Mechanisms of Genome Surveillance in Pluripotency

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TABLE OF CONTENT

ABBREVIATIONS

BSO	L-buthionine-sulfoximine
C_P	Crossing point
ddC	2′-3′-dideoxycytidine
DDR	DNA damage response
DMF	Dimethyl fumarate
DNA	Deoxyribonucleic acid
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FGF	Fibroblast growth factor
Fig.	Figure
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSH-OEt	Glutathione ethylester
GSSG	Oxidized glutathione (disulfide)
GST	Glutathione transferase
h	Hours
HDF	Human dermal fibroblast
iPSC	Induced pluripotent stem cell
kb	kilo bases
LORD-Q	Long-run RT-PCR based DNA damage quantification
M	Molar
MGST	Microsomal glutathione transferase
min	Minutes
mRNA	messenger ribonucleic acid
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
PCR	Polymerase chain reaction
PRX	Peroxiredoxin
PSC	Pluripotent stem cell
qPCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
ssDNA	Single stranded Deoxyribonucleic acid
UV	Ultraviolet light

LIST OF PUBLICATIONS

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ZUSAMMENFASSUNG

Krebserkrankungen stellen weltweit eine der häufigsten Todesursachen dar, wobei die Zahl der Krebserkrankten stetig ansteigt. Ein Drittel aller Krebstode lässt sich auf die fünf Risikofaktoren Tabak- und Alkoholkonsum, mangelnde Bewegung, zu geringer Konsum von Gemüse und Obst sowie einen zu hohen *body mass index* zurückführen. Jeder dieser Faktoren erhöht nachweislich die intrazellulären Konzentrationen reaktiver Sauerstoffspezies (ROS). Im Überschuss vorhanden, können diese Moleküle beträchtlichen Schaden in lebenden Zellen anrichten, indem sie Lipide, Proteine oder DNS-Basen oxidieren. Wird das so geschädigte Genom nicht vollständig und korrekt repariert, können Mutationen entstehen, die zur Tumorgenese oder zum Fortschreiten der Krankheit beitragen können.

Von besonderer Bedeutung ist in diesem Zusammenhang die Sicherung der genetischen Integrität für pluripotente Zellen. Diese Stammzellen sind in der Lage sich unlimitiert zu teilen und in Zellen aller drei Keimblätter auszudifferenzieren. Diese Fähigkeit birgt jedoch auch das Risiko, dass gegebenenfalls auftretende Entartungen vergleichsweise schnell über den ganzen Organismus zu streuen vermögen.

Durch ihre Pluripotenz verfügen Stammzellen über ein enormes therapeutisches Potenzial. Bevor jedoch ein klinischer Einsatz in Erwägung gezogen werden kann, ist es unerlässlich etliche Sicherheitsfragen bezüglich der Gewährleistung der genetischen Stabilität dieser Zellen zu beantworten und die zugrundeliegenden, noch unzureichend erforschten Mechanismen aufzuklären.

Aus diesem Grunde befasst sich diese Studie mit der Fragestellung welche Mechanismen induzierten pluripotenten Stammzellen (iPS) zur Verfügung stehen, um ihre genomische Integrität zu wahren. Früheren Studien zufolge verfügen Stammzellen über eine erhöhte DNS-Reparaturkapazität sowie eine geringe Apoptose-Hemmschwelle. Letzteres wurde auf pro-apoptotisches, prä-aktiviertes BAX zurückgeführt, welches zum Golgi-Apparat transloziert werde. Diese Arbeit bestätigt die schnelle Apoptoseinduktion der iPS-Zellen, widerlegt jedoch die bisherige Begründung und weist stattdessen als Auslöser eine geringe Apoptose-Hemmschwelle aufgrund geringer Konzentrationen anti-apoptotischer Proteine nach.

Außerdem wird erstmals ein zusätzlicher Mechanismus zum Schutz der genetischen Information in iPS-Zellen identifiziert: Mittels der neuen RT-PCR basierten Methode zur DNA-Schadensquantifizierung LORD-Q wird die DNS-Schadensanfälligkeit von humanen iPS-Zellen untersucht und gezeigt, dass mit dem Status der Pluripotenz eine reduzierte Anfälligkeit gegenüber genotoxischen Stimulanzien einhergeht. Der zu Grunde liegende Mechanismus wird aufgeklärt und gezeigt, dass erhöhte Level des antioxidativen Moleküls Glutathion und der Glutathionperoxidase 2 hauptverantwortlich für die zelltypspezifische ROS-Abwehr in humanen iPS-Zellen sind.

Des Weiteren erweitert diese Studie die LORD-Q Methode um die Möglichkeit einer simultanen Messung von DNA-Schäden und der mitochondriellen Kopienzahl.
Diese Weiterentwicklung der Methode wird genutzt, um den Einfluss der Kopienzahl des mitochondriellen Genoms auf dessen Anfälligkeit gegenüber genotoxischen Schäden zu untersuchen. Es wird gezeigt, dass geschädigte mitochondrielle DNS abgebaut wird, worauf eine hyperkompensatorische DNS-Replikation folgt. Eine größere Menge mitochondrieller DNS pro Zelle, scheint diese zwar teilweise vor UV-Strahlung zu schützen, jedoch nicht mit einem generell höheren Schutz der DNS verknüpft zu sein.
Zudem wird die erweiterte LORD-Q Methode an Gewebeproben etabliert und ihre Funktionalität demonstriert, was ihre Anwendbarkeit auf viele weitere Forschungsbereiche ausweitet.
Zusammengefasst entschlüsselt die vorliegende Studie grundlegende Mechanismen zur Erhaltung der genomischen Stabilität und etabliert durch die Weiterentwicklung der LORD-Q Methode ein neues und vielseitig einsetzbares Werkzeug für zukünftige Forschungsprojekte.

ABSTRACT

Cancer is a leading cause of death worldwide and global numbers of cases are growing rapidly. One third of all cancer deaths can be attributed to exposure to the five leading risk factors tobacco and alcohol consumption, high body mass index, lack of physical activity, as well as low vegetable and fruit intake, which all have been proven to increase concentrations of intracellular reactive oxygen species (ROS). If produced in excess, these highly reactive molecules cause severe damage by oxidizing lipids, proteins and ribonucleic acids, thereby impairing the genomic integrity of the cell. If not repaired correctly, resulting DNA lesions lead to mutations, which in turn can promote tumorigenesis and cancer progression.

Maintaining genomic integrity is especially crucial to pluripotent stem cells (PSCs), since they are able to proliferate unlimitedly and to differentiate into cells of all three germ layers. This potential yet also bears the risk of spreading possibly malignant cells throughout the body. However, due to their pluripotency, PSCs also hold great therapeutic potential. To assess the possibilities of clinical use, however, safety issues concerning the maintenance of genomic integrity in PSCs have to be addressed and underlying mechanisms are to be explored thoroughly.

This study addresses the question which mechanisms induced PSCs are harnessing to secure their genomic information. Previous work has identified enhanced DNA repair capacities and low apoptosis thresholds in PSCs. This study shows that induced pluripotent stem cells (iPSCs) rapidly undergo apoptosis upon genotoxic stimulation. Contrarily to previous reports, this readiness to undergo apoptosis is attributed to a low apoptosis-threshold due to low expression of anti-apoptotic proteins (mitochondrial priming) rather than pre-activated pro-apoptotic BAX.

Further a novel, additional mechanism of securing genomic information is identified: The novel long-run RT-PCR based DNA damage quantification (LORD-Q) method is utilized to measure DNA damage and to reveal a decreased DNA damage susceptibility of human iPSCs in comparison to parental fibroblasts as well as other cell lines. Human iPSCs are demonstrated to hold a cell-type specific enhancement of the antioxidative defense system, which significantly reduces ROS-induced DNA damage. The antioxidative molecule glutathione (GSH) and the GSH-dependent

glutathione peroxidase 2 (GPX2) are identified as two key players in this mechanism of DNA protection.

Furthermore this study extends the applicability of the LORD-Q method by providing the possibility of simultaneous measurement of the mitochondrial copy number and DNA damage.

This new implementation is made use of to examine the influence of the mitochondrial DNA (mtDNA) copy number on DNA damage susceptibility. Damaged mtDNA is shown to be degraded upon genotoxic insults, followed by a phase of hyper-compensatory DNA replication. Higher amounts of mitochondrial DNA however are revealed to only provide protection against genotoxic UV radiation, but not in a general way.

Lastly, the extended LORD-Q method is demonstrated to be suitable for analysis of tissue samples, thereby providing a tool for a broad range of research.

In summary this study reveals some of the fundamental mechanisms of genome surveillance and by extending the applicability of the LORD-Q assay provides a novel, powerful multiplex tool to further work in a broad field of research.

INTRODUCTION

Cancer is the second most common cause of death in Germany and accountable for over 8 million deaths per year worldwide (Stewart et al., 2014). While survival rates of cancer patients in Germany are increasing, prognoses are rather poor in low-income countries and global cases of cancer are estimated to increase by 70% within the next two decades (Allemani et al., 2015).

Approximately one third of global cancer deaths are associated to the five leading risk factors tobacco and alcohol consumption, high body mass index, lack of physical activity, as well as low vegetable and fruit intake (Stewart et al., 2014). All of these factors have been shown to increase intracellular concentrations of reactive oxygen species (ROS) (Brinkmann and Brixius, 2013; Hernández et al., 2016; de Kok et al., 2010; Rani et al., 2016; Vu et al., 2016).

Oxidative Stress

There are two groups of ROS: The superoxide anion (O_2^-), the hydroxyl radical (HO•), peroxy radicals (ROO•), and alkoxy radicals (RO•) of lipids are radical oxygen species. Singlet oxygen ($^1O_2$), ozone (O_3), the hypochlorite anion (OCl^-), peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2) and other peroxides are non-radical oxygen species. Both are generated by partial reduction of O_2, decay of water induced by ionizing radiation, or follow-up reactions of other ROS.

Increased ROS levels are a common accompaniment of mutations within genes of the respiratory chain's proteins, cellular stress or environmental impact (Fato et al., 2008). Also many genotoxic drugs, such as bleomycin harm DNA indirectly by mediating ROS production (Wallach-Dayan et al., 2006). Besides causing strand breaks and catalyzing wavelength-dependent photochemical reactions yielding cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoadducts (Ikehata and Ono, 2011), UV radiation also activates cellular oxygen to singlet oxygen via riboflavin and causes the formation of hydrogen peroxide in the presence of tryptophan (McCormick et al., 1976). Also other redox-cofactors like NADH or porphyrin were shown to generate ROS by transferring electrons when irradiated with UV light (Peak et al., 1984; Walrant and Santus, 1974).

Under non-pathological conditions ROS are produced as byproducts of metabolic processes such as oxidative phosphorylation or in response to cytokines (Ray et al., 2012). ROS fulfill distinctive roles in metabolic and immunological pathways and serve as signaling agents of the cellular anti stress response (Lagouge and Larsson, 2013; Ray et al., 2012). If produced in excess due to pathological conditions, such as deregulation of metabolic processes, however, they can cause severe damage (Di Meo et al., 2016). In this case, termed oxidative stress, high levels of ROS are present in the cell and oxidize proteins, lipids and DNA bases. Therefore, cells possess various antioxidant systems to keep ROS concentrations low.

Antioxidants

Oxidative damage has been shown to cause premature ageing, deregulation of the immune system, impaired cell proliferation and regenerative potential, neurological diseases, various types of cancer and contributes to a large array of diseases in newborns (Denu and Hematti, 2016; Lephart, 2016; Ozsurekci and Aykac, 2016; Salim, 2016). ROS-induced mitochondrial DNA (mtDNA) damage has also been reported to contribute to the development of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (Bender et al., 2006; Lovell and Markesbery, 2007).

To prevent oxidative damage, cells hold various antioxidant systems to capture and neutralize ROS. *Inter alia* these systems are composed of chaperones, detoxifying enzymes and ROS-scavenging small molecule antioxidants, such as ascorbate, ubiquinone, α-tocopherol and glutathione (GSH).

Glutathione is a tripeptide (γ-glutamylcysteinylglycine) exhibiting a free thiol group, which is susceptible to oxidation and successively dimerizes with a second GSH molecule by forming a disulfide bond. GSH is also used by GSH-dependent enzymes to enzymatically reduce ROS, unintended disulfide bonds or to detoxify xenobiotics.

Besides catalase and superoxide dismutases, which detoxify hydrogen peroxide and superoxide radical anions, the three types of GSH-dependent enzymes are major contributors to the cellular antioxidative defense:

First, GSH peroxidases (GPXs) reduce peroxides by oxidizing two molecules of GSH, forming the GSSG dimer (Morris et al., 2014). The majority of human GPXs are selenoenzymes with a highly reactive selenocysteine in the catalytic domain (Dayer

et al., 2008). This Se-Cys residue is oxidized during the reaction and is subsequently regenerated utilizing GSH (Flohe et al., 1973).

Peroxiredoxins (PRXs) are also an important part of the antioxidant system relying on a similar mechanism. However, PRXs are not classified as GSH-dependent enzymes, though their oxidized form can be reduced by GSH as well (Dayer et al., 2008).

GPX- or PRX-dependent detoxification of ROS results in a shift in the cellular GSH/GSSG ratio, which can be measured and serves as a scale for the oxidative state of the cell (Fahey et al., 1975; Zitka et al., 2012).

GSSH produced by detoxification of ROS can be recycled by GSH reductase. This enzyme keeps the GSH/GSSG ratio at around 50:1 in cultured cells by reducing GSSG to two molecules of GSH, thereby oxidizing $NADPH/H^+$ (Dringen and Hamprecht, 1996). GSH reductase is the sole member of the second type of GSH-dependent enzymes.

The third type is the super-family of GSH transferases (GSTs) consisting of multiple families, located in different cellular compartments (Singhal et al., 2015). Most GSTs are soluble proteins. Some, such as the microsomal GST (MGST), are membrane associated, however, they share the function and substrate specificity of their cytosolic counterparts (Board and Menon, 2013): GSTs utilize GSH to form conjugates with a broad range of xenobiotics or ROS. Subsequently the conjugates are excreted (Pacifici et al., 1987). Therefore, GST-dependent conjugation of GSH, in contrast to ROS detoxification by GPXs, leads to a depletion of cellular GSH content.

This depletion of intracellular GSH concentration was shown to raise cellular levels of ROS, which under physiological conditions are tightly controlled to prevent oxidative damage (Nelson et al., 1999). Intracellular ROS are a major source of oxidative DNA lesions such as abasic sites and single strand breaks. More than 20 ROS-mediated types of DNA damage have been reported to date (Cooke et al., 2003).

Oxidative DNA Damage

DNA damage can be caused by various stimuli, such as alkylating, intercalating or crosslinking chemicals. Deamination of DNA bases, depurination or depyrimidation can occur spontaneously or induced by UV or ionizing radiation. Double strand breaks can be caused by a collapse of the replication fork due to excessive starts of replication or following irradiation (Garinis et al., 2005). However, ROS pertain to the most common genotoxic agents present in the cell, causing a wide range of oxidative lesions.

Hydroxyl radicals can add to double bonds of DNA bases, thereby introducing new hydroxyl or carbonyl groups and altering base pairing. One of the most common and well studied adducts is 8-hydroxydeoxyguanosine (8OHdG), also known as 8-oxo-7,8-dihydroguanine (8-oxoguanine) nucleotide. It is formed in the DNA as well as in cellular nucleotide pools. Free 8OHdG is integrated into newly synthesized nucleic acid and pairs with C equally well than with A, which leads to G-T base exchange (Shibutani et al., 1991).

Nearly 10^5 DNA modifications are removed correctly per day and cell under physiological conditions (Ciccia and Elledge, 2010). However, if repair mechanisms fail to correctly restore all damaged sites of the genome, insufficient or incorrect repair can cause mutations. Accumulation of such mutations promotes tumorigenesis and cancer development (Cha and Yim, 2013; Hwang et al., 2009; Toussaint et al., 2000). Hence, to maintain genome stability, DNA damage can lead to apoptosis induction, cell cycle arrest or cellular senescence, which prevent mutations from spreading to daughter cells (Linke et al., 1997b; Lowe et al., 1993).

DNA Damage Response & Apoptosis

Alterations in the genome can lead to tumorigenesis or inheritable cancer susceptibility (Huang, 2012). Therefore, eukaryotic cells possess various mechanisms to prevent malignant mutations. The DNA damage response (DDR) pathway is responsible for sensing DNA damage and initiating repair, inducing cell cycle arrest or apoptosis.

The DDR comprises several proteins, sensing DNA damage or genotoxic substances. Most DDR proteins induce fast responses via post-transcriptional modifications, such as phosphorylation (Ciccia and Elledge, 2010). Others, such as ATM and CHK2, trigger transcriptional responses via activation of p53, which mediates cell cycle arrest, senescence or apoptosis (Zhou and Elledge, 2000). This activation is induced in a cyclic manner and regulated by a negative feedback loop, resulting in a repeated checkpoint for genome integrity (Batchelor et al., 2009). P53 regulates the pro-apoptotic Bcl-2 proteins PUMA and BAX, triggering the intrinsic apoptosis pathway in case of insufficient DNA repair by permeabilization of the outer mitochondrial membrane (Riley et al., 2008). Thereby cytochrome c is released, which subsequently forms the apoptosome with Apaf-1, which binds to caspase 9 via a caspase recognition domain and activates the protease (Yuan and Akey, 2013). Caspase 9, an initiator caspase, proteolytically activates effector caspases, which are responsible for actual cell death by inducing the classic hallmarks of apoptosis, such as cell shrinkage, loss of membrane asymmetry, membrane blebbing, cytosolic acidification and DNA fragmentation (Chang and Yang, 2000).

The majority of human cancers exhibit mutations within the tumor suppressor gene TP53, impairing efficiency of apoptosis induction (Essmann and Schulze-Osthoff, 2012). Mutations in the apoptosis pathways can lead to higher tolerance of DNA damage and consecutive increase of mutation accumulation. Therefore, efficient apoptosis induction is crucial to ensure genomic stability.

Stem Cells

Genome stability is especially crucial to cells with high proliferative potential, due to an increased risk of mutation inheritance. Therefore, stem cells undergo rapid apoptosis in case of DNA damage (TeSlaa et al., 2016).

Human embryonic stem cells (ESCs) are able to proliferate *de facto* unlimitedly and can be cultivated in a pluripotent state. Pluripotent stem cells can be differentiated into cells of every germ layer. Therefore, human ESCs represent a great hope in regenerative medicine for curing various diseases, such as leukemia or cardiac infarction (Koudstaal et al., 2013). However, human ESCs cannot be obtained without harming human embryos. Hence, research on human ESCs as well as their therapeutic use is discussed controversially due to ethical issues and restricted by law in many countries such as Germany and France.

Pluripotent stem cells can also be obtained by somatic cell nuclear transfer (SCNT) by enucleation of oocytes and consecutive fusion with somatic cells (Gurdon, 1962; Tachibana et al., 2013). Since this method could be used for human cloning, ethical discussions concerning its use are highly controversial (Ethics Committee of the American Society for Reproductive Medicine and Ethics Committee of the American Society for Reproductive Medicine, 2016).

In the past decade, however, regenerative medicine has taken a huge step, following the first reprogramming of differentiated cells into a pluripotent, stem cell-like state (Aoi, 2016). These induced pluripotent stem cells (iPSCs) were first generated in 2006 from mouse embryonic fibroblasts (MEFs) using retroviral vectors to ectopically express the four transcription factors Oct4, Sox2, c-Myc and Klf-4 (Takahashi and Yamanaka, 2006). The resulting cells have been reported to exhibit properties similar to ESCs in terms of morphology, proliferation rate, gene expression pattern, expression of surface antigens, teratoma formation and the ability to produce adult chimeras (Okita et al., 2010; Takahashi et al., 2007). To date these four 'Yamanaka factors' have successfully been used to reprogram human adult fibroblasts, as well as other cell types to iPSCs (Liu et al., 2010; Seki et al., 2010; Takahashi et al., 2007; Zhou et al., 2012).

While non-autologous cells used in stem cell therapy cause immune responses in patients, iPSCs are isogenic and therefore rarely show immune rejection (Araki et al., 2013; Okita et al., 2011). Immunogenicity has been observed in undifferentiated

iPSCs, forming teratomas, which however is most likely to be based on immune responses against tumorigenic cells (Kaneko and Yamanaka, 2013). Nevertheless, this topic is discussed controversially.

Since iPSCs are isogenic to their progenitor cells, they can also be employed for personalized drug screening (Scott et al., 2013) or *in vitro* models of genetically predisposed diseases in tissues which cannot be sampled, such as e.g. brain samples in cases of Alzheimer's disease (Carlessi et al., 2013).

Yet, an important question for future clinical use in respect of transplantation might be whether iPSCs can secure their genomic stability and prevent tumorigenesis (Sarig and Rotter, 2011).

As retroviral vectors used for reprogramming insert into the genome at random places, they might insert into important genes and cause non-silent mutations. Therefore, various non-DNA-integrating protocols, such as adenoviral, transposon/transposase, synthetic RNA and protein delivery systems were established to prevent mutations (Kim et al., 2009; Stadtfeld et al., 2008; Warren et al., 2010; Woltjen et al., 2009).

Also reactivation of transgenic c-Myc was shown to cause tumor development (Okita et al., 2007). Therefore, alternate reprogramming protocols were established using only three or less factors (Giorgetti et al., 2009; Nakagawa et al., 2008).

Another major safety concern regards genomic instability due to susceptibility to mutations (Ronen and Benvenisty, 2012), which are acquired by DNA repair failure following DNA damage. Evidence was provided of human iPSCs as well as human ESCs lacking G1/S phase cell cycle arrest which could prevent them from proliferation while their DNA is damaged (Momčilović et al., 2011). Conversely, both cell types are more likely to undergo apoptosis than isogenic fibroblasts when treated with DNA-damaging agents, such as ionizing radiation, and exhibit elevated DNA repair capacities compared to differentiated cells (Luo et al., 2012; Momčilović et al., 2009). So far, however, little is known about DNA damage prevention in human iPSCs.

Mitochondrial Copy Number

Most cell types contain large numbers of copies of the mitochondrial genome (Reznik et al., 2016). This 16 kilobase mitochondrial DNA is more susceptible to mutations than genomic DNA, due to high concentrations of ROS in mitochondria. Though the mitochondrial DNA polymerase γ has been shown to exhibit an exonuclease activity for proof reading, it is still susceptible to misincorporation of dNTP analogs (Kaguni, 2004). Also mtDNA was shown to be rapidly degraded following exposure to DNA intercalating agents such as ethidium bromide (Desjardins et al., 1985; Swerdlow et al., 2006).

In addition, the DNA repair capacity in mitochondria is rather poor in comparison to the nucleus (Bohr and Anson, 1999; Sawyer and Van Houten, 1999).

Exposure to radiation and high levels of oxidative stress leads to elevated numbers of mtDNA copies in human cells (Lee et al., 2000; Zhang et al., 2009), suggesting a mechanism of protection through abundance of information.

Interestingly, stem cells possess only a low mitochondrial DNA copy number (St. John, 2016), though mitochondrial integrity is essential to maintain pluripotency (Facucho-Oliveira et al., 2007). This suggests a specific DNA defense mechanism in stem cells.

However, stem cells are not the only cell type in need of mtDNA protection. Changes in the mtDNA copy number were associated with cancer development (van Gisbergen et al., 2015). Also failure to maintain mitochondrial genome integrity has been linked to the process of ageing, impaired fertility, and childhood autism (Chen et al., 2015; Finkel and Holbrook, 2000; Hendriks et al., 2015; Otten and Smeets, 2015).

The LORD-Q Method

Increasing interest in the field of DNA damage and mitochondrial DNA content rises the necessity of new methods, suitable for high-throughput analysis of both traits. Current qPCR protocols to measure the mtDNA copy number are fast and capable of analyzing samples in large quantities. However, most methods to determine DNA damage rates either require large amounts of DNA or labor- and cost-intensive normalization.

Recently the long-run real-time PCR-based DNA damage quantification (LORD-Q) method was described to resolve these drawbacks (Lehle et al., 2014). The authors improved the detection range of the previous semi-long run PCR method (Rothfuss et al., 2010) and extended the applicability to nuclear DNA, resulting in a highly sensitive method, capable of high-throughput measurement. The LORD-Q method uses internal reference samples and requires no measurement of standards for normalization purposes. DNA damage levels can be determined in distinct gene loci of >3kb in mtDNA and/or nuclear DNA, allowing analysis of locus-dependent damage susceptibility.

AIMS OF RESEARCH

DNA damage caused by *e.g.* ROS can lead to mutations, which are responsible for tumorigenesis and cancer development (Nyström et al., 2012). Therefore, maintaining genomic integrity is especially crucial to pluripotent cells due to their high proliferation rates and potential to differentiate into cells of every germ layer and thereby spreading oncogenic mutations throughout the whole organism.

Consequently, it was the aim of this study to investigate the susceptibility of human iPSCs to DNA damage and to reveal the underlying mechanisms of genome surveillance in human pluripotent stem cells.

Human iPSCs were reported to secure their genomic stability by undergoing rapid apoptosis following DNA damage due to pre-activated BAX at the Golgi (Dumitru et al., 2012). However, the H1 human ESC line was shown to lack pre-activated BAX but to show sensitivity to apoptosis nonetheless (Liu et al., 2014). Therefore, it was aimed to challenge this hypothesis and identify further mechanisms explaining the apoptosis-sensitivity of PSCs.

Despite undergoing rapid apoptosis, human iPSCs were reported to suffer less initial DNA damage than isogenic fibroblasts after exposition to ROS or ROS-inducing stimuli like bleomycin or UV radiation (Lehle et al., 2014; Luo et al., 2012). Therefore, it seemed likely that pluripotent cells possess increased antioxidative defense. Hence, the question arose which defense mechanisms might be responsible for the improved protection of human iPSC genomes. This study aimed to elucidate the role of antioxidants in the cell-type specific protection of human iPSC genomes.

Also DNA damage rates as well as levels of antioxidants in human iPSCs were to be compared to those of various other cell types to test the hypothesis that human iPSCs possess a cell-type specific defense against DNA damaging ROS.

Further it was to be investigated whether this protection was solely limited to the pluripotent state of reprogrammed cells or if redifferentiated cells suffer lower rates of DNA damage as well.

With age ROS levels throughout the body rise, causing mtDNA integrity to decline (Balaban et al., 2005). Resulting oxidative stress can further enhance ROS generation, creating a vicious circle (Indo et al., 2007). With growing life expectancy this causes a major health threat in society.
However, elevated ROS levels also induce an increase in replication of mitochondrial DNA (Lee et al., 2000), suggesting a mechanism of protection-by-abundance.
The second part of this study aimed to test this hypothesis. Therefore, mtDNA copy number and mtDNA damage rates in various cell lines were to be measured and compared.

The LORD-Q method provided an ideal tool to conduct this study, due to its capability of fast analysis of small samples in a microtiter format. However, the protocol was limited to analyze DNA damage. Therefore, the applicability of the LORD-Q method had to be widened to a multiplex assay, also assessing the mtDNA copy number.
Also, the LORD-Q method had only been shown to be applicable to the analysis of *in vitro* samples so far. Therefore, finally a proof-of-principle study on tissue samples was to be conducted.

RESULTS & DISCUSSION

Many of the known main causes of cancer such as the five main risk factors tobacco smoke, alcohol, overweight and lack of exercise or a healthy diet as well as others, such as UV or gamma radiation, are known to result in increased oxidative stress (Brinkmann and Brixius, 2013; Hernández et al., 2016; Kam and Banati, 2013; de Kok et al., 2010; McCormick et al., 1976; Rani et al., 2016; Rinnerthaler et al., 2015; Vu et al., 2016).

Oxidative stress represents an imbalance of ROS concentrations and the capacity of the cellular antioxidant systems to detoxify these potentially harmful molecules. Excess ROS oxidize proteins, membrane lipids and cause DNA lesions, which can lead to mutations, responsible for tumorigenesis and cancer development (Nyström et al., 2012). Therefore, maintaining genomic integrity is of upmost importance to all cells.

Mitochondrial Priming in Human iPSCs

It is especially crucial to pluripotent cells to secure genomic stability due to their high proliferation rates. In combination with PSCs' potential to differentiate into cells of each of the three germ layers, they could spread potentially oncogenic mutations throughout the body. Also every mutation could impair their pluripotency and tissue renewal capacity (Facucho-Oliveira et al., 2007; Tapia and Schöler, 2010).

Continuously dividing cells, such as cancer cells, suffer from replication stress, which causes DNA damage (Jones et al., 2013). Since human iPSCs also exhibit high proliferation rates, they are likely to be susceptible to replication stress as well. However, human iPSCs were shown to exhibit higher DNA repair capacities, compared to differentiated cells (Luo et al., 2012).

Also PSCs secure their genomic stability by undergoing rapid apoptosis following DNA damage (Liu et al., 2013). Human PSCs were reported to accomplish this by being primed for cell death due to pre-activated BAX at the Golgi (Dumitru et al., 2012). However, certain pluripotent cell lines, such as the H1 human ESC line, were shown to lack pre-activated BAX but not sensitivity to apoptosis (Liu et al., 2014).

In the current study immunohistochemistry revealed BAX not to be located at the Golgi but equally distributed in the cytosol in non-apoptotic human induced PSCs (Fig. S2; Dannenmann et al., 2015).

While two independent lines of human iPSCs showed resistance to stimuli of the extrinsic apoptosis pathway (Fig. 1; Dannenmann et al., 2015) due to low expression levels of death receptors (Fig. 2; Dannenmann et al., 2015), both iPSC lines L1 and L2 exhibited low apoptosis thresholds in comparison to isogenic fibroblasts for stimuli activating the mitochondrial apoptosis pathway. This increased sensitivity was also observed following treatment with agents inducing ER stress or Golgi disassembly (Fig. 1; Dannenmann et al., 2015), whereas human ESCs only were reported to exhibit low apoptosis thresholds confined to DNA-damaging stimuli (Dumitru et al., 2012). This suggested an altered balance between pro- and antiapoptotic BCL-2 proteins in human iPSCs, which is termed mitochondrial priming.

Under basal conditions, iPSCs showed an accumulation of the tumor suppressor p53, which activates transcription of multiple pro-apoptotic BCL-2 proteins. Also expression levels of several p53 target genes such as BAK, BIM, and NOXA exhibited strong upregulation, indicating high mitochondrial priming in human iPSCs compared to differentiated cells (Fig. 2; Dannenmann et al., 2015).

Low DNA Damage Rates in Human iPSCs

Presumably several mechanisms secure an efficient maintenance of DNA integrity in pluripotent stem cells. It was reported that e.g. in comparison to differentiated cells, ESCs display increased expression of certain DNA repair enzymes, involved in DNA double-strand break repair (Saretzki et al., 2008). Also a previous study in human ESCs reported low levels of oxidative DNA lesions, such as 8OHdG (Maynard et al., 2008). This suggested an increased expression of the primary base excision repair enzyme 8OHdG glycosylase, which is required for removing this DNA lesion. However, the authors did not find elevated activities of this enzyme. Therefore, it seemed likely that rather oxidative DNA lesions might be prevented in pluripotent stem cells.

To test this hypothesis and to further elucidate genome surveillance in human iPSCs, the highly sensitive LORD-Q (long-run real-time PCR-based DNA damage

quantification) method was harnessed, which detects gene locus-specific DNA lesions of both the nuclear and mitochondrial genome.
Due to enhanced mitochondrial priming in human iPSCs, examination of DNA damage following long exposition to genotoxic substances proved difficult. Also, the increased DNA repair capacity could interfere with the measurement of DNA damage acquisition. Therefore, the current study focused on DNA damage induced by hydrogen peroxide and UVC radiation. Both are well known to induce oxidative stress in cells within seconds upon exposure.
Initial damage rates, representing the state of the DNA immediately after stimulation were examined. Effects of DNA repair as well as DNA fragmentation due to apoptosis induction therefore could be neglected.

UV irradiation caused similar rates of DNA lesions in mitochondrial and nuclear DNA. Contrarily hydrogen peroxide treatment mainly caused lesions within mitochondrial DNA. This finding indicates that radiation penetrates cellular barriers, such as the nuclear membrane, and takes effect in all cellular compartments to a similar extent. In contrast, hydrogen peroxide might not permeate the nuclear membrane as efficiently and take effect mainly within the mitochondria, which it might access more easily. It might also affect the mitochondrial oxidative stress levels by interfering with the electron transfer at the respiratory chain.

Interestingly, treatment with both UVC irradiation as well as hydrogen peroxide caused human iPSCs to acquire less DNA damage compared to isogenic fibroblasts, in mitochondrial as well as nuclear DNA (Fig. 3; Dannenmann et al., 2015). These results were confirmed using commercially available immunosorbent assays detecting lesions specific to UV and hydrogen peroxide treatment, respectively.
Moreover, human iPSCs displayed low DNA damage levels following genotoxic treatments in comparison with various tumor cell lines, suggesting a special mechanism of DNA protection in human iPSCs (Fig. 4; Dannenmann et al., 2015).
Remarkably, this DNA damage protection is rapidly lost upon differentiation (Fig. 3; Dannenmann et al., 2015). Elevated DNA damage rates in differentiated human iPSCs indicate that the proposed protection of human iPSC genomes is only maintained in the pluripotent state.

The reduced susceptibility of human iPSCs to DNA damage as compared to isogenic fibroblasts as well as to other cell lines was observed following treatment with genotoxic agents, known to cause oxidative stress (Ikehata and Ono, 2011). Therefore, DNA damage rates were examined for correlation with concentrations of cellular ROS.

In general, oxidative stress levels in human iPSCs were observed to be drastically lower, as compared to their parental HDFs, even though human iPSCs were expected to suffer more stress due to high replication rates (Jones et al., 2013). This finding was consistent with previous studies, which revealed that stem cells maintain low ROS levels by reduced oxygen consumption and low mitochondrial biogenesis. ESC cultures generate reduced amounts of ROS, compared to most somatic cell types (Armstrong et al., 2010). This can be explained by PSCs lower rates of oxidative phosphorylation and mitochondrial biogenesis (Prigione and Adjaye, 2010).

FACS analysis also revealed a lower increase of ROS levels in human iPSCs upon hydrogen peroxide treatment, as compared to fibroblasts (Fig. 5; Dannenmann et al., 2015). This suggested that hiPS cells prevent oxidative DNA damage by increased antioxidative defense mechanisms.

Increased Antioxidative Defense in Human iPSCs

Human iPS cells acquired less initial DNA damage post stimulation with UV radiation and hydrogen peroxide, respectively, compared to fibroblasts. They also suffered less oxidative stress upon stimulation. This led to the assumption that human iPSCs hold special mechanisms to protect their genome from high ROS loads and DNA damage.

Human cells harbor several antioxidants, which could be part of the enhanced antioxidative defense capacity of human iPSCs. The most important of which is glutathione.
Indeed enzymatic analysis of GSx (GSH and GSSG) content (Tietze, 1969) revealed significantly elevated levels of glutathione in human iPSCs compared to HDFs (Fig. 5; Dannenmann et al., 2015). This finding strongly supported the idea that glutathione might be involved in increased antioxidative defense capacities in human

iPSCs. Further examination, using a combination of the glutathione biosynthesis inhibitor buthionine sulfoximine (Griffith and Meister, 1979) and dimethyl fumarate, which decreases cellular glutathione levels by crosslinking it and mediating excretion (Nelson et al., 1999) successfully depleted >80% of cellular glutathione in two iPSC and HDF lines, respectively (Fig. 5; Dannenmann et al., 2015).
Alteration of cellular glutathione levels has been reported to induce apoptosis (Boivin et al., 2011; Circu and Aw, 2008). However, apoptosis and necrosis rates, respectively, showed no significant increase within the time of experimentation (data not shown).
Depletion of cellular glutathione, however, significantly elevated the increase of intracellular ROS levels upon stimulation with hydrogen peroxide (Fig. 5; Dannenmann et al., 2015).
Both, GSH concentrations as well as ROS levels were partially rescued by adding GSH-O-ethyl ester to the medium during depletion.

Expectedly, depletion of cellular GSH concentrations and the resulting elevated ROS levels led to higher frequencies of DNA lesions. However, elevation of ROS levels as well as initial DNA damage rates upon hydrogen peroxide treatment were found to be lower in iPSC lines as compared to their parental HDF lines, despite glutathione-depletion (Fig. 5; Dannenmann et al., 2015). Glutathione depletion did not lead to equal amounts of DNA lesions or ROS levels in human iPSCs and fibroblasts.
This suggested glutathione to play a role in keeping cellular ROS levels low. The results of this study, however, also indicated that elevated glutathione concentrations were not the sole reason for the increased DNA damage protection in human iPSCs compared to HDFs.

RNA expression analysis of a wide variety of antioxidative genes revealed upregulation of peroxiredoxin expression as well as several glutathione associated genes (Fig. 6; Dannenmann et al., 2015). *GPX2* and *GSTA2* showed several thousand-fold upregulation in human iPSCs, compared to HDFs. Also expression levels of these two genes were found to be higher in iPSCs than in a wide range of cell lines (Fig. 7; Dannenmann et al., 2015).
Gene expression analysis of cells at different stages of glutathione depletion also revealed lower cellular glutathione levels to cause an increase in gene expression of

glutathione reductase (*GSR)* and different glutathione S-transferases (*GST*s) (data not shown). Upregulation of *GSR* might compensate decreased glutathione levels to keep cellular ROS levels low. Higher expression of *GST*s suggests a cellular response to high xenobiotic loads, aiming for a fast excretion.
These results indicated that glutathione might not be a sole key player in this safeguard mechanism of iPSCs, but play a major role as part of the glutathione antioxidant system.

Since mRNA expression patterns revealed high levels of *GPX2* and *GSTA2* in iPSCs compared to HDFs, the respective genes were efficiently knocked down by RNAi in iPSCs (Fig. 7; Dannenmann et al., 2015). IPS cells lacking GPX2 exhibited elevated mtDNA damage upon hydrogen peroxide treatment compared to cells treated with non-targeting siRNA and hydrogen peroxide. Expectedly, elevated DNA damage rates in GPX2 depleted iPSCs increased drastically upon exposition to DMF and BSO.
Human iPS cells seemingly are able to partially compensate for depletion of GSH content or low GPX2 expression, due to high levels of the corresponding enzyme or substrate, respectively. However, the combined depletion of both, GSH and GPX2 content, resulted in significantly elevated oxidative stress and DNA damage levels in human iPSCs (Fig. 7; Dannenmann et al., 2015).
Surprisingly, knock-down of GSTA2 showed no significant effect on DNA damage rates (Fig. 7; Dannenmann et al., 2015).

Since GPX2 knock-down, in combination with depletion of cellular glutathione, was able to overcome DNA damage protection in human iPSCs, the effects of GPX2 overexpression were examined in HDFs.
GPX2-overexpressing HDFs showed a significantly increased resistance to oxidative stress and corresponding DNA damage (Fig. 7; Dannenmann et al., 2015). This is consistent with previous data, showing that GPX2 overexpression reduces the ROS-mediated apoptosis induction in breast cancer cells (Yan and Chen, 2006).

These results provide strong evidence for a cell-type specific antioxidative defense against DNA damage in human iPSCs. Also the glutathione antioxidant system was

shown to play an important role in human iPSC genome protection, with high levels of GSH and GPX2 being hallmarks of this defense system.
However, depletion of both, GSH and GPX2, could not equal DNA damage in iPSCs and their non-pluripotent parenting cells. Therefore, an involvement of other antioxidants, such as peroxiredoxins, is very likely. This assumption is supported by the finding that several antioxidative proteins showed elevated expression levels in human iPSCs.

Potential of Human iPSCs in Regenerative Medicine

Genomic instability is the main point of criticism against the use of iPSCs in regenerative medicine, since elevated occurrence of mutations could promote malignant outgrowth of transplanted cells.
Early research in the field of iPSC genome stability suggested high frequencies of mutations, aberrations, chromosome trisomies, and fluctuations in telomere elongation (Ronen and Benvenisty, 2012). While most unintended genome alterations were attributed to the reprogramming process itself (Ji et al., 2012), the risk of prolonged in vitro culturing and the general susceptibility of iPSCs to oncogenic mutations was discussed controversially.
More recent reprogramming protocols were able to partially solve this problem by eliminating risk factors such as transgenic cMyc expression and reported mutation levels following reprogramming to be similar to those in parental cells (Bai et al., 2013; Yoshihara et al., 2016).
Besides using only well-characterized host cells for reprogramming to avoid the use of potentially aberrant cells and utilizing rather genome preserving methods of reprogramming, it remained of major importance to elucidate the efficiency and underlying mechanisms of genome surveillance in iPSCs.
In combination mitochondrial priming, increased repair capacity and antioxidative DNA protection constitute three pillars of genome surveillance in human iPSCs: This study shows that human iPSCs hold an enhanced antioxidant defense, which effectively prevents DNA lesions. Occurring DNA damage is repaired quickly due to their high repair capacity. However, if high levels of DNA damage are induced, mitochondrial priming prompts a strong and rapid apoptotic response, causing damaged cells to die, thereby efficiently preventing inheritance of mutations. This

system secures genome integrity, pluripotency and tissue renewal capacities, which were shown all to be affected by mutations.

These results represent an important step on the way to clinical application of human iPSCs. However, it still remains of major importance to further secure the genomic integrity during reprogramming. Also sequencing of parenting cells as well as reprogrammed cells seems crucial to ensure safety. This could be accomplished by next generation sequencing technologies, which however are still financially inefficient on a genome-wide basis (Yoshihara et al., 2016).

Another topic yet to be addressed is the epigenomic stability of iPSCs (Lund et al., 2012).

Consequently, while the identification of the genome protective mechanisms in iPSCs mark an essential step to therapeutic application of iPSCs in transplantation, it is still a long way to go. However, their use in individual drug screening and patient-specific toxicity tests has already made them a powerful tool in personalized medicine (Matsa et al., 2016). IPSCs in combination with various differentiation methods provide a platform for a broad range of research, such as immunological and virological studies on non-primary tissue, which had previously been limited to tumor cell lines (Kuadkitkan et al., 2016).

Thus, iPSCs already constitute an important tool in basic research and harbor great potential for future use in regenerative medicine, which however will need further research to take account of safety concerns.

Effects of the Mitochondrial Copy Number on DNA Damage

Healthy human cells are mostly diploid, with the exception of oocytes and sperm cells which are haploid. Therefore, they contain one or two copies of genomic DNA each. However, the copy number of the mitochondrial genome strongly varies across different cell types, spanning from roughly 100 in sperm to over 150 000 in mature oocytes (Wai et al., 2010). The circular 16 kilo base genome usually comprises around 1% of cellular DNA and is packed by roughly 100 mitochondrial transcription factor A (TFAM) proteins per DNA molecule (Herrera et al., 2015). Mitochondrial DNA content was shown to be regulated by proteolytic degradation of TFAM (Matsushima et al., 2010).

Changes in mitochondrial copy numbers are tightly associated with the process of ageing, as well as cardiovascular disease and other prevalent diseases (Finkel and Holbrook, 2000; Herrera et al., 2015). Decreased mitochondrial DNA (mtDNA) copy numbers were associated with a reduction of fertility in maternal oocytes (Hendriks et al., 2015). Recent studies have also associated altered mtDNA copy number with childhood autism and various types of cancer (Chen et al., 2015; Reznik et al., 2016).

Besides maintaining genomic integrity, also securing mitochondrial soundness therefore is crucial to all cells. However, mitochondrial DNA is more susceptible to mutations in comparison to genomic DNA, due to an inferior DNA repair capacity compared to nuclear DNA (Sawyer and Van Houten, 1999). Also mitochondria are exposed to higher concentrations of ROS, which even increase with age (Balaban et al., 2005). ROS are known to cause mtDNA damage, which then can enhance further ROS production, resulting in a vicious circle (Indo et al., 2007).

Elevated ROS levels were reported to induce replication of mitochondrial DNA (Lee et al., 2000). This raised the question whether cells react to genotoxic threats by replicating intact mitochondrial DNA to strive for a protection-by-abundance mechanism.

To check whether there is a correlation between DNA damage rates and mtDNA copy number, a fast and sensitive screening method, capable of measuring DNA damage and mitochondrial copy number in high throughput was needed.
The recently developed LORD-Q assay is a highly sensitive method, measuring DNA damage levels in distinct gene loci in mtDNA and nuclear DNA in a microtiter format (Lehle et al., 2014).
This study extends the applicability of LORD-Q by simultaneous determination of the mitochondrial copy number (Dannenmann et al., 2017). This additional measurement of the mtDNA copy number is not accompanied by any further effort or cost.
When the DNA damage in a genomic as well as a mitochondrial locus is assessed, the shift between exponential phases of the nuclear and mitochondrial short fragment amplifications corresponds to the difference of mitochondrial and nuclear genome copies present in the cell. The mitochondrial copy number can therefore be calculated, using the respective C_p values and ploidity of the examined cells (Fig. 1; Dannenmann et al., 2017).

The dual measuring method LORD-Q was harnessed to answer the question whether there is a correlation between DNA damage rates and mtDNA copy number. Therefore, ten common cancer cell lines of the NCI60 panel were treated with ROS-inducing stimuli, indicating a negative correlation between mtDNA copy number and mitochondrial DNA damage levels after ROS inducing irradiation (Fig. 2; Dannenmann et al., 2017). Cells with higher quantities of mtDNA showed less susceptibility to DNA damage induced by oxidative stress. This finding supported the idea of a protection-via-abundance mechanism.
However, this idea seemed contradictory to previous findings of this study. Human iPSCs were shown to be more resistant to oxidative stress, as compared to their differentiated counterparts or their parental dermal fibroblasts (see above). However, analysis of mitochondrial copy numbers in these cell types revealed very low levels of mtDNA in pluripotent stem cells, while mtDNA copy number were rising during differentiation (Fig. 2; Dannenmann et al., 2017). Thus, abundance in DNA content did not seem to be a sole key player in the protection of DNA.
Since human iPSCs possess a cell-type specific antioxidative defense system, this could be true for other cell types as well. Therefore, the effect of cellular mtDNA content was to be examined independently from of cell-type specific effects.

Treatment with ethidium bromide is well known to inhibit mitochondrial, but not nuclear DNA replication. Thereby, continuous exposure to ethidium bromide reduces mitochondrial copy numbers in various cell types within a few days or even completely eliminates mtDNA over the course of a few weeks (Olgun and Akman, 2007). Cells which show a complete loss of mtDNA are known as rho° cells.

Utilizing ethidium bromide, mitochondrial copy numbers were depleted in Jurkat cells. Since a loss of mtDNA however prevents examination of DNA damage rates in mtDNA, cells were maintained at a level of mtDNA content reduction of roughly 80-90% (Supplemental Fig. 1; Dannenmann et al., 2017). As a result, mitochondrial mass in depleted cells decreased by 62%. These cells were termed pseudo rho° cells.

The susceptibility to ROS-induced DNA damage of pseudo rho° and non-depleted Jurkat cells were compared. Interestingly, no significant difference in mtDNA damage rates was observed following hydrogen peroxide treatment.

To exclude artificial effects of high doses of ethidium bromide, results were reproduced using cells, depleted of mtDNA using the polymerase γ inhibitor 2′-3′-dideoxycytidine (ddC).

The lack of a significant difference in DNA damage levels strongly contradicted the idea of a protection-by-abundance mechanism. Further it suggested the mitochondrial copy number not to play any role in ROS-mediated DNA damage prevention at all but rather being limited to radiation induced DNA lesions.

Cells depleted of mitochondrial DNA have been reported to be less susceptible to the chemotherapeutic drug bleomycin. Bleomycin causes DNA damage in mitochondria in a ROS dependent manner (Brar et al., 2012).

Thus, ROS levels were measured in bleomycin treated pseudo rho^0 Jurkat cells, revealing an impaired increase in ROS levels during stimulation compared to non-mtDNA-depleted cells, regardless which depleting agent had been used (Fig. 2; Dannenmann et al., 2017).

Congruently with previous reports, lower lesion rates were observed in mitochondrial DNA of pseudo rho^0 cells, as compared to non-depleted cells while nuclear DNA damage levels in pseudo rho^0 and control cells showed no significant differences.

These results indicate that variations in mitochondrial DNA content can in fact influence the resistance against DNA lesions. However this observation did strongly

depend on the mechanism of the respective genotoxic stimulus. Yet DNA damage susceptibility seems not to be generally affected by the mtDNA copy number.
In regards of the process of ageing, as well as the various pathologies, the mtDNA copy number has been associated with, it remains an important question for future research to further elucidate in which cases variation of the mitochondrial copy number is cause, concomitant, or consequence of a disease. This question has to be addressed individually for each pathology in order to examine possible approaches of therapeutic treatment.
The LORD-Q assay provides a powerful tool to elucidate this question. However the applicability of the LORD-Q method so far had only been tested under *in vitro* conditions and the initial report on the method had only described primers for analysis of human cells (Lehle et al., 2014).

LORD-Q Analysis of *ex vivo* Samples

LORD-Q represents a wide-ranging measuring tool, due to the possibility to assess DNA damage in distinct gene loci within the nuclear and mitochondrial genome as well. However, the LORD-Q method would be of much greater versatility if it was able to assess DNA damage not only in human cells, but also in common model organisms, such as the murine system. Also, it would be useful if patient samples could be analyzed directly, without the need to outgrow and culture the cells *in vitro*.
To examine the applicability of the LORD-Q assay to analysis of tissue samples, various murine tissue types were analyzed following genotoxic treatment.
Ionizing radiation is a common method to induce DNA damage and changes in mtDNA copy number (Malakhova et al., 2005; Zhang et al., 2009). Therefore, mice were irradiated with 0, 3 and 6 Gy, respectively. LORD-Q analysis of samples of the brain, spleen, liver and bone marrow of irradiated and non-irradiated control mice revealed an initial increase of lesions in all irradiated samples (Fig. 3; Dannenmann et al., 2017). A correlation between irradiation dosages and detected DNA lesions was observed in mitochondrial as well as nuclear DNA. One day post treatment DNA damage was repaired nearly completely in all organs examined. These results are consistent with previous studies, showing almost complete DNA repair within the first 24 hours (Sawyer and Van Houten, 1999).

Interestingly, DNA damage rates were observed to be higher in nuclear DNA, as compared to mitochondrial DNA of the respective tissues. Nuclear DNA is usually considered to be packed in a denser state, as compared to mitochondrial DNA, which implies an inferior protection of the latter in case of exposition to radiation. This result however suggests mechanisms in favor of genome protection against DNA damage caused by irradiation to be present in mitochondria. One way to protect the mitochondrial genome would be by rapid degradation of damaged DNA strands, which is exactly what was found by examining changes in mitochondrial copy numbers following irradiation: An initial decrease of mitochondrial copy numbers was observed within three hours following stimulation. Subsequent restoration of cellular mtDNA content was accomplished at 24 hours post irradiation and accompanied by hyper compensation. While 3 Gy caused mitochondrial copy numbers to rise above initial levels mainly in brain tissue, a dosage of 6 Gy induced hyper compensation in all tissues, raising mtDNA copy numbers by up to more than 200% in liver tissue.
This ionizing radiation induced mtDNA synthesis has been discussed to be a delayed compensatory reaction of cells with heavily damaged mitochondrial DNA (Malakhova et al., 2005; Sawyer and Van Houten, 1999), which is consistent with the results described in this study.

Applicability of the LORD-Q Method

The results of this study demonstrate the capability of the LORD-Q method to assess DNA damage levels and mitochondrial copy numbers simultaneously.
DNA damage as well as the mitochondrial copy number have been shown to promote and being hallmarks of the process of ageing (Finkel and Holbrook, 2000; Lephart, 2016), tumorigenesis, cancer progression and drug resistance (van Gisbergen et al., 2015; Nyström et al., 2012; Reznik et al., 2016; Stewart et al., 2014) and various pathologies, such as neurodegenerative diseases (Lovell and Markesbery, 2007; Ramassamy et al., 1999).
The ability to measure both, DNA damage and mitochondrial copy numbers, showcase the LORD-Q assay as ideal tool for research in the field of ageing as well as for high-throughput screenings to evaluate the effectiveness of chemotherapeutic drugs or new candidates for drug development.

The results of the analysis of murine *ex vivo* samples met expectations (Dannenmann et al., 2017) and were consistent with current literature (Malakhova et al., 2005; Sawyer and Van Houten, 1999; Zhang et al., 2009), showing the suitability of the method to simultaneously measure DNA damage and mitochondrial copy numbers in multiple types of murine tissue samples and suggesting applicability for research in a wide range of model organisms.

Furthermore, the applicability of the LORD-Q assay to examine human tissue was tested successfully (unpublished data). LORD-Q is capable to reliably detect DNA damage and mitochondrial copy number changes in human *ex vivo* samples as well.

Since mitochondrial copy numbers were reported to be of prognostic value in some cancers, such as lung cancer (Xu et al., 2013), the LORD-Q method therefore provides the possibility to screen patient samples fast and economically.

Another possible field of application for the LORD-Q method is current research in reproductive medicine. With increasing interest in this subject, cryopreservation of semen as well as oocytes is becoming a growing field of research.

With the critical points of cryopreservation in reproductive medicine being impairment of DNA integrity and mitochondrial copy number alterations (Hendriks et al., 2015), there is increasing interest in finding novel cryoprotective agents. However, oocytes used for screening these substances are a scarce resource. Therefore, common methods of DNA damage quantification, which require large quantities of sample DNA are not applicable to this specific task. The LORD-Q method however could provide a suitable tool with the need for comparably low amounts of DNA. Also the LORD-Q assay allows analysis of mtDNA separately from nuclear DNA to examine the specific role of mitochondrial DNA damage, which possibly causes the alterations in mitochondrial copy numbers of oocytes following cryopreservation. Further the novel method measures the mitochondrial copy number simultaneously without the need for additional sample DNA, thereby covering both analyses with just one measurement per sample and minimizing the use of scarce resources such as oocytes.

In summary, the extended LORD-Q method is a fast and labor-effective method of high sensitivity, applicable to research in a wide range of flourishing fields.

Summary & Outlook

In the current study human iPSCs were shown to protect their genomic integrity by a system based on not two but three pillars: Firstly, pluripotent stem cells exhibit enhanced DNA repair capacities (Luo et al., 2012; Maynard et al., 2008). Secondly, this study reveals pluripotent stem cells being primed for apoptosis, which is in line with previous research (Liu et al., 2013). This priming has previously been attributed to pre-activated BAX, located to the Golgi. However, no translocation of BAX was found during this study. Rather a low expression of anti-apoptotic proteins was shown to be responsible for the readiness of PSCs to undergo rapid apoptosis.

The third pillar of genome protection in iPSCs was shown to be an enhanced antioxidative defense system, which seems to be specific to iPSCs and is rapidly lost upon differentiation. This protection against ROS-induced DNA damage mainly comprises elevated levels of cellular glutathione and glutathione peroxidase 2. In addition, other antioxidative molecules seem to play a role in this enhanced antioxidative system as well. It will remain a target of future research to identify these other contributors and to further elucidate which pathways are linking these genes to the status of pluripotency.

Better understanding of the ways in which human iPSCs secure their genomic stability is of major importance to assess the risk of iPSCs in clinical use. This study has provided important information on this topic but further research will be required to fully evaluate all safety concerns.

Further in this study the applicability of the novel DNA damage measuring LORD-Q method was extended to tissue analysis. Due to the low requirement for sample DNA quantities of LORD-Q, this bypasses current restrictions in clinical use, as well as the use on small model organisms without pooling samples. Also the potential of the assay to simultaneously measure the mitochondrial copy number without the need for additional measurement or sample material was demonstrated.

This new implementation was harnessed to examine the influence of the mtDNA copy number on DNA damage susceptibility. Mitochondrial DNA was shown to react to genotoxic insults by degradation of damaged DNA strands, followed by a hyper-compensatory synthesis of mtDNA. Higher amounts of mitochondrial DNA however

did not always provide protection against genotoxic stimuli. This way of defense-via-abundance rather only provided protection in specific cases, such as UV irradiation.
Concerning the increasing number of traits, being associated with mtDNA copy number alteration (Chen et al., 2015; Reznik et al., 2016; St. John, 2016; Wai et al., 2010), future research will need to clarify in which cases the mtDNA copy number is affected by, concomitant, or even cause of the symptoms, which have been associated with it.

CONTRIBUTIONS

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

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Project planning and experimentation of this publication was started by Dr. Simon Lehle. The results depicted in figure 1, 2, 4 and S1 were obtained by him and Ayline Kübler under his supervision. Dr. Dominic Hildebrand supported the project with advice in critical points. Mirjam Fröschl assisted me on performing the experiments shown in figure 5. Paula Grondona, Vera Schmid and Katharina Holzer were student assistants under the supervision of me and Dr. Simon Lehle and contributed to the experimentation underlying figure 4. Dr. Frank Essmann assisted and advised me while performing the experiments for figure S2. Dr. Oliver Rothfuss and Prof. Dr. Klaus Schulze-Osthoff supervised the project and raised the funding. The manuscript was written by me and Dr. Simon Lehle and revised by Klaus Schulze-Osthoff.

"**Genome surveillance in pluripotent stem cells: low apoptosis threshold and efficient antioxidant defense**"

Mol Cell Oncol 2016 *3*, e1052183

This publication is based on the data of the article named above and was written by me and Prof. Dr. Klaus Schulze-Osthoff. Dr. Frank Essmann assisted me in creating the schematic figure 1.

"Simultaneous quantification of DNA-damage and mitochondrial copy number by Long-run DNA-damage quantification (LORD-Q)"

Oncotarget 2017 (accepted manuscript)

Figure 2A shows reevaluated data, partially obtained by Dr. Simon Lehle. Sebastian Lorscheid performed the mouse work and Prof. Dr. Stephan M. Huber performed the irradiation of the mice. Prof. Dr. Klaus Schulze-Osthoff supervised the project and raised the funding.

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APPENDIX

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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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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

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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 H_2O_2, 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 H_2O_2 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/cm^2, their somatic precursor cells remained viable even after a high-dose UVC treatment of 100 mJ/cm^2 (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 antiapoptotic BCL-2 protein members, including *BCL2*, *BCLX*, *BCLW*, and *A1*. In contrast,

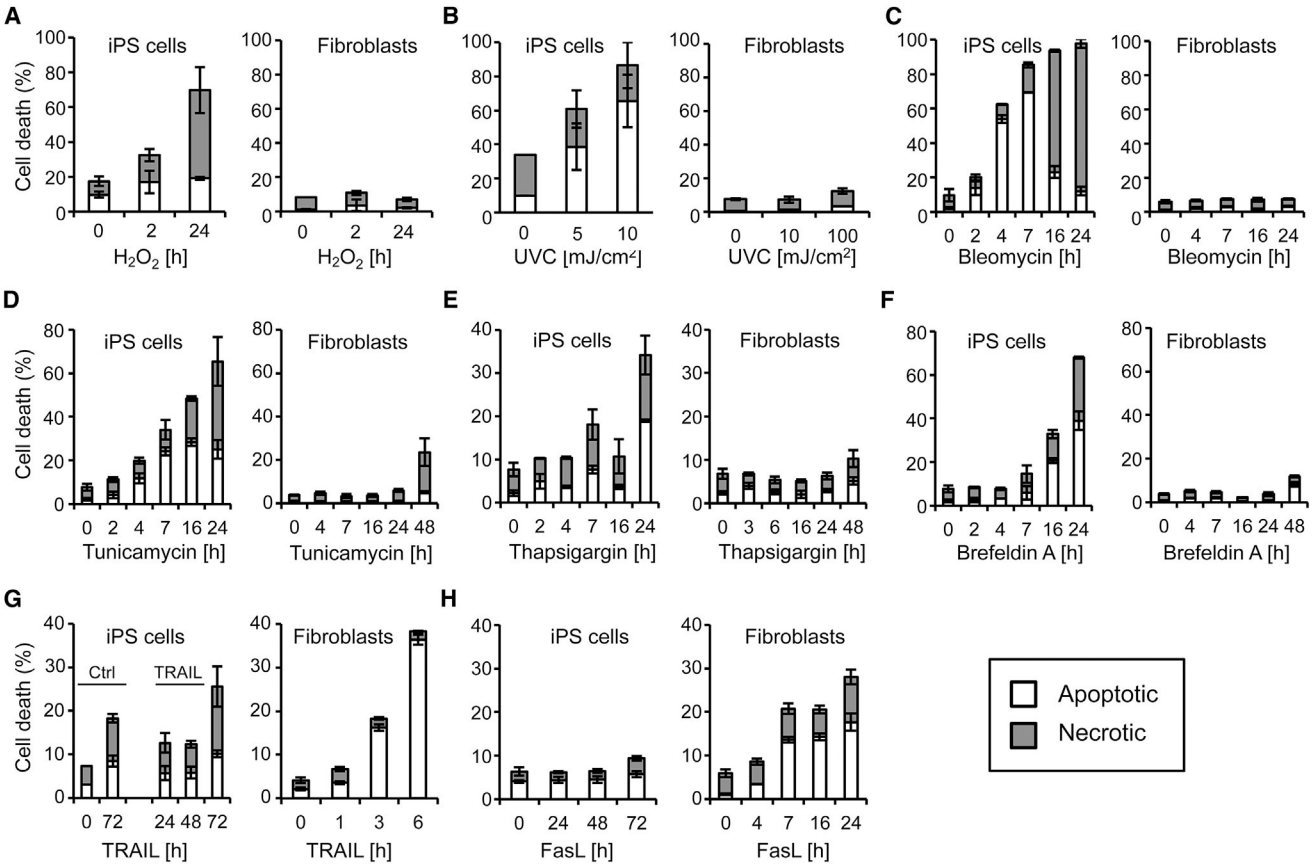

Figure 1. Human iPSCs Are Highly Susceptible to Stress Stimuli Triggering the Mitochondrial Death Pathway

(A–H) The human iPSC lines L1 and L2 and corresponding primary fibroblasts were treated for the indicated time with 500 μM H_2O_2 (A), 50 μM bleomycin (C), 6 μM tunicamycin (D), 1 μM thapsigargin (E), 1 μM brefeldin A (F), 50 ng/ml TRAIL (G), and 50 ng/ml FasL (H). In addition, cells were stimulated with the indicated dose of UVC 6 hr prior to cell death analysis (B). Apoptosis and necrosis rates were measured by annexin-V/PI staining. Results represent means ± SD from three independent experiments. ***$p < 0.001$.

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-X_L, 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

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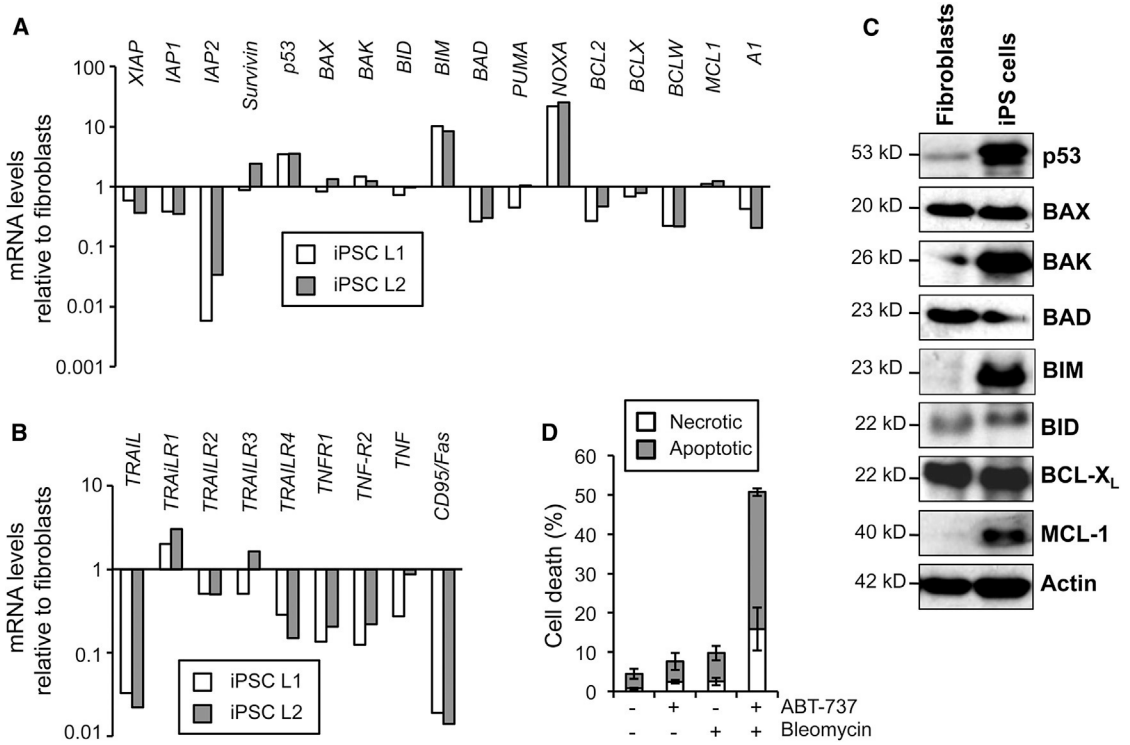

Figure 2. Human iPSCs Display Increased Mitochondrial Priming

(A and B) The iPSC lines L1 and L2 were analyzed for mRNA expression of the indicated regulators of the intrinsic (A) and extrinsic (B) apoptosis pathways relative to L2 fibroblast mRNA levels. *GAPDH* was used as reference. A representative experiment is shown.

(C) Western blot analysis shows p53 and the indicated members of the BCL-2 family in L2 iPSCs and fibroblasts.

(D) The BH3 mimetic BCL-2 inhibitor ABT-737 sensitizes fibroblasts to DNA damage-induced cell death. L2 fibroblasts were treated for 24 hr in the presence or absence of bleomycin (70 μM) and ABT-737 (25 μM). Apoptosis and necrosis were measured by annexin-V/PI staining. Results show the mean ± SD from three independent experiments.

(Lehle et al., 2014). 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). Also, we analyzed mtDNA damage and the occurrence of 8-hydroxydeoxyguanosine (8-oxo-dG), a mutagenic DNA lesion, in response to H_2O_2 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 FGF2. When fibroblasts and undifferentiated and differentiated iPSCs were exposed to different H_2O_2 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.

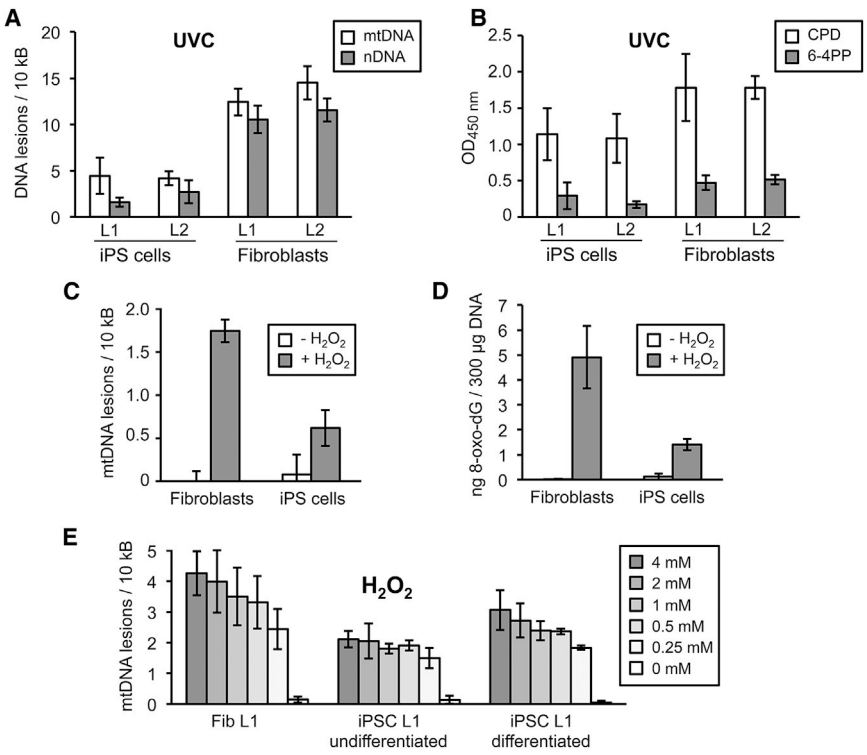

Figure 3. Human iPSCs Are Protected against Mitochondrial and Nuclear DNA Damage

(A) The iPSC lines L1 and L2 and corresponding fibroblasts were irradiated with 20 mJ/cm^2 UVC light. Immediately after irradiation, damage of mitochondrial (mtDNA) and nuclear DNA (nDNA; *GAPDH* locus) was determined by LORD-Q analysis. Results show mean values ± SD from three independent experiments. $p = 0.005$ (L1 mtDNA), $p < 0.001$ (L2 mtDNA, L1 nDNA, and L2 nDNA).

(B) Following UVC irradiation (20 mJ/cm^2) of iPSCs and fibroblasts, DNA samples were analyzed for cyclobutane pyrimidine dimers (CPD) and pyrimidine(6-4)pyrimidone photoproducts (6-4PP) by ELISA. Results show mean values ± SD from three independent experiments. $p < 0.05$.

(C) The iPSCs and fibroblasts were exposed to 5 mM H_2O_2 for 5 min before mtDNA damage was measured by LORD-Q analysis. Results show mean values ± SD, which were obtained from L1 and L2 iPSCs and the corresponding fibroblasts in three independent experiments. $p < 0.002$.

(D) The iPSCs and fibroblasts were exposed to 5 mM H_2O_2 for 5 min before DNA samples were analyzed for 8-oxo-dG lesions. Results show mean values ± SD, which were obtained from L1 and L2 iPSCs and the corresponding fibroblasts in three independent experiments. $p < 0.001$.

(E) L1 fibroblasts, undifferentiated iPSCs, and iPSCs that had been differentiated by 30 days of incubation in the presence of FCS and absence of FGF2 were treated for 5 min with the indicated concentrations of H_2O_2. mtDNA damage rates were measured by LORD-Q analysis and are given as mean ± SD from three independent experiments. Comparison of whole datasets of undifferentiated iPSCs with data of fibroblasts and differentiated iPSCs resulted in p values of $p < 0.001$ and $p = 0.002$, respectively.

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 H_2O_2, 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 dihydrorhodamine 123 staining and FACS analysis. As shown in Figure 5D, both cell types displayed considerable ROS level increases after H_2O_2 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.

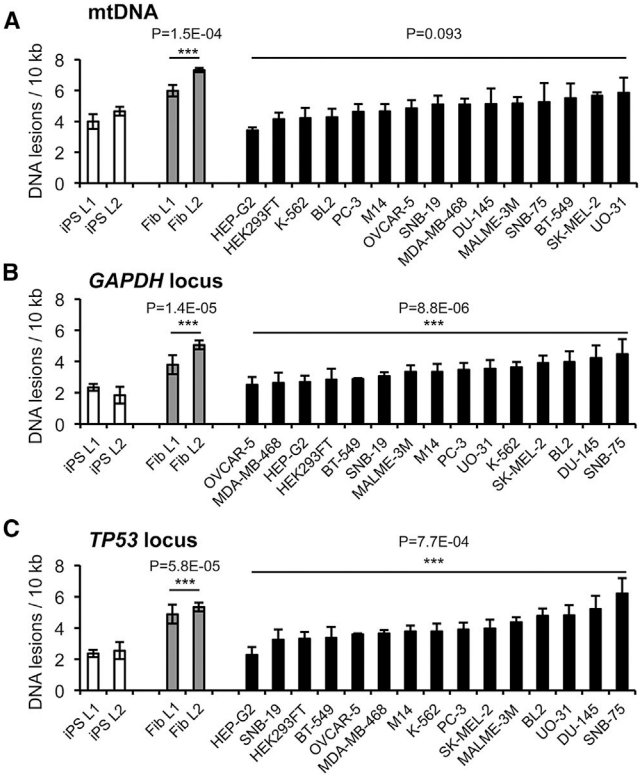

Figure 4. Comparison of DNA Damage Rates in iPSCs, Fibroblasts, and Tumor Cell Lines

(A–C) The human iPSC lines L1 and L2, corresponding fibroblasts, and several human cell lines of different tumor entities were irradiated with 10 mJ/cm^2 UVC light. Immediately after irradiation, cells were harvested and mtDNA damage (A) and nDNA damage in the *GAPDH* (B) and *TP53* locus (C) were quantified using LORD-Q analysis. P values indicate statistical significance between iPSCs and fibroblast and tumor cell lines, respectively.

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 H_2O_2 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 H_2O_2 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 H_2O_2 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

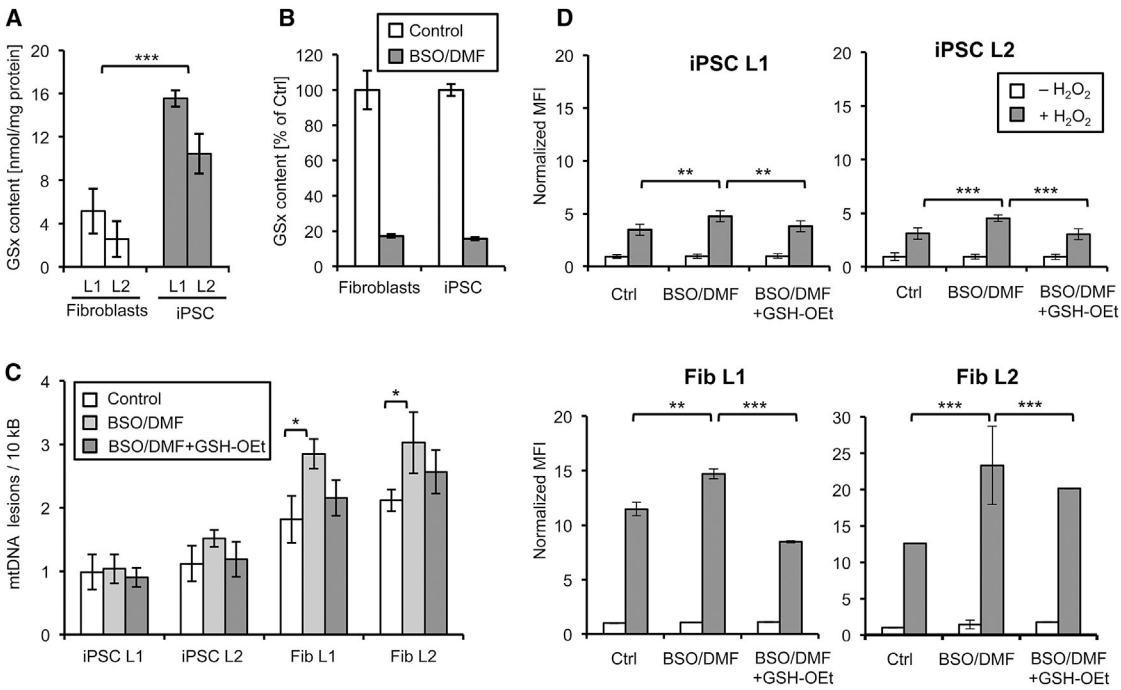

Figure 5. Human iPSCs Have High GSH Levels and Decreased Oxidative Stress

(A) Analysis of total GSH (GSx) levels in iPSCs and fibroblasts. Values show means ± SD from three independent experiments.

(B) Combined treatment of iPSCs and fibroblasts with 100 μM DMF and 100 μM BSO for 1 hr strongly reduces GSH levels. Values show means ± SD from three independent experiments.

(C) Effect of GSH depletion on H_2O_2-induced mtDNA damage. GSH-depleted iPSCs and fibroblasts were treated for 5 min with 2 mM H_2O_2 before mtDNA damage was determined by LORD-Q analysis. GSH depletion significantly increased H_2O_2-induced mtDNA lesions in fibroblasts, but not in iPSCs. Sensitization was partially reversed by supplementation with 2 mM GSH-OEt. Results show means ± SD from three independent experiments.

(D) Oxidative stress levels in iPSCs and fibroblasts. GSH-depleted, GSH-OEt-supplemented, BSO/DMF-treated, and control cells were stained with dihydrorhodamine 123 prior to H_2O_2 exposure (2 mM, 5 min) and analyzed by flow cytometry. ROS levels are shown as values of normalized mean fluorescence intensity (MFI) from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

threshold. 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), 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

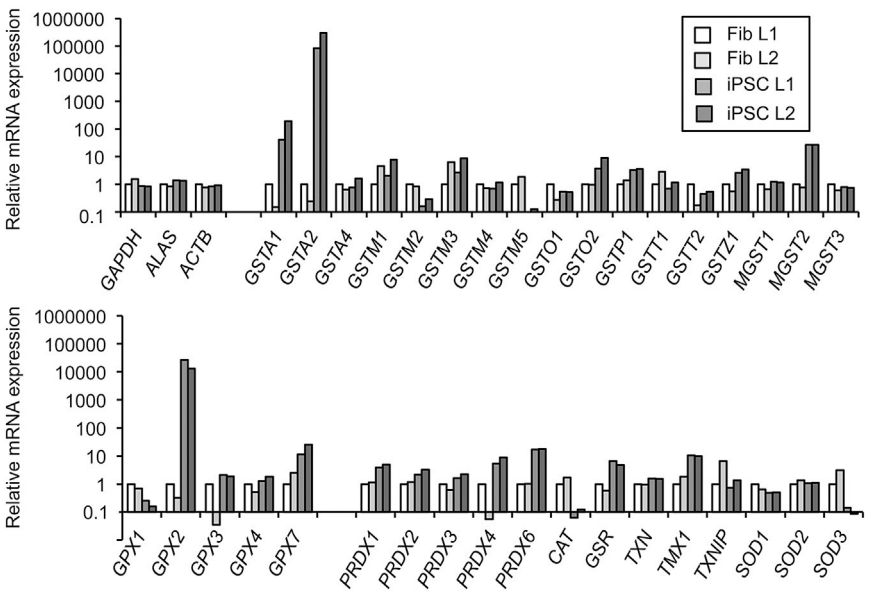

Figure 6. Increased mRNA Expression of Antioxidant and Detoxifying Enzymes in iPSCs

The iPSCs and fibroblasts were screened for the differential expression of factors involved in cellular ROS detoxification by qRT-PCR. mRNA expression in L1 fibroblasts was set as 1. A representative dataset is shown. *GAPDH*, GAPDH glyceraldehyde-3-phosphate dehydrogenase; *ALAS*, aminolevulinate synthase; *ACTB*, beta-actin; *GST*, glutathione S-transferase; *MGST*, microsomal GST; *GPX*, glutathione peroxidase; PRDX, peroxiredoxin; *CAT*, catalase; *GSR*, GSH reductase; *TXN*, thioredoxin; *TMX1*, thioredoxin-related transmembrane protein 1; *TXNIP*, thioredoxin-interacting protein; *SOD*, superoxide dismutase.

fibroblasts. Also, in response to treatment with H_2O_2 and UVC, oxidative nucleotide modifications such as cyclobutane pyrimidine dimers, (6-4) photoproducts, and 8-hydroxydeoxyguanosine (8-oxo-dG) were detected less frequently. Thus, our data are consistent with the hypothesis that pluripotent stem cells have superior DNA maintenance 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 interesting to note that a previous study in human ESCs (Maynard 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 activities of 8-oxoguanine glycosylase, the primary base excision repair enzyme required for removing this mutagenic DNA lesion. These results indicate that base excision repair is presumably not elevated in ESCs, but rather the occurrence of oxidative DNA lesions is prevented.

Our study shows that iPSCs are highly proficient in antioxidant defense, which is presumably responsible for the low frequency of oxidative DNA lesions in both the mitochondrial 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; Facucho-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 lesions. 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 glutathione 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).

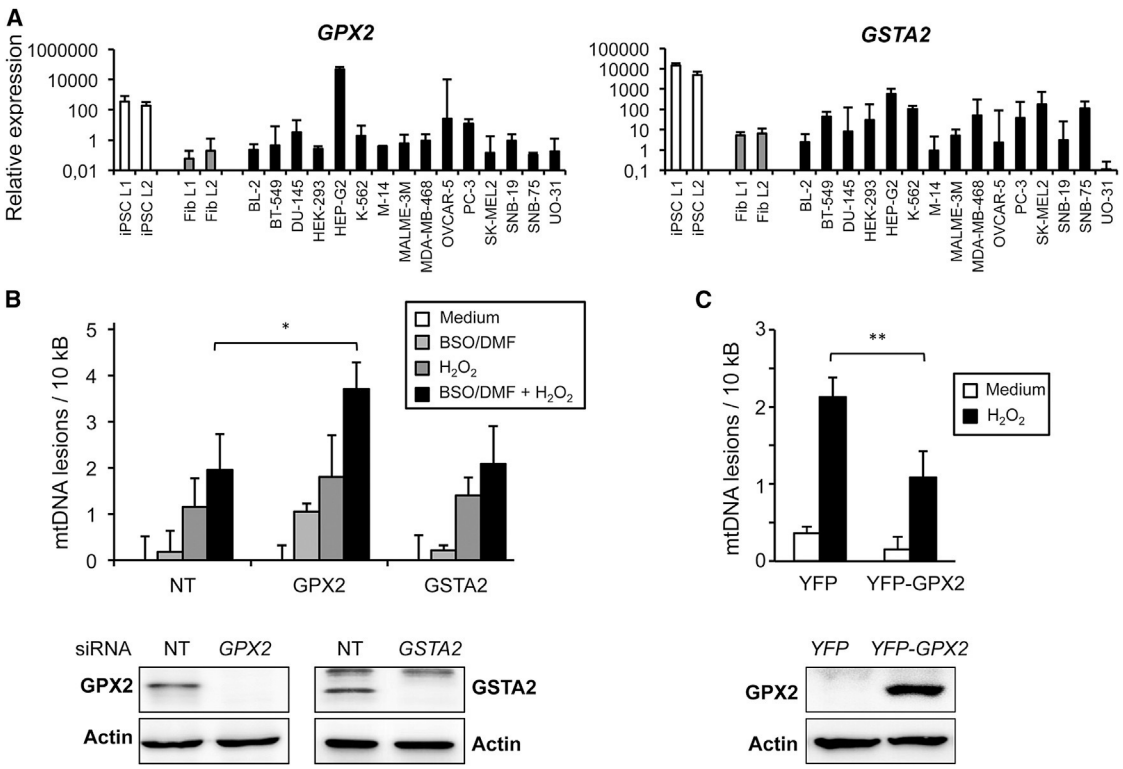

Figure 7. GPX2 and GSH Contribute to DNA Damage Protection in iPSCs

(A) Transcript levels of *GPX2* and *GSTA2* in iPSCs, fibroblasts, and cell lines of different tumor entities. The mRNA levels of the two GSH-dependent enzymes were analyzed by qRT-PCR relative to *GAPDH*. A representative dataset is shown.

(B) Combined GSH depletion and *GPX2* knockdown impair DNA damage protection of iPSCs. L2 iPSCs were incubated with 25 nM *GPX2*-specific, *GSTA2*-specific, or non-targeting (NT) siRNA. After 72 hr, GSx was depleted by the addition of BSO/DMF for 1 hr, followed by exposure to 2 mM H_2O_2 for 5 min. (Top) Measurement of mtDNA damage using the LORD-Q assay is shown. Results represent means ± SD of three independent experiments. *$p < 0.05$. (Bottom) Confirmation of the GPX2 and GSTA2 knockdown by western blotting is shown.

(C) Overexpression of GPX2 is sufficient to confer increased resistance to DNA damage in fibroblasts. L2 fibroblasts were transduced with lentiviral vectors for YFP-coupled GPX2 or the YFP control. Then, 48 hr post-infection, cells were treated with 2 mM H_2O_2 for 5 min. (Top) Measurement of mtDNA damage by LORD-Q analysis is shown. Results represent means ± SD from three independent experiments. $p = 0.002$. (Bottom) Confirmation of GPX2 overexpression by western blotting using an anti-GPX2 antibody is shown.

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, indicating that, in addition to GPX2, further antioxidant mechanisms might be involved in the protection against 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-Flohé 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,

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 endowance 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 with 4% formaldehyde. For detection of active BAX at the Golgi, cells were fixed in formaldehyde, permeabilized in blocking buffer, and stained with conformation-specific anti-BAX-NT and the Golgi marker anti-GM130.

Cell Death Analysis

Cells were stimulated with the indicated genotoxic and proapoptotic agents, including bleomycin (Medac), hydrogen peroxide, thapsigargin, tunicamycin, brefeldin A (all from Sigma), as well as recombinant TRAIL and FasL (both from Enzo Life Sciences). After cell harvest using trypsin for differentiated cells and accutase (Millipore) for iPSCs, supernatants containing dead 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, 1×10^6 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 Stratalinker 2400 (Stratagene). Bleomycin exposure was carried out in 300 μl PBS/F for 20 min. H_2O_2 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 LORD-Q analyses as described previously (Lehle et al., 2014). Briefly, whole-cell DNA was isolated from genotoxin-exposed and control samples using the DNeasy Blood & Tissue kit (QIAGEN) and diluted to 10 ng/μl 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 20× 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 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 $2^{-\Delta\Delta C}{}_{T}$ 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 C_p values were averaged.

Manipulation and Analysis of Cellular GSH Content

Total cellular GSH (GSx; c[GSx] = c[GSH] + 2 × c[GSSG]) content was determined as described previously (Tietze, 1969). Briefly, 10^5 to 10^6 cells were lysed in ice-cold 1% 5-sulfosiacylic acid. After 30-min incubation on ice, lysates were centrifuged (10 min, 20,000 × *g*) and the supernatants were used for GSx 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 5,5′-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. GSx concentrations were calculated using standard curves, normalized for protein content and expressed as nmol GSx/mg protein. Depletion of cellular GSx 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) (Ghoreschi et al., 2011).

Determination of ROS Levels

To measure intracellular ROS, 3 × 10^5 cells were stained with 1 µM dihydrorhodamine 123 (Sigma) in PBS for 10 min at 37°C. Subsequently, cells were pelletized and resuspended in PBS. Stimulation with 2 mM H_2O_2 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 × 10^5–10^6) 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 F.E. performed experiments and assembled the data.

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Stem Cell Reports, Volume *4*

Supplemental Information

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

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Supplementary Figures

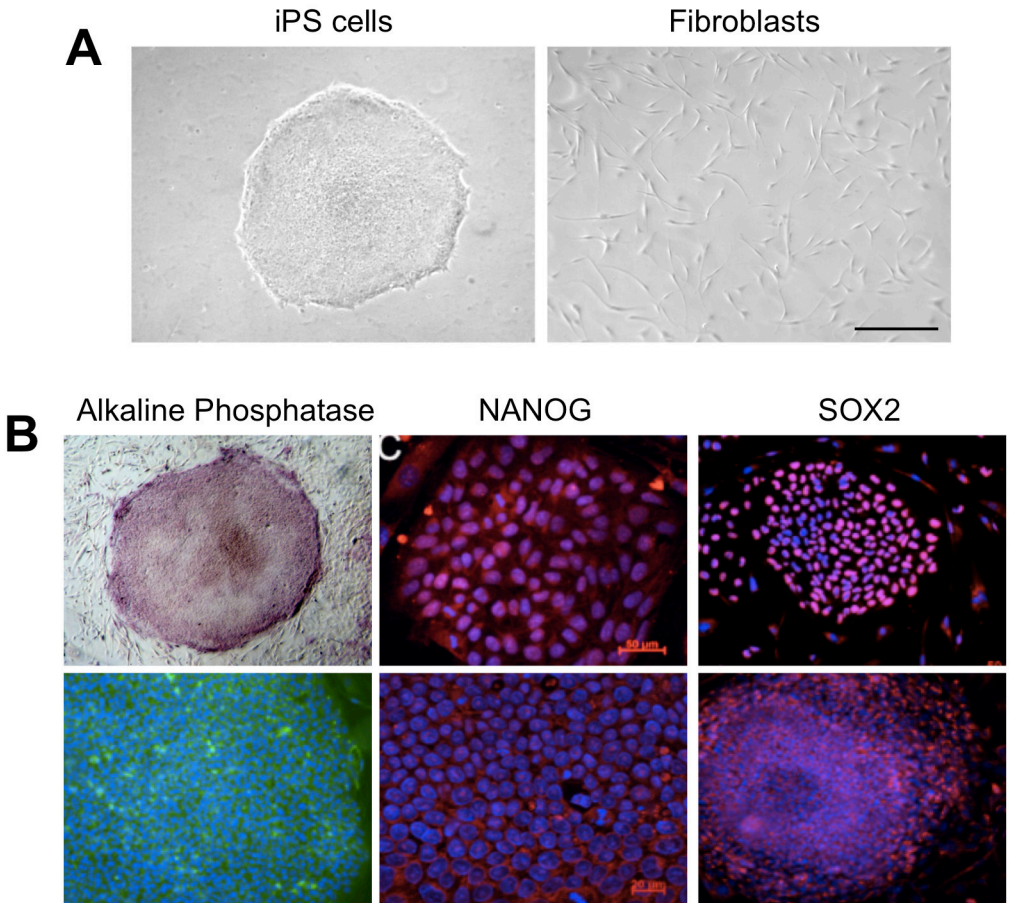

Figure S1: Characterization of employed human iPS cells and fibroblasts.

(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.

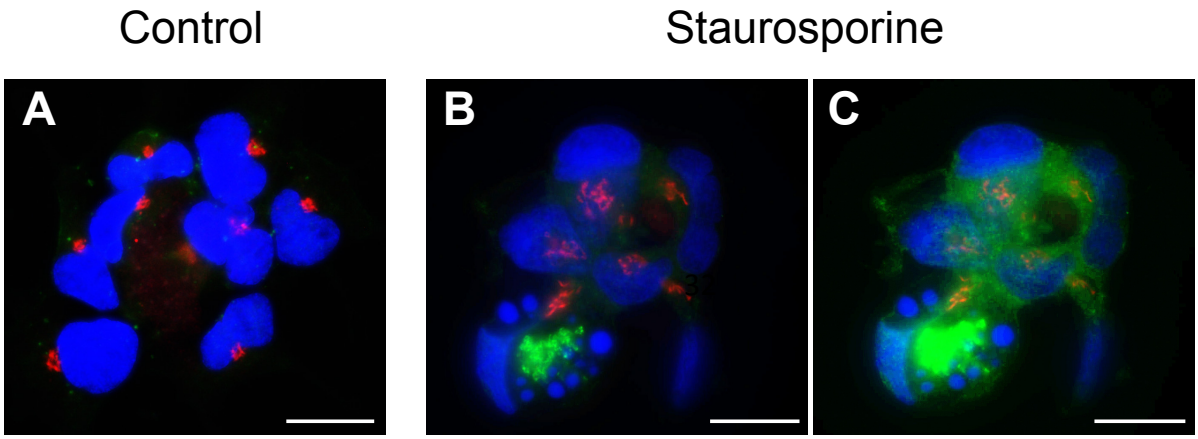

Figure S2: Human iPS cells do not maintain a constitutively active form of BAX at the Golgi.

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'-GGCCACAGCACTTAAACACA-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'-GCCTCACTCCTTTTGCAGAC-3'
			GAPDH.R (4128)	5'-GTCTTCTGGGTGGCAGTGAT-3'
nDNA: *TP53* (L)	3075	1.649	*TP53*.F	5'-CATAACCGCAAATGGGAAAC-3'
			TP53.R (3075)	5'-CGGGACGTGAAAGGTTAGAA-3'
nDNA: *TP53* (S)	45	1.991	*TP53*.F	5'-CATAACCGCAAATGGGAAAC-3'
			TP53.R (45)	5'-CGTCCTTTTGATGGCCTTT-3'

Table S3: Primers used in qRT-PCR experiments.

ACTB.F	5'-CATGTACGTTGCTATCCAGGC-3'
ACTB.R	5'-CTCCTTAATGTCACGCACGAT-3'
ALAS1.F	5'-CGCCGCTGCCCATTCTTAT-3'
ALAS1.R	5'-TCTGTTGGACCTTGGCCTTAG-3'
CAT.F	5'-TGTTGCTGGAGAATCGGGTTC-3'
CAT.R	5'-TCCCAGTTACCATCTTCTGTGTA-3'
GAPDH.F	5'-GGAGCGAGATCCCTCCAAAAT-3'
GAPDH.R	5'-GGCTGTTGTCATACTTCTCATGG-3'
GCLC.F	5'-GGAGGAAACCAAGCGCCAT-3'
GCLC.R	5'-CTTGACGGCGTGGTAGATGT-3'
GCLM.F	5'-TGTCTTGGAATGCACTGTATCTC-3'
GCLM.R	5'-CCCAGTAAGGCTGTAAATGCTC-3'
GDF3.F	5'-GCCATCAAAGAAAGGGAACA-3'
GDF3.R	5'-TCTGGCACAGGTGTCTTCAG-3'
GPX1.F	5'-CAGTCGGTGTATGCCTTCTCG-3'
GPX1.R	5'-GAGGGACGCCACATTCTCG-3'
GPX2.F	5'-GGTAGATTTCAATACGTTCCGGG-3'
GPX2.R	5'-TGACAGTTCTCCTGATGTCCAAA-3'
GPX3.F	5'-AGAGCCGGGGACAAGAGAA-3'
GPX3.R	5'-ATTTGCCAGCATACTGCTTGA-3'
GPX4.F	5'-GAGGCAAGACCGAAGTAAACTAC-3'
GPX4.R	5'-CCGAACTGGTTACACGGGAA-3'
GPX7.F	5'-CCCACCACTTTAACGTGCTC-3'
GPX7.R	5'-GGCAAAGCTCTCAATCTCCTT-3'
GSR.F	5'-TTCCAGAATACCAACGTCAAAGG-3'
GSR.R	5'-GTTTTCGGCCAGCAGCTATTG-3'
GSTA1.F	5'-CTGCCCGTATGTCCACCTG-3'
GSTA1.R	5'-AGCTCCTCGACGTAGTAGAGA-3'
GSTA2.F	5'-TACTCCAATATACGGGGCAGAA-3'
GSTA2.R	5'-TCCTCAGGTTGACTAAAGGGC-3'
GSTA4.F	5'-CCGGATGGAGTCCGTGAGAT-3'
GSTA4.R	5'-GGGCACTTGTTGGAACAGC-3'
GSTM1.F	5'-TCTGCCCTACTTGATTGATGGG-3'
GSTM1.R	5'-TCCACACGAATCTTCTCCTCT-3'
GSTM2.F	5'-TGTGCGGGGAATCAGAAAAGG-3'
GSTM2.R	5'-CTGGGTCATAGCAGAGTTTGG-3'
GSTM3.F	5'-TCGTGCGAGTCGTCTATGGT-3'
GSTM3.R	5'-TCTCCTCATAAGAGGTATCCGTG-3'
GSTM4.F	5'-AGAGGAGAAGATTCGTGTGGA-3'
GSTM4.R	5'-TGCTGCATCATTGTAGGAAGTT-3'
GSTM5.F	5'-CCATCCTGCGCTACATTGC-3'
GSTM5.R	5'-CCAGCTCCATGTGGTTATCCAT-3'
GSTO1.F	5'-GAACGGCTGGAAGCAATGAAG-3'
GSTO1.R	5'-TGCCATCCACAGTTTCAGTTT-3'

GSTO2.F	5'-TGCCCCTATTCTCACAGGACC-3'
GSTO2.R	5'-TCCAGGTACTCACAAGCAATAAC-3'
GSTT1.F	5'-TGCCGCGCTGTTTACATCTT-3'
GSTT1.R	5'-GTGCTGACCTTTAATCAGATCCA-3'
GSTT2.F	5'-TGGCATCCCCTTAGAGCTG-3'
GSTT2.R	5'-CTTGAGCGTCGGCAGTTTC-3'
GSTZ1.F	5'-GCCCAGAACGCCATCACTT-3'
GSTZ1.R	5'-CTACACAGTATATGCCCGCTG-3'
MGST1.F	5'-ATGACAGAGTAGAACGTGTACGC-3'
MGST1.R	5'-TACAGGAGGCCAATTCCAAGA-3'
MGST2.F	5'-TCGGCCTGTCAGCAAAGTTAT-3'
MGST2.R	5'-TGCCCGAAATACTCTCTCAAAC-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'-GAAGCTGTCGGACTACAAAGG-3'
PRDX2.R	5'-TCGGTGGGGCACACAAAAG-3'
PRDX3.F	5'-GAGACTACGGTGTGCTGTTAGA-3'
PRDX3.R	5'-GTTGACGCTCAAATGCTTGATG-3'
PRDX4.F	5'-AGAGGAGTGCCACTTCTACG-3'
PRDX4.R	5'-GGAAATCTTCGCTTTGCTTAGGT-3'
PRDX6.F	5'-GTTGCCACCCCAGTTGATTG-3'
PRDX6.R	5'-TGAAGACTCCTTTCGGGAAAAGT-3'
SOD1.F	5'-GGTGGGCCAAAGGATGAAGAG-3'
SOD1.R	5'-CCACAAGCCAAACGACTTCC-3'
SOD2.F	5'-GCTCCGGTTTTGGGGTATCTG-3'
SOD2.R	5'-GCGTTGATGTGAGGTTCCAG-3'
SOD3.F	5'-ATGCTGGCGCTACTGTGTTC-3'
SOD3.R	5'-CTCCGCCGAGTCAGAGTTG-3'
TMX1.F	5'-AGTATGTCAGCACTCTTTCAGC-3'
TMX1.R	5'-CACACTGGCAATCCAAGGTCT-3'
TXN.F	5'-GTGAAGCAGATCGAGAGCAAG-3'
TXN.R	5'-CGTGGCTGAGAAGTCAACTACTA-3'
TXNIP.F	5'-GGTCTTTAACGACCCTGAAAAGG-3'
TXNIP.R	5'-ACACGAGTAACTTCACACACCT-3'

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AUTHOR'S VIEW

Genome surveillance in pluripotent stem cells: Low apoptosis threshold and efficient antioxidant defense

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ABSTRACT
Pluripotent stem cells must be endowed with efficient genome surveillance. Here we describe the multiple mechanisms that ensure their genome integrity, including high susceptibility to apoptosis and efficient prevention of DNA lesions. In induced pluripotent stem cells, apoptosis hypersensitivity is mediated by increased expression of proapoptotic BCL-2 protein, whereas DNA damage is prevented by the upregulation of several antioxidant enzymes. Antioxidants might be therefore employed for safer stem cell therapies.

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Antioxidant; apoptosis; Bcl-2; DNA damage; genome surveillance; glutathione; LORD-Q; oxidative stress; pluripotent stem cell

Pluripotent stem cells have the capacity to self-renew and to differentiate into all cell types of the organism. Therefore, maintenance of their genomic stability must be stringently controlled, as any genetic alteration could impair their functionality and tissue renewal. Mutations can also favor uncontrolled proliferation or predispose cells to further mutations associated with cancer development. In fact, there is evidence that embryonic and induced pluripotent stem cells (iPSCs) have enhanced tumorigenic potential and share several features with cancer cells.[1] Because they originate from differentiated somatic cells, potential genomic instability and tumorigenicity are relevant concerns for iPSCs. A better understanding of genomic surveillance mechanisms is therefore crucial with regard to future therapeutic applications of iPSCs.

To date, the mechanisms that maintain genomic integrity in human iPSCs have been largely elusive. We and others recently found that genome surveillance in pluripotent stem cells is basically achieved by 2 mechanisms, namely a very low accumulation of DNA lesions and a hypersensitivity to apoptosis, which enables rapid removal of cells once DNA damage has occurred.[2,3] Compared to differentiated cells such as fibroblasts, iPSCs were found to be exceptionally sensitive to apoptosis and readily died even after exposure to low-damage doses of genotoxic agents. Unlike human embryonic stem cells,[4] in iPSCs this apoptosis hypersensitivity is not restricted to genotoxic insults, but is also observed after treatment with agents causing Golgi or ER stress. Interestingly, as a result of the low expression level of death receptors iPSCs are largely resistant to stimuli activating the extrinsic apoptosis pathway. Thus, iPSCs display a selective sensitivity to the mitochondrial death pathway.

To explore the mechanisms underlying this apoptosis-prone state of iPSCs, we investigated the expression of various apoptosis regulators. Transcript levels of members of the inhibitor-of-apoptosis protein family, including *XIAP*, *BIRC2,* and particularly *BIRC3*, were considerably reduced in iPSCs compared to fibroblasts. Human embryonic stem cells have been previously reported to be primed for apoptosis by the presence of constitutively active BAX at the Golgi that can rapidly translocate to mitochondria upon genotoxic insults.[4] In human iPSCs, however, BAX is evenly distributed in the cytosol but not localized at the Golgi.[2] Nevertheless, in line with constitutive expression of p53 (TP53), mRNA expression of proapoptotic p53 target genes of the BCL-2 family including *BAK1*, *BIM* (*BCL2L11*), and *NOXA* (*PMAIP1*) is strongly upregulated, whereas the levels of several antiapoptotic regulators including *BCL2*, *BCLX* (*BCL2L1*), *BCLW* (*BCL2L2*), and *BCL2A1* are reduced. Thus, iPSCs reveal increased mitochondrial priming and a strong p53 response, resulting in a shift of the balance of from anti- to proapoptotic BCL-2 proteins.

Even the reprogramming of somatic cells to iPSCs is thought to cause DNA damage, which might explain why the absence of p53 improves reprogramming efficiency. Other sources of DNA damage include replication stress or reactive oxygen species (ROS) generated during mitochondrial respiration.[5] To assess DNA damage in iPSCs we employed the highly sensitive LORD-Q (long-run real-time PCR-based DNA-damage quantification) method, which detects gene locus-specific DNA lesions of both the mitochondrial and nuclear genome.[6] Interestingly, upon exposure to various genotoxic conditions, the accumulation of nuclear and mitochondrial (mt) DNA lesions was significantly lower in iPSCs than in fibroblasts.[2] Moreover, comparison with a large panel of tumor cell lines revealed less frequent DNA damage in iPSCs than in transformed cells. Remarkably, when undifferentiated and differentiated iPSCs were exposed to genotoxins, differentiated iPSCs clearly

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displayed enhanced DNA damage compared to their undifferentiated counterparts, suggesting that protection of pluripotent stem cells against DNA damage is rapidly lost upon differentiation.[2]

The reduced DNA damage in iPSCs could be mediated by increased expression of DNA repair genes. 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. Indeed, measurement of the levels of glutathione (GSH), the most important cellular antioxidant, revealed 3- to 4-fold elevated levels of GSH in iPSCs compared to fibroblasts. Notably, the increase in ROS levels in fibroblasts was up to 10-fold higher, suggesting that oxidative stress is efficiently prevented in iPSCs.

In addition to GSH, iPSCs upregulate the expression of several antioxidant enzymes. For instance, we found that several glutathione S-transferases (GSTs), which act as antioxidant and detoxifying enzymes, were upregulated in iPSCs compared to their somatic precursor cells.[2] Most prominent was *GSTA2*, transcript levels of which were more than 80,000-fold higher in iPSCs than in primary fibroblasts. In addition, iPSCs revealed more than 10,000-fold higher mRNA expression of glutathione peroxidase 2 (*GPX2*). Furthermore, expression of several peroxiredoxins, which scavenge ROS and organic hydroperoxides, and of glutathione reductase was considerably elevated, further supporting the potent antioxidant status of iPSCs.

In GSH-depleted cells knockdown of *GPX2*, but not of *GSTA2*, rendered the cells significantly more vulnerable to DNA damage following hydrogen peroxide exposure.[2] *Vice versa*, ectopic overexpression of GPX2 in fibroblasts was sufficient to confer DNA protection. Interestingly, the expression of GPX2 has been implicated in the proliferation and self-renewal capacity of gastrointestinal crypt stem cells and malignant epithelial cells.[7] *GPX2* expression is largely controlled by NRF2, an antioxidant transcription factor, which can considerably improve the self-renewal capacity of stem cells.[7,8] It is thus conceivable that the strong antioxidant defense is not only involved in genome surveillance, but also required for self-renewal and delayed differentiation of iPSCs.

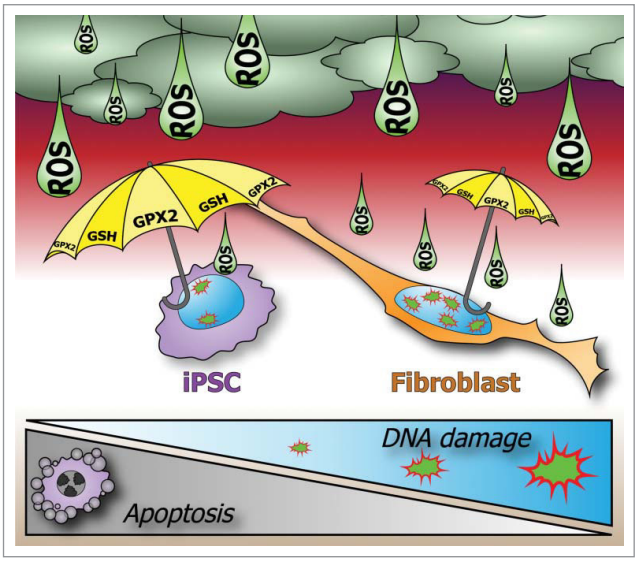

Figure 1. Maintenance of genome integrity in pluripotent stem cells. Pluripotency requires strict genome surveillance to prevent transmission of mutations. Induced pluripotent stem cells (iPSCs) maintain genomic integrity through hypersensitivity to apoptosis and strong protection from DNA damage. Unlike fibroblasts, iPSCs upregulate glutathione peroxidase-2 (GPX2) and glutathione (GSH), which scavenge reactive oxygen species (ROS) and prevent DNA damage.

Several lines of evidence suggest that the occurrence of mtDNA lesions in particular must be prevented for the maintenance of pluripotency. This is for exemplified in mice with defective proof-reading by DNA polymerase γ; these mice exhibit not only an accumulation of mtDNA mutations, but also demonstrate stem cell exhaustion and premature aging.[9] Moreover, iPSCs reveal reduced mitochondrial biogenesis and appear to rely more on anaerobic, rather than aerobic, mitochondrial respiration.[10] Thus, stem cells maintain low ROS levels not only by their high antioxidant activity, but also by reduced oxygen consumption.

In conclusion, studies by us and others suggest that pluripotent stem cells are able to defend their genomic integrity through their exceptional hypersensitivity to apoptosis as well as by maintaining low ROS levels to prevent DNA damage (Fig. 1). Antioxidant supplementation might therefore improve the safety of iPSCs for future therapeutic applications.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Simultaneous quantification of DNA damage and mitochondrial copy number by long-run DNA-damage quantification (LORD-Q)

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ABSTRACT

DNA damage and changes in the mitochondrial DNA content have been implicated in ageing and cancer development. To prevent genomic instability and tumorigenesis, cells must maintain the integrity of their nuclear and mitochondrial DNA. Advances in the research of DNA damage protection and genomic stability, however, also depend on the availability of techniques that can reliably quantify alterations of mitochondrial DNA copy numbers and DNA lesions in an accurate high-throughput manner. Unfortunately, no such method has been established yet. Here, we describe the high-sensitivity long-run real-time PCR technique for DNA-damage quantification (LORD-Q) and its suitability to simultaneously measure DNA damage rates and mitochondrial DNA copy numbers in cultured cells and tissue samples. Using the LORD-Q multiplex assay, we exemplarily show that the mitochondrial DNA content does not directly affect DNA damage susceptibility, but influences the efficacy of certain anticancer drugs. Hence, LORD-Q provides a fast and precise method to assess DNA lesions, DNA repair and mtDNA replication as well as their role in a variety of pathological settings.

INTRODUCTION

Human cells possess hundreds of mitochondria containing a circular genome of approximately 16 kilobase (kb) pairs. Although most mitochondria harbor several copies of mitochondrial DNA (mtDNA), replication can be highly variable and regulated in a cell type-specific manner. Moreover, mtDNA is highly susceptible to mutation, due to the continuous production of reactive oxygen species (ROS) during respiration, which cause oxidative lesions in the mtDNA [1]. Additionally, the DNA repair capacity for mtDNA is lower than that for nuclear DNA. Maintaining the integrity of mtDNA, however, is of utmost importance, since failures to properly repair mtDNA damage have been linked to ageing and a variety of human diseases [2]. Several studies have associated altered mtDNA copy numbers (mtDNAcn) with various types of cancer as well as with anti-cancer drug resistance [3–5]. Interestingly, the mtDNAcn has been proposed as a prognostic factor in lung cancer and also influences the risk of various other tumors, such as lymphomas or soft tissue sarcomas [6, 7]. Besides cancer, neurodegenerative diseases and depressive disorders have been associated with altered mtDNA content and DNA damage [8]. Furthermore, mtDNAcn alterations have been linked with impaired female fertility and childhood autism [9–11].

The growing interest in genotoxicity testing and research of mitochondrial dysfunctions in human disease has created an increasing demand for fast, robust and quantitative analytical methods for the assessment of DNA damage and mtDNA content. Unfortunately, most common methods are slow and cumbersome or have considerable limitations, as they are unsuitable for the sequence-specific detection of DNA lesions. Many methods to quantify DNA

damage also depend on large amounts of sample DNA or labor-intensive normalization procedures, making these methods inapplicable for high-throughput analyses. Recently, we developed the long-run real-time PCR-based DNA damage quantification (LORD-Q) method [12]. LORD-Q is a PCR-based assay that relies on the principle that DNA lesions stall DNA polymerase, resulting in a decrease in the amount of PCR product. There are several advantages of using the LORD-Q assay. First, the assay is highly sensitive and sequence-specific, because it is primer-based and allows analysis of any gene *locus* in both the mitochondrial and nuclear genome. Moreover, LORD-Q needs very little sample DNA and enables the high-throughput quantification of DNA damage in long DNA templates of distinct gene *loci* of > 3 kb.

In the present study, we describe an optimized flexible LORD-Q procedure that can be used to simultaneously determine the number of DNA lesions and mtDNAcn. Using this adapted LORD-Q assay we investigated the relationship between mtDNAcn alterations and DNA damage in human cells following exposure to different genotoxic insults. Furthermore, we show that LORD-Q is capable to detect both DNA damage and mtDNAcn changes in tissue samples. Thereby, LORD-Q is not only suitable for genotoxicity testing and assessment of DNA repair processes, but also useful to assess pathological processes or drug actions that differentially affect copy number and lesions of mitochondrial or nuclear genomes.

RESULTS AND DISCUSSION

LORD-Q allows the simultaneous measurement of nuclear and mitochondrial DNA damage and mitochondrial DNA copy number

Due to an established role of mitochondrial dysfunction in aging, cancer and various other diseases as well as the recognized association between mtDNA damage and replication, it is important to elucidate the mechanisms underlying the maintenance of mtDNA integrity and mtDNA synthesis. Thus, development of methods to gene-specifically quantify DNA damage and the mtDNA copy number (mtDNAcn) might help to elucidate disease mechanisms and to provide targets for clinical interventions. So far, however, most conventional methods to detect DNA damage are of low sensitivity or detect DNA lesions in a global and sequence-independent manner.

We recently developed the LORD-Q assay, which allows the accurate quantification of DNA damage in distinct gene *loci* for the high-throughput assessment of DNA repair processes, genotoxicity testing and many other applications [12]. By using a novel rapid high-fidelity DNA polymerase, a second-generation fluorescent DNA dye, and by optimizing the PCR parameters, we were able to considerably increase the sensitivity of DNA damage detection and to establish a protocol for the quantification of DNA lesions in mitochondrial and nuclear probes of up to 4 kb length.

The present study describes an improved LORD-Q multiplex assay, which allows the simultaneous measurement of mtDNAcn together with the sequence-specific quantification of mitochondrial and nuclear DNA damage. The LORD-Q assay is based on the principle that DNA damage impedes DNA polymerase in the PCR reaction, resulting in decreased amounts of PCR product (Figure 1). For the assay, two fragments of different length are amplified in a real-time PCR reaction. A long DNA fragment of 3–4 kb from mitochondrial or nuclear DNA serves as experimental probe to detect DNA lesions. Since PCR amplification of this DNA template is inhibited by DNA lesions, the amount of the PCR amplification is inversely proportional to the amount of DNA damage. Assuming that DNA lesions are roughly distributed randomly and that each DNA base can be either damaged or undamaged, the formula used to calculate the DNA lesion rate can be derived from a Bernoulli equation. In order to calculate the probability of a single base being damaged (x), an undamaged reference is required. Because the probability of a DNA lesion to occur is proportional to the length of the fragment, a short fragment of ~ 50 to 70 bp, is assumed to be undamaged and serves as a normalization control.

$$\binom{Length}{0}(1-x)^{Length} = (1-x)^{Length}$$

Subsequent data analysis is based on the measured crossing point (Cp) values for the long and the small fragment. Thus, the Cp values for amplification of the short (S) and the long fragment (L) of each sample, the amplification efficiencies (E) of the corresponding primer pairs and the amplicon length in bp of the long fragment allow the calculation of the average number of DNA lesions per 10 kb in the respective sample. An additional undamaged control sample serves as reference (Ref) for normalization of the results.

$$\frac{\text{Lesions}}{10kb} = \left[\left(\frac{\frac{E_L^{Cp_L}}{E_S^{Cp_S}}}{\left(\frac{E_L^{Cp_L}}{E_S^{Cp_S}}\right)_{Ref}} \right)^{\left(\frac{1}{length_L}\right)} - 1 \right] \times 10\,000$$

In the present study we extended the LORD-Q method to further investigate the link between DNA damage and mtDNAcn. If the LORD-Q assay is performed for a *locus* of both the mitochondrial and nuclear DNA, the shift between the exponential phases of the short fragment amplifications corresponds to the difference of mitochondrial and nuclear genome copies. Therefore, C_p

values of the short reference fragments can be utilized to calculate mtDNAcn, provided that the ploidy p of the relevant gene in the sample is known.

$$mtDNAcn = p \times 2^{(Cp_{S(n)} - Cp_{S(mt)})}$$

Mitochondrial DNA copy number correlates with mitochondrial DNA damage

Oxidative stress is a continuous threat to the integrity of mtDNA. ROS trigger mtDNA damage that in a vicious circle can further enhance ROS generation [2, 13]. Moreover, elevated ROS levels induce replication of mtDNA [14], suggesting a protection-by-abundance mechanism that compensates for defects in mitochondria with damaged mtDNA. Therefore, we investigated a potential correlation of the copy number of mtDNA with DNA damage rates.

We first employed the LORD-Q method to analyze samples from human dermal fibroblasts (HDFs) and 9 cancer cell lines of the NCI60 panel representing different tumor entities. When cells were irradiated with UVC light to induce DNA damage, we generally detected less mtDNA damage in cells with higher mtDNAcn as compared to cells with less mtDNA copies (Figure 2A). For instance, HepG2 hepatoma cells, which contain almost 6000 mtDNA copies per cell, revealed the lowest number of mtDNA lesions, whereas U0-31 renal carcinoma cells with the lowest mtDNAcn were significantly more vulnerable to UVC-induced mtDNA damage. Hence, in this setting UVC-induced mtDNA damage appears to be inversely correlated with the number of mtDNA copies.

Several mechanisms are crucial for the maintenance of DNA integrity, including efficient DNA repair systems and high levels of antioxidants that inhibit genotoxic ROS formation [15–17]. To prevent harmful mutations, the maintenance of genomic stability must be very stringent. Pluripotent stem cells are particularly resistant to DNA damage due to their high levels of glutathione and strong expression of antioxidant enzymes, such as glutathione peroxidase-2 [18, 19]. We therefore compared the occurrence of mtDNA lesions in HDFs and human induced pluripotent stem cells (HiPSCs) that were either naïve or induced to undergo undirected differentiation by culture in the absence of FGF2. Indeed, a short-term exposure to hydrogen peroxide induced significantly less mtDNA damage in naïve HiPSCs compared to differentiated HiPSCs or HDFs (Figure 2B). In line with their reduced mitochondrial biogenesis [19], naïve HiPSCs contained lower mtDNAcn than differentiated HiPSCs or HDFs (Figure 2C). Thus, in contrast to the results described above in UV-irradiated cancer cells, in these cells the occurrence of oxidative stress-induced mtDNA damage appeared to correlate with mtDNAcn.

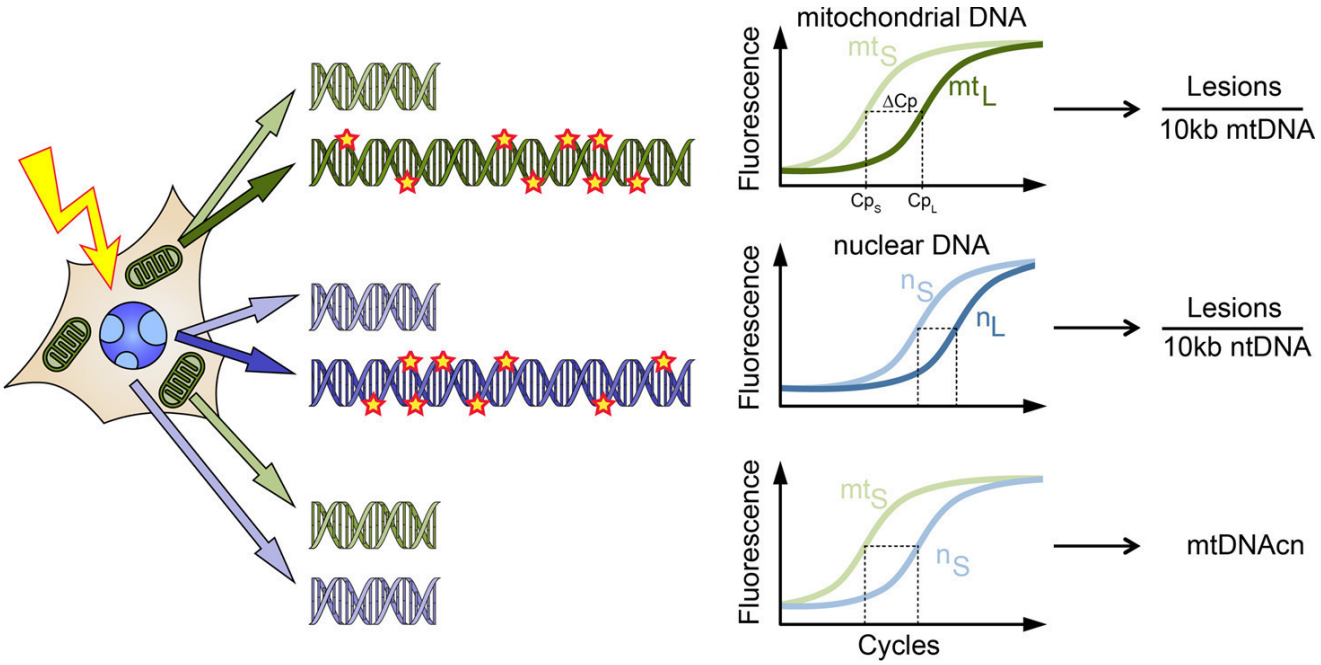

Figure 1: Schematic illustration of the LORD-Q assay allowing the simultaneous quantification of lesions in long DNA probes of mitochondrial (mt) and nuclear (n) genomes and the mtDNA copy number (mtDNAcn). Following treatment, e.g. with genotoxic agents, whole-cell DNA is isolated. The PCR amplification of long (L) template DNA sequences of 3–4 kb is inhibited by polymerase-stalling DNA lesions, resulting in a delayed exponential phase in the real-time PCR, which can be detected by the fluorescence signal of the DNA dye ResoLight. In contrast, short (S) probes of 40–70 bp, which serve as reference templates, are assumed to remain undamaged and are therefore amplified normally. Thereby, the average occurrence of DNA lesions can be calculated. Additionally, mtDNAcn can be calculated from the difference in Cp values of the short fragments of mitochondrial and nuclear DNA, taking the ploidy of the respective sample into account.

To further examine the relationship between mtDNAcn and DNA damage susceptibility, we depleted Jurkat cells of mtDNA (Figure 2D). Generation of mtDNA-depleted pseudo-ρ^0 cells was accomplished either by incubation of cells with 2′,3′-dideoxycytidine (ddC), which is converted to the DNA polymerase γ inhibitor ddCTP, or by incubation with ethidium bromide, which intercalates into mtDNA and thereby inhibits mtDNA replication and transcription. Incubation of cells with either of these agents for 7 days resulted in a strong dose- and time-dependent depletion of mtDNA (Supplementary Figure 1). The depletion of mtDNA was associated with a drop of mitochondrial mass by 38%, as determined by flow cytometric staining with MitoTracker Green FM (Supplementary Figure 1). Importantly, when cells were treated with the chemotherapeutic drug bleomycin, Jurkat wild-type cells displayed increased ROS production and significantly more mtDNA damage compared to their mtDNA-depleted counterparts (Figure 2E, 2F). Intriguingly, the damage of nuclear DNA of pseudo-ρ^0 cells was comparable to wild-type cells (Figure 2G). In line with previous studies [20], these results suggest that bleomycin preferentially induces mitochondrial rather than nuclear DNA lesions and that depletion of mtDNA can confer drug resistance. Moreover, bleomycin-induced ROS production appeared to correlate with the copy number and damage of the mtDNA. Whether mtDNAcn and mtDNA damage are causally linked to the formation and therapy resistance of tumors is still under debate and remains to be investigated in more detail in future studies. Certainly, LORD-Q will be a powerful method to provide further insight into these processes.

Efficient analysis of tissue samples by LORD-Q

Traditional methods to determine DNA damage often require large quantities of sample DNA. The resulting need for large amounts of tissue limits the possibility to assess DNA damage in smaller organisms without pooling samples from several individuals. The requirement of large amounts of DNA also restricts analysis of clinical samples with limited availability. So far, the suitability of LORD-Q has only been tested in DNA samples derived from human cell culture [12]. As LORD-Q requires only nanogram quantities of isolated DNA, we tested its applicability for the simultaneous analysis of mtDNA damage and copy number in murine tissue samples. For the animal experiments we chose γ-irradiation as a genotoxic stimulus, as it was reported to induce DNA damage and changes in mtDNAcn [21, 22].

We γ-irradiated mice with 3 Gy or 6 Gy and isolated samples of whole-body irradiated and control mice from several organs that differ in their radiation sensitivity, i.e. brain, spleen, liver and bone marrow. To account for different DNA repair capacities, the tissue samples were isolated from the mice 3 and 24 hours post-irradiation. After excision of the organs and isolation of bone marrow, we analyzed DNA damage in mitochondrial and nuclear DNA and determined mtDNAcn using the LORD-Q assay.

Different organs of the irradiated mice exhibited different levels of DNA damage compared to samples from non-irradiated animals. In brain tissue, mtDNA showed a dose-dependent increase of 2–3 lesions per 10 kb 3 hours post-irradiation, which were mostly repaired within 24 hours (Figure 3A). Nuclear DNA of brain tissue displayed higher lesion rates (4 – 5 per 10 kb) as compared to mtDNA. The mtDNAcn in irradiated brain cells showed no significant change within the first 3 hours post-treatment, however, 24 hours after irradiation mtDNAcn was increased more than 2-fold as compared to untreated control cells (Figure 3A).

Compared to brain, we detected very low mtDNA lesion rates in spleen, whereas nuclear DNA damage was comparable in both tissues (Figure 3B). Nuclear DNA lesions in spleen, however, seemed to be more efficiently repaired compared to brain samples. The copy numbers of mtDNA showed an initial decrease 3 hours post-irradiation and increased in a dose-dependent manner during 24 hours post-irradiation (Figure 3B). Higher doses of ionizing radiation and DNA damage clearly resulted in enhanced replication of mtDNA.

Liver tissue showed comparably high lesion rates in mtDNA 3 hours post-treatment, which were efficiently repaired within 24 hours (Figure 3C). Nuclear DNA damage levels were slightly higher than in mtDNA, but comparable to nuclear DNA damage in the brain. The copy numbers of mtDNA initially decreased, but became elevated up to 3-fold after 24 hours (Figure 3C). Bone marrow accumulated relatively few mtDNA lesions, and also nuclear DNA appeared less vulnerable compared to other tissues (Figure 3D). Following an initial decay, mtDNAcn in bone marrow were restored within 24 hours. Thus, LORD-Q demonstrates a dose-dependent increase of lesions in both the mitochondrial and nuclear DNA. After 24 hours DNA lesion rates in all samples were only marginally higher compared to the untreated controls, reflecting an efficient DNA repair.

Altogether, this study demonstrates a high suitability of LORD-Q for the simultaneous quantification of DNA damage and mtDNAcn in cultured cells as well as in tissue samples. Both DNA damage and mtDNAcn alterations have been associated with cancer formation, altered tumor metabolism and resistance to anticancer drugs. Hence, LORD-Q provides a valuable tool for the evaluation of new chemotherapeutic drugs targeting these parameters. Abnormalities of mtDNAcn have been also proposed as a prognostic marker in certain tumors, which could be conveniently analyzed in more detail using the LORD-Q method. In addition, the fields of ageing research and reproduction medicine might benefit from a sensitive assay capable of simultaneously measuring DNA damage

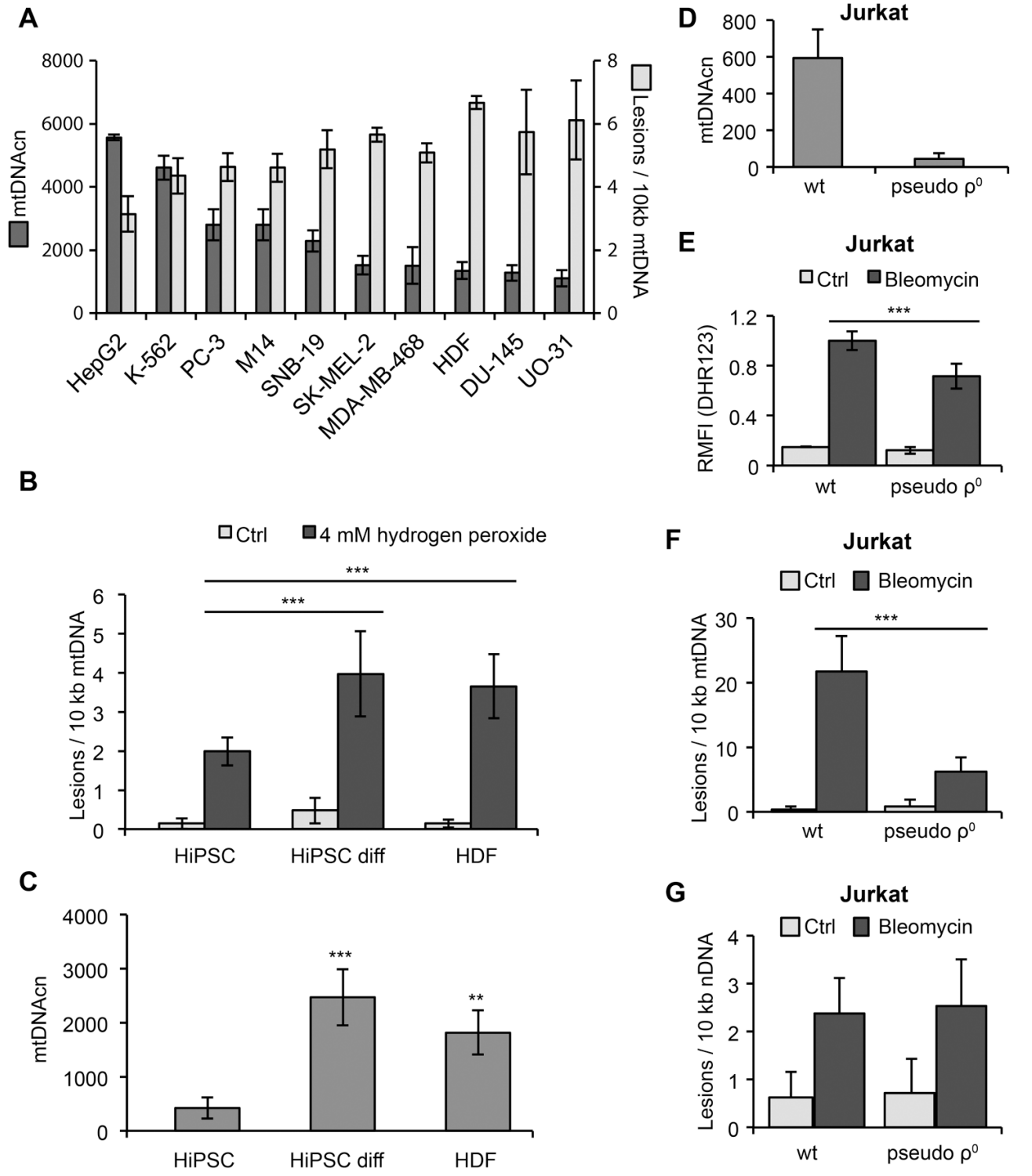

Figure 2: Correlation of mtDNA damage and copy number. (**A**) Human dermal fibroblasts (HDF) and 9 different cancer cell lines from the NCI60 panel were irradiated with 10 mJ/cm^2 UVC light and harvested immediately after irradiation. Damage of mtDNA and mtDNAcn were determined by LORD-Q analysis. (**B**) HiPSCs are less vulnerable to mtDNA damage than progenitor HDFs or differentiated HiPSCs after treatment with hydrogen peroxide for 5 min. (**C**) Reduced mtDNAcn of HiPSCs compared to HDFs and differentiated HiPSCs. (**D**) Depletion of mtDNA in Jurkat T cells cultured in the presence of 100 ng/mL ethidium bromide for 7 days. (**E**) Detection of ROS in wild-type (wt) and pseudo-ρ^0 Jurkat cells following 1 hour of treatment with 100 µM bleomycin. The results were normalized to bleomycin-treated wild-type cells and indicate the relative median fluorescence intensity (RMFI) after staining with dihydrorhodamine 123 (DHR123). (**F**) Reduced mtDNA lesions in Jurkat pseudo-ρ^0 cells compared to wild-type cells following 20 min of treatment with 100 µM bleomycin. (**G**) Lesion rates in nuclear DNA of Jurkat wild-type and pseudo-ρ^0 cells following 20 min of treatment with 100 µM bleomycin do not significantly differ.

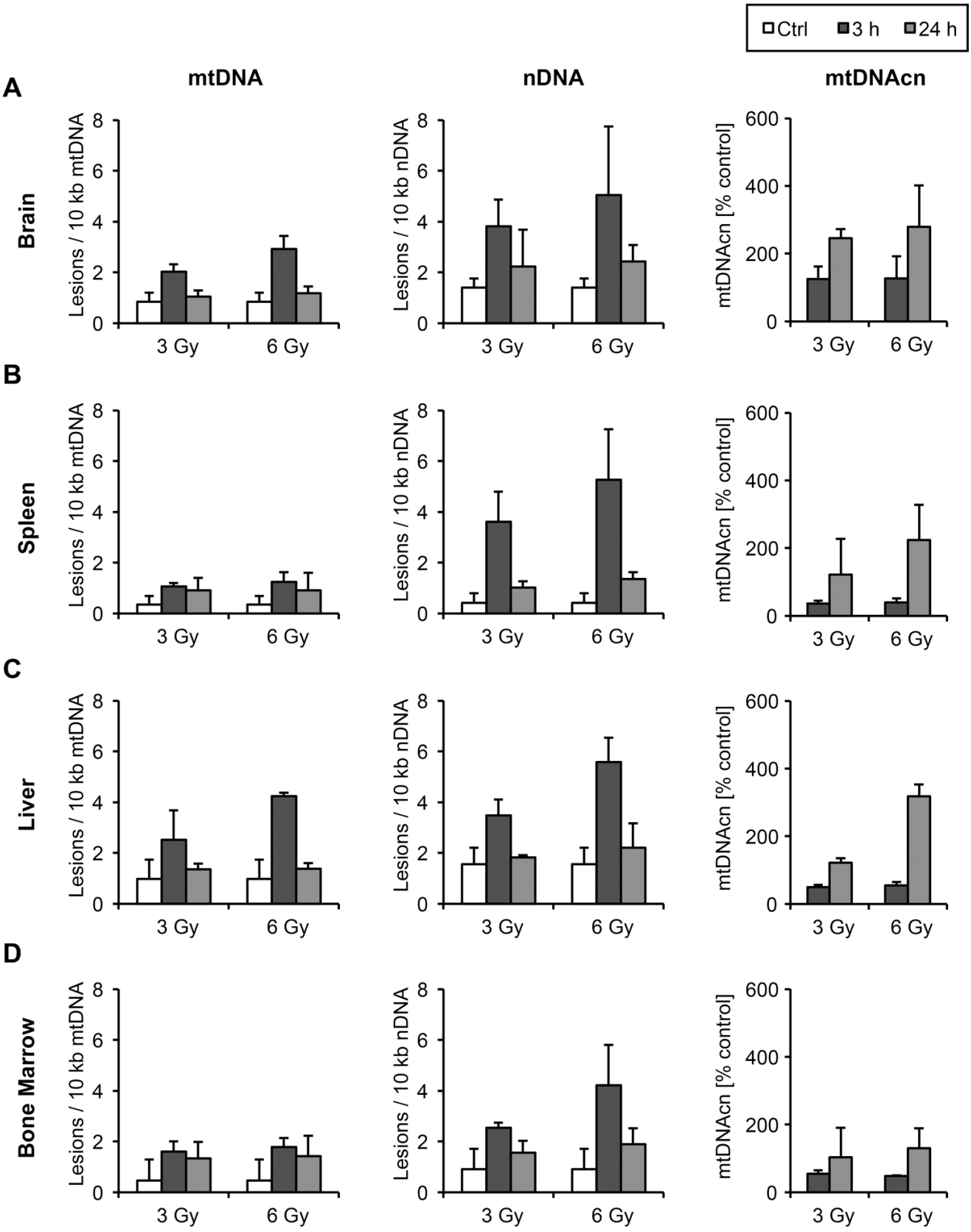

Figure 3: Mitochondrial and nuclear DNA damage and mtDNAcn in mice following ionizing irradiation. Mice were exposed to 3 Gy and 6 Gy of ionizing irradiation, before mitochondrial and nuclear DNA lesions as well as alterations of mtDNAcn were measured by LORD-Q in isolated brain (**A**), spleen (**B**), liver (**C**) and bone marrow (**D**) at 3 and 24 hours post-irradiation ($n \geq 3$). The mtDNAcn is given as the percentage relative to the respective tissue of untreated control mice.

and mtDNA ploidy. Finally, LORD-Q might be a valuable tool for large-scale genotoxicity screening programs, for instance for the European Union REACH regulation, which addresses the impact of chemicals on both human health and the environment. Thus, LORD-Q is a unique technique that enables the sequence-specific quantification of DNA damage and mtDNA copies and thereby allows numerous applications in oncology, toxicology and pharmaceutical research.

MATERIALS AND METHODS

Animal experimentation

Male C57/BL6 mice were used at 6 to 11 weeks of age. Ionizing irradiation was performed using a Philips SL25 linear accelerator (Philips; Amsterdam, The Netherlands) with irradiation rates of 1.3 Gy per minute. Mouse work was performed in accordance with the German law guidelines of animal care, as permitted by regional authorities (Regierungspräsidium Tübingen, no. IB 1/16). Organs were excised at the indicated times post-irradiation and bone marrow was isolated as described [23].

Cell culture and genotoxic treatment

Human Jurkat T lymphocytes were depleted of mtDNA by cultivation of cells in the presence of 2′-3′-dideoxycytidine (ddC) or ethidium bromide [24, 25]. The selection medium was further supplemented with 1 mM sodium pyruvate and 100 µg/mL uridine. Human induced pluripotent stem cells (HiPSCs) were generated by transduction of human dermal fibroblasts (HDFs) with the Yamanaka retroviral cocktail for the expression of OCT4, SOX2, KLF4 and c-MYC reprogramming factors. Cells were cultured and validated for stem cell properties as described [12]. Undirected differentiation of HiPSCs was performed by culture in the presence of FCS and absence of FGF2 for 30 days. Cells were treated with hydrogen peroxide and bleomycin or UV-irradiated as described [18].

DNA isolation

DNA was purified from cultured cells and tissue using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). Tissue lysis in ATL buffer (Qiagen) was carried out for 30 min. DNA concentration was determined spectrometrically using a Nanodrop 1000 photometer (Peqlab, Erlangen, Germany). DNA was diluted to 5 ng/µL with buffer AE prior to use.

Flow cytometry

To measure intracellular ROS levels, cells were stained with 1 µM dihydrorhodamine 123 (Sigma-Aldrich; St. Louis, MO) and analyzed by flow cytometry [18, 26]. Relative cellular content of mitochondria was determined using MitoTracker Green FM (Thermo Fisher, Waltham, MA) essentially as described [27].

LORD-Q assay

Measurement of mitochondrial and nuclear DNA damage was performed by the LORD-Q method as originally described [12]. For each analyzed genomic or mitochondrial gene *locus* a long DNA fragment (~3000 – 4000 bp) was used as sensor for DNA lesions and an internally nested short fragment (~50 – 70 bp) that was considered as undamaged served as reference. The nested intact reference was efficiently PCR-amplified, whereas amplification of the large sensor is inhibited by DNA lesions including abasic sites, thymine dimers, strand breaks and oxidative lesions [12]. Upon induction of DNA damage, the exponential amplification phase for the damage-sensitive large fragment is reached later as compared to the nested intact reference. The difference in crossing point values (Cp) therefore allows calculation of the average incidence of lesions per bp.

Initially, the replication efficiency of each primer pair was determined using a standard dilution of whole-cell DNA as described [12]. Then, real-time PCR was carried out in 96-well or 384-well plates with each well containing a reaction volume of 20 µL and 10 µL, respectively. The reaction mixture contained 2.5 ng/µL isolated sample DNA, 1 x KAPA2G HS Polymerase ReadyMix (Peqlab), 0.0016 x ResoLight dye (Roche, Basel, Switzerland) and 500 nM of HPLC-purified forward and reverse primers (Sigma-Aldrich), respectively.

Cycling conditions were as follows: a pre-incubation phase of 5 min at 95°C was followed by up to 60 cycles of 10 s at 95°C, 10 s at 60°C and 1 s at 72°C for small amplicons or 135 s for large amplicons, respectively. The reactions were carried out in a LightCycler 480 II system (Roche). Samples were measured in triplicates (96-well plates) or quadruplicates (384-well plates). C_p values were calculated using the LightCycler 480 software and replicates were averaged. All primers were designed to match the requirements of LORD-Q and are listed in Supplementary Table 1. For detection of nuclear DNA damage promoter sequences of the human and murine *COL1A1 locus* were analyzed. Data is depicted as mean ± standard deviation from at least three independent experiments.

Author contributions

B.D. and K.S.-O. conceived and designed the study. B.D., K.S.-O and F.E. wrote the paper. B.D. and S.Le. performed the LORD-Q and data analyses. S.Lo. did the mouse work. S.M.H. performed the irradiation.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FUNDING

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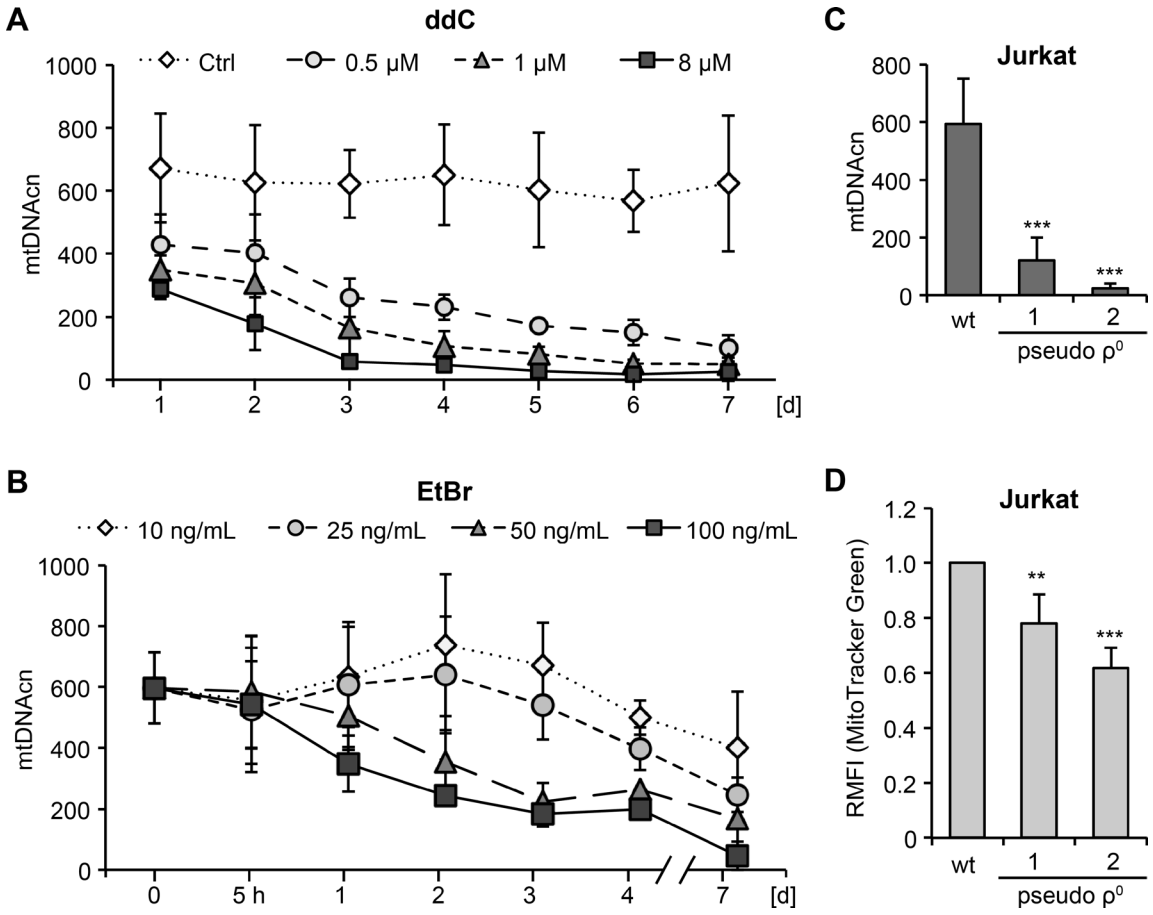
Simultaneous quantification of DNA damage and mitochondrial copy number by long-run DNA-damage quantification (LORD-Q)

SUPPLEMENTARY MATERIALS



Supplementary Figure 1: Depletion of mtDNA in Jurkat T cells. Cells were cultured in the presence of the indicated concentrations of (**A**) dideoxycytidine (ddC) or (**B**) ethidium bromide (EtBr), resulting in an up to 90% reduction of mtDNAcn within the first 3 days. (**C**) mtDNAcn of two pseudo-ρ^0 Jurkat cell lines that were independently generated by incubation with dideoxycytidine or ethidium bromide. (**D**) The Jurkat pseudo-ρ^0 cell clones were incubated with the mitochondria-specific dye MitoTracker Green FM and analyzed by flow cytometry. The reduction of fluorescence intensity indicating a loss of mitochondrial mass correlated with a drop of the mtDNAcn.

Supplementary Table 1: LORD-Q primers applied in DNA damage quantification experiments

Locus	Base pairs	Primer Denotation	Primer Sequence
mtDNA (L) human	3724	H.mtDNA.L.F	5′-ATCGTAGCCTTCTCCACTTC-3′
		H.mtDNA.R	5′-TGGTTAGGCTGGTGTTAGGG-3′
mtDNA (S) human	50	H.mtDNA.S.F	5′-GGCCACAGCACTTAAACACA-3′
		H.mtDNA.R	5′-TGGTTAGGCTGGTGTTAGGG-3′
mtDNA (L) mouse	3921	MM.mtDNA.F	5′-TCCTACTGGTCCGATTCCAC-3′
		MM.mtDNA.L.R	5′-CGGTCTATGGAGGTTTGCAT-3′
mtDNA (S) mouse	74	MM.mtDNA.F	5′-TCCTACTGGTCCGATTCCAC-3′
		MM.mtDNA.S.R	5′-GGCTCCGAGGCAAAGTATAG-3′
COL1A1 (L) human	3578	H.COL1A1.L.F	5′-ATTATCGGGACATCGGTGAA-3′
		H.COL1A1.R	5′-CCACCAAAGCTTTCTTCTGC-3′
COL1A1 (S) human	52	H.COL1A1.S.F	5′-TGCAGGGTGAGAAACATGAC-3′
		H.COL1A1.R	5′-CCACCAAAGCTTTCTTCTGC-3′
Col1a1 (L) murine	2637	MM.col1a1.L1.F	5′-CCGTTTGTCCCATTACTGCT-3′
		MM.col1a1.L1.R	5′-AGCAAGGACGAGGACTTTGA-3′
Col1a1 (S) murine	60	MM.col1a1.S.F	5′-AAAGTGGGAATCTGGACACG-3′
		MM.col1a1.S.R	5′-CAGAGGCCTTATTTCATTTTCG-3′