Chromosomal Stability during Stem Cell Culture: Recent Status and Its Implications

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Abstract

One of the key issues impeding the application of human embryonic stem cells (hESCs) in regenerative medicine is the occurrence of karyotypic instability of hESCs during long term culture. Stem cells grown in culture are exposed to strong selection pressures that often result in genomic alterations, varying in size from point mutations to large chromosomal aberrations, trisomies and monosomies. Different culturing conditions may affect the nature and the frequency of the acquired aberrations; however no culturing technique is immune to genomic instability. The current techniques to evaluate the genomic integrity of stem cells are mostly based on direct cytogenetic and DNA-based analyses; traditional karyotype, spectral karyotyping, array-based molecular analyses of genomic integrity etc. The potential use of aberrant or unstable stem cells in cell therapy is obviously hindered as there is increased tumorigenicity risk. It is then important to optimize culturing conditions, cryo-storage and monitoring systems to be applied to the newly derived as well as existing cell lines to decipher any genetic and epigenetic alterations. It was also found that reduced oxygen to physiological levels and additions of antioxidants can be employed as possible strategies to lower oxidative stress and decrease chances of chromosomal transformation. The manual and mechanical dissection are preferentially used during hESCs sub-culturing as being less aggressive and better preserve the genome integrity.

Key words: Stem cell, genomic instability, tumorigenicity risk, DNA based analysis.

1. Introduction

Stem cells are characterized by the ability to self renewal through mitotic cell division and differentiating into a diverse range of specialized cell types (Puri et al., 2014). Embryonic stem cells (ESCs) are undifferentiated, unspecialized and self-renewable cells that can be maintained in vitro in the same undifferentiated status over extended periods of culture. At present scientists are intensively studying the fundamental properties of stem cells to determine precisely how stem cells remain unspecialized and self renewing for many years and to identify the signals that cause stem cells to become specialized cells. It is supposed to a variety of growth factors and nutrients which not only help the growth and multiplication of cells, but nourishes embryonic stem cells. However, growth factors are naturally occurring regulatory molecules, which bind to receptors on the cell surface (Puri et al., 2014). Providing specific culture conditions, ES cells can be maintained in undifferentiated state for a prolonged period of time (Gardner, 1997; Wang et al., 1997). An important characteristic of ES cells is their ability to undergo a spontaneous differentiation in vitro forming a variability of progenitors and differentiated cells in response to specific signals (Doetschman et al., 1985; Bautch et al., 1996). Stem cells can be identified on the basis of its different characteristics. Cherney et al. (1993) gave some criteria for the identification of stem cells. e. g. High nucleus: cytoplasm ratio, Immortal cell lines, differentiation in culture in absence of cytokine LIF, karyotypic analysis, in vivo produce...
teratocarcinoma, chimeras etc. ESCs are characterized by the expression of specific transcription factors (OCT-4, SOX2, and NANOG) and surface markers (TRA-1-60, TRA-1-81, SSEA-3 and SSEA-4 in hESCs) by high telomerase expression and alkaline phosphatase activity (Rebuzzini et al., 2011). The growing popularity of hESC is based on two important properties: first, their pluripotent capability, i.e. their potential to differentiate into every cell type in the human body, which makes them suitable for almost countless applications; second, their putative long-term stability of pluripotency and karyotype, which gives promise of high reproducibility (Hanson and Caisander, 2005).

One of the key issues impeding the application of hESCs in regenerative medicine is the occurrence of karyotypic instability of hESCs during long term culture (Chung et al., 2011). Stem cells grown in culture are exposed to strong selection pressures that often results in genomic alterations, varying in size from point mutations through copy number changes in small genomic elements, amplification of repetitive sequences and retro-element mobility, to large chromosomal aberrations, trisomies and monosomies (Lefort et al., 2009). Stem cells acquire genomic changes throughout their expansion in culture. Much attention has been drawn in recent years to the genomic aberrations acquired by human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), from the resolution of point mutations to the resolution of whole-chromosome trisomies. It is important to note that genomic instability is not a characteristic of human stem cells only but it is a common phenomenon in mouse stem cells as well (Ben-David and Benvenisty, 2012; Pasí et al., 2011; Quinlan et al., 2011) and it is likely to be found in stem cell cultures of all the species used in biomedical research. It is thus considered, for most practical purposes, a “necessary evil” that does not seriously compromise the utility of aberrant cells in basic science (Ben-David and Benvenisty, 2012).

2. History
The stem cell era began in the early 1980s when mouse ES cells were successfully derived (Evans and Kaufman, 1981, Martin, 1981). It took more than a decade to obtain ESC lines from blastocysts of the primate rhesus monkey (Thomson et al., 1995), human (Thomson et al., 1998), dog (Hayes et al., 2008) and rat (Li et al., 2008; Buehr et al., 2008). In a study of blastocysts, Hardarson and co-workers in 2003 found that only 42% of blastocysts originating from surplus embryos were chromosomally normal. Of the abnormal embryos, most were mosaic, i.e. with a mixture of diploid and aneuploid cells. But it has recently been suggested that hESC may be prone to acquire chromosomal anomalies while being cultured continuously in vitro. This could be due to the in vitro environment itself and to the mode of culture conditions. By comparison of stem cell lines expanded with either enzymatic or mechanical passage, Mitalipova et al. (2005) found indications of perpetuation of aneuploid cell populations and also of quantitative changes in gene expression when using the enzymatic method (Hanson and Caisander, 2005).

3. Causes of Chromosomal Instabilities
The variety of culture protocols applied in different laboratories working with ESCs may be the source of variations in cell differentiation and genome stability (Rebuzzini et al., 2011). Different culturing conditions, such as media composition (whether of animal or artificial origin), the presence of the feeder layer, cell passaging techniques and freeze-thaw cycles, may affect the nature and the frequency of the acquired aberrations; however, no culturing technique is immune to genomic instability (Ben-David and Benvenisty, 2012). In vitro culture causes significant chromosomal instability in hematopoietic cells and that this effect appears to be attributable to oxidative stress imposed on the cells. It has been shown that high oxygen concentrations increase reactive oxygen species (ROS) levels and oxidative stress, which in turn leads to an increased incidence of genomic abnormalities in cultured cardiac stem cells and ESCs (Liu et al., 2012).

ESCs are derived and maintained in vitro with a co-culture protocol on a feeder layer of mitotically inactivated fibroblast monolayers or immortalized fibroblasts. Puri et al. (2013) propagated in vitro derived buffalo (Bubalus bubalis) embryonic stem cells on mitomycin-C inactivated murine fibroblast monolayer. Besides the feeder layers, stem cells are cultured on supportive synthetic matrices i.e., gelatin, fibronectin or matrigel-TM. It was observed that genetic alterations were observed both in mESC and hESC lines using the former or later. A fundamental component of the ESC medium is the serum of animal (calf or bovine) or artificial (knockout serum replacement of defined composition) origin. Despite the type of serum used, the genomic stability seems compromised (Rebuzzini et al., 2011). The most frequent karyotypic changes so far reported involved chromosome trisomy, indicating that chromatid separation during mitosis may be prone to error in hESCs. Inefficiency of G2 decatenation checkpoint and uncoupling of checkpoint-apoptosis in hESCs might be a likely source of karyotypic abnormalities. This clearly suggests that an altered apoptotic mechanism caused by in vitro culture environments may have great
impact on the preservation of genetic integrity of hESCs. The aneuploidy observed in hESCs is also most likely driven by the stresses induced by variable environmental conditions to which these cells are exposed in culture. Exposure of hESCs to ambient oxygen tension (20%) could promote abnormalities by causing damage to chromosomes (shortening telomeres) and mitochondrial DNA (Chung et al., 1996). However, the resolution is much lower for the identification of intra-chromosomal duplications and deletions (Meisner and Johnson, 2008), especially when compared to that of the molecular methods described in the next paragraphs. In order to detect sub-microscopic chromosomal aberrations beyond the detection threshold of SKY, complementary fluorescent in-vitro hybridization (FISH) may be applied. However, a suspected region of interest has to be determined in advance, in order to make this method useful. Array-based molecular analyses of genomic integrity (also called “virtual karyotypes”) exhibit a dramatically improved detection resolution, of 20 Kbp to 1 Mb (depending on the probe density on the array (Speicher and Carter, 2005). The best known of these techniques are array-comparative genomic hybridization (aCGH) and single nucleotide polymorphism (SNP) array. In aCGH, the test sample and a normal reference sample are differentially labeled with different fluorophores, and are hybridized to thousands (or even millions) of probes. The ratio of fluorescence intensity is then measured, enabling to detect copy number changes along the genome. Similarly, SNP array is also based on labeling DNA fragments and hybridizing them on the array to allele-specific oligonucleotide probes. SNP array has a couple of advantages over aCGH: it can detect loss of heterozygosity (LOH) events, and it can detect polyploidy (that is, the addition of an entire set of chromosomes, which cannot be detected in aCGH because test and control samples contain the same proportion of haploid sets per hybridized DNA) (Lefort et al., 2009). However, both techniques also have limitations when compared to cytogenetic methods: (a) they are more expensive; (b) their sensitivity is rather low as they are based on DNA extracted from the cell population, genomic aberrations can be detected in a mosaic culture only if present at over 20% of the cells (Lefort et al., 2009; Meisner and Johnson, 2008); and (c) they cannot detect balanced translocations and inversions. The integrity of stem cells can also be examined by whole genome sequencing, resulting in an extremely high single-base resolution, and the ability to detect a change as subtle as point mutation (Gore et al., 1996). However, the sensitivity of the method is quite high, since single cells are examined. Usually, 20 metaphases are observed, resulting in a detection threshold of >5% (that is, when over 5% of the cells in the population harbor a chromosomal aberration, it is likely to be detected). While it is relatively economical, karyotyping is still technically challenging and requires skilled personnel.

Spectral karyotyping (SKY) is a molecular cytogenetic technique that improves the resolution of the traditional karyotype (Schrock et al., 1996). Chromosome-specific DNA sequences are labelled with different fluorophores, generating unique fluorescent probes for each pair of chromosomes. The individually colored chromosomes are then visualized, and genomic changes can be detected. SKY is as sensitive as traditional karyotype, but is somewhat more expensive. Its resolution is higher than that of G-banding, and gets to about 2 Mb for the identification of translocations and complex karyotypes (Schrock et al., 2008).

4. Common Methods for Evaluation of Genomic Integrity

The current techniques to evaluate the genomic integrity of stem cells are mostly based on direct cytogenetic and DNA-based analyses. The most commonly used technique, by far, is the traditional karyotype analysis. Karyotyping is based on arresting the cells during cell division, followed by their staining, usually with Giemsa staining (G-banding). Based on the cytogenetic characteristics of the individual chromosomes at metaphase (e.g. size, centromeric location, unique pattern of light and dark bands), chromosomal abnormalities can be observed under the microscope. The average resolution of G-banding is about 10 Mb, depending on the region of interest (Meisner and Johnson, 2008; Speicher and Carter, 2005). The sensitivity of the method is quite high, since single cells are examined. Usually, 20 metaphases are observed, resulting in a detection threshold of >5% (that is, when over 5% of the cells in the population harbor a chromosomal aberration, it is likely to be detected). While it is relatively economical, karyotyping is still technically challenging and requires skilled personnel.

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based on the gene expression patterns of the cells. This methodology is implemented for the accurate evaluation of the genomic integrity of human PSCs (hESCs and iPSCs) (Mayshar et al., 2010), human multipotent stem cells (neural, mesenchymal and hematopoietic stem cells) (Ben-David et al., 2011) and mouse pluripotent stem cells (ESCs, iPSCs and epiblast stem cells) (Ben-David and Benvenisty, 2012).

5. Consequences of Chromosomal Aberrations in Stem Cell Research

The implications are far-reaching and encompass all of the potential applications of these cells. The potential use of aberrant or unstable stem cells in cell therapy is obviously hindered as there is increased tumorigenicity risk, the cells are inadequate due to perturbed expression pattern, they have limited differentiation capacity, and by possible less number of the differentiated cells (Ben-David and Benvenisty, 2012). The most prominent promise of stem cells is their potential to serve in cell-based therapy. Given that genomic aberrations are a characteristic of practically all cancer types, where they serve both as a driving force and as a by-product of tumorigenesis, these aberrations may increase the tumorigenicity of the cells, preventing their clinical implementations (Ben-David and Benvenisty, 2011; Ben-David et al., 2010; Goldring et al., 2011). Their use in drug screening studies is problematic, as the aberrant expression patterns and cellular properties may affect their sensitivity to drugs, leading to both false positive and false negative “hits”. Their use for disease modelling is jeopardized due to artificial effects induced by the aneuploidy, and due to a possible failure to accurately recapitulate the disease-specific cells and phenotypes.

6. Methods to Reduce Chromosomal Instabilities

It is then important to optimize culturing conditions, cryostorage and monitoring systems to be applied to the newly derived as well as existing cell lines to decipher any genetic and epigenetic alterations, which may have taken place. In a publication (Herszfeld et al., 2006) better results were obtained in the production of more stable hESCs when a serum replacement was used, likely because the use of artificial serum avoids the uncertainty of its composition which is frequently observed with animal-derived sera. The technique used to detach ESCs for passaging also seems to play a major role in the maintenance of their genomic stability. The manual and mechanical dissection are preferentially used during hESCs sub-culturing, as, being less aggressive, they better preserve the genome integrity (Buzzard et al., 2004; Mitalipova et al., 2005). It was also found that reduced oxygen to physiological levels and additions of antioxidants can be employed as possible strategies to lower oxidative stress and decrease chances of chromosomal transformation (Liu et al., 2012).

7. Conclusion

One of the key issues impeding the application of hESCs in regenerative medicine is the occurrence of karyotypic instability of hESCs during long term culture. Different culturing conditions may affect the nature and the frequency of the acquired aberrations; however, no culturing technique is immune to genomic instability. The current techniques to evaluate the genomic integrity of stem cells are mostly based on direct cytogenetic and DNA-based analyses. The implications are far-reaching and encompass all of the potential applications of these cells. The potential use of aberrant or instable stem cells in cell therapy is obviously hindered as there is increased tumorigenicity risk. It is then important to optimize culturing conditions, cryo-storage and monitoring systems to be applied to the newly derived as well as existing cell lines to decipher any genetic and epigenetic alterations.

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