitivity. In support of this notion is the finding that the H1 ESC line displays the typical apoptosis hypersensitivity, but lacks expression of active BAX at the Golgi (Dumitru et al., 2012). Moreover, we found that, also in human iPSCs, BAX was evenly distributed in the cytosol, but was not localized at the Golgi (Figure S2). Nevertheless, our results showed that, even under basal conditions, iPSCs reveal a strong accumulation of p53, a tumor suppressor, which also acts as a barrier to somatic cell reprogramming (Tapia and Schöler, 2010). p53 activates the transcription of multiple genes involved in apoptosis, in particular pro-apoptotic BCL-2 proteins. Indeed, compared to fibroblasts, expression of several p53 target genes including BAK, BIM, and NOXA was strongly upregulated, indicating that iPSCs exhibit high mitochondrial priming compared to differentiated cells. Fibroblasts, however, could be sensitized to DNA damage-induced apoptosis using ABT-737, an inhibitor of antiapoptotic BCL-2 proteins.

In addition to apoptosis, we investigated the occurrence of DNA lesions in response to genotoxic insults. Using LORD-Q analyses, a novel sensitive technique to quantify DNA lesions (Lehle et al., 2014), we found that, despite their elevated apoptosis sensitivity, iPSCs accumulated significantly less DNA lesions than differentiated...
fibroblasts. Also, in response to treatment with H_2O_2 and UVC, oxidative nucleotide modifications such as cy-clobutane pyrimidine dimers, (6-4) photoproducts, and 8-hydroxydeoxyguanosine (8-oxo-dG) were detected less frequently. Thus, our data are consistent with the hypoth-
thesis that pluripotent stem cells have superior DNA mainte-
nance responses. Interestingly, we observed that this DNA damage protection was rapidly lost during differentiation 
of iPSCs.

There are presumably several mechanisms that 
contribute to an efficient maintenance of DNA integrity 
in pluripotent stem cells, including the prevention of 
DNA damage and the removal of DNA lesions. Compared 
to differentiated cells, ESCs display a moderate increase (approximately 2- to 3-fold) in the expression of certain 
DNA repair enzymes of the homologous recombination 
and non-homologous end-joining pathways, which repair 
DNA double-strand breaks (Saretzki et al., 2008). It is inter-
esting to note that a previous study in human ESCs (May-
nard et al., 2008) found decreased levels of oxidative 
DNA lesions, such as 8-oxo-dG, which we assessed as an 
additional marker of DNA damage in iPSCs. Although the 
decreased 8-oxo-dG levels are suggestive of a more efficient 
repair of this lesion, the authors did not find elevated activ-
ities of 8-oxoguanine glycosylase, the primary base exci-
sion repair enzyme required for removing this mutagenic 
DNA lesion. These results indicate that base excision repair is presumable not elevated in ESCs, but rather the occurrence 
of oxidative DNA lesions is prevented.

Our study shows that iPSCs are highly proficient in anti-
oxidant defense, which is presumably responsible for the 
low frequency of oxidative DNA lesions in both the mito-
chondrial and nuclear genome. Notably, previous non-
quantitative proteomic studies revealed an abundant 
expression of antioxidant enzymes, in particular several 
peroxiredoxins in human ESCs (Baharvand et al., 2006). Furthermore, ESC cultures generate fewer ROS than most 
somatic cell types, because of their lower reliance on 
oxidative phosphorylation and limited mitochondrial 
biogenesis (Prigione et al., 2010; Armstrong et al., 2010). Structural analyses of mitochondria in human ESCs 
demonstrated an immature network characterized by few 
organelles with poorly developed cristae, which however 
increase during differentiation (St John et al., 2006; Facu-
cho-Oliveira et al., 2007). Thus, stem cells maintain low 
ROS levels not only by high antioxidant activity, but also 
by reduced oxygen consumption and low mitochondrial 
biogenesis. Our PCR-based LORD-Q method enabled us 
to specifically assess not only nuclear but also mtDNA le-
ssions. Several lines of evidence suggest that, in particular, 
the occurrence of mtDNA lesions must be prevented for 
the maintenance of pluripotency. For instance, studies in 
a mouse model with high levels of mtDNA mutations due 
to a proof-reading defect of DNA polymerase γ (mtDNA 
mutator mice) established causal relationships among the 
accumulation of mtDNA mutations, stem cell exhaustion, 
and premature aging (reviewed in Baines et al., 2014).

In our study, we found that the level of GSH, the most 
important cellular antioxidant, was elevated up to 4-fold 
in iPSCs compared to somatic fibroblasts. In addition, the 
mRNA levels of several peroxiredoxins, GSTs, and gluta-
thione reductase were considerably increased. All these 
enzymes might be involved in antioxidant defense and 
detoxification, a finding reminiscent of the expression of 
aldehyde dehydrogenase-1, which is often used as marker 
of cancer stem cells (Ginestier et al., 2007).
Most notable was our finding that expression of two GSH-dependent enzymes, GSTA2 and GPX2, was elevated more than 80,000- and 10,000-fold, respectively, compared to fibroblasts. We therefore investigated their possible contribution to the maintenance of genomic integrity. Our data show that overexpression of GPX2 in fibroblasts can significantly increase resistance to oxidative stress-induced DNA damage. Moreover, using RNAi, we found that knockdown of GPX2, but not GSTA2, could overcome DNA damage protection in iPSCs. However, combined depletion of GSH was required for sensitization to DNA damage in iPSCs. This assumption is supported by our finding that the expression of several antioxidant enzymes was strongly increased in iPSCs.

Although GPX2 is known as a gastrointestinal GSH peroxidase, it also is expressed in other tissues (Brigelius-Flohe and Kipp, 2012). Interestingly, its expression level is increased in intestinal crypt stem cells and malignant epithelial cells, suggesting a role in proliferation and self-renewal. It also was found that GPX2 overexpression alleviates the apoptotic response of breast cancer cells to oxidative stress (Yan and Chen, 2006). The GPX2 promoter is activated by the Wnt pathway that is highly active in iPSCs and ESCs (Kipp et al., 2012). Like many antioxidant enzymes, GPX2 expression is further controlled by transcription factor NRF2 (Banning et al., 2005), which very recently has been implicated also in the self-renewal capacity of ESCs (Jang et al., 2014). It is thus conceivable that the high antioxidant defense of iPSCs is not only involved in genomic stability, but also required for self-renewal.

![Figure 7. GPX2 and GSH Contribute to DNA Damage Protection in iPSCs](image-url)
replicative immortality, and the delay of differentiation of iPSCs.

In summary, our study brings together two seemingly separate processes that accompany the function of iPSCs. Human iPSCs are able to defend their genomic integrity by maintaining low levels of ROS through a combination of enhanced removal and limited production of these molecules. In addition to the endowment with superior DNA repair systems, iPSCs display high mitochondrial priming and apoptosis sensitivity, once DNA damage has occurred. These processes might not only ensure genomic stability and prevent transmission of mutations to the progeny, but also might be important for a possible therapeutic application of iPSCs.

EXPERIMENTAL PROCEDURES

Cell Culture

Human iPSCs were generated as described (Takahashi et al., 2007; Lehle et al., 2014) by transduction of dermal fibroblasts with the Yamanaka retroviral cocktail, and grown on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells in hES medium containing Knockout DMEM, 20% serum replacement (all from Life Technologies), 2 mM glutamine, non-essential amino acids, 25 μM 2-mercaptoethanol, and 5 ng/ml FGF2 (PeproTech). Human fibroblasts were cultured in RPMI-1640 supplemented with 10% FCS, 2 mM glutamine, and 2 mM sodium pyruvate. All other cell lines were obtained from ATCC and grown in the recommended media. Culture media were supplemented with penicillin/streptomycin.

Microscopy

For characterization of the iPSCs, cells were stained for the expression of pluripotency markers, including NANOG, SOX2, TRA1-60, TRA1-81, and SSEA-4. To this end, cells were fixed with ice-cold acetone/methanol (1:1) for 5 min, washed with PBS, and incubated for 1 hr in blocking buffer (4% BSA, 0.05% saponin in PBS) at room temperature. The primary antibodies (listed in Table S1) were incubated at 4°C overnight. After washing the cells thrice in blocking buffer, the appropriate Alexafluor-coupled secondary antibody was applied for 1 hr. Cells were washed again in PBS and incubated afterward in PBS containing 100 ng/ml DAPI for 5 min. Coverslips were mounted in fluorescence mounting medium and analyzed using a DMI6000 fluorescence microscope (Leica). Alkaline phosphatase activity was determined by the Alkaline Phosphatase Detection kit (Millipore) after fixation of cells and the detached cells were combined. Subsequently, cells were stained with FITC-annexin-V and PI following the instructions of the manufacturer (BD Biosciences) and analyzed by flow cytometry. Cells with positive annexin-V but negative PI staining were considered apoptotic, whereas double-positive cells were considered necrotic.

Induction and Detection of DNA Damage

For induction of DNA damage, adherent somatic cells were singularized using trypsin/EDTA. The iPSCs were detached by accutase in the presence of the ROCK inhibitor Y-27632 (10 μM, Wako Pure Chemicals Industries) for 10 min at 37°C. Then, 1 × 10⁶ cells were resuspended in PBS supplemented with 5% FCS (PBS/F). UVC irradiation was carried out in 100-mm culture dishes and 10 ml PBS/F using a Stratagene 2400 (Stratagene). Bleomycin exposure was carried out in 300 μl PBS/F for 20 min. H₂O₂ treatment was performed in 1 ml PBS for 5 min at 37°C. Immediately after stimulation, cells were collected by centrifugation and snap-frozen in liquid nitrogen.

Detection and quantification of DNA damage was performed by ELISA or LAMP-sequences as described previously (Lehle et al., 2014). Briefly, whole-cell DNA was isolated from genotoxic-exposed and control samples using the DNeasy Blood & Tissue kit (QIAGEN) and diluted to 10 ng/ml by the addition of suitable volumes of elution buffer. Subsequently, 5 μl diluted sample DNA was added to 15 μl master mix consisting of 10 μl 2× KAPA2G Fast Hot Start Polymerase, 1 μl sense and 1 μl antisense primer (containing each 10 pmol of the respective oligonucleotide), 0.05 μl 20X LightCycler 480 ResoLight Dye (Roche), and 2.95 μl high-performance liquid chromatography (HPLC)-grade water. For each analyzed genomic locus, a long DNA damage sensor fragment and a short reference DNA fragment were amplified in two separate real-time PCR runs (5 min at 95°C pre-heating phase followed by 50 PCR cycles: 10 s 95°C, 10 s 60°C, 2:15 min [long fragments] or 1 s [short fragments] at 72°C). For nuclear DNA damage determination, the GAPDH and TP53 loci were analyzed (see Table S2 for primer sequences). Calculation of detected lesions per 10 kb was performed as described previously (Lehle et al., 2014). DNA lesions such as cyclobutane pyrimidine dimers, (6-4) photoproducts, and 8-hydroxydeoxyguanosine were measured using ELISA kits from Cell Biolabs.

Western Blot Analyses

Cells were washed in ice-cold PBS and resuspended in 100–200 μl RIPA buffer supplemented with 1× Mini Complete Protease Inhibitor cocktail (Roche). Protein concentrations were determined by the BCA assay and 10–50 μg protein per lane was loaded onto SDS-PAGE gels. After electrophoresis, proteins were transferred onto polyvinylidenedifluoride membranes (Amersham Biosciences). Membranes were blocked in PBS containing 4% BSA and 0.05% Tween-20 for 1 hr, followed by an overnight incubation with the primary antibodies (listed in Table S1) in blocking buffer at 4°C. After washing the membrane thrice in Tris-buffered saline (TBS)/0.05% Tween, peroxidase-coupled secondary antibodies were visualized with horseradish peroxidase (HRP)-coupled secondary antibodies (Amersham Biosciences) and chemiluminescence (ECL).
were applied for 1 hr. Proteins were visualized using enhanced chemiluminescence (ECL) reagents (Amersham Biosciences).

qRT-PCR
For relative expression analysis, RNA was isolated from cell pellets using the RNeasy Mini Kit (Qiagen). After reverse transcription (Transcriptor First Strand cDNA Synthesis kit, Roche), cDNA levels were analyzed in a real-time PCR approach. Experiments were carried out in 96- or 384-well plates on a LightCycler 480 II system (Roche). Per reaction (96-well plates: 20 μl; 384-well plates: 10 μl reaction), 10 μl 2× SYBR Green master mix (Fermentas), each 10 pmol sense and antisense primer (see Table S3 for primer sequences), 10 ng of sample cDNA, and HPLC-grade water (ad 20 μl) were used. The real-time PCR program comprised a 5-min heating phase (95°C) followed by 35 to 50 cycles (10 s at 95°C, 10 s at 60°C, 10 s at 72°C). Relative transcript levels were calculated using the 2-ΔΔCT method (Livak and Schmittgen, 2001). Glyceraldehyde dehydrogenase (GAPDH), beta-actin (ACTB), and delta-aminolevulinate synthetase 1 (ALAS1) were used as reference genes. Each sample was analyzed in triplicate and the resulting C₀ values were averaged.

Manipulation and Analysis of Cellular GSH Content
Total cellular GSH (GSSx; c[GSSx] = c[GSH] + 2 × c[GS-SG]) content was determined as described previously (Tietze, 1969). Briefly, 10³ to 10⁶ cells were lysed in ice-cold 1% 5-sulfosalicylic acid. After 30-min incubation on ice, lysates were centrifuged (10 min, 20,000 x g) and the supernatants were used for GSSx determination, while the pellets were analyzed for protein content by the BCA assay. Then, 10 μl supernatants were transferred to a 96-well plate and mixed with 100 μl reaction solution containing 0.64 μl glutathione reductase (Sigma) solution, 400 μM NADPH, 300 μM of the colorimetric dye S,5′-dithiobis-(2-nitrobenzoic acid), and 2 mM EDTA in 100 mM sodium phosphate buffer (pH 7.5). Subsequently, the absorption at 412 nm was followed for 10 to 15 min and the slopes of the resulting curves were determined. GSSx concentrations were calculated using standard curves, normalized for protein content and expressed as nmol GSSx/mg protein. Depletion of cellular GSSx pools was achieved by co-incubation of the cells with 100 μM DMF (Sigma) and 100 μM BSO (Sigma) for up to 4 hr (Boivin et al., 2011). GSH repletion was performed by co-incubation of cells with BSO/DMF and 2 mM GSH-OEt (Sigma) (Ghorbani et al., 2011).

Determination of ROS Levels
To measure intracellular ROS, 3 × 10⁵ cells were stained with 1 μM dihydrodorhadamine 123 (Sigma) in PBS for 10 min at 37°C. Subsequently, cells were pelleted and resuspended in PBS. Stimulation with 2 mM H₂O₂ was performed for 5 min at 37°C. After centrifugation, cells were resuspended in PBS/F and analyzed by flow cytometry.

Gene Knockdown via siRNA
The iPSCs (5 × 10³–10⁴) were harvested with accutase supplemented with 10 μM ROCK inhibitor Y-27632 as described above. Subsequently, the protocol of Ma et al. (2010) was followed. Briefly, cells were resuspended in 100 μl OPTIMEM medium (Invitrogen) supplemented with 400 nM SMARTpool siRNA (Dharmacon) and 5 μl lipofectamine 2000 (Invitrogen). The cell suspension was incubated for 5 min at 37°C, then diluted with 1.5 ml culture medium and transferred into six-well plates coated with feeder MEFs. Cells were harvested 72 hr post-transfection and stimulated with the indicated genotoxic agents.

Overexpression of GPX2 in Human Dermal Fibroblasts
A third-generation lentiviral expression system was used to overexpress YFP-tagged GPX2 and YFP, respectively, in human dermal fibroblasts. The lentiviral vectors pLV.YFP and pLV.YFP-GPX2 were kindly provided by O. Kranenburg (University Medical Center, Utrecht, the Netherlands) and co-transfected with pMDLg, pRSV-Rev, and pMD2.G vector constructs (Addgene) into HEK293 cells using jetPEI (Polyplus). Virus was harvested 2 days post-transfection. The fibroblasts were incubated with virus-containing medium for 24 hr, and infected cells were selected with 5 μg/ml puromycin 48 hr following infection.

SUPPLEMENTAL INFORMATION
Supplemental Information includes two figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.04.004.

AUTHOR CONTRIBUTIONS
B.D.S.L., O.R., and K.S.-O. conceived and designed the study, performed data analysis, and wrote the paper. B.D., S.L., D.G.H., A.K., P.G., V.S., K.H., M.F., and E.E. performed experiments and assembled the data.

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High Glutathione and Glutathione Peroxidase-2 Levels Mediate Cell-Type-Specific DNA Damage Protection in Human Induced Pluripotent Stem Cells

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Figure S1: Characterization of employed human iPS cells and fibroblasts.

(A) Microscopical images of human dermal fibroblasts and iPS cells that were generated by retroviral transduction with OCT4, SOX2, KLF4 and c-MYC. Scale bar = 200 µM.

(B) Alkaline phosphatase staining and expression of pluripotency markers NANOG, SOX2, TRA1-60, TRA1-81, and SSEA-4. Nuclei were visualized by blue DAPI staining. Exemplarily, L2 iPS cells (P25) and fibroblasts (P7) are shown.
Figure S2: Human iPS cells do not maintain a constitutively active form of BAX at the Golgi.

Human iPS (iPSC L1) cells were either left untreated (A) or stimulated with 100 nM staurosporine (B, C) for 2 h to undergo apoptosis. Immunohistochemistry reveals that active Bax (green staining) is hardly detectable in healthy untreated cells and not present at the Golgi (red staining). An apoptotic cell shown in (B) reveals typical BAX clustering and nuclear fragmentation (blue staining). A brighter illumination of the picture (C) shows in pre-apoptotic cells an even cytosolic distribution of BAX but no localization at the Golgi. Scale bar = 20 µM.

For indirect immunofluorescence cells were fixed in 4% formaldehyde and permeabilized in immunofluorescence buffer (PBS, 4% BSA, 0.05% saponin) for 1 h followed by over-night incubation at 4°C with conformation-specific anti-BAX-NT and the Golgi marker anti-GM130. Subsequently cells were washed and secondary antibodies were applied for 3 h at RT. Nuclei were stained for 5 min in PBS containing DAPI (10 ng/mL).
## Supplementary Tables

### Table S1: List of primary antibodies.

| Specificity                  | Origin | Provider                  | Cat #/Address               |
|-----------------------------|--------|---------------------------|----------------------------|
| Actin                       | Mouse  | Sigma                     | A2228                      |
| Anti-mouse Alexa Fluor 488  | Chicken| Invitrogen                | A-21200                    |
| Anti-mouse Alexa Fluor 568  | Rabbit | Invitrogen                | A-11061                    |
| Anti-mouse Alexa Fluor 647  | Chicken| Invitrogen                | A-21463                    |
| Anti-rabbit Alexa Fluor 594 | Chicken| Invitrogen                | A-21442                    |
| Anti-rat Alexa Fluor 488    | Chicken| Invitrogen                | A-21470                    |
| BAD                         | Mouse  | BD Transduction Laboratories | 610391                   |
| BAK                         | Rabbit | Millipore (Upstate)       | 06-536                     |
| BAX                         | Mouse  | Trevigen                  | 2281-MC                    |
| BAX-NT (active BAX)         | Rabbit | Millipore (Upstate)       | 06-499                     |
| BCL-2                       | Mouse  | Santa Cruz                | sc-7382                    |
| Bcl-X                       | Rabbit | BD                        | 610212                     |
| Bid                         | Goat   | R&D                       | AF860                      |
| BIM                         | Rabbit | Stressgen                 | ADI-AAP-330-E              |
| GM130                       | Rabbit | Abcam                     | EP892Y                     |
| GPX2                        | Rabbit | Anna P. Kipp              | DIFE, Postdam-Rehbrücke, Germany |
| GSTA2                       | Rabbit | John Hayes                | University of Dundee, UK   |
| MCL-1                       | Mouse  | BD Pharmingen             | 559027                     |
| Nanog                       | Rabbit | Abcam                     | ab21624                    |
| p53                         | Mouse  | Calbiochem                | OP43                       |
| Sox2                        | Rabbit | Abcam                     | ab59776                    |
| SSEA-4                      | Rat    | Chemicon                  | MAB4303                    |
| TRA1-60                     | Mouse  | Chemicon                  | MAB4360                    |
| TRA1-81                     | Mouse  | Chemicon                  | MAB4381                    |
Table S2: LORD-Q primers applied in DNA damage quantification experiments.

| Locus          | Base pairs | Efficiency | Primer Denotation | Primer Sequence                  |
|----------------|------------|------------|-------------------|----------------------------------|
| mtDNA (L)      | 3724       | 1.643      | CL5.F             | 5'-ATCGTAGCCTTCTCCACTTC-3'        |
|                |            |            | AS2.R             | 5'-TGGTTAGGCTGGTGTTAGGG-3'        |
| mtDNA (S)      | 50         | 1.989      | AS2.F             | 5'-GGCCACAGCCTTAAACACA-3'         |
|                |            |            | AS2.R             | 5'-TGGTTAGGCTGGTGTTAGGG-3'        |
| nDNA: GAPDH (L)| 3653       | 1.660      | GAPDH.F (1598)    | 5'-AGTCCCCAGAAACAGGAGGT-3'        |
|                |            |            | GAPDH.R (5250)    | 5'-GGCTGAGCTCCACTAACCAG-3'        |
| nDNA: GAPDH (S)| 45         | 1.995      | GAPDH.F (4076)    | 5'-GCCTCACTCCTTTTGCAAGC-3'        |
|                |            |            | GAPDH.R (4128)    | 5'-GTCTTCTGGGTGGCAGGT-3'          |
| nDNA: TP53 (L) | 3075       | 1.649      | TP53.F            | 5'-ATAAACCAGCAAATGGGAAAC-3'       |
|                |            |            | TP53.R (3075)     | 5'-GGGACGTGAAAGGTGTTAGAA-3'       |
| nDNA: TP53 (S) | 45         | 1.991      | TP53.F            | 5'-ATAAACCAGCAAATGGGAAAC-3'       |
|                |            |            | TP53.R (45)       | 5'-CGTCCTTTTGATGGCCTTT-3'         |
Table S3: Primers used in qRT-PCR experiments.

| Gene   | Forward Primer | Reverse Primer |
|--------|----------------|----------------|
| ACTB   | 5'-CATGTACGTTGCTATCCAGGC-3' | 5'-CTCCCTAATGTCAGCAGAT-3' |
| ALAS1  | 5'-CGCCGCTGCCCATTTTAT-3' | 5'-TCTTGGACCTTGCCCTTAG-3' |
| CAT    | 5'-TGCCTGCTGATCTCTCGGTTCC-3' | 5'-ATCCACGGATCTCCCTCAGAT-3' |
| GAPDH  | 5'-GGAGCGAGATCCCTCCAAAAT-3' | 5'-GGCTGTTGTCATACTTCTCATG-3' |
| GCLC   | 5'-GGAGGAAACCAAGCGCCAT-3' | 5'-CTTGGACGGCGTGGTAGATGT-3' |
| GCLM   | 5'-TGTCTTGGAATGCACTGTATCTC-3' | 5'-CCCAGTAAGGCTGTAAATGCT-3' |
| GDF3   | 5'-GCCATCAAAGAAAGGGAACA-3' | 5'-TCTGGCACAGGTGTCTTCAG-3' |
| GPX1   | 5'-AGAGCCGGGGGACCGAGAGA-3' | 5'-ATTTGCCAGCCTTCTGTTA-3' |
| GPX2   | 5'-AGAGCCGGGGGACCGAGAGA-3' | 5'-ATTTGCCAGCCTTCTGTTA-3' |
| GPX3   | 5'-AGAGCCGGGGGACCGAGAGA-3' | 5'-ATTTGCCAGCCTTCTGTTA-3' |
| GPX4   | 5'-AGAGCCGGGGGACCGAGAGA-3' | 5'-ATTTGCCAGCCTTCTGTTA-3' |
| GSR    | 5'-TTCCAGAACTACCCAGACTGCAAAG-3' | 5'-GGCTGTTGTCATACTTCTCAT-3' |
| GSTA1  | 5'-CTGGGCAAGACCGAAGTAACTC-3' | 5'-AGCTCCTGCTTCTGATACAGG-3' |
| GSTA2  | 5'-CTGGGCAAGACCGAAGTAACTC-3' | 5'-AGCTCCTGCTTCTGATACAGG-3' |
| GSTA4  | 5'-AGAGCCGGGGGACCGAGAGA-3' | 5'-ATTTGCCAGCCTTCTGTTA-3' |
| GSTM1  | 5'-CTGGGCAAGACCGAAGTAACTC-3' | 5'-AGCTCCTGCTTCTGATACAGG-3' |
| GSTM2  | 5'-CTGGGCAAGACCGAAGTAACTC-3' | 5'-AGCTCCTGCTTCTGATACAGG-3' |
| GSTM3  | 5'-CTGGGCAAGACCGAAGTAACTC-3' | 5'-AGCTCCTGCTTCTGATACAGG-3' |
| GSTM4  | 5'-CTGGGCAAGACCGAAGTAACTC-3' | 5'-AGCTCCTGCTTCTGATACAGG-3' |
| GSTM5  | 5'-CTGGGCAAGACCGAAGTAACTC-3' | 5'-AGCTCCTGCTTCTGATACAGG-3' |
| GSTO1  | 5'-CTGGGCAAGACCGAAGTAACTC-3' | 5'-AGCTCCTGCTTCTGATACAGG-3' |
GSTO2.F  5'-TGCCCCCTATTCTCACAGGACC-3'
GSTO2.R  5'-TCCAGGTACTCACAAGCAATAAC-3'
GSTT1.F  5'-TGCCGCGCTGTTTACATCTT-3'
GSTT1.R  5'-GTGCTGACCTTTAATCAGATCCA-3'
GSTT2.F  5'-TGGCATCCCCCTTAGAGCTG-3'
GSTT2.R  5'-CTTGAGCGTCGGCAGTTTC-3'
GSTZ1.F  5'-GCCCAGAACGCCATCACTT-3'
GSTZ1.R  5'-CTACACAGTATATGCCCGCTG-3'
MGST1.F  5'-ATGACAGAGTGAGATGTACGC-3'
MGST1.R  5'-TACAGGAGGCCAATTCCAAGA-3'
MGST2.F  5'-TCGGCCTGTCAGCAAAGTTAT-3'
MGST2.R  5'-CTTGAGCGTCGGCAGTTTC-3'
MGST3.F  5'-GGCCCACCTAGCCATCAATG-3'
MGST3.R  5'-CGCTGAATGCAGTTGAAGATGT-3'
NANOG.F  5'-ACTCTCCAACATCCTGAACCTC-3'
NANOG.R  5'-GCCTTCTGCGTCACACCA-3'
PRDX1.F  5'-CATTCCTTTGGTATCAGACCCG-3'
PRDX1.R  5'-CCCTGAACGAGATGCCTTCAT-3'
PRDX2.F  5'-GAGACTACGGTGTGCTGTTAGA-3'
PRDX2.R  5'-GGAAATCTTCGCTTTGCTTAGGT-3'
PRDX3.F  5'-AGAGGAGTGCCACTTCTACG-3'
PRDX3.R  5'-GGTGGGCCAAAGGATGAAGAG-3'
PRDX4.F  5'-AGAGGAGTGCCACTTCTACG-3'
PRDX4.R  5'-GGAAATCTTCGCTTTGCTTAGGT-3'
PRDX6.F  5'-GTTGCCACCCCAGTTGATTG-3'
PRDX6.R  5'-TGAAGACTCTCCTTTCCGGAAAGT-3'
SOD1.F  5'-GGTGGGCAAGAGATGAAGAG-3'
SOD1.R  5'-CCACAAGACCAAACGACTTCC-3'
SOD2.F  5'-GCTCCGCTTGGGGTGATCTG-3'
SOD2.R  5'-GCGTTGATGTGAGGTTCCAG-3'
SOD3.F  5'-ATGCTGGCGCTACTGTGTTC-3'
SOD3.R  5'-CTCCGCCGAGTCAGAGTTG-3'
TMX1.F  5'-AGTATGTCAGCAGTCTTTCAAGC-3'
TMX1.R  5'-CACACTGGCAATCCAAAGGTCT-3'
TXN.F  5'-GTGAAGCAGATCGAGAGCAAG-3'
TXN.R  5'-CGTGGCTGAGAAGTCAACTCA-3'
TXNIP.F  5'-GGTCTTTAAGCACCCTGAAAAGG-3'
TXNIP.R  5'-ACACGAGTAACCTCACAACCT-3'