Chapter 5
Stem Cells and DNA Repair Capacity: Muse Stem Cells Are Among the Best Performers

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Abstract Stem cells persist for long periods in the body and experience many intrinsic and extrinsic stresses. For this reason, they present a powerful and effective DNA repair system in order to properly fix DNA damage and avoid the onset of a degenerative process, such as neoplastic transformation or aging. In this chapter, we compare the DNA repair ability of pluripotent stem cells (ESCs, iPSCs, and Muse cells) and other adult stem cells. We also describe personal investigations showing a robust and effective capacity of Muse cells in sensing and repairing DNA following chemical and physical stress. Muse cells can repair DNA through base and nucleotide excision repair mechanisms, BER and NER, respectively. Furthermore, they present a pronounced capacity in repairing double-strand breaks by the nonhomologous end joining (NHEJ) process. The studies addressing the role of DNA damage repair in the biology of stem cells are of paramount importance for comprehension of their functions and, also, for setting up effective and safe stem cell-based therapy.

Keywords Senescence · Apoptosis · DNA damage · DNA repair · Embryonic stem cells · Adult stem cells

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5.1 Cellular Intrinsic and Extrinsic Stress

Cells experience several types of intrinsic and extrinsic stresses for the entire duration of their life. DNA replication and cell metabolism are the principal intrinsic stressors [1]. Cells are continuously exposed to reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl-free radicals, superoxide anion radicals, and singlet oxygen. ROS derive from the imbalanced intracellular reduction of oxygen or by mitochondrial respiratory activity. Low concentrations of intracellular ROS are not deleterious and act in several signaling pathways, but a sharp increase in ROS level produces oxidative stress that damages proteins, lipids, and DNA. Oxidation of nucleotides may induce DNA mutations, and thousands of them are damaged per day per cell [2].

DNA replication is a vulnerable event in the cell's life. The double helix DNA molecule is quite stable, but during duplication, DNA unwinds and becomes single stranded. The borders between single and double strands (replication forks) present an intrinsically labile structure; moreover, these regions are histone-free. This occurrence renders replication forks open to attack by chemical or physical damaging agents that may induce DNA mutations. Telomeres are the physical ends of linear chromosomes and contain repetitive sequences. These sequences shorten after every cell replication and are another vulnerable region of DNA that may undergo mutation and alterations [3].

Hundreds of chemical and physical genotoxic agents are present in the environment and may act as extrinsic stress factors for cells. Ionizing radiations, UV rays, and high electromagnetic fields are among the most frequent and dangerous physical agents. Prooxidant molecules, factors that produce DNA deamination or alkylation, are chemical agents that are very hazardous and may induce many types of DNA damages [4].

5.1.1 How Do Cells Cope with DNA Damage?

After a stress event that induces DNA damage, cells have to eliminate and/or reduce the possibility they will undergo neoplastic transformation. A correct DNA damage sensing and repair system may recover damaged cells. Cells have different mechanisms to repair DNA (Fig. 5.1). Base pair excision repair (BER) system removes single-nucleotide mutations, such as methylation on O^8 of guanine. DNA glycosylases recognize mutated base moiety of nucleotides of the lesion and cleave the N-glycosidic bond to the damaged base, leaving an apurinic/apyrimidinic site (AP). The AP endonucleases cleave an AP site and leave a nucleotide gap that is filled by combined action of DNA polymerases and ligases. Nucleotide excision repair (NER) is the more versatile system to remove single-strand mutation. It does not recognize a mutated base; rather, it identifies distortions in the double helix DNA structure that are caused by the presence of damaged nucleotides. In this way, almost any kind of mutation can be repaired. NER may eliminate pyrimidine dimers created by UV irradiation. A protein complex runs along DNA to find distortions associated

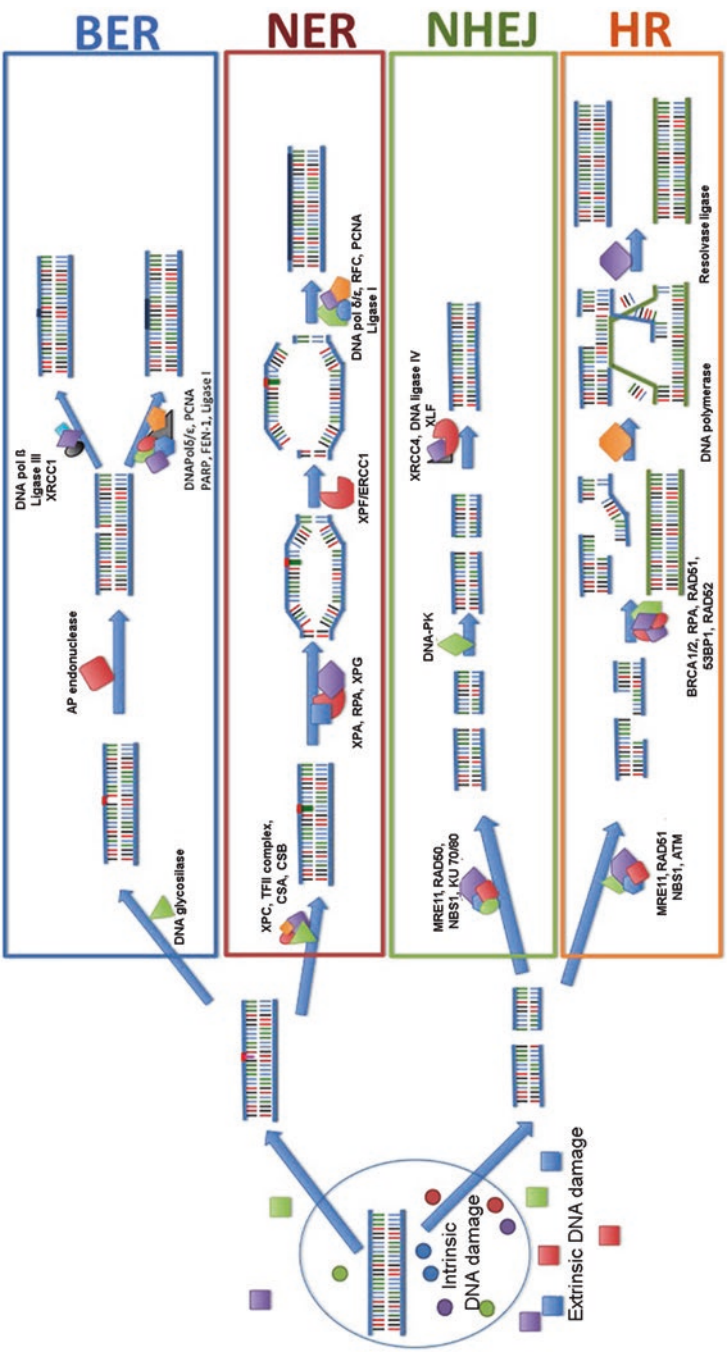

Fig. 5.1 DNA damage sensing and repair
Every day, cells receive hundreds of extrinsic and intrinsic DNA injuries that may induce single- or double-strand breaks: SSB and DSB, respectively. SSB are repaired by BER and NER, while DSB are repaired by NHEJ and HR. Figure 5.1 shows the principal factors that participate in sensing and repairing DNA. See main text for further details

with damaged nucleotides. An endonuclease activity is activated when the complex reaches a mutated base. Endonuclease enzymes remove a stretch of DNA containing the damaged bases, and then DNA polymerase and ligase fill the gap. Throughout DNA, global genomic NER repairs both transcribed and silent mutations. For many types of mutations, NER restores the transcriptionally active genes faster than non-transcribed ones. This activity is called transcription-coupled NER (TC-NER). It is a way for a cell to preserve more active genes; indeed, mutations occurring in silent DNA regions may be less harmful than those present in actively transcribed genes. The DNA mismatch repair system (MMR) identifies and repairs erroneous nucleotide insertion, deletion, and misincorporation that can occur during DNA duplication and recombination. Some genotoxic agents may induce double-strand breaks (DSB). This type of DNA damage is the most dangerous one because, in single-strand breaks (SSB), the intact helix serves as a template for DNA repair. This cannot occur when both helices are broken. Cells have developed two DSB repair systems: the homologous recombination (HR) and the non-end joining recombination (NHEJ). The NHEJ system has a protein complex that recognizes double-strand breaks; this system recruits proteins to remove damaged nucleotides and end-processing enzymes (DNA polymerases, ligases). The NHEJ process is an error-prone mechanism that repairs DSB but can insert DNA mutations. However, it is preferable to no repair in many circumstances. During HR repair, nucleotide sequences are exchanged between two similar or identical molecules of DNA. Interaction of a broken DNA present in a chromosome with the intact corresponding sequence on the homologous chromosome allows perfect DNA repair [5, 6].

Repair mechanisms have to provide a way to restore full cell functions. Alternatively, cells showing unrepairable DNA trigger apoptosis or a senescence process [1, 7, 8]. Apoptosis is a programmed cell death phenomenon aiming at the elimination of damaged or unnecessary cells from a tissue. Senescence is a permanent cell cycle arrest that is associated with loss of cellular functions and onset of pro-inflammatory cell phenotype. Both apoptosis and senescence are part of physiological activities contributing to tissue homeostasis and renewal. Nevertheless, persistent stress stimuli (either intrinsic or extrinsic) may promote massive DNA damage and hence apoptosis or senescence that can have a profound pathological consequence on an organism [1, 7, 8].

Stem cells, lineage-committed cells, and terminally differentiated cells have different needs for DNA repair. The latter ones permanently exit the cell cycle and replicate their genomes no more. For this reason, their repair mechanisms are focused on fixing damage present mainly on transcribed genes rather than constantly scanning the entire genome. Indeed, in these cells, a considerable part of the DNA is well protected from genotoxic agents because it is in compact heterochromatin. Only transcribed genes are located in euchromatin that is less densely packed to allow access to RNA polymerase complexes. This status, anyway, renders DNA more vulnerable to damages.

On the other hand, committed precursors and stem cells are cycling cells that can experience DNA replication errors and have less heterochromatin than differentiated cells. In addition, given their long life, stem cells may suffer from several rounds of intrinsic and extrinsic stresses.

5.2 DNA Repair in Embryonic Stem Cells and Induced Pluripotent Stem Cells

Embryonic stem cells (ESCs) originate from the inner cell mass of mammalian embryo blastocyst. ESCs are pluripotent stem cells and can differentiate into all cell types of the three primary germ layers: ectoderm, endoderm, and mesoderm [9]. The stability of the ESC genome is highly desirable because a mutation can have profound consequences on the organism's development. Indeed, the mutation rate of ESCs is far lower than in differentiated cells. As an example, the mutation frequency for the hypoxanthine-guanine phosphoribosyltransferase gene (*Hprt*) is lower than 10^{-8} per base pair per year, while in mouse embryonic fibroblasts (MEFs), it is around 10^{-5}. The frequency for the adenine phosphoribosyltransferase gene (*Aprt*) is 10^{-6} in ESCs and 10^{-4} in MEFs [10–12].

The low frequency of mutation is related to an efficient DNA repair system. Following ionizing irradiation, peroxide hydrogen, or psoralen treatments, ESCs are more proficient in repairing DNA by BER and NER than somatic cells. This is related to an increased expression of genes involved in DNA repair [13]. ESCs can also cope with DSB. It is reasonable to hypothesize that ESCs prefer to repair DSB by the high-fidelity HR rather than by the error-prone NHEJ, given the importance of a stable genome for these cells. In agreement with this hypothesis, scientists proved that ESCs spend roughly 70/75% of their life in S-phase when the HR is active [14]. Others showed that the resolution of RAD51 foci, involved in the HR pathway, is highly active in differentiated cells (astrocytes) and not in stem cells (neural progenitor cells and ESCs) [15]. In a model of induced DSB by the conditional expression of an endonuclease, Francis and Richardson showed that somatic cells repair DNA either by HR or NHEJ, while in ESCs the activity of NHEJ is negligible [16].

Nevertheless, there are many studies reporting that ESCs express proteins belonging to the NHEJ pathway. Adam and coauthors evidenced that ATM-independent, high-fidelity NHEJ predominates in human ESCs [17]. This system seems to be DNA-PKs independent, while the classic NHEJ is not. The factors and features of NHEJ repair change through differentiation: it becomes more error prone as differentiation proceeds. The existence of a high-fidelity NHEJ may explain why ESCs could use it safely. Others, however, challenged the idea that ESCs may use such a system. Bogomazova and coauthors showed evidence that in the G_2 phase, the human ESCs use the error-prone NHEJ [18]. These authors treated ESCs with X-rays and found several repairs that occurred through chromatid exchanges. These derived from NHEJ that misrejoined DNA breaks. ESCs with misrepaired DNA were prone to apoptosis, and authors hypothesize that ESCs use NHEJ repair and consequent apoptosis as a strategy to preserve genome stability. Indeed, cells that cannot properly repair their DNA by HR are quickly eliminated through programmed cell death.

Adult somatic cells can be induced to reprogram their biological features and become induced pluripotent stem cells (iPSCs). These cells show high similarity with ESCs since they can produce mesodermal, endodermal, and ectodermal deriva-

tives. The iPSCs, like ESCs, are mitotically active and can self-renew. Currently, iPSCs are used in clinical trials for cell therapy or are used as in vitro models for the studies of several diseases [19, 20].

An in-depth analysis of Fan and collaborators suggested that ESCs and iPSCs use overlapping strategies to cope with DNA damage. Both upregulate the BER and NER following oxidative stress and activate the HR and NHEJ pathways after heavy genotoxic injuries. These authors also showed that ESCs ensure genomic stability by having a low apoptotic threshold in response to DNA damage. The iPSCs evidenced a partial apoptotic response [21].

5.2.1 DNA Repair in Adult Somatic Stem Cells

Among the different types of adult stem cells, the DDR has been deeply investigated only in hematopoietic stem cells (HSCs) and neural stem cells (NSCs). In general, stem cells were more proficient in repairing DNA with respect to mature cells. Nevertheless, in some types of damage or specific conditions, scientists did not find significant differences between stem cells and their progeny.

Bracker and collaborators studied the DNA repair capacity of HSCs in comparison with that of committed and mature cells belonging to the lymphohematopoietic system. They noted a decline in repair capacity during commitment and differentiation. The elimination of DNA adducts, the resealing of repair gaps, and the resistance to DNA-reactive drugs were higher in HSCs compared to progenitors and differentiated cells. This was in agreement with increased expression of DDR genes in HSCs [22].

Other researchers evaluated NER in several human acute myeloid leukemia cell lines, before and after differentiation into macrophage-like cells. They found that repair of cisplatin crosslinks in mature cells was robustly reduced compared to progenitors. While some UV-induced damages were repaired with the same efficiency [23], HSCs and their differentiated derivatives showed no difference in the capacity to repair methylation DNA damage since treatment of cells with methylating agent N-methyl-N-nitrosourea (MNU) induced similar levels of apoptosis in stem cells and their progeny. This is in spite of a general trend toward the high expression of several DNA repair genes in HSCs compared to their differentiated counterparts. The overexpressed genes belonged to the NER, BER, MMR, and DSB repair pathways [24].

Of interest, several studies evidenced that the expression of genes involved in DNA repair declines with age. This phenomenon is accompanied by a progressive accumulation of DNA damage and leads to adult stem cell exhaustion. This is supposed to be one of the principal aging mechanisms. Studies of Nijnik and collaborators strengthen this hypothesis. Mice hypomorphic ligase IV mutation showed diminished double-strand break repair by NHEJ. This mutation induces progressive exhaustion of HSCs and bone marrow cellularity during aging and negatively affects stem cell function in tissue culture and transplantation [25].

A study carried out on NSCs and their differentiated derivatives showed that these stem cells were highly proficient in removing 8-oxoguanine from DNA by means of BER. This damage is one the most common DNA injuries induced by an oxidative attack and accumulates at high levels in the genome. The high capacity of NSCs in repairing this damage compared to differentiated cells suggested that NSCs are highly susceptible to oxidative stress [26].

NER activity in repairing UV-induced DNA lesions was analyzed in mature human neurons and their precursor NT2 cells. Removal of UV damage was lower in neurons than in the NT2 cells [27].

A finding of Nowak and collaborators evidenced that following irradiation of a developing mouse brain with a 2Gy dose of gamma rays, neural progenitors went into apoptosis, while neurons did not and survived. Analysis of gamma histone H2AX, a marker of the DNA damage repair process, indicated that the level of DNA damage was equivalent in the two different cell types. Kinetics of gamma H2AX staining evidenced that the DDR system repaired DNA damage in neural progenitor's DNA more slowly than in neurons. This was in agreement with high radiosensitivity of progenitor cells. Also, in this case, the suicide strategy of neural precursors appears to be a strategy to preserve genome stability [28].

5.3 Repair Capacity of Muse Cells

The mesenchymal stromal cells (MSCs) are found in the stroma of almost every organ. Their presence in bone marrow, umbilical cords, and adipose tissues is higher than in other organs. MSCs are heterogeneous because they are composed of distinct cell populations: stem cells, committed progenitors, and mature cells. Many investigators reported that stem cells of MSCs can give origin to progeny having a mesodermal phenotype such as osteocytes, chondrocytes, adipocytes, and muscle cells [29]. In recent years, Dezawa and collaborators identified a pluripotent stem cell population within MSCs that they named multilineage-differentiating stress enduring (Muse) cells, given their stress tolerance. These cells can differentiate in endodermal, ectodermal, and mesodermal derivatives. They express genes of pluripotency such as OCT3/4, SOX2, and NANOG. Muse cells present on their surface the pluripotency antigen SSEA3 that is used to isolate them from MSCs [30, 31]. Recently, Muse cells are considered endogenous reparative stem cells that contribute to tissue repair at serious damage as well as to daily minute repair for maintenance of tissue homeostasis. Since damaged tissue is hostile microenvironment for cells and the main function of Muse cells is to repair tissues, their capacities for high DNA repair and resistance to senescence/apoptosis seem reasonable and rational [32].

Several studies proved that the therapeutic potential of Muse cells is comparable to that of ESCs without the ethical issues that these last cells pose to public opinion. Furthermore, the use of Muse cells may challenge the current applications of iPSCs because Muse cells are naturally found in mammalian organs, while iPSCs are artificially created. In this scenario, it is of paramount importance to evaluate if Muse

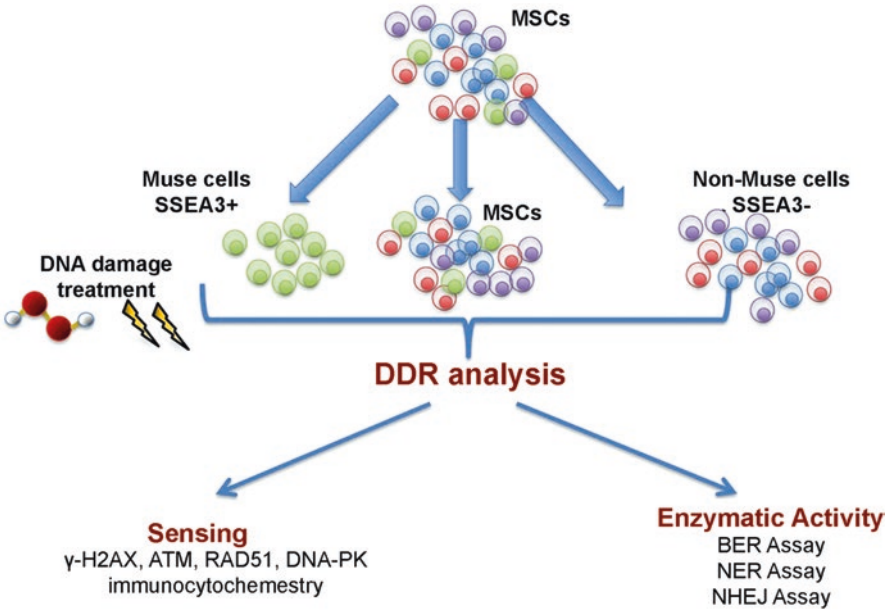

Fig. 5.2 Experimental workflow
We performed research on bone marrow MSCs and their SSEA3 positive and negative fractions, Muse, and non-Muse cells, respectively. Cells were treated with H_2O_2 and UV rays to induce DNA injuries. 1, 6, and 48 h following stress treatments, cells were collected for DDR analysis. We evaluated DNA damage sensing by immunochemistry detection of ATM, RAD51, DNA-PK, and γ-H2AX. We also analyzed the enzymatic activity of DNA repair enzymes by BER, NER, and NHEJ biological assays

cells have a high ability to cope with DNA damage as several reports have demonstrated for ESCs and partially for iPSCs.

Our research group evaluated DNA repair capacity of Muse cells in comparison with MSCs [33]. We analyzed the activation of the DNA damage checkpoint and repair system following the induction of chemical (H_2O_2) and physical (UV rays) stresses (Fig. 5.2).

We studied three different cell populations: naïve MSCs and their SSEA3 positive (Muse cells) and negative (non-Muse cells) subpopulations.

Muse cells showed better protection from chemical and physical damages than non-Muse cells and MSCs. This resulted in resistance to senescence and apoptosis, which are phenomena that cells trigger when damaged DNA is unrepairable.

DNA damage triggers cell cycle arrest and the induction of DNA repair machinery. The ataxia-telangiectasia mutated kinase (ATM) coordinates DNA repair activities by recruiting to damaged foci several DNA repair enzymes. ATM is one of the first proteins activated following genomic injuries, and if DDR works properly, ATM activation is switched off some hours later [34]. In Muse cells, we observed an increase in ATM activation soon after H_2O_2 treatment or UV irradiation and a

decline to basal level by 48 h following stresses. The ATM activation of MSCs occurred at a level higher than in Muse cells and did not decline to basal values even 2 days after induction of DNA damages. In non-Muse cells, the activation of ATM did not occur properly.

Our investigation further continued by studying the expression of RAD51 and DNA-PK, which are two fundamental downstream effectors of ATM. RAD51 is part of HR, while DNA-PK is a component of NHEJ [35].

The treatment of Muse cells with H_2O_2 or UV rays produced an increase of RAD-51(+) and DNA-PK(+) quickly after injuries and then a return to basal levels. In MSCs, the activation of RAD51 and DNA-PK took place accurately, but again, no decline to basal level was detected. Of interest, in non-Muse cells, this activation did not occur. These results suggest that only Muse cells repaired DNA damages correctly. The analysis γ-H2AX staining validated this hypothesis.

Following the activation of ATM, the histone protein H2AX is activated by phosphorylation (γ-H2AX). The phosphorylated H2AX contributes to recruitment of DNA repair factors. Soon after DNA injury, the presence of γ-H2AX foci indicated regions with damaged DNA that is undergoing repair. The permanence of active γ-H2AX foci evidences that DNA has been unrepaired or misrepaired [36].

Muse cells presented several H2AX foci soon after treatments with H_2O_2 or UV rays. Two days post-stresses, we still observed H2AX foci in cells that were in the G_1 phase, but this percentage was far lower than those detected in non-Muse cells and in MSCs.

The DDR system relies on sensing factors that identify damaged regions of DNA and then recruit enzymes involved in repair of different types of injuries. We evaluated the enzymatic activities involved in repairing SSB by BER and NER and DSB by NHEJ. The proficiency of Muse cells in correcting SSB by NER and BER was equivalent to those observed in MSCs and non-Muse cells, while NHEJ was significantly higher in Muse cells.

The high activity of NHEJ in Muse cells could be related to the fact that this is the only mechanism that repairs DSB in every cell cycle phase. Further investigations should demonstrate whether, in Muse cells, NHEJ is error prone or a high-fidelity system.

After DNA damage cells activate a quick response by triggering the repair proteins already present in the nucleus. Then, cell may switch on a late response to cope future harmful stimuli. This can be obtained inducing changes in mRNA expression of genes implicated in DNA repair.

After DNA injury, Muse cells changed the mRNA expression of several genes involved in NER, BER, mismatch repair (MMR), NHEJ, and homologous recombination (HR) to cope with DNA repair. These changes occurred soon after damage and persisted 48 h later. Non-Muse cells showed minimal modifications of gene expression only soon after DNA damage (1 h) [33]. Changes in the mRNA expression of genes belonging to DNA damage and repair pathways suggest that in Muse cells is active an efficient adaptive response mechanism. This phenomenon implies that a minimal priming dose of a DNA damaging agent can protect cells against a larger second dose given several hours or days later by the activation of a repair process.

5.4 Conclusions

Mechanisms that properly cope with environmental stress are a typical feature of stem cells. Muse cells present a quick and effective DDR. The rapidity in detecting damaged foci and fixing damages may explain the effectiveness of this process. The study of DDR is of paramount importance for comprehension of stem cell functions and for setting up effective and safe stem cell-based therapy. In summary, Muse cells appear to have high DDR capacity as already evidenced for ESCs and iPSCs, and this is important for therapeutic applications. However, ESCs and iPSCs are not endogenous, and they don't reside in normal living body. They are consistently exogenous cells. Muse cells are naturally existing endogenous stem cells, normally distributing in the connective tissue of every organ, peripheral blood, and the bone marrow. They may represent a better alternative to ESCs and iPSCs in cell therapy.

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