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Concise Review: Genomic Instability in Human Stem Cells: Current Status and Future Challenges

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ABSTRACT

Genomic instability is recognized as one of the most important hurdles in the expanding field of stem cell-based therapies. In the recent years, an accumulating body of evidence has shown that human stem cells undergo a diverse program of biological changes upon ex vivo cultivation that include numerical and structural chromosomal abnormalities, point mutations, variation of telomere length, and epigenetic instability. As the field moves forward, the growing awareness of the risk factors associated with human genome plasticity strongly advocates for the use of extensive genetic screening as part of a quality control platform to attest to the safety of stem cell-based products. Here we present a timely and comprehensive review that addresses the current status and emerging trends of the field, ultimately underscoring the need to implement new regulatory standards able to streamline the route to therapeutic applications. STEM CELLS *2014;32:2824–2832*

INTRODUCTION

The broad field of Regenerative Medicine brings the exciting promise of using stem cells and/or their progeny to replace injured tissues damaged by disease, either through the cell's integration (engraftment) into the target tissue and/or the cell's ability to deliver soluble signaling factors. Stem cells can be derived from multiple tissues, namely from embryonic and adult sources. Human embryonic stem cells (hESCs) were first derived from the inner cell mass of blastocysts [1] and are known for their self-renewal capacity and pluripotency, being able to give rise to all types of cells that develop from the three germ layers of the embryo (mesoderm, endoderm, and ectoderm). hESCs hold great promise for replacement therapies, disease modeling, and drug screening, but the last recent years have brought to light a disturbing amount of data regarding the onset of chromosomal aberrations, which together with significant ethical issues, have hampered research and the clinical application of these cells. In 2006, Takahashi and Yamanaka demonstrated the feasibility of reprogramming somatic cells into an embryonic-like state by ectopic coexpression of defined transcription factors [2]. The enthusiasm in obtaining these so-called induced pluripotent stem cells (iPSCs), thereby avoiding embryo destruction *ex utero*, somehow overshadowed the high mutation rate associated with the reprogramming process [3]. Based on the current state of knowledge, hESCs and human iPSCs (hiPSCs) show subtle differences at the genetic, epigenetic, and transcriptional level. It is an open issue, however, whether such differences are meaningful or simply the result of, for example, using different culture conditions.

Alternatively, in the recent years, human adult stem cells such as hematopoietic stem cells (HSCs), mesenchymal stem/stromal cells (MSCs), neural stem cells (NSCs), epithelial stem cells or skin stem cells have been found in different niches within the organism throughout adulthood, providing an alternative source of quiescent progenitors able to support tissue maintenance and regeneration. Some of these multipotent stem cells, such as HSCs or MSCs, can also be found in neonatal tissues such as the placenta or the umbilical cord blood. However, as in pluripotent stem cells, an increasing body of evidence has revealed a time-dependent accumulation of genetic abnormalities and transformation during ex vivo expansion.

In this context, it is important to note that regardless of the cell type considered, quality control during ex vivo expansion becomes critical for a safer clinical implementation of stem cell therapies. In their *Reflection Paper on Stem Cell-Based Medicinal Products*, the European Medicines Agency (EMA) highlighted the tumorigenic potential associated with manipulation steps

and culture of pluripotent and somatic cells and made recommendations on performing cytogenetic analysis and evaluating parameters such as telomerase activity, proliferative capacity, and senescence status [4]. Similar concerns have been addressed by the International Stem Cell Banking Initiative (ISCBI) [5], who envisages the creation of a global network of standardized good practices for stem cell banking and distribution. In this matter, the US Food and Drug Administration (FDA) has stepped up its oversight of the increasing number of clinics usually operating under poorly regulated jurisdictions and offering unproven treatments against a myriad of pathologies (reviewed in [6]). The lack of a sound and reliable scientific follow-up has in some cases led to fatal outcomes [7].

Herein, we provide a brief summary on methods for evaluating genomic integrity, followed by an up-to-date and comprehensive review of the findings reported thus far concerning genomic instability in hESCs, hiPSCs, and human adult stem cells. Research bottlenecks and future trends are also discussed.

Common Methods for Evaluating Genomic Integrity–A Brief Overview

The most frequently used techniques to evaluate genomic integrity essentially rely on cytogenetic and DNA-based analyses. Conventional karyotyping has been considered as the gold standard for the detection of aneuploidy, polyploidy, and other large chromosomal imbalances. It typically involves the banding of metaphase-arrested chromosomes with Giemsa stain (G-banding), which can then be analyzed by ordinary bright-field microscopy. Karyotypes of Giemsa-stained chromosomes can be described according to the International System for Human Cytogenetic Nomenclature (ISCN) [8]. Although some optimizations have been proposed [9], conventional karyotyping is however a lengthy procedure that requires skilled personnel, being constrained by a low average resolution (typically $>$3 Mb), by the difficulties in deciphering complex rearrangements using a monochrome banding pattern, and by the need to obtain a high number of metaphases. Moreover, it is now clear that some subkaryotypic variants cannot be dismissed as they can have serious implications from the clinical point of view. Taken together, these shortcomings have contributed to major advances in the field of molecular cytogenetics, particularly through the use of higher resolution nonisotopic approaches such as in situ hybridization-based technologies. One example is that of fluorescent in situ hybridization (FISH). Developed in the early 1980s [10], FISH essentially relies on the use of directly or indirectly labeled probes to detect specific DNA target sequences by means of fluorescence in metaphase chromosomes (resolution of 1–2 Mb), interphase nuclei (50 kb to 1 Mb), or DNA fibers (10–500 kb). Due to its high sensitivity, cost effectiveness, and reproducibility, FISH quickly gained a widespread recognition in biology and medicine and has proven invaluable for a multitude of purposes [11]. Some examples include the analysis of chromosomal aberrations in nondividing cells, 3D chromosome organization studies, gene mapping, DNA replication/recombination studies, disease characterization and diagnosis, among others. FISH has however a major downfall of only being able to detect known genetic aberrations and of being limited in its genome-wide application, not allowing for a comprehensive screening of chromosomal aberrations. Such limitations were greatly circumvented with the capability to hybridize and image multiple differentially labeled DNA probes allowing the visualization of all 24 human chromosomes (22 autosomes, X and Y chromosomes), each in a different color and in a single step. This resulted in the development of several new FISH-based techniques such as spectral karyotyping (SKY) [12] and multiplex-FISH (M-FISH) [13]. Both techniques differ in their image acquisition mode: SKY relies on a one-step image acquisition through a customized multiband optical filter, whereas M-FISH uses a set of fluorochrome-specific optical filters. Limitations of these techniques include the prerequisite of metaphase cells, a typical low resolution (around 1–3 Mb), and their inability to detect intrachromosomal rearrangements.

Another popular technique is comparative genomic hybridization (CGH) [14], which in recent years has provided unparalleled insights into oncological research and in the detection of aberrations in fetal and neonatal genomes. CGH uses a test and a control genome, which are differentially labeled with fluorochromes (e.g., green color for the test and red for the control) and competitively hybridized to metaphase chromosomes. The fluorescence ratio of the test genome relative to the control is then examined along each chromosome, providing information on DNA regions with gains (elevated green-to-red ratios) or losses (reduced ratios) of genetic material. CGH has however some limitations, namely its relatively low resolution (5–10 Mb), and the fact that it cannot detect balanced rearrangements, such as inversions, or reciprocal, or Robertsonian translocations. The CGH principle has also been coupled to microarray technology (array-CGH) using bacterial artificial chromosomes (BACs) (150–200 kb in size), cDNAs (0.5–2 kb), polymerase chain reaction (PCR) products (0.1–1.5 kb), and oligonucleotides (25–80 bp) as interrogating probes [15–18]. The maximum level of resolution of array-CGH technology is a function of the length, distribution, and spacing between probes and is typically limited to 50–100 kb for BACs and 1–10 kb for oligonucleotide probes. Other array-based platforms allow single nucleotide polymorphism (SNP) detection, and apart from providing information on copy number variants (CNVs), have the advantage of revealing loss of heterozygosity or segmental uniparental disomy.

We now stand at the point where next generation sequencing technologies are maturing, which allow mapping the landscape of rearrangements to the bp level, although at the cost of less flexibility and more demanding computational power (reviewed in [19]). In the expanding stem cell field, the use of these techniques has been and will continue to be central to the identification and characterization of culture-acquired abnormalities, shedding light on the real magnitude of genome maintenance challenges.

Genomic Instability in hESCs

hESCs have been increasingly considered as valuable tools for replacing injured tissue and for the potential treatment of a wide variety of disorders. Their use has sparked controversy mainly because current methods of obtaining hESCs require the destruction of human blastocysts, for example, from

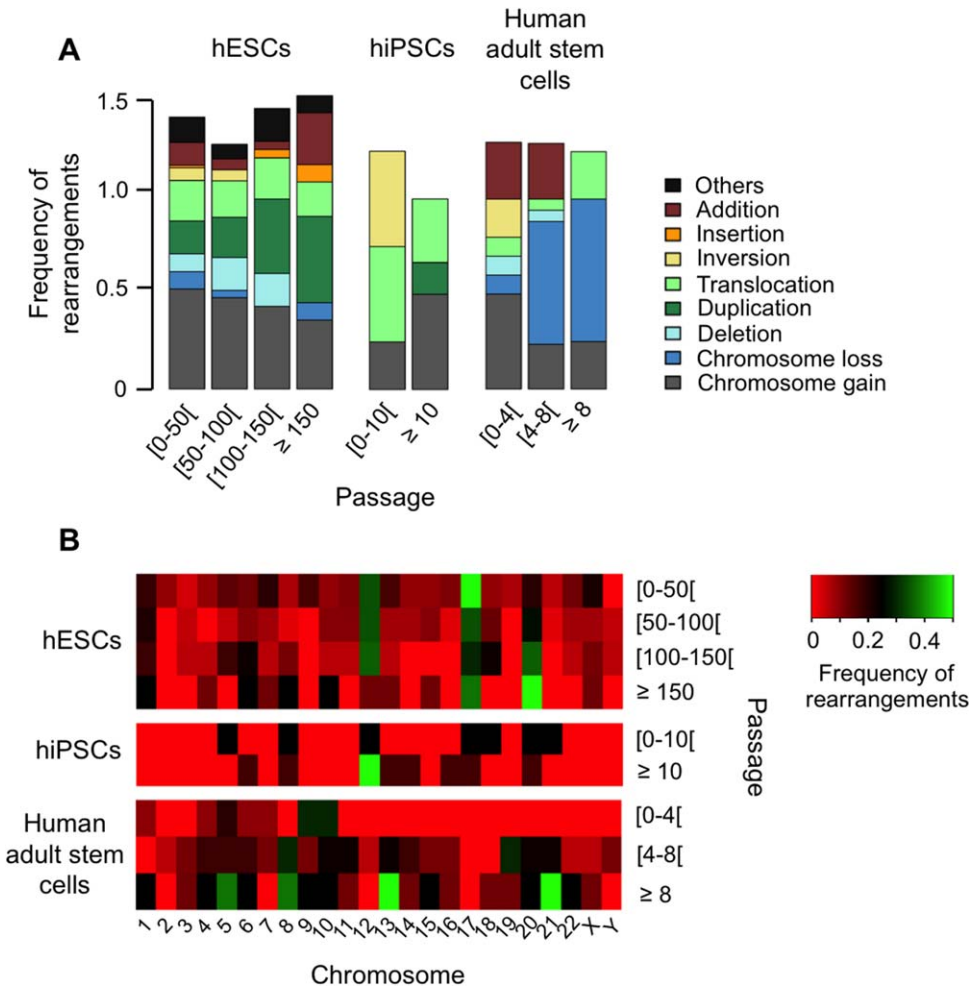

Figure 1. Average frequency of rearrangements among aberrant human stem cell lines. **(A):** Variation in the average frequency of numerical and structural rearrangements in hESCs, hiPSCs, and human adult stem cells at different passage intervals. **(B):** Variation in the average frequency of rearrangements in hESCs, hiPSCs, and human adult stem cells at different passage intervals and for each chromosome. Further details on this meta-analysis are available as Supporting Information. Abbreviations: hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells

surplus in vitro fertilization embryos or aborted fetuses. These derivation approaches have fueled clashing views on the moral standing of the preimplantation embryo. Those who object to embryo destruction often base their arguments on symbolism or potentiality, according to which a preimplantation human embryo has the potential to develop into a fully developed human and consequently should be accorded equal rights, interests, and moral status. On the other side of the spectrum, there is the argument that the blastocyst is merely composed of a clump of undifferentiated cells, with no nervous system discernible, and is thus too premature in development to hold any interests or rights. In light of the therapeutic potential of these cells, researchers, policymakers, and ethics specialists should continue to seek as much common ground between the different arguments.

In addition to ethical concerns, there are additional roadblocks that must be addressed before considering a move to the clinic. These concern the increasing body of evidence describing chromosomal abnormalities that appear during in vitro culture, which prompts the question of how safe these cells are for human applications. This is an important concern since a normal karyotype is essential not only to assure the maintenance of hESC properties in vitro but also to prevent adverse effects *in vivo*. Genetically abnormal cells typically arise during culture adaptation and often show an increased growth rate and higher propensity for acquiring a malignant transformation [20]. Apparently, hESCs show a biased predisposition to aneuploidies (mainly gains) of chromosomes 12 (particularly 12p) [20–27], 17 (usually 17q) [20, 22, 23, 25, 27–30], 20 (particularly 20q) [20–22, 25, 27–29, 31], and X [20, 22, 25, 28, 32] (Fig. 1). Trisomy of chromosome 12 has been shown to increase the proliferative potential of hESCs, to promote cell division with multiple spindles and originate tumor-like tissues *in vivo* [26]. On the other hand, duplication of 20q11.21 ranging in size from 0.55 to 4.6 Mb [21, 33] is known to affect genes such as *ID1* and *BCL2L1*, the latter encoding the antiapoptotic protein BCL-X, recently found to be responsible for the strong selective advantage of this duplication [31, 34]. Gain of 20q11.21 is also commonly observed in a variety of human cancers [35, 36]. Loss of chromosome X has been reported [28], but gains are apparently more frequent [20]. In a recent report, combined

chromosome counting, FISH, and SKY, revealed 18%–35% of mosaic aneuploidy consistently present in hESC (and hiPSC) cultures independently of passage number, culture technique, or laboratory [37]. Mosaicism apparently arises in a stochastic fashion and seems to be either responsible for phenotypic heterogeneity or subsequent clonal aneuploidies that lead to loss of pluripotency and increased tumorigenicity [37]. In two other studies the use of higher resolution approaches such as CGH or SNP arrays allowed the determination of numerous CNVs ranging in size from 20 kb to 3 Mb in different chromosomes, affecting cancer-related genes and leading to altered gene expression profiles [22, 38]. Differentiation also seems to rapidly select genomically aberrant cells, meaning that mutation detection efforts should go on beyond the pluripotent state [39].

Interestingly, some hESC lines seem inherently more prone to acquire mutations than others. This biased mutation vulnerability seems to relate either to embryo source or to the environmental conditions to which these cells are exposed [24]. The latter include the type of feeder layer used, medium composition, technique used for cell passaging, and freeze-thawing [30, 40, 41]. Also, the use of physiologic hypoxia (~2%) instead of atmospheric oxygen (21%, referred to as normoxia) has been associated with a decreased content of genetic abnormalities [42]. In addition, hESCs expanded on microcarriers to high densities have been shown to preserve their pluripotency and a normal karyotype for several months [43, 44]. In this regard, finding a reliable biomarker for the identification of senescent and genetically abnormal hESCs would facilitate the development and validation of culture conditions by reducing the volume of cytogenetic analyses needed. In one of such attempts, Herszfeld et al. [45] identified the tumor necrosis factor receptor CD30 as a suitable candidate, since it appeared to be overexpressed in karyotypically abnormal hESCs in comparison to normal cultures. Later on, two independent studies have challenged this interpretation of the data by essentially showing that the ascorbate present in the Knockout Serum Replacement (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) medium was the sole agent responsible for inducing CD30 expression through enhanced CpG promoter demethylation [46, 47]. In these cases, CD30 expression led to inhibition of apoptosis, enhanced growth, and could contribute to support the survival of cells having accumulated genetic lesions [47].

The integrity of the mitochondrial genome and the impact of both point and large-scale homoplasmic and heteroplasmic mutations on the bioenergetic performance of mitochondria have so far received little attention. Nevertheless, this scenario appears to be gaining increasing relevance. About 22% in a list of nine late-passage hESC lines have been reported to carry heteroplasmic mutations, the majority of them being nonsynonymous and located in coding regions of the mitochondrial genome [29]. In another study, all the 16 hESC lines screened contained large-scale mitochondrial DNA (mtDNA) deletions at an average mutation load of 23%, which could be detected as soon as Passage (P) 3–6 and after terminal differentiation [48].

In view of the promising clinical role envisaged for hESCs, the aforementioned findings underline the need for a regular and thorough genetic analysis, capable of deepening our current knowledge on the biology of these cells and helping to improve culture conditions. Despite the substantial hurdles that have been pointed out in this section, hESCs are slowly finding their way into the clinical setting. Advanced Cell Technology is currently conducting FDA-approved clinical trials with hESCs (see for example [49]) after Geron Corporation discontinued in November 2011 its Phase 1 clinical trial focusing on the safety of hESC-derived oligodendrocyte progenitors for spinal cord injury [50]. Two years later, Asterias Biotherapeutics, a subsidiary of the biotechnology company BioTime, acquired Geron's assets, and it seems likely that it will launch further hESC-based trials within the next years [51].

Genetic Instability in iPSCs

In their 2006 and 2007 groundbreaking contributions, Yamanaka's team was able to reprogram somatic cells to a hESC-like state, through the ectopic coexpression of four transcription factors [2, 52]. Yet, a plethora of subsequent articles addressing questions of similarity between hiPSCs and hESCs soon made clear by the significant number of differences detected that more time would be needed before attaining a completely safe, efficient, and mature therapeutic product.

The occurrence of cytogenetic imbalances in hiPSC lines seems to be independent of the reprogramming procedure, not enriched in stem cell or cancer-related genes, and similar in type and frequency to those found in hESC cultures [53]. In a similar way to hESCs, trisomies 12 and 20q were predominant in hiPSCs [53–55], but contrary to the former, trisomies 17 and X were seldom observed [53–55] (Fig. 1B). In another study, it was shown that CNVs are generated de novo and overrepresented in early-passage hiPSCs, relative to intermediate-passage hiPSCs, fibroblasts, and hESCs [56]. While most of these CNVs are apparently disadvantageous and negatively selected over time in culture, a minority does persist. Also, the observation that approximately six point mutations are present per iPS cell exome, particularly in cancer-related genes, suggests the introduction of complete exome or genome sequencing as a useful post-reprogramming quality-control procedure to ensure that deleterious point mutations are not present [57]. The total point mutation load of hiPSCs results from pre-existing mutations in parental cells (19%), in vitro passaging (7%), and from the cellular reprogramming process itself (74%) [3]. In line with these findings, hiPSCs also seem to form teratomas faster and with higher aggressiveness than hESCs, irrespective of the site of administration or engraftment [58]. Two new studies have observed that mitigation of oxidative stress during reprogramming and early passaging by using antioxidant supplements in the growth media was able to reduce genome instability in hiPSCs [59, 60].

Monitoring the onset of mtDNA mutations during reprogramming is also of vital importance. In a recent work, several hiPSC lines were found to harbor homoplasmic and heteroplasmic mutations not reported to be present in the parental cells, and having a nonsynonymous versus synonymous ratio equal to or higher than one [61]. Although the majority of these changes were haplogroup-specific, a minor percentage was found to be cancer-associated or not previously identified in dedicated databases [61]. Interestingly, it has also been shown that mitochondrial ultra structure can be re-established even in aged reprogrammed cells harboring chromosomal aberrations [62].

Apart from the aforementioned genetic abnormalities, hiPSC induction from committed cells has also been linked to a defective epigenetic reversion, and several findings contributed to spark the debate on their epigenetic safety. First, hiPSCs seem to retain to some extent the epigenetic signature of the tissue of origin, thus skewing their differentiation potential [63]. Second, the stoichiometry of the reprogramming factors does affect the epigenetic state of the cell [64]. Third, several changes in genomic imprinting or neoplastic-related DNA methylation modifications have been detected in hiPSCs which may cause abnormal differentiation or transformation [65, 66].

That said, the successful move of hiPSCs into the fast-paced field of regenerative medicine will largely depend on the implementation of rigorous quality control standards and on the improvement of reprogramming techniques built on nonintegrating molecules. We anticipate that novel integration-free strategies, based for example on proteins and RNA [67] or chemical induction [67, 68] will address some of the concerns raised by the reprogramming methods based on the integration of transgenes into the genome via viral systems.

Genomic Instability in Adult Stem Cells

Adult stem cells are rare, postnatal, and mostly quiescent multipotent tissue-specific cells, which play a pivotal role in regeneration and repair. Among the organs and mature tissues known to harbor adult stem cells (*e.g.*, fat, gastrointestinal tract, skeletal muscle, heart, liver, as well as neonatal tissues such as umbilical cord blood and placenta), the bone marrow (BM) has been for many years the primary reservoir of both HSC and MSC populations. Over the last three decades, a wealth of exciting research findings on the immunomodulatory, antiapoptotic, proangiogenic, and anti-inflammatory properties of MSCs appears to lend further support to their use as a promising cell source in the treatment of immune disorders and in tissue repair. At the time of writing, the U.S. National Institutes of Health ClinicalTrials.gov website [69] listed 122 clinical studies using human MSCs, which have been completed or are under way. Among the completed trials, many have successfully demonstrated the efficacy of using autologous or allogeneic MSCs, for example, in rescuing BM function after chemotherapy sessions in hemato-oncological settings, treatment of graft-versus-host-disease, and acute myocardial infarction, among others.

With the increasing demand of human adult stem cells for both research and clinical purposes (typically 1–5 million cells per kg of body weight are required per treatment, www.clinicaltrials.gov), it becomes of utmost importance to bridge the gap between the need to expand the cells in vitro and the capability of harnessing the factors underlying replicative senescence. Adult stem cells are known to have a limited lifespan in vitro and to enter replicative senescence almost undetectably upon starting in vitro culturing [70]. This process is typically characterized by a gradual decrease in proliferative and differentiation capacity, morphological changes (cells typically display enlarged, flattened, and more irregular shapes), high levels of tumor suppressors (p16, p21, p53, RB), and accelerated telomere erosion (average loss of 1 kb per 10 population doublings [PDs]) [71]. For efficiency and safety reasons, the number of PDs should be kept at reasonably low levels (typically below 20) if clinical applications are envisaged [72]. And despite the fact that adult stem cells typically divide less frequently than pluripotent stem cells, they are also prone to acquire chromosomal aberrations during expansion in culture. For instance, MSCs as well as NSCs also acquire large chromosomal alterations at a lower or similar frequency (respectively 4 and 9%) than ESCs (9%), and once acquired, they are able to take over the culture in as few as 6–7 passages [73]. Monosomy 13 is recurrent in MSCs, while trisomy 19 and 7 occur more frequently in NSCs [73, 74] (Fig. 1). Aneuploidy may be observed early in the culture and have some degree of donor dependency [75], but its involvement in cell transformation remains controversial [75–78]. Long-term cultured human NSCs (passage 17) were shown to undergo spontaneous transformation to tumor-initiating cells and to exhibit abnormal karyotypes and tandem repeat instability [79]. Recently, a recurrent jumping translocation of chromosome 1q has been shown to allow hESC-derived NSCs to bypass senescence and to impair engraftment in rat brains [80]. Since this aberration had previously been associated with hematologic disorders and brain tumors, the authors suggest regular monitoring of neural derivatives [80].

Human adipose-derived stem/stromal cells (hASCs) are apparently more resilient to genomic changes even when cultured for long periods of time (6 months) [81]. Minor pericentromeric or telomeric/subtelomeric variations were occasionally detected at early passages but subsequently eliminated from culture. More recent data are less indicative of such stability, showing that expansion under standard culture conditions gradually increases the accumulation of aneuploid cells (chromosomes 8, 11, and 17), the latter being observed as soon as P2 [82].

In the case of umbilical cord blood-derived CD34+ stem/progenitor cells which contain HSCs obtained from healthy donors, karyotypic abnormalities were also detected upon ex vivo expansion as soon as day 7, although not displaying evidence of neoplastic transformation [83]. In another study performed with endothelial progenitors, tetraploidies and aneuploidies were detected in early passages (P2-P4) [84]. In some cases it has been observed a donor-age-dependent increase in genomic damage accompanied by a decrease in functional capacity and DNA repair capability particularly through a loss of fidelity and efficacy of the nonhomologous end-joining (NHEJ) pathway [85, 86]. Accordingly, a decrease in proliferation and occurrence of karyotypic abnormalities with transformation seems particularly frequent in human adult stem cells obtained from aged donors (typically more than 60 years old) [71, 87, 88].

Multiple studies have been carried out to address the effect of hypoxic preconditioning on the genomic instability of human adult stem cells, often with contradictory results. Some authors point to enhanced structural instability and aneuploidy events at early passages (P1–P7) under low oxygen (5% O_2) [87]. Others describe physiological O_2 concentrations (1%–7%) to significantly reduce or prevent chromosomal aberrations [89–92]. Rodríguez-Jiménez et al. [93] have shown that low oxygen environments (1% O_2) repress the mismatch repair system through epigenetic chromatin inactivation and diminished SP1 binding, resulting in increased microsatellite

instability (MSI) in mouse NSCs and human BM MSCs as soon as 6 hours. Similarly, our group has also provided evidence for a concerted downregulation of the DNA repair machinery and subsequent increase in MSI in human BM MSCs and hASCs under 2% O_2 in comparison to normoxic conditions [94]. We also observed that hASCs react more slowly than BM MSCs to low oxygen environments in terms of changes in expression of DNA repair genes and mitochondrial performance. The regulation of telomerase gene expression by hypoxia has also been addressed in adult stem cells. These cells are typically telomerase-low/negative [95], but contrasting results have been reported [96]. Tsai et al. [92] have shown that human BM MSCs expanded under hypoxic conditions (1% O_2) by up to 100 PDs had greater telomerase activity and telomere length than cells expanded under normoxic conditions. Routine procedures such as cell passaging, when extended to a near confluence stage, allow a generalized cell contact to take place and accelerate senescence independent of telomere shortening and p53 activation [97].

Challenges Ahead

Despite the remarkable advances seen in the last few years in the broad field of stem cell research, it is yet to be perceived in full detail the real extent to which ex vivo manipulation increases cell mutation load. One thing is certain: restraining the clinical use of cells solely to a low passage number will help to mitigate to a certain point the risk of adverse effects but should not be regarded *per se* as a guarantee of genomic integrity. It is important to realize that genomic mutations always occur naturally in any cell culture, and, more important than aiming for a mutation-free genome, is to distinguish between clinically harmless and deleterious mutations. For example, potentially deleterious mutations may be observed only during short periods of time, and be associated with physiological processes of the cell such as differentiation [98].

The control of genomic instability and the suppression of deleterious mutations is probably one of the most significant obstacles for the translation of stem cell therapies to the clinic and will require the development of new strategies to adequately monitor and/or alleviate this phenomenon. Recent achievements have given important steps toward such goal. For example, the growing sophistication of cellular engineering technologies allowing fast and precise genome editing and mutation correction offer exciting perspectives and will certainly boost the clinical use of cell-based therapies. Some examples include zinc finger nucleases or transcription activator-like effector nucleases which can be used as customization tools to generate site-specific double strand breaks in the target locus, followed by NHEJ and homologous recombination (reviewed in [99]). In terms of mutation mapping, a protocol has recently been proposed that uses expression-based data for purposes of cell karyotyping (e-karyotyping). The rationale for this technique is based on the fact that alterations in genomic regions will drive expression changes in genes located within or in the close vicinity of those regions. The resolution of this technique is dependent on cell type and microarray platform but is still comparable to that of cytogenetic methods (roughly 10 Mb) [100]. Dedicated and standardized tests for evaluation of telomere length variation during cell culture also seem to be looming in the near future, with some examples involving FISH [101] and real-time PCR [102, 103].

From the clinical point of view, there is a growing interest in the use of massive parallel sequencing approaches for a more detailed and comprehensive rendering of the onset and progression of certain malignancies. Recent advances in the safety assessment of stem cell-based therapies are allowing researchers to follow the evolutionary trajectory from ancestral premalignant clone reservoirs to fully established clonal lineages. One illustrative example relates with the increased risk of relapse for patients undergoing autologous or allogeneic stem cell transplantation for the treatment of acute myeloid leukemia and myelodysplasia. In a recent report, preleukemic somatic clones were successfully identified in healthy donor cells by exome sequencing [104], highlighting the need for the use of analytical tools that allow examining the identity and presence of potentially detrimental lesions at the single-cell level. In this context, it will be interesting to understand in greater detail the genetic determinants that underlie the origin of tumor-initiating cells and their putative hierarchical relation with normal stem cells.

From a bioengineering standpoint, there is a pressing need to implement a fast route toward more robust and standardized platforms to cultivate stem cells at a production scale. In particular, this can be accomplished by making use of more efficient bioreactor configurations and by avoiding the use of poorly defined culture media and contaminant xenogeneic-derived components. In this context, karyotypic abnormalities were found to be more frequent when cells are cultivated using serum-free media [45], which calls for continued efforts to improve defined culture media. On the same line, systemic studies become necessary to understand the oxygen consumption characteristics of the culture and the physiological effects exerted on the different cell types particularly in scenarios of nonphysiological or fluctuating oxygen tensions (*e.g.*, during cell manipulation). Indeed, many of the discrepancies observed in the literature, may be attributable to the lack of use of dedicated pO_2-controlled flow hoods, inaccurate pO_2 measurements, preculture conditions used, medium composition, type of cell, and donor characteristics. Moreover, it is worth noticing that in the past 25 years, 18%–36% of the cultures performed within the frame of independent studies were in fact contaminated or misidentified [105]. This disturbing finding, which has been recurrently pointed out [105–108] but often ignored by several parties, demands for a serious and concerted effort to verify cell-line identity and avoid inconsistent or flawed results.

Many of the abovementioned issues underscore the need for an international program of standardization and uniformity among cell therapies, for example, focusing on aspects such as culture conditions, differentiation, product labeling and storage. Moreover, it is not perfectly clear how regulatory agencies, within the frame of such consensus standards, will adapt their guidelines to the different models of cell therapy that are expected to emerge. We anticipate that some degree of regulatory flexibility will be needed in order to adapt to the different cell types and their inherent variability, to the different extents of cell manipulation, or to the need for cell storage during indeterminate periods of time. Also, it remains to be clarified the acceptable threshold number of passages

able to grant the maintenance of genomic stability, or at least, able to guarantee that despite any lesions eventually present, they will not pose a safety risk to the patient. To debate on these questions, we propose that an international multidisciplinary meeting should be held, as a forum for fostering a constructive dialogue between stakeholders, such as regulatory authorities, researchers, pharmaceutical companies, funders and patient groups. This would represent an early-stage effort to seek a common understanding on relevant topics, and hopefully to lay the ground for future key recommendations that would guide the development of cell therapies.

Conclusion

In conclusion, further improvements in the upcoming years will unfold around improving cellular product consistency, process development, quality monitoring, and uniformity. As cell therapies move toward the clinic setting, there is a pressing need to ensure proper safety standards and to establish fully defined criteria able to pinpoint deleterious variants. Much of this process will require a large and concerted effort by the scientific community directed toward the categorization and functional interpretation of large volumes of data. As our understanding of the balance between health benefits, risk and ethical issues matures, stem cell therapies will move a step closer towards a scenario of full clinical implementation.

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Author Contributions

P.H.O.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; C.L.d.S. and J.M.S.C.: conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflict of interests.

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