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Induced Pluripotency by Defined Factors: Prey of Oxidative Stress

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ABSTRACT

Reprogramming somatic cells to pluripotency (induced pluripotent stem cells, iPSCs) via forced expression of defined factors has become one of the most fascinating areas in biomedical research because it holds a tremendous application potential for cell therapy, disease modeling and drug screening applications. However, cellular reprogramming is a very inefficient and metabolically demanding process commonly associated with genomic instability of the resulting iPSCs. Low reprogramming efficiency and presence of *de novo* genomic aberrations in iPSCs may hamper their downstream applications. Here, we review mounting studies that have tackled reprogramming efficiency and genome stability of iPSCs. In particular, we focus on the effect of oxidative stress on cellular reprogramming. We will discuss how oxidative stress influences cellular reprogramming and the mechanisms by which antioxidants promote reprogramming efficiency and preserve genome integrity of iPSCs. A reduction of oxidative stress is expected to augment reprogramming efficiency and concomitantly promote the genomic integrity of the resulting iPSCs, eventually facilitating the implementation of cellular reprogramming for downstream applications.
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INTRODUCTION

Nuclear reprogramming to pluripotent state was first achieved by transfer of the nucleus of a somatic cell into an enucleated egg cell almost two decades ago [1]. While factors exerting the reprogramming effect by nuclear transfer method remain mysterious, the group of Shinya Yamanaka discovered that a set of defined transcription factors (Oct4, Sox2, Klf4 and c-Myc, hereafter referred as OSKM) can remarkably induce somatic cells to become pluripotent stem cells (PSCs), termed iPSCs [2, 3]. This groundbreaking technology marks the advent of a new era in biomedical research and has broad application potential in personalized regenerative medicine, disease modeling as well as drug screening. However, conversion of somatic cells to iPSCs by forced expression of defined factors is a very inefficient process by which a somatic cell has to overcome various barriers to reach the pluripotent state [4].

During embryonic development, a totipotent fertilized oocyte undergoes the first cell fate determination by giving rise to trophoblast and pluripotent cells in the inner cell mass (ICM) of the blastocyst embryo [5]. Cells in the ICM become epiblast after implantation which sequentially differentiates into somatic cells of three germ layers [5]. Unique cellular properties of PSCs such as indefinite self-renewal and pluripotency are orchestrated by sophisticated regulatory network comprising various transcription factors, signaling pathways and epigenetic modifiers [6, 7]. Lineage specification of PSCs is accompanied by progressive silencing of the molecular program operating in PSCs through repressive epigenetic modifications such as DNA methylation and histone deacetylation etc [6, 7]. Thus, reprogramming barriers exist largely due to intrinsic differences between somatic cells and PSCs with respect to epigenetic landscape and signaling pathway engagement. Conversion of somatic cells back to PSCs requires resetting the epigenome of somatic cells to activate the molecular signature unique to PSCs [8]. Indeed, various studies have demonstrated that removal of repressive epigenetic marks robustly boost reprogramming efficiency [9-12]. In addition to epigenetic mechanisms, a number of signaling pathways such as Wnt, GSK3 and MEK are implicated in the maintenance of self-renewal and pluripotency of PSCs and activation of these pathways in the target somatic cell facilitates reprogramming process [13-15]. On the other hand, there are further barriers hampering reprogramming which seem to be associated with detrimental effects directly imposed on the target cells upon forced expression of reprogramming factors. In this review, we will focus on reprogramming barriers arising from forced expression of exogenous factors rather than genomic/epigenomic signatures intrinsically different between somatic cells and PSCs.

Although great attention is given to identification of molecular roadblocks for the purpose of improving reprogramming efficiency, genome integrity of iPSCs has emerged as another controversial aspect underlying reprogramming technology. Genome-wide genomic studies of iPSCs generated by different methods reveal the presence of diverse genomic aberrations including aneuploidy, large-scale sub-chromosomal aberrations, copy number variations (CNVs) and sequence variants (substitutions and indels) [16]. Evidence indicates that both pre-existing mutations in the starting somatic cell population [17, 18] and acquisition of *de novo* mutations during generation and culturing of iPSCs [19-21] contribute to the somatic mutations found in the established iPSCs, suggesting that introduction of reprogramming factors undermines not only reprogramming efficiency, but also genomic stability of iPSCs. Mounting studies have reported elevated levels of reactive oxygen species (ROS) after forced expression of reprogramming factors and the detrimental effect of oxidative stress on reprogramming efficiency and genomic integrity of iPSCs [22-24]. In this review, we focus on oxidative stress as one of the consequences of forced expression of reprogramming factors. We discuss how oxidative stress occurs during reprogramming and the mechanism by which it compromises both efficiency and genome integrity of iPSCs.

Oxidative stress impedes cellular reprogramming

ROS are generated as byproducts of aerobic metabolism in the mitochondria [25]. Oxidative phosphorylation produces ATP to fuel the biological functions of cells via electron transport chain [26]. At the end of mitochondrial respiration, most of oxygen is reduced to become water. However, a small fraction of oxygen is incompletely reduced forming reactive oxygen such as superoxide radical [27]. Under physiological conditions, ROS are scavenged by cell's antioxidant defense system including superoxide dismutase (SOD), catalase and glutathione peroxidase [28]. These enzymes catalyze the conversion of superoxide anion into hydrogen peroxide which is further converted into oxygen and water [29, 30]. However, the equilibrium will be disrupted in certain circumstances such as oncogene activation where ROS generation exceeds the capacity of the cellular antioxidant defense system to detoxify excessive ROS [31]. Activation of oncogenes will robustly accelerate cell proliferation, which imposes great metabolic demand on energy and raw materials for protein, nucleic acid and lipid biosynthesis within the cells. Such a metabolic gear-up may easily increase the engagement of electron transport chain for energy production, which can result in more leakage of electrons into the cytoplasm as ROS. High level of ROS can cause damage to DNA, RNA, proteins and lipids and eventually lead to cellular senescence or death [32].

Interestingly, nuclear reprogramming by defined factors resembles the tumorigenesis process with respect to transformation of somatic cells of finite life span into

immortal iPSCs [33]. Most, if not all, reprogramming factors such as c-Myc and Klf4 are well-known oncogenes [34, 35]. Oct4 and Sox2 also possess oncogenic potential when expressed in the right cellular context [36-38]. Forced expression of Oct4 has been shown to block differentiation and promote cellular dysplasia in epithelial tissues [36], whereas Sox2 is amplified in lung and esophageal squamous-cell carcinomas and plays a role in controlling tumour initiation and cancer stem cell functions [37, 38]. Previous studies have demonstrated that forced expression of reprogramming factors induces DNA damage and p53 activation leading to apoptosis and senescence [39-42]. Importantly, alleviation of apoptosis and senescence by suppressing p53 can robustly increase the reprogramming efficiency, suggesting that they are roadblocks to reprogramming [39-42]. This may be in part due to the excessive production of damaging ROS as a result of reprogramming factors overexpression (Figure 1). In line with this, Esteban and colleagues are the first to report that forced expression of OSK, but not OSKM, elevates ROS levels at early stage of reprogramming of murine cells [22]. Intriguingly, they however found that supplementation of medium with antioxidants such as Vitamin C (Vc) augments the reprogramming efficiency with both OSK and OSKM by alleviating senescence [22]. They suggest that although oxidative damage may contribute to low reprogramming efficiency, the effect of Vc is not exclusively mediated by its antioxidant function. Thus subsequent studies from this group further demonstrated that Vc treatment facilitates the reprogramming of murine cells with OSK by modulating epigenetic modifiers including H3K36 demethylases, Jhdm1a/1b, and TET1 hydroxylase [43, 44]. This underscores a role for Vc in improving the efficiency of murine cell reprogramming beyond its antioxidant function (Figure 1). Despite that the maintenance of human ESCs as well as the in vivo stem cell function of cord blood $CD34^+$ cells have been reported to rely on a mitochondrial response [33, 45], the impact of oxidative stress and antioxidant in human cell reprogramming is less studied. Prigione et al has reported that the mitochondria in the somatic cells revert to an immature state and lower levels of ROS are produced in immature mitochondria as they depend more on anaerobic respiration [46]. Human iPSCs exhibit a lower level of oxidative DNA damage due to less dependence on mitochondrial respiration for energy production [46]. This study suggests that anaerobic metabolism largely independent of mitochondria may promote reprogramming efficiency. In support of this, Yamanaka's group has demonstrated that hypoxia enhances the generation of both mouse and human iPSCs [47]. Furthermore, the group of Adjaye has shown that HIF1αmodulates human cellular reprogramming by promoting glycolytic shift [48]. However, the immediate mitochondrial response has not been examined in the somatic cells upon forced expression of reprogramming factors in these studies. We have recently reported that OSKM elevates ROS level in human fibroblasts and treatment with N-acetyl-cysteine (NAC), a potent antioxidant, reduces ROS level in OSKM- and c-MYC-, but not OSK-, transduced fibroblasts [24]. Unlike the murine system where OSK increases ROS levels, c-MYC is the major factor responsible for the increased ROS levels in the transduced human fibroblasts. The discrepancy could be attributed to differential responses of human and murine cells to exogenous factors. NAC does not increase the efficiency of reprogramming human fibroblasts [24] likely because the commonly used media (knockout serum replacement) for derivation and expansion of iPSCs already contains high-levels of basal antioxidant Vc [22]. However, its effect on oxidative stress remains unclear due to its non-antioxidant functions of Vc. Further research on reprogramming with antioxidant (NAC, Coenzyme Q, etc) treatment or modulation of ROS scavenging enzymes such as SOD, which are thought to quench or detoxify ROS exclusively, in reprogramming culture media lacking antioxidants should address the question. Furthermore, patient-specific iPSCs constitute a unique tool to understand the cellular and molecular mechanisms underlying disease pathogenesis. Thus, the generation and re-differentiation of iPSCs from patients suffering from metabolic diseases, some of which affecting the synthesis of master cellular antioxidants (i.e. Coenzyme Q) [49] should shed light on the role of antioxidants in cell reprogramming and oxidative stress.

Oxidative stress contributes to genomic aberrations in iPSCs

Regardless of the reprogramming methods and combinations of reprogramming factors used for derivation of iPSCs, the presence of various types of genomic aberrations in iPSCs poses a safety and scientific concern on the use of iPSCs for the clinical and research purposes [16]. Although genomic aberrations of iPSCs may have their origins in the pre-existing mutations in the parental cells or be acquired during iPSCs culturing [16, 18, 50, 51, 52], we have reported that reprogramming-induced genomic aberrations constitute a major proportion of the total mutational load observed in iPSCs [18]. This suggests that reprogramming itself is mutagenic, which is likely in part due to the elevated level of ROS as a result of forced expression of the reprogramming factors (Figure 1). Oxidative stress can cause damage to various cellular structures and the DNA is particularly susceptible to oxidative lesions [53]. ROS can result in the modification of individual nucleotide bases (such as the mutagenic 8-oxoguanine), single- and double-strand breaks [31], and telomere attrition [54].

In response to different types of DNA damage, cells have developed multiple repair mechanisms to protect the genome from their deleterious effect [55]. Among various types of genomic lesions, DNA double strand breaks (DSBs) are the most cytotoxic ones. In general, there are two types of DSBs repair pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). While NHEJ involves direct ligation of

the break ends therefore is error-prone, HR requires sister chromatin for exchange and is error-free [56]. Therefore, the choice of DSBs repair pathway influences the genome integrity. The presence of various genomic aberrations in iPSCs suggests that DNA damage occurring during reprogramming is not properly repaired. Blasco's group has provided the first evidence to demonstrate that introduction of reprogramming factors induces DSBs in mouse embryonic fibroblasts (MEFs) and DSBs persist in the resulting iPSCs as shown by the presence of gammaH2AX foci, a marker of DSBs [40]. Mouse iPSCs generated from p53 deficient MEFs retained more DSBs. This suggests that forced expression of reprogramming factors incur DNA damage and engagement of DNA damage response (DDR) mediated by p53 activation helps to maintain the genomic integrity of iPSCs [40]. Müller et al., has also confirmed that forced expression of reprogramming factors induces DNA damage and activates Fanconi anemia (FA) pathway which is necessary for reprogramming [57]. Since viral transgene integration can also cause DNA damage [58], González et al employed an inducible reprogramming system to further confirm that ectopic expression of reprogramming factors causes DNA damage and HR repair pathway is required for reprogramming as deficiency of HR repair pathway impairs reprogramming efficiency [59]. Taken together, it suggests that reprogramming is mutagenic and DNA damage repair pathways are important to mitigate the DNA damage and preserve the genome integrity of iPSCs. We have provided direct evidence indicating that forced expression of reprogramming factors causes DSBs in human cells as shown by gammaH2AX staining [24]. Treatment with antioxidant NAC reduces the number of foci in the cells, supporting that ROS contributes to the formation of DSBs during reprogramming [24]. Furthermore, genotoxicity mediated by ROS is an important promoter of genomic aberrations in iPSCs as our results demonstrate that supplementation with antioxidants NAC and Vc during reprogramming reduces *de novo* CNVs in iPSCs [24] (Figure 1). Importantly, a previous study from Hochedlinger's group reported that Vc improves genomic quality of mouse iPSCs by preventing loss of Dlk1-Dio3 imprinting and facilitating generation of all-iPS cell mice [23]. This improvement is attributed to the reduction in DNA methylation during reprogramming. However, it is possible that Vc treatment may also reduce genomic aberrations in mouse iPSCs which could contribute to the generation of all-iPS cell mice.

Our study has demonstrated that treatment with antioxidants NAC and Vc reduces, but does not eliminate, *de novo* CNVs in iPSCs [24]. Interestingly, the treatment has no effect on point-mutations. Firstly, it suggests that antioxidant supplementation is insufficient to quench ROS levels during reprogramming. Metabolic diversion of mitochondrion-dependent aerobic respiration to anaerobic glycolysis through hypoxia or modulation of HIF1αexpression likely reduces the generation of ROS and minimizes their damaging effect when combined with antioxidant treatment during reprogramming. On the other hand, overexpression of ROS scavenging enzymes such as SOD in the cells together with antioxidant supplementation during reprogramming may represent another strategy to detoxify ROS and exert protective effect. Secondly, it is likely that certain types of cells are more susceptible to DNA damage and accumulation of genomic aberrations during reprogramming possibly due to low expression levels of DNA damage repair molecules. Adjaye's group has demonstrated that aged donor-derived human iPSCs possess various chromosomal aberrations that are absent in young donor-derived iPSCs by karyotyping analysis [60]. It suggests that the age of human donor cells constitutes a parameter to influence genomic integrity of the resultant iPSCs. Therefore, boosting DNA damage repair capacity of the cells by overexpression of genes in various repair pathways, in particular oxidative DNA damage repair pathway, may be useful to further safeguard genome integrity of iPSCs. Base-excision repair consists of MTH1, OGG1 and MUTYH enzymes which prevent mutations associated with 8-oxoguanine (8-oxoG), a common product of oxidative damage to DNA [61]. While MTH1 hydrolyses 8-oxo-dGTP and remove it from the nucleotide pool to prevent its incorporation into DNA by polymerases [62], OGG1 excises 8-oxoG from the 8-oxoG•C base pair and MUTYH removes the inappropriate A in the mismatched 8-oxoG•A base pair [63]. It will be interesting to examine whether overexpression of MTH1, OGG1 and MUTYH in the cells during reprogramming will help to preserve the genome integrity of iPSCs. Thirdly, factors other than ROS may also contribute to genomic aberrations. It will be important to identify these factors to further optimize reprogramming process for derivation of iPSCs with better genome stability. For instance, activation of oncogene can also cause replication stress which may lead to DNA damage and genomic aberrations in the cells [64]. Therefore, targeting replication stress potentially occurring after forced expression of the reprogramming factor may further improve the genomic integrity of iPSCs.

SUMMARY

Low reprogramming efficiency and genome instability of iPSCs are two unsolved stumbling blocks associated with cellular reprogramming. Although low reprogramming efficiency can be largely attributed to the intrinsic differences in molecular and cellular characteristics between somatic cells and PSCs, introduction of reprogramming factors itself paradoxically constitutes another important aspect hampering reprogramming efficiency. This is, at least in part, because reprogramming factors possess oncogenic properties and induce oxidative stress (Figure 1). Elevated ROS levels cause damage to various cellular components, in particular DNA, eliciting DDR leading to cell cycle arrest. Depending on the extent of DNA damage, cells may either undergo apoptosis/senescence or re-enter the cell cycle if the damage

is corrected by the repair pathways. However, inappropriate repair of the DNA damage may leave errors on the DNA and introduce genomic abnormalities (Figure 1). Thus, oxidative stress as a result of forced expression of reprogramming factors appears to be a common culprit that undermines both reprogramming efficiency and genome integrity of iPSCs. Thus, treatment with antioxidants comprising NAC and Vc among others improves reprogramming efficiency and reduces genomic aberrations of iPSCs (Figure 1). However, it is unlikely that antioxidants exert the effect by exclusively scavenging ROS since Vc possess non-antioxidant function such as regulating epigenetic enzymes (Figure 1). Although it remains to be determined the impact of other strategies reported to increase reprogramming efficiency on safeguarding genome integrity of iPSCs, it warrants the supplementation of reprogramming media with antioxidants for the one-stone-two-bird effect. Given that antioxidant treatment does not eliminate genomic aberrations of iPSCs, it suggests that other factors in addition to oxidative stress also contribute to the mutational load of iPSCs. Identification and targeting of such factors in combination with antioxidant treatment shall further increase genomic quality of iPSCs. Robust reprogramming efficiency together with preservation of genome integrity of iPSCs will be essential to the application of reprogramming technology in future cell-replacement therapy, disease modeling and drug screening approaches.

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AUTHOR CONTRIBUTIONS

S.Q.: Manuscript writing; Z.F.: Manuscript writing; D.W.: Manuscript writing; P.M.: Manuscript writing; K.Y.: Manuscript writing, Financial support; J.J.: Conception and design, Financial support, Final approval of manuscript

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Figure 1. Mechanisms by which antioxidants promote reprogramming efficiency and genome integrity of iPSCs. Forced expression of reprogramming factors increases ROS levels generated from mitochondria which causes DNA damage and undermines both reprogramming efficiency and genomic integrity of iPSCs. Antioxidants such as Vc can promote reprogramming efficiency and safeguard genome stability of iPSCs by quenching ROS as well as exerting non-antioxidant functions including modulating the epigenetic modifiers Jdhm1a/1b and Tet1 which in turn influence DNA methylation and histone modifications (H3K36me2/3, H3K4m3 and H3 acetylation).