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REVIEW

Achilles' heel of pluripotent stem cells: genetic, genomic and epigenetic variations during prolonged culture

Paola Rebuzzini[1,2] · Maurizio Zuccotti[3] · Carlo Alberto Redi[1,2,4] · Silvia Garagna[1,2]

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Abstract Pluripotent stem cells differentiate into almost any specialized adult cell type of an organism. PSCs can be derived either from the inner cell mass of a blastocyst—giving rise to embryonic stem cells—or after reprogramming of somatic terminally differentiated cells to obtain ES-like cells, named induced pluripotent stem cells. The potential use of these cells in the clinic, for investigating in vitro early embryonic development or for screening the effects of new drugs or xenobiotics, depends on capability to maintain their genome integrity during prolonged culture and differentiation. Both human and mouse PSCs are prone to genomic and (epi)genetic instability during in vitro culture, a feature that seriously limits their real potential use. Culture-induced variations of specific chromosomes or genes, are almost all unpredictable and, as a whole, differ among independent cell lines. They may arise at different culture passages, suggesting the absence of a safe passage number maintaining genome integrity and rendering the control of genomic stability mandatory since the very early culture passages. The present review highlights the urgency for further studies on the mechanisms involved in determining (epi)genetic and chromosome instability, exploiting the knowledge acquired earlier on other cell types.

Keywords Embryonic stem cells · Induced pluripotent stem cells · Aneuploidy · Regenerative medicine · Tissue engineering · Cell therapy

✉ Paola Rebuzzini
paola.rebuzzini@unipv.it

✉ Maurizio Zuccotti
maurizio.zuccotti@unipr.it

✉ Silvia Garagna
silvia.garagna@unipv.it

[1] Laboratorio di Biologia dello Sviluppo, Dipartimento di Biologia e Biotecnologie 'Lazzaro Spallanzani', Università degli Studi di Pavia, Via Ferrata 9, 27100 Pavia, Italy

[2] Center for Health Technologies (C.H.T.), Università degli Studi di Pavia, Via Ferrata 1, Pavia, Italy

[3] Unita' di Anatomia, Istologia ed Embriologia, Dipartimento di Scienze Biomediche, Biotecnologiche e Traslazionali (S.BI.BI.T.), Università degli Studi di Parma, Via Volturno 39, 43100 Parma, Italy

[4] Fondazione I.R.C.C.S. Policlinico San Matteo, Piazzale Golgi, 19, 27100 Pavia, Italy

Introduction

Pluripotent stem cells (PSCs) have the potential to differentiate into almost any specialized adult cell type of an organism. PSCs can be derived from the inner cell mass (ICM) of a preimplantation blastocyst [1–3], giving rise to embryonic stem cell (ESC) lines. ESCs are undifferentiated and self-renewing cells that, once successfully established in vitro, preserve features and developmental potential (pluripotency) of the founder cells of the embryo. Almost a decade ago, Yamanaka's group, enforcing the ectopic expression of four key transcription factors *Oct-4*, *Sox2*, *Klf4*, and c-*Myc*, reprogrammed for the first time somatic terminally differentiated fibroblast cells to pluripotency [4, 5]. These cells, called induced pluripotent stem cells (iPSCs), show similar self-renewal and differentiation potential of ESCs [4, 6, 7]. Since then, many other reprogramming methods have been proposed which entail either the genome integration of reprogramming genes (single cassette reprogramming vectors with Cre-Lox mediated transgene excision) or not (Adenovirus, Sendai Virus, PiggyBac, minicircle vectors, episomal plasmids), the overexpression of reprogramming proteins (proteins and

mRNA delivery), the modulation of the expression of pluripotency or reprogramming genes (miRNA) and epigenetic chromatin remodeling (e.g., hydroxamic acid, trichostatin A and valproic acid) [8].

The potential use of these cells in regenerative medicine and tissue replacement, for investigating in vitro early embryonic development, screening the effects of new drugs or xenobiotics, relies on their capability to maintain genetic integrity during prolonged culture and differentiation [9]. Although PSCs have specialized mechanisms to preserve their genome integrity, such as facilitated apoptosis, a $\sim$100 times lower mutation frequency compared to differentiated cells, high efficient DNA repair mechanisms and antioxidant defense [10, 11], they accumulate genetic and epigenetic abnormalities when maintained in long-term culture in vitro [for reviews, see 12, 13]. The diversity of culture protocols among independent laboratories perplexes the identification of the causes of such variations, although the presence or absence of a feeder layer, the animal or artificial source of serum and the different methods used for cell passaging seem to be major factors affecting the maintenance of a correct genome integrity [14].

In this review, we present a detailed overview of the alterations observed during long-term culture of human or mouse ESCs and iPSCs. We grouped these numerous alterations into five main types, such as chromosome number and structural variations, microsatellite instability, epigenetic instability and aberrations of mitochondrial DNA; also, for each of them we gave a synthesis of the known causal mechanisms.

Chromosome number variations

The loss of euploidy in PSCs during long-term culture has been widely reported by several independent laboratories. Using classical and molecular cytogenetic techniques, gain or loss of entire chromosomes has been described in both human and mouse ESC lines, with a strong species specificity. Gain of specific chromosomes appears to confer growth advantage and higher culture adaptation to cells carrying numerical variations (see paragraph "Chromosome structural variations").

IPSC lines maintain a normal karyotype during the very early culture passages [5, 15], but, as for ESCs, they display chromosome number variability during prolonged culture.

Gain of entire chromosomes 12, 17, 20 or, to a lesser extent, 16 and X (particularly in female cell lines) frequently occurs in human ESCs (hESCs) [16–22], representing about 50 % of all aberrations observed [18]. A progressive tendency to chromosome gain occurs during culture and thus it is advisable to avoid their use beyond passage 70 [23].

Since the first significant study reporting the presence of alterations in the chromosome make-up of mouse ESC (mESC) lines [24], it has become evident that chromosome number variations are a general feature of mESCs. More recently, cytogenetic analyses performed on 540 [25] and 97 [26] independent mESC lines show, by 20 passages, that about half of the cell lines has modal chromosomal numbers of 41, 42 or 39 and about 25 % of all aberrations are trisomies of chromosomes 8 and 11. These same recurrent abnormalities are also described in other cell lines together with other numerical random aberrations [25–32].

As for ESCs, genetic variations in iPSCs may originate either early from individual starting cells or during prolonged culture, but they may be caused also by the reprogramming process itself. Large scale screenings of more than 320 lines show that about 10–20 % of either mouse or human iPSC lines contain numerous chromosomal aberrations [20, 21, 33–35] and of these, about 40-45 % are recurrent and involve full trisomy of chromosomes 8, 12 or X or aneuploidy of chromosomes 6 (in 1.4 % of the cell lines analyzed), 11 (16.9 %), 12 (5.63 %), 19 (1.41 %) and Y (7.04 %) [21, 30, 33, 36–40]. The recurrence of these specific aberrations suggests a process of culture adaptation during reprogramming [33], perhaps conferring a selective and/or proliferative advantage.

Mechanisms involved in the generation of chromosome number variations

In eukaryotic cells, four main mechanisms have been described to control spindle assembly, microtubule/kinetochore coupling, centrosome duplication and sister chromatid cohesion during cell replication ("Box 1"). The failure of one or more of them is the main cause of chromosome number variations described in cancer and differentiated cells [41, 42]. Up to date, an abnormal number of centrosomes, with the formation of multipolar spindles, has been the sole mechanism described as possible cause of chromosome number variability in hESCs [43–45]. To this regard, an abnormal number of centrosomes, associated with an altered expression level of mitotic spindle-related molecules such as Aurora A, CDK2 [44], BUB1, CENPE and MAD2 [45] results in the formation of multipolar spindles and centrosome number variability in 12 hESC lines derived from normal or parthenogenetic embryos, respectively.

Chromosome structural variations

The presence of structural variations (SVs; duplications, deletions, insertions, inversions, translocations, and complex rearrangements) in PSCs has been extensively

reported making use of array comparative genomic hybridization. The most recurrent SVs detected are chromosome deletions, which cause loss of heterozygosity, and duplications, also defined as copy number variations (CNVs) [46]. Importantly, the presence of an additional copy of some specific genes (see below) confers to cells resistance to apoptosis, growth and/or survival advantage, leading to culture adaptation.

Following prolonged culture, hESC lines display several SVs, among which frequent additional segments of chromosomes 1, 12, 17 or 20 and deletions of chromosomes 8, 10, 16, 18 and 22 have been described (Table 1). Interestingly, additional SVs of chromosomes 1, 12, 17 or 20 have also been described in cancer cells in association with growth and/or survival advantage. Specifically, the duplication of the 12p13 band, containing the cell-cycle regulator *CCND2,* the pluripotency marker genes *DPPA3* (*Stella-Related*), *GDF3* and *NANOG*, has been associated with proliferative advantage and culture adaptation [17]. Proliferative advantage and culture adaptation in cancer cells is also associated with gain of chromosome 17q, containing *BIRC5*, encoding for the anti-apoptotic gene *Survivin* [47], and the miRNA has-mir-21, which regulates the anti-apoptotic gene *Bcl2* [48, 49]. The duplicated 20q11.21 region contains the stemness gene *DNMT3B*, *TPX2* and *KIF3B*, involved in the cell cycle, *ID1* in cell growth, *BCL2L1* and *PDRG1* in cell survival and the immature form of the micro-RNA miR-1825 [50–53].

Several random SVs were detected in 49 (38.0 %) out of 129 mESC samples, revealing a high frequency of chromosomal aberrations. In 5 out of 129 mESC lines, the most recurrent aberration, acquired within 12 passages of culture, is the gain of 11qE2, a region that is fully syntenic with human 17q25, the latter a common aberration detected in hPSCs. Also, deletions of chromosomes 10qB and 14qC-14qE have been observed in 13.5 and 4.8 % of the mESC lines analyzed, respectively [30]. In addition, duplications of specific regions on chromosome 1, 2 and 12 were described in other mESC lines [54].

Comprehensive genome-wide analyses revealed that both hiPSCs and miPSCs accumulate a number of SVs higher than ESCs (during more than 50 passages) [18, 40, 55–59]. Whilst certain SVs may arise in both ESCs and iPSCs, some are specific of iPSCs and are recurrent (as found in ESCs) and shared by more that 25 % of all hiPSC lines analyzed; instead, others result from the amplification of 1q31.1, 8q24.3 and 17q21.1 [60] unique to hiPSCs. Loss of 8q24.3 region, observed in 12 % of cell lines analyzed, is found specifically in hiPSC lines [21].

In miPSCs, both gain and loss of SVs are variable among different cell lines and distributed along the majority of chromosomes [59].

Mechanisms involved in the generation of structural variations

The main processes, whose failure leads to chromosome deletions or duplications, are the homologous recombination (HR)—a repair mechanism that requires the presence of DNA sequence identity between chromosomes—and the

Table 1 Summary of SVs described in hESC lines during prolonged *in vitro* culture

Structural variations	Aberration	Putative candidates		References
		Genes	miRNA	
1q11–1p36 1q31.3	Gain	*MCL1, IL6R, PSMD4, PSMB4, UBE2Q, UBAP2L, RBM8A, RPS27, PIAS3, POLR3C, HIST2H2AA, LASS2, MRPL9, JTB, HAX1, SHC1, APH-1A, BCL9, ZNF364, SSR2, MAPBPIP, CCT3, MEF2D, C1orf85*	hsa-mir-9-1	[22, 66, 182]
Partial chromosome 12 or isochromosome 12p	Gain	*NANOG, DPPA3 (Stella-Related), GDF3, CCND2*	*n.d.*	[17, 22, 183]
17q11–q12, 17q21–17q23.2, 17q25–qter	Gain	*BIRC5*	miRNA has-mir-21	[17, 22]
20q11.21	Gain	*DNMT3B, TPX2, KIF3B, BCL2L1 PDRG1*	*n.d.*	[22, 66–68]
8q24.3	Loss	*n.d.*	*n.d.*	[18, 21, 46, 53, 184]
10p13pter	Loss	*n.d.*	*n.d.*	
16q	Loss	*n.d.*	*n.d.*	
18q21qter	Loss	*n.d.*	*n.d.*	
22q13qter	Loss	*n.d.*	*n.d.*	

n.d. not detected

non-homologous recombination (NHR), which, by contrast, either employs micro-homology or does not require homology. These processes are highly conserved in higher eukaryotes and among different species and are active in PSCs [61, 62]. Repair of DNA double strand breaks (DSBs) by HR could be the source of deletions arising in PSCs during prolonged culture, whereas several NHR subpathways ("Box 2") [non-allelic homologous recombination (NAHR), non-homologous end-joining (NHEJ), microhomology-mediated break-induced replication (MMBIR) and fork stalling and template switching (FoSTeS)] seem to be specifically involved in the generation of duplications [63, 64].

One or more of these mechanisms are directly involved in the onset of SVs in PSCs. The recurrent gain of the 20q11-13 region, in several hESC lines (i.e., SA01, H1, VUB05, H7 and HSF1), might be caused by error-prone HR events, as reported for the synthenic mouse 2H region, 'hypermutable' for the presence of microsatellite D2MIT140 [65–68]. In addition, NAHR and NHEJ in hESM01, hESM01f and HS27 hESC lines are responsible for the mis-rejoining of DSBs, resulting in SVs [46].

In E14, AB2.2 and JM8 mESC lines, NAHR is responsible of the appearance of frequent CNVs during routine culture [69]. More recently, in TC1 and *Xrcc4*$^{-/-}$ mESC lines, FoSTeS and MMBIR were proposed as candidate mechanisms for CNVs, as well as other more complex rearrangements [70].

Both hiPSC and miPSC lines harbor higher levels of SVs than ESCs [21, 33, 40, 55–57, 59], likely originated by NHEJ, FoSTeS or MMBIR, which appear the prevalent mechanisms during reprogramming and culture adaptation [57, 58].

Microsatellites instability

Microsatellites are highly polymorphic stretches of repetitive DNA regions, disseminated throughout the genome and very abundant in eukaryotes. Microsatellite instability (MSI) is a common hallmark observed in tumor cells, which display additions or deletions of repeated units [71]. To this regard, only a very recent paper tackles the occurrence of MSI in PSCs when maintained in culture for a long period. Specifically, ten hESC lines that were analyzed for 122 microsatellites dispersed over the whole genome, screened at early, medium and late passages from passage 7 to 334, showed that 2 out of 10 hESC lines accumulate altered (both deletions and duplications) microsatellites patterns, involving about 1–20 % of the total number of alleles, indicating very low and fluctuating frequency of MSI. The novel alleles observed could take over during prolonged culture as passenger mutations, without conferring a selective advantage [53].

Mechanisms involved in microsatellite instability

In cancer cells, the main cause of MSI is the inactivation or deficiency in the post-replicative mismatch repair (MMR) process [72] ("Box 3"), due to the failure of some key MMR genes. This failure was not observed in hESC lines, whose expression of MMR genes and proteins resulted unaltered [53].

Epigenetic instability

DNA methylation and histone tail modification are the major epigenetic processes that contribute to regulate gene expression in early developmental phases [for reviews, see 73, 74]. During prolonged in vitro culture, an increasing number of studies reported the accumulation of epigenetic alterations in PSCs [18, 66, 75–77]. These alterations dramatically affect their pluripotency status [78, 79] and, persisting during differentiation, are associated with aberrant phenotypes [80]. Following, we will summarize some alterations of the epigenetic status of PSCs, comprising modifications in imprinted genes, DNA and histone methylation and X-chromosome inactivation (XCI) [81, 82].

Imprinted genes

In several hESC and hiPSC lines, an epigenetic drift in the methylation pattern of some paternally (e.g., *H19*, *PEG1*, *SNRPN* and *IGF2*) or maternally (e.g., *MEG3*, *IGF2R* and *SLC22A18*) imprinted genes has been observed during prolonged culture in many independent laboratories [83–87]. These data were recently reinforced by a very comprehensive study that, analyzing more than 200 hPSC lines, demonstrated an overall variable allelic expression of several imprinted loci (*DIRAS3*, *NAP1L5*, *MEST*, *H19*, *ZIM2/PEG3*, *PLAGL1*, *GRB10* and *GNAS*) compared to somatic cells [88].

In several mESC lines, the epigenetic status of *H19*, *Peg1, Snrpn* and *Igf2r* imprinted genes significantly changes during about 30 in vitro passages [89]. Epigenetic changes have also been reported in individual mESC subclones derived from the same cell line and independently expanded [90].

Altered epigenetic profile of imprinted genes, e.g. *H19*, *MEST, IGF2R*, *SNRPN*, which during mammalian development are involved in the regulation of cell division [91, 92] may confer to ESCs a selective growth advantage [81].

DNA and histone methylation

Cytosines methylation changes in CpG dinucleotides may arise both during the earliest stages of derivation and during in vitro expansion [76]. The *RASSF1*, *DcR1* and *PTPN6* genes, selected for their altered methylation in cancer, showed methylation modifications in their promoter during long-term hESCs culture (from 22 to 175 passages) [66]. Variations of DNA methylation levels of the same and of other genes (*CBLN4*, *GPC3*, *RASSF1*, *RalGDS/AF-6* and *glypican 3*) were reported in other hESC lines by other groups, suggesting a high susceptibility of these loci to culture-induced changes [18, 75]. More recently, high-resolution techniques brought to light that 20 independent hESC lines have an almost identical DNA methylation pattern at both single CpG sites and CpG islands, but displayed high variability when the analysis was extended to global DNA methylation and transcription. Various genes exhibited transcriptional variations, while having demethylated promoters, whereas others showed high variability in DNA methylation of their promoters but expression stability [79].

Aberrant DNA methylation was also reported for few mESC lines. Abnormal hypermethylation pattern of various CpG islands was shown to cause dysregulated expression of genes involved in various processes of differentiation. Also, a strong correlation between abnormal methylation and density of H3K27me3 (associated with transcription repression; [93]) at specific loci was evidenced, H3K27me3 that mediates the recruitment of Dnmts to these specific sites [94]. Fractions of both h- and m-ESC populations have high levels of both H3K4me3 (associated with transcription activation; [93]) and H3K27me3 (bivalent chromatin) at lineage-specific loci [95], strongly influencing the differentiation potential of the cell line. This heterogeneity complicates the analysis of the epigenetic regulation in PSCs, in particular during prolonged culture.

The analysis of DNA methylation in iPSCs is complicated by the difficulty to discriminate between the impact of the reprogramming process itself, which requires a global remodeling of the epigenetic status, and the culture conditions. When compared to ESC line profiles, many loci or CpG islands resulted hypomethylated, whereas others, being incompletely reprogrammed, were variable among different iPSC lines [82]. Interestingly, these differences are reduced following long-term culture (>40 passages), suggesting continuous reprogramming of the iPSC population [96].

X-chromosome inactivation

X-chromosome inactivation (XCI) is the functional heterochromatinization and silencing of one of the two X chromosomes in female cells, an event that occurs very early during mammalian female embryonic development. The X-inactive specific transcript (XIST) drives X chromosome silencing, regulating the initiation of random XCI in the inner cell mass of an embryo [97].

Several papers report variable status of XCI in female hESCs. Numerous hESC lines exhibit different states of XCI, together with a heterogeneity among cells of the same cell line [77, 99]. In brief, in all hESC lines analyzed, three different classes of the X inactivation status were identified (Fig. 1). Cells with both active X chromosomes (Xa) (Class I) express very low or undetectable XIST RNA. During differentiation they upregulate XIST and undergo XCI, as described also for mESCs. These cells easily switch to class II, in which the expression of the XIST RNA leads to the random inactivation of one of the two X chromosomes (Xi). Class II cells may switch to class III where the expression of XIST is lost, leading to partial reactivation of some Xi-linked genes [77]. However, XIST is not the sole player involved in the XCI process. XCI and XIST up-regulation occurs in combination with trimethylation of H3K27 on the inactive X [100]. In hESCs, Class I cells show neither H3K27me3 nor XIST foci, class II cells have H3K27me3 domains in all $XIST^{+}$ cells, whereas class III cells do not display H3K27me3 at all. Thus, XIST clouds correlate perfectly with hypermethylation of H3K27, and loss of XIST in class III cells always induces the depletion of H3K27me3 on the inactive X [77]. The X chromosome status was found to be hyper-variable both in different hESC lines and among subculture or different culture passages in the same hESC line [77, 101]. It has been clearly demonstrated that the transit along the three classes of the X chromosome status typically occurs with continuous passage and during long-term culture. Under optimal culture conditions, active X chromosome status can be stably maintained in female hESCs for more than 100 passages [98]; however,

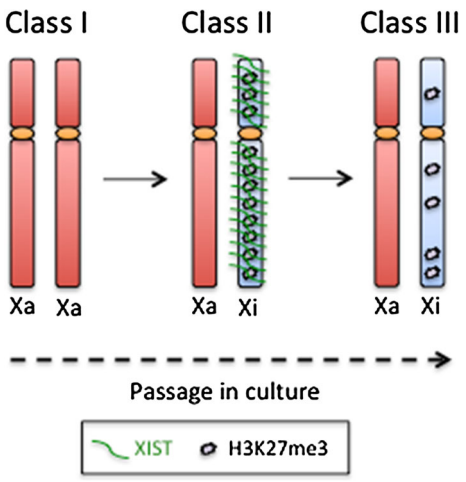

Fig. 1 Variations in X chromosome inactivation status in hESC lines during long-term culture

suboptimal culture conditions (i.e., 20 % O_2 concentration, harsh freeze–thaw cycles, high level of cell death during passages) induce XCI instability [102, 103].

During reprogramming of somatic cells to iPSCs, the acquisition of pluripotency should be coupled with X chromosome reactivation. As for hESCs, hiPSC showed variable XCI pattern [82] with non-random X inactivation in low passage female hiPSCs, suggesting the maintenance of the X-inactivation pattern of the somatic founder cells [104, 105] or the presence of two active X chromosomes [106, 107]. Erosion of the XCI status during culture, due to loss of both XIST expression and foci with H3K27me3, has also been reported [105].

The variable pattern of XCI observed in hPSCs is probably an adaptive response of cells to the suboptimal in vitro culture conditions. However, the skewed pattern leads to an altered expression of X-linked genes, event that dramatically affects the undifferentiated status and the differentiation potential of the cell lines [102].

To our knowledge, no specific alterations in XCI have been described in mESC or miPSC lines. Female mESCs normally have two active X chromosomes (Xa) [108, 109] likely maintained by the Oct4, Sox2 and Nanog pluripotency regulating factors which bind to intron 1 of *Xist* and repress its activity [110, 111].

Mechanisms involved in epigenetic instability

The several enzymes in DNA/histone methylation ("Box 4") and their cofactors [for a review 112] can be causal to the epigenetic instability. Nevertheless, up to date, the mechanisms producing aberrant epigenetic patterns observed in PSCs during long-term culture are unknown. DNA methylation, although critical during differentiation, does not appear to be essential for the maintenance of the undifferentiated status, since the triple knockout Dnmt1$^{-/-}$/Dnmt3a$^{-/-}$/Dnmt3b$^{-/-}$ mESC line proliferates, maintains pluripotency and retains their ability to differentiate [113]. It has been suggested that ESCs maintain stable heterochromatin and chromosomes by an epigenetic mechanism independent of CpG methylation. To this regard, previous studies have shown that ESCs have specific epigenetic regulatory mechanisms, namely hyperdynamic architectural chromatin proteins that bind loosely to chromatin. This state may contribute to maintain chromatin in a global open and plastic state [114, 115].

Aberrations of mitochondrial DNA

Human and mouse PSCs possess few and immature mitochondria, similar to those found in preimplantation embryos [116]. The integrity of mitochondrial DNA (mtDNA) has been scarcely investigated and its alterations have been described in few hPSC lines. Specifically, heteroplasmic missense or nonsense point mutations in NADH dehydrogenases 1, 2 and 4 and in ATPase genes were found in two out of nine (22 %) late-passage hESC lines, mutations that impair the proteins function [66]. More recently, large mtDNA deletions (ranged between 4973 and 8876 bp) were identified in 16 hESC lines cultivated from passage 3–334. The deletions observed may be present since the early passages after derivation and dispersed over the whole mitochondrial genome. Also, several mutated mtDNA molecules can be present in a single hESC line [117].

Homoplasmic and heteroplasmic single base mutations (substitutions, insertions and deletions) were also identified in four hiPSC lines reprogrammed from fibroblast of young and healthy donors [118]. Up to date, no information is currently available on the mtDNA integrity in mouse ESCs and iPSCs.

Mechanisms involved in the generation of mtDNA alterations

mtDNA deletions can arise during the replication phase, through slipped-strand mispairing between direct homologous base-pairs [119]. Although mitochondria possess active repair mechanisms, mtDNA has a high mutation rate, 100- to 1000-fold higher than nuclear DNA [120], probably due to the stressor environment of the mitochondria. The occurrence of point mutations in mtDNA has been extensively described, due to a failure of Base Excision Repair pathway that does not repair the oxidative DNA lesions [121].

Conclusions

Following the derivation of the first hESC line in 1998 [3] and, in 2006, of iPSCs [4], PSCs have represented a great promise for future therapeutic and clinical applications. However, PSCs are prone to genomic and (epi)genetic instability during in vitro culture [31, 122, 123], a feature that seriously limits their real potential use. Culture-induced aberrations of specific chromosomes or genes are almost all unpredictable. Although aberrations of chromosomes 12, 17 and X for hPSCs or 8 and 11 for mPSCs are recurrent in several different cell lines, as a whole, aberrations differ among independent cell lines and may arise at different culture passages. Regarding the effects that aberrations might have on PSCs, several studies indicate that their stemness is not affected [29, 31, 124–126]. However, upon differentiation, in the presence of specific abnormalities such as trisomy of chromosome 12,

transplanted hPSCs increase their tumorigenicity [127]. Similarly, in the presence of trisomy of chromosome 21, the electrophysiological features of hESCs-derived cardiomyocytes are impaired [128] or the degeneration of mESCs-derived neurons is increased [129].

For successful therapeutical applications, the goal is the generation of Good Manufacturing Practice-grade PSCs. Two are the levels of intervention that are urgently needed: (1) to establish protocols that eliminate or minimize (epi)genetic and chromosome instability during culture, by acting, for iPSCs, on the reprogramming process itself (e.g., episomal plasmids, mRNA and microRNA technologies) and, for all PSCs, on the culture environmental conditions (e.g., animal feeder- and serum-free culture, reduction of ROS levels by inducing HIF pathway, increased levels of CHK1 or nucleoside or vitamin C supplementation, to reduce replication stress) [130, 131]; promising methods rely on engineered in vitro culture techniques that result in a downstream improvement of the karyotype stability [132–135] and quality of cell differentiation [136, 137]. (2) The absence of a safe passage number makes mandatory the monitoring of the genome integrity in PSCs, with the establishment of efficient and systematic methods, beyond the conventional banding techniques, spectral karyotyping, multicolor fluorescent in situ hybridization and array-based comparative genomic hybridization [32]. To this regard, an important contribution might derive from high-throughput microscopy, which allows the rapid and automated acquisition of numerous images, and the exome sequencing technique, the latter a cost-effective alternative to whole-genome sequencing [130]. Furthermore, the present review highlights the urgency for further studies on the mechanisms involved in determining (epi)genetic and chromosome instability, exploiting the knowledge acquired earlier on other cell types.

Box 1

The spindle assembly checkpoint (SAC) [for a review see 138] controls erroneous attachments of chromosomes to the mitotic spindle, to ensure proper chromosome segregation [139]. Defects or dysfunction in proteins involved in SAC lead to chromosome instability (onset of aneuploidies or deletions) in daughter cells [140, 141].

Similarly, microtubule/kinetochore coupling (attachment of spindle microtubules to the kinetochore) is crucial for the correct segregation of chromosomes during anaphase. Erroneous coupling (monotelic, syntelic or merotelic attachment) is the most common cause of genomic instability [142, 143], resulting in chromosome missegregation, mitotic delay and in aneuploid cell progeny [144–146]. Merotelic kinetochore attachment is the most frequent cause of aneuploidy in mitotic cells [147] and it is the primary described mechanism of chromosome instability in tumor cells [148]. The chromosomal passenger complex is responsible for the correction of these anchoring errors and its abolishment induces accumulation of errors in chromosome anchoring, with the consequent onset of abnormalities [149–151].

Aberrant centrosome duplication may adversely affect the maintenance of cell polarity and an abnormal number of centrosomes lead to multipolar spindles, resulting in chromosomal segregation abnormalities and aneuploidy of daughter cells [152, 153]. Several cell cycle kinases operate during the centriole duplication [154–156]. Among these, the Polo-like kinase 4 (Plk4) plays a key role in centrosome cycle [157], a process whose disruption causes aneuploidy [158, 159].

Sister chromatid cohesion is the process by which sister chromatids are paired and held together for alignment on the metaphase spindle. Cohesin is a chromosomal protein complex which regulates the correct distribution of the chromatid into the two daughter cells [160]. The lack or altered expression of cohesins induces premature sister separation or failed chromosome disjunction at the beginning of anaphase [161, 162], leading to cell death or aneuploidy [42].

Box 2

NAHR is one of the best-characterized recombination-based mechanisms in primates and it is responsible for recurrent CNVs. In this process, two regions of DNA with high sequence similarity are used for the repair of a DNA double strand break [163]. NAHR is mostly mediated by 10–300 kb long interspersed low-copy repeats [highly homologous sequences (95–97 %)] [164, 165] or short microsatellite with recombination hotspots that facilitate the occurrence of a misalignment during the strand invasion step [63].

NHEJ, MMBIR, FoSTeS are involved in the generation of CNVs, particularly in the formation of non-recurrent variation in copy number of portions of DNA [166]. NHEJ, together with HR, is one of the major mechanisms used by eukaryotic cells to repair DSBs [167], but some authors proposed this mechanism to explain chromosome duplication [168, 169]. MMBIR is involved in repairing single double-strand ends when portions of single-stranded DNA are present and share microhomology with the 3′ single-strand end from the collapsed fork [63]. Similarly, the replication-based mechanism FoSTeS acts when a replication fork stalls for the presence of a DNA lesion, allowing the lagging strand to detach and move to a region of micro-homology on a neighboring replication fork [170].

Box 3

Repetitive DNA regions are more prone than others to accumulate DNA replication errors and the failure of some key MMR genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*) or the minor accuracy of the process cause the accumulation of unrepaired mitotic errors. A replication slippage of the DNA polymerase occurs through five main steps: (1) DNA polymerase tackles a direct repeat during the DNA replication process; (2) DNA polymerase arrests and is, for a short time, released from the template strand; (3) the newly synthesized strand detaches from the template strand and pairs with another direct repeat upstream, forming a loop; (4) DNA polymerase, returning on the template strand, restarts with the normal replication process, but during the course of reassembling, the polymerase slips and repeats the insertion of dNTPs that were previously added. This faulty event causes the insertion of new nucleotides. Usually, MMR proteins recognize the temporary insertion–deletion loop, but when they do not work properly, this loop results in frame-shift mutations [171–173].

Box 4

The principal molecules involved in cytosines methylation are the DNA methyltransferases (Dnmt). Dnmt1, Dnmt3a, Dnmt3b and Dnmt3L are a family of proteins implicated in the maintenance of the correct DNA methylation status. Dnmt1, localizing to DNA replication foci and showing a strong affinity for hemi-methylated DNA, plays a major role in the maintenance of methylation in dividing cells [174, 175]. Dnmt3a and Dnmt3b, working on unmethylated as well as on hemi-methylated substrates, possess a de novo methylation activity [176, 177]. Dnmt3L (*Dnmt3*-like) methyltransferase is a regulator of DNA methylation, it lacks the catalytic functional domains and stimulates de novo methylation by Dnmt3a and Dnmt3b [178, 179]. In mESCs, Dnmt3a and Dnmt3b proteins are physically associated and act synergistically to methylate the promoters of pluripotency genes (i.e., *Oct4*, *Nanog*) during differentiation processes [180].

Covalent histone tails modifications govern the conformation of the chromatin and influence the gene transcription by promoting or restricting the recruitment of gene regulatory proteins [181]. Histones can be methylated on lysine (K) and arginine (R) residues and H3 and H4 histone tails are the main targets of methylation [for a review, 112]. Several histone-modifying enzymes including lysine or arginine methyltransferases, serine-threonine kinases, and acetyltransferases are involved in this process [93].

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