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DNA damage and repair in the differentiation of stem cells and cells of connective cell lineages: A trigger or a complication?

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ABSTRACT

The review summarizes literature data on the role of DNA breaks and DNA repair in the differentiation of pluripotent stem cells (PSC) and connective cell lineages. PSC, including embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC), are rapidly dividing cells with highly active DNA damage response (DDR) mechanisms to ensure the stability and integrity of the DNA. In PSCs, the most common DDR mechanism is error-free homologous recombination (HR) that is primarily active during the S phase of the cell cycle, whereas in quiescent, slow-dividing or non-dividing tissue progenitors and terminally differentiated cells, error-prone non-homologous end joining (NHEJ) mechanism of the double-strand break (DSB) repair is dominating. Thus, it seems that reprogramming and differentiation induce DNA strand breaks in stem cells which itself may trigger the differentiation process. Somatic cell reprogramming to iPSCs is preceded by a transient increase of the DSBs induced presumably by the caspase-dependent DNase or reactive oxygen species. In general, pluripotent stem cells possess stronger DNA repair systems compared to differentiated cells. Nonetheless, during a prolonged cell culture propagation, DNA breaks can accumulate due to the DNA polymerase stalling. Consequently, the DNA damage might trigger the differentiation of stem cells or replicative senescence of somatic cells. The differentiation process *per* se is often accompanied by a decrease in the DNA repair capacity. Thus, the differentiation might be triggered by DNA breaks, alternatively, the breaks can be a consequence of the decay in the DNA repair capacity of differentiated cells.

Key words: DNA breaks; DNA repair; differentiation; stem cells; connective tissue.

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Introduction

DNA damage can be induced by external stressors such as physical factors or genotoxic agents. Moreover, the internal DNA damage can be induced by a metabolic activity resulting in reactive oxygen species (ROS) generation, hydroxylation, deamination, and S-adenosylmethionine alkylation or action of endogenous nucleases.[1] The base excision repair (BER) excises and replaces damaged bases as well as recognizes single strand breaks (SSB) whereas DNA lesions that distort the double helix are repaired by the nucleotide excision repair (NER). Double strand breaks are repaired by the non-homologous end joining (NHEJ) or homologous recombination (HR), whereas DNA replication mistakes are corrected by a mismatch repair (MMR). All above mentioned DNA repair pathways are active in stem cells.[1,2] Although DNA damage is considered to be mostly a consequence of the action of genotoxic agents, leading either to apoptosis, aging or mutations, numerous data indicate the involvement of such DNA modifications in physiological processes – for example, differentiation. We have summarized the literature on the role of DNA breaks in differentiation more than a decennium ago.[3] Ever since the importance of DNA breaks in the function of neurons,[4] immune response,[5,6] and spermatogenesis[6] has been confirmed several times and mechanisms of the DNA breakage have been elucidated. During meiosis, the double-strand breaks (DSB) are induced by the SPO11 enzyme, whereas the DSBs for V(D)J recombination are induced by the RAG and repaired *via* a non-homologous end-joining mechanism (NHEJ). The process of the B-cell receptor diversification is initiated by the Activation-Induced Cytidine-Deaminase (AID).[6] DNA breakage triggers also muscle cell differentiation, in this case, breaks are induced by the caspase-activated DNase, an enzyme that is mostly involved in the process of apoptosis. The DNA breaks were mapped in the promoter of the p21 gene.[7-10]

In recent years, methods of DNA damage detection have substantially progressed. For example, protocols of the most common method, the comet assay, have been improved enabling studies of different forms of DNA damage and increasing the reproducibility of the data.[11] Novel methods enable to induce DNA strand breaks and to follow their repair in a single site of the genome or to study the distribution of the breaks in the whole genome determining the "breakome" using the next-generation sequencing.[12] For instance, spermatogenesis-related DSB were localized by applying this approach.[6,13,14] The DNA breaks involved in a V(D)J recombination and induced by a RAG were mapped also using the next generation sequencing.[15] In the present review we aimed to summarize the latest literature data on the role of DNA strand breaks in the pluripotent stem cell and connective tissue stem cell proliferation and differentiation.

Embryonic stem cells

Maintenance of the integrity of the embryonic stem cell (ESC) DNA is important for use of these cells in tissue engineering, to avoid the risk of tumour development. Numerous studies on DNA integrity and DNA damage repair (DDR) capacity of the ESCs have been performed. The results provide evidence that human ESCs manifest greater precision and efficiency of the DSB repair compared to somatic cell lines derived from these stem cells.[12] Efficiency of the DSB repair is achieved due to the active homologous recombination in ESCs.[16] The activity of caspases increases during the differentiation of the ESCs.[17] It was hypothesized that the transient increase of the caspase activity with the following activation of the caspase-dependent DNAse is necessary for stem cell differentiation.[18] Chromosome aberrations seen in ESCs usually appear in late passages, which could be attributed to the culture adaptation.[19]

Induced pluripotent stem cells

The very reprogramming of the human dermal fibroblasts to generate pluripotent stem cells (iPSCs) is accompanied by a transient accumulation of DSB discovered utilizing the histone gamma-H2AX (γH2AX) immunocytochemical detection.[20] Taking into account the increased activity of apoptotic caspases during this process, the DNA strand breaks could be induced by the caspase-activated DNase.[21] According to an alternative point of view, reprogramming of a somatic cell into iPSC is not a natural process and it leads to the accelerated rate of cell division, which increases the oxidative stress and the accumulation of the DNA damage due to the elevated ROS.[22] Strikingly, a reprogramming of differentiated cells was observed after a transient increase in the DNA breaks, induced by zeocin, in an organism evolutionary very remote from the humans, namely the moss *Physcomitrella patens*. The DNA damage induced Stem Cell-Inducing Factor 1 (STEMIN1) promoter activation in some leaf cells and DNA-strand-break-induced reprogramming required the DNA damage sensor ATR kinase.[23] A rather high level of the γH2AX foci, co-localizing with the replication sites, was found in iPSCs.[24] It is supposed that DSBs in this process arise due to the replicative stress and a DNA-end resection as a step of homologous recombination is important for the reprogramming.[25]

Chromosome aberrations are frequently seen in iPSC clones and it is assumed that the reprogramming process itself is "mutagenic".[19,26] On the contrary, the rate of DNA mutations is higher in early passage iPSCs, indicating that the reprogramming itself is the cause of genetic instability. DSBs introduced by the elevated ROS during reprogramming are mainly repaired by the error-prone NHEJ. Interestingly, the DSB damage was abrogated by adding an antioxidant N-acetyl-cysteine to the cell culture medium during the reprogramming. The karyotype remained stable during further propagation of the established iPSC clones.[26] Shimada and colleagues studied the balance between DDR and apoptosis in 2 Gy irradiated iPSCs, fibroblast cell line, and neural progenitor cells (NPCs).[27] They observed that 4 hours after irradiation, γ-H2AX foci remained at higher levels in iPSCs (>50%) than in fibroblasts (>30%) and NPCs (>20%). Moreover, the TUNEL assay confirmed the apoptotic DNA breaks in 40% of iPSCs compared to none in fibroblasts. Altogether, these results indicate that iPSCs with DNA damage are removed from the cell population by apoptosis to ensure the genetic stability of the iPSCs.[27] However, according to others, radioresistance of the iPSCs is comparable to that of the dermal fibroblasts and exceeds radioresistance of the human ESCs.[28]

DNA damage by nitric oxide (NO) released from a NO-donor molecule triggers differentiation of the iPSC into NPCs and further to astrocytes and neurons. The differentiation is coupled to the decrease of the double-break DNA repair efficiency *via* the homologous recombination mechanism.[29]

DNA stability in connective tissue stem cells and progenitors

Mesenchymal stromal cells (MSCs) are fibroblast-shaped, plastic adherent cells, capable of differentiation into adipocytes,

osteocytes, and chondrocytes that reside in the stroma of the connective tissues.[30] The function of MSCs is to maintain tissue integrity and homeostasis by responding to the tissue damage either through a paracrine action or by differentiation into mesodermal cell types.[30] Due to the regenerative and immunomodulatory properties, MSCs are tested in numerous clinical trials to treat graft-versus-host disease, musculoskeletal disorders, and diverse inflammatory conditions. MSCs are found at a frequency of 0.1-0.01% in the bone marrow aspirate, therefore a rigorous *in vitro* expansion step to obtain enough cells for treatment is needed, which might compromise the genomic integrity of MSCs.[31,32]

Preserving MSC genome integrity by DDR in stress situations is crucial for the maintenance of the MSC physiological functions and to avoid carcinogenesis.

ESCs preferentially use the efficient HR-mediated DSB repair, contrary to NHEJ-mediated DSB repair used by somatic cells.[33] It is generally assumed that NHEJ is active prior to a cell division whereas HR takes place mainly during the S and G_2 phases of the cell cycle.[2] During *in vitro* expansion of MSCs, the activity of NHEJ was the predominant mechanism of DSB repair independently of the passage whereas the baseline activity of HR was reduced at passage 12.[34] Similarly, Bao *et al*. showed that several genes involved in DNA repair by HR, namely *BRCA1, Rad54* and *Rad51,* were downregulated in late passage MSCs.[35]

Generally, MSCs are considered relatively resistant to radiation-induced DNA damage and DNA-damaging agents.[36,37] MSCs were able to recover 48 hours after treatment with 25 μM etoposide as showed by a normalization of the γ-H2AX foci count. NHEJ was the predominant repair mechanism as the expression of *KU70*, *KU80*, and *DNA-PK* remained unaltered.[34] Interestingly, Po Kuei Wu *et al*. found that G_2/M arrest increased in early passage MSCs following irradiation, thus indicating towards HR as a predominant DSB repair mechanism, whereas late passage MSCs were arrested in the G_0/G_1 phase that would point to NHEJ as a main DSB repair mechanism.[36] MSCs from different sources demonstrated variable radiation resistance, for example, adipose tissue-derived MSCs showed less DNA damage in comet assay and they were more efficient in DNA repair following irradiation compared to gingival MSCs and umbilical cord-derived MSCs.[38] Remarkably, bone marrow MSCs recovered after exposure to 30 Gy and 60 Gy, and could be propagated *in vitro* for 16 weeks. The DNA repair mechanisms were unaltered between irradiated and non-irradiated cells, however irradiated MSCs entered in accelerated replicative senescence.[39] Thus, cellular senescence could be considered as a persistent DNA damage response activation.[40] In response to an accumulation of the DNA damage events, MSCs become senescent, and subsequently, the colony-forming activity, differentiation properties, and secretome is altered. It has been shown that MSC aging decreases osteogenesis and favors adipogenesis during senile osteoporosis. The molecular mechanism behind impaired osteogenesis in aged MSCs is linked to the decreased expression of the transcription factor Runx2 that plays an important role in the initiation of osteogenesis. On the contrary, the transcription factor PPARγ that regulates adipogenesis is upregulated in aged MSCs.[40] Based on the fact that the Runx family of proteins is involved in the regulation of genomic integrity, a possible interaction between Runx2 and DDR has been suggested.[41]

Interestingly, the cell-free DNA (cfDNA), a GC-rich DNA fraction that circulates throughout the bloodstream, can induce both SSB and DSB in human Adipose tissue-derived mesenchymal stem cells (haMSCs), revealed by comet assay and detection of the γH2AX foci. The DNA damage was followed by an increase in the expression of the DNA repair and anti-apoptotic genes. Finally, the cells manifested changes in morphology and gene expression, characteristic of adipocyte differentiation.[42] The authors attribute DNA breakage to the oxidative stress induced by the cfDNA. Interestingly, treatment with cfDNA provokes expression of pro-apoptotic pathways in differentiated cells, but the anti-stress response in the stem cells.[43]

In summary, MSCs possess a high DNA repair activity that render them resistant to DNA damage by physical and chemical factors (Figure 1). Nevertheless, the accumulation of DNA damage during lifetime or prolonged *ex vivo* expansion may lead to cell cycle arrest and early senescence that might have implications on MSC fate decision and secretory profile.

Adipocyte differentiation

Induction of the haMSCs to differentiate into adipocytes was followed by an accumulation of the DNA damage, revealed by the alkaline comet assay (sum of the SSB, DSB, and alkali-labile sites) and Fpg-sensitive sites (oxidized bases). Adipogenic differentiation was followed also by a decrease in the repair of DNA lesions induced by hydrogen peroxide.[44] Increase of DNA breaks in non-apoptotic pre-adipocytes on early stages of the adipocyte differentiation was observed also by flow cytometry of nucleoids.[45] Additionally, the increase of the DSB repair efficiency during the differentiation of preadipocytes to adipocytes was detected using both pulse-field electrophoresis and γH2AX foci after induced DNA damage. The authors concluded that the non-homologous end joining mechanism is more active in adipocytes compared to pre-adipocytes.[46] It is supposed that adipocyte differentiation is regulated by poly(ADP-ribose)polymerase-1, a sensor of DNA breakage.[47] In conclusion, elevated DNA damage is a root cause of adipocyte senescence, which plays a determining role in the development of obesity and insulin resistance.[48]

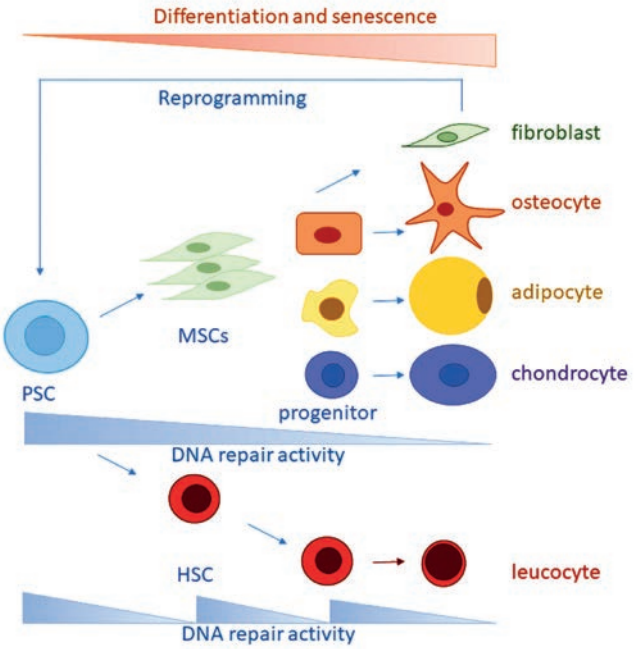

Figure 1. The correlation of the DNA repair activity and the cell differentiation state. PSC, pluripotent stem cell; MSCs, mesenchymal stromal cells; HSC, hematopoietic stem cell.

DNA damage response in osteogenesis

The function of bone marrow MSCs is to give rise to osteoblasts that further differentiate into osteocytes. MSCs' ability to differentiate into osteoblasts diminishes with old age, triggering a loss of bone mass and osteoporosis. The decreased bone regeneration capacity can be partly attributed to the cell senescence caused by the accumulation of DNA damage.[39] Moreover, Oliver *et al.* showed that following γ-irradiation-induced DNA damage, DDR activity quantified by γ-H2AX foci was more pronounced in undifferentiated haMSCs compared to the differentiated osteoblasts. The DNA damage during the osteogenic differentiation resulted in the apoptosis and death of differentiated cells.[49] A 50% decline in transcription factor Osterix1 expressing osteoblast progenitor cells with age was noted in a mouse model. Additionally, the markers of DNA damage and senescence, such as γH2AX foci, G_1 cell cycle arrest, phosphorylation of p53, were increased in osteoblasts from old mice, suggesting that DNA damage correlates with aging.[50] The microenvironment of the aging bone contains elevated levels of ROS, inflammatory cytokines, and free fatty acids that inevitably lead to the MSC senescence and the impaired osteogenic differentiation. It has been suggested that the presence of ROS and pro-inflammatory cytokines provides a favorable environment for adipogenesis and fat accumulation, and impairs bone regeneration, thus leading to the development of osteoporosis.[51]

DNA damage response in chondrogenesis

During bone formation, MSCs differentiate into two chondrocyte subtypes: round, low proliferating Sox9 expressing chondrocytes and high proliferating chondrocytes, that mature and become hypertrophic chondrocytes, producing mineralized extracellular matrix.[52] In physiological conditions, chondrocyte differentiation is affected by aging, metabolic syndrome, and obesity that cause cellular senescence and inflammation. Oxidative stress leads to telomere erosion, and increased expression of p53 and cyclin-dependent-kinase (CDK) inhibitors, p21 and p16INK4a (p16).[53,54] Moreover, senescent chondrocytes acquire a senescence-associated secretory phenotype characterized by a production of pro-inflammatory cytokines and matrix degrading enzymes, which further impair cartilage regeneration. It was shown that both, DNA damage and mitogenic stimuli by growth factors, are required to induce the persistent senescence in chondrocytes.[55] The increased chondrocyte senescence correlates with the development of osteoarthritis - one of the most common complications in the aging population in Western countries.[54,56]

iPSC-derived chondrocytes provide an alternative source for joint regeneration; therefore, the estimation of DNA stability and integrity is crucial before a therapeutic application. The differentiation process may induce differentiation-associated stress and increase DNA damage in iPSCs-derived cells.[57] To study the effect of irradiation on DNA damage, the DSBs and DDR were compared between iPSCs, iPSC-derived chondrocytes, and mature chondrocytes.[58] Chondrocytes demonstrated resistance to irradiation-induced DSBs, as less than 10% of cells were positive for γH2AX foci following irradiation at doses 1, 2, and 5 Gy. On the contrary, the percentage of γH2AX foci in iPSCs and iPSC-derived chondrocytes was approximately 40% before the irradiation, whereas the percentage of γH2AX foci reached 90% for iPSCs and 60% for iPSC-derived chondrocytes 9 hours after the 5 Gy exposure, respectively. Moreover, iPSC-derived chondrocytes upregulated NHEJ and HR mechanisms, and the number of γH2AX positive cells decreased to the baseline level 24 hours post 5 Gy irradiation, while iPSCs went to apoptosis. Altogether, iPSC-derived chondrocytes showed much higher DDR activity than mature chondrocytes, yet iPSC-derived chondrocytes were more prone to senescence compared to mature chondrocytes.[58]

DNA damage response in hematopoetic stem cells

Hematopoietic stem cells (HSCs) regenerate the blood system throughout the life. However, DNA damage accumulates in these cells during aging due to an attenuation of the DNA repair systems, especially in quiescent HSCs. It has been shown that recruitment to the cell cycle upregulates DNA repair systems in HSCs.[59] The DNA damage in aging HSCs manifests as DSB, and even point mutations and chromosome aberrations. Both ROS and stalled replication forks provoke the damage. Interestingly, in organisms the HSCs occupy niches with low oxygen concentration to minimize the ROS-induced damage. In some species niches of the HSCs are sheltered by melanocytes to avoid UV-light induced damage.[60,61]

In HSCs, the upregulation of growth arrest and DNA damage-inducible 45 alpha (GADD45A) factor in response to the cell damage results in an enhanced HSC differentiation.[62,63] On the contrary, the overexpression of the DNA-repair protein BRCA1 blocks the differentiation of the HSCs.[64] DNA repair capacity is further modified during myeloid differentiation: it is decreased after HSCs differentiate to monocytes, and expression of several DNA repair enzymes is blocked. Nevertheless, after monocytes differentiate into macrophages, the DNA repair capacity is restored.[65] Thus, DNA repair pathways appear to be differently regulated in differentiated and undifferentiated hematopoietic cells.[66] Role of the DNA strand breaks in lymphocyte differentiation merits a separate analysis and the topic has been recently reviewed in detail.[5,6]

Conclusions

PSC, including ESC and iPSC, are rapidly dividing cells with highly active DDR mechanisms to ensure the stability and integrity of DNA. In PSCs, the most common DDR mechanism is error-free HR that is primarily active during the S phase of the cell cycle, whereas in quiescent, slow-dividing, or non-dividing tissue progenitors and terminally differentiated cells, error-prone NHEJ mechanism of DSB repair is dominating. Thus, it seems that reprogramming and differentiation induce DNA breaks in stem cells which itself may trigger the differentiation process. Somatic cell reprogramming to iPSC is preceded by a transient increase of the DSB induced presumably by a caspase-dependent DNase or ROS. In general, pluripotent stem cells possess more robust DNA repair system activity compared to differentiated cells. Nevertheless, during a prolonged cell culture propagation, the DNA breaks can accumulate due to the DNA polymerase stalling. Consequently, DNA damage might trigger differentiation of stem cells or replicative senescence of somatic cells. The differentiation process *per se* is often accompanied by a decrease in the DNA repair capacity. Thus, the differentiation might be triggered by DNA strand breaks, alternatively, the breaks can be a consequence of the decay in the DNA repair capacity of differentiated cells. Altogether, the activity of DNA repair mechanisms may vary in different cell types, and the functional outcome of such differences could lead to either differentiation or senescence, or even geneti-

cally unstable cells that would be the least favourable scenario. Understanding the fundamental principles of DNA repair mechanisms could provide new prospects for the guided cell differentiation and disease prevention.

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