Quality control

Genome maintenance in pluripotent stem cells

Uri Weissbein, Nissim Benvenisty, and Uri Ben-David

Stem Cell Unit, Department of Genetics, Silberman Institute of Life Sciences, The Hebrew University, Jerusalem 91904, Israel

Pluripotent stem cells (PSCs) must maintain their proper genomic content in order to preserve appropriate self-renewal and differentiation capacities. However, their prolonged in vitro propagation, as well as the environmental culture conditions, present serious challenges to genome maintenance. Recent work has been focused on potential means to alleviate the genomic insults experienced by PSCs, and to detect them as soon as they arise, in order to prevent the detrimental consequences of these genomic aberrations on PSC application in basic research and regenerative medicine.

Pluripotent stem cells (PSCs) can be obtained from the inner cell mass of the embryonic blastocyst, resulting in embryonic stem cells (ESCs), or by reprogramming somatic cells into a pluripotent state (iPSCs). Pluripotent cells can self-renew indefinitely without losing their cellular identity, and can also differentiate into all the different cell types of the embryo. Importantly, while the latter trait is an inherent characteristic of pluripotent cells by definition, the former is actually a culture artifact, as pluripotent cells exist only transiently in vivo. Maintaining a proper genomic content is crucial for proper embryonic development in vivo, and is also critical for most applications of PSCs, such as cell therapy, disease modeling, and research of early development. Hence, it is important to understand the genome maintenance challenges that PSCs cope with, to characterize the recurrent genomic aberrations that they acquire, and to identify their functional consequences, in order to monitor, and potentially minimize, these genomic abnormalities.

Genomic abnormalities in PSCs

Cultured PSCs can acquire genomic abnormalities ranging in size from full chromosome aneuploidy to single nucleotide point mutations. The typical aberrations of both human and mouse PSCs, and the potential sources for these recurrent aberrations, have been extensively studied in recent years (Lund et al., 2012; Liang and Zhang, 2013). In this part of the review, we will discuss the main findings regarding genomic instability of mouse and human PSCs (summarized in Table 1).

Large chromosomal aberrations. Soon after the derivation of mouse ESCs (mESCs), attempts to generate chimeric mice faced the problem of low germ cell transmission efficiency. Further research uncovered that mESCs tend to acquire large chromosomal abnormalities when maintained in culture for many passages. These aberrant cells rarely contributed to the germ line after their injection into mouse blastocysts (Liu et al., 1997). Intense research, based at first on GIEMSA staining, and later on more advanced methods such as SNP arrays, gene expression profiling, and DNA sequencing, revealed recurrent characteristic aberrations in mouse and human PSCs. Two recent studies have estimated that ∼10% of human PSC (hPSC) cell lines exhibit at least one large chromosomal aberration (Ben-David et al., 2011; Taapken et al., 2011). These estimations referred to large chromosomal aberrations that already appear in most metaphases (that is, are prevalent in culture). A study by the International Stem Cell Initiative found that 34% of the cell lines showed more than 2 out of 30 metaphases with identical abnormalities (Amps et al., 2011). Trisomies of chromosomes 12 and 17 and gain of chromosome X are the most common large aberrations in hPSCs (Brimble et al., 2004; Draper et al., 2004; Baker et al., 2007; Mayshar et al., 2010; Amps et al., 2011; Ben-David et al., 2011; Laurent et al., 2011; Martins-Taylor et al., 2011; Taapken et al., 2011). In the mouse, it was revealed that over one third of the mESC samples had large chromosomal genetic aberrations, mainly trisomies of chromosomes 8 and 11. Interestingly, the distal half of mouse chromosome 11 is completely syntenic to human chromosome 17, whereas other aberrations seem to be species specific (Ben-David and Benvenisty, 2012b). Comparing mouse and human aberration prevalence indicates that mPSCs tend to acquire more genetic chromosomal changes than hPSCs. However, it is important to note that mESCs were derived 17 years before their human counterparts, so popular cell lines have since spent much more time in culture. Also of note, whereas trisomies of

Correspondence to Nissim Benvenisty: nissimb@cc.huji.ac.il; or Uri Ben-David: uri.ben-david@mail.huji.ac.il

Abbreviations used in this paper: CNV, copy number variation; DSB, double-strand break; ESC, embryonic stem cell; hPSC, human PSC; iPSC, induced PSC; IR, ionizing radiation; mESC, mouse ESC; NHEJ, nonhomologous end joining; PSC, pluripotent stem cell; ROS, reactive oxygen species; SNV, single nucleotide variation.

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Table 1. **Genomic abnormalities observed in mouse and human PSCs**

Aberration type		Mouse PSCs	Human PSCs	Comparison of human and mouse PSCs	Origin	Gene enrichment	Likely mechanism of formation
Chromosomal aberrations	Recurrent aberrations	Gains 8 and 11 Deletions 10qB and 14q [a, b, c, d, e, f]	Gains 1, 12, 17, 20 and X [g, h, i, j, k, l, m, n]	Human chromosome 17 is completely syntenic to the distal half of mouse chromosome 11 [c]	Most aberrations arise in culture during propagation (culture adaptation) [i, n]	Difficult to analyze, as aberrations contain multiple genes	Defects in chromosomal segregation during cell division
	Frequency of aberrations	~38% in mESCs ~23% in miPSCs [c] ~25% of the aberrations involve chromosomes 8 and 11 [c]	~32–34% in hESCs [i, n] ~20% in hiPSCs [n] ~50% of the aberrations involve chromosomes 1,12,17 or 20 [i]				
Subchromosomal aberrations and copy number alterations	Recurrent aberrations	Gains within chromosome 8 [o] Multiple deletions (including in 14q) [c, p]	20q11.21 and 12p13.31 [i, m, q, r, s]	Small chromosomal aberrations occur both in mouse and human ESCs, but no syntenic recurrent aberrations have been identified.	Most CNVs arise from selection for rare populations in the parental cells during reprogramming or culturing [u, v, w] The total number of CNVs decreases in culture [t]	Specific genes have been suggested, such as BCL2L1 [i] and NANOG [m, n] May be associated with pluripotency pseudogenes, cancer-related genes, and genes within common fragile sites [m, o, t]	Defects in DNA damage response and replication stress
	Frequency of aberrations	?	Average of 109 CNVs per hiPSC line and 55 CNVs per hESC line [t] 10–25% of hESCs display the recurrent amplification of 20q11.21 [i, m] 13% of hESCs display the recurrent amplification of 12p13.31 [m]				
Single nucleotide variations (SNVs)	Recurrent aberrations	Not identified	Not identified	One study identified the same variants in four mouse iPS clones. This has not been observed in human PSCs. [w]	Most SNVs can be traced back to the parental cells	Shared SNVs were not observed between different iPS cell lines derived from the same somatic fibroblasts	Replication defects
	Frequency of aberrations	~11 point mutations in coding regions per clone [w]	~6 point mutations in coding regions per clone [v, x, y]				

[a]Liu et al., 1997; [b]Brimble et al., 2004; [c]Ben-David and Benvenisty, 2012b; [d]Liang et al., 2008; [e]Sugawara et al., 2006; [f]Sommer et al., 2010; [g]Ben-David et al., 2011; [h]Taapken et al., 2011; [i]Amps et al., 2011; [j]Draper et al., 2004; [k]Baker et al., 2007; [l]Martins-Taylo et al., 2011; [m]Laurent et al., 2011; [n]Mayshar et al., 2010; [o]Pasi et al., 2011; [p]Arlt et al., 2012; [q]Närvä et al., 2010; [r]Lefort et al., 2008; [s]Werbowetski-Ogilvie et al., 2009; [t]Hussein et al., 2011; [u]Abyzov et al., 2012; [v]Gore et al., 2011; [w]Young et al., 2012; [x]Cheng et al., 2012; [y]Ruiz et al., 2013.

autosomal chromosomes are common in both species, recurrent monosomies have been observed only in the sex chromosomes.

Abnormal karyotype is generally perceived as a consequence of culture adaptation due to positive selection (Draper et al., 2004; Baker et al., 2007). There is a positive correlation between abnormal karyotype and passage number, although abnormal karyotype can sometimes be found in low passage cultures, and vice versa (Mayshar et al., 2010; Taapken et al., 2011). In addition, only a few types of aneuploidies are commonly found in late-passage PSCs, suggesting that most chromosomal aberrations cannot easily take over the culture. The ability of specific aneuploid cells to outcompete the diploid cells in culture is probably driven by elevated expression of genes found on the gained chromosomes (Baker et al., 2007; Blum et al., 2009; Mayshar et al., 2010; Ben-David and Benvenisty, 2012b). However, as large chromosomal aberrations harbor hundreds to thousands of genes, it is difficult to pinpoint the exact gene(s) that provide them with a selection advantage.

Subchromosomal aberrations and copy number alterations. Subchromosomal aberrations encompass small chromosomal regions on the mega-base scale, whereas copy number alterations are usually much smaller, on the kilo-base scale. Such changes are frequently observed in both mouse and human PSCs, are not easily detected, and may have important functional consequences. During reprogramming, small chromosomal aberrations can arise de novo or can be amplified from a small population of aberrant parental somatic cells. DNA array studies showed that low-passage hiPSC lines harbor more copy number variations (CNVs) than their parental fibroblast populations and late-passage hiPSCs, suggesting that CNVs are either introduced during the reprogramming process or fixed in the population due to the clonal nature of this process, but then most of them soon disappear, as they are disadvantageous (Hussein et al., 2011; Laurent et al., 2011). Studies that applied whole-genome sequencing technologies to hPSCs have argued that most, if not all, CNVs can already be detected at low frequency in the parental somatic cells (Abyzov et al., 2012; Cheng et al., 2012). Regardless of their exact origin, a subset of these reprogramming-associated aberrations rapidly outcompete their normal counterparts and take over the culture (Hussein et al., 2011).

Interestingly, reprogramming has been associated with deletions in genomic areas that contain tumor suppressors, whereas culture adaptation of hESCs and hiPSCs has been associated with duplication of oncogenes (Laurent et al., 2011). Early-passage, but not late-passage, hiPSCs were found to harbor deletions in genes important for maintaining an undifferentiated state (Hussein et al., 2011). Reprogramming-induced deletions were also enriched in common fragile sites, which are known to create double-strand breaks (DSBs) upon replication stress (Schwartz et al., 2006), in both human (Hussein et al., 2011) and mouse (Ben-David and Benvenisty, 2012b). Two small chromosomal aberrations are repeatedly observed in hPSCs during prolonged culturing. The amplification of chromosome 20q11.21 was observed in many independent experiments (Lefort et al., 2008; Werbowetski-Ogilvie et al., 2009; Närvä et al., 2010; Amps et al., 2011; Laurent et al., 2011) and is estimated to be present in ∼14.5% of hPSC lines (Lund et al., 2012). Interestingly, aberrations of chromosome 12p, which are frequently observed in human PSCs, are also frequent in many subtypes of germ cell tumors (Oosterhuis and Looijenga, 2005), suggesting that this recurrent aberration may be advantageous, in a cell lineage–dependent manner, both in vitro and in vivo (Ben-David et al., 2011). In mouse PSCs, small deletions were frequently identified in chromosomes 10q and 14q (Liang et al., 2008; Ben-David and Benvenisty, 2012b), and the prevalence of CNV accumulation significantly increased after replication stress (Arlt et al., 2012).

Point mutations. Several studies have tried to identify single nucleotide variations (SNVs) during reprogramming using whole-genome or exome sequencing technologies. In human cells, an average of 5–6 mutations in coding regions per clone (when compared with the parental cells) was reported (Gore et al., 2011; Cheng et al., 2012; Ruiz et al., 2013), whereas an average of 11 such mutations was identified in mouse cells (Young et al., 2012). More than a thousand mutations per clone were detected in noncoding regions. Interestingly, although one study reported that most mutations appeared during the reprogramming process (Ji et al., 2012), most of the reports showed that most mutations originate from the parental cell line (Gore et al., 2011; Cheng et al., 2012; Ruiz et al., 2013). As with the origin of CNVs, limitations in detection make it difficult to determine whether "novel" SNVs are already present at the cell of origin population at an undetectable prevalence.

If recurrent point mutations exist in iPSC colonies, this could imply selective advantage of these mutations during reprogramming. One report on miPSCs was able to identify a recurrent set of point mutations in all four miPSC clones tested (Young et al., 2012); however, none of the studies with hiPSCs could detect any recurrent SNV, suggesting that no single mutation significantly tends to arise during successful reprogramming (Gore et al., 2011; Cheng et al., 2012; Ruiz et al., 2013). Moreover, analyses of the mutations that did arise spontaneously, or were induced experimentally, in hiPSC lines argued by and large against selective advantage conferred by any of these mutations (Ruiz et al., 2013). Although it thus seems that there are no "hot spots" for such mutations, it is important to bear in mind that only few studies have addressed the issue of point mutations in PSCs, with the largest one using 22 iPSC genomes (Gore et al., 2011). These findings thus remain to be confirmed in much larger datasets, such as those used for the study of CNVs and chromosomal aberrations. As whole-genome sequencing technologies advance rapidly, more iPSC genomes will soon be sequenced, enabling us to answer this question more confidently.

DNA integrity challenges in PSCs

Cell cycle and checkpoints. Pluripotent cells undergo a substantially shorter cell cycle than committed and differentiated cells (Stead et al., 2002; Becker et al., 2006; Bárta et al., 2013; Calder et al., 2013). In human cells, the length of the cell cycle increases dramatically upon lineage commitment (Becker et al., 2006; Calder et al., 2013). The short cell cycle observed in PSCs is mainly due to a truncated G1 phase: pluripotent cells spend ∼65% of the cell cycle time in S phase and only ∼15%

in G1, whereas differentiated cells spend ∼40% of the cell cycle time in G1 phase (Becker et al., 2006). Somatic cells reprogrammed into iPSCs begin to proliferate rapidly and acquire a short cell cycle similar to that of ESCs, supporting the notion that rapid cell divisions are a key property of PSCs (Ghule et al., 2011; Ruiz et al., 2011). Moreover, manipulating the cell cycle of hPSCs by altering the activity level of cyclin D–CDK4/6 can enhance differentiation and direct cell fate choice (Pauklin and Vallier, 2013), suggesting a causal relationship between cell cycle and differentiation.

The numerous successive rounds of DNA replication impose a major hurdle for the DNA replication machinery and for the successful maintenance of the genomic content. The process of culture adaptation, which often involves chromosomal changes (as discussed in the previous section), is also accompanied by a marked increase in the proliferation rate of the cells (Werbowetski-Ogilvie et al., 2009). A direct measurement of cell cycle length in short- and long-term cultured hESCs has revealed a reduction in the cell cycle length (Bárta et al., 2013). Another study reported that in culture-adapted hESCs a larger fraction of the cells are in S phase at any given time (Yang et al., 2008). Rapid proliferation could thus be both a cause and a consequence of genomic aberrations.

Eukaryotic cells use a set of checkpoints in order to ensure a proper transition through the cell cycle phases. The G1/S checkpoint's role is to prevent cells with damaged DNA from entering the S phase. Mouse ESCs lack the G1/S checkpoint (Aladjem et al., 1998; Hong and Stambrook, 2004), and most studies in hESCs also reported the absence of the G1/S checkpoint upon ionizing radiation (IR) or replication stress (Filion et al., 2009; Momcilovic et al., 2010; Desmarais et al., 2012). However, one report could detect activation of the G1/S checkpoint upon ultraviolet (UV) radiation. Interestingly, the G1/S arrest was achieved in that study only through inhibition of CDK2 by CHK2 phosphorylation of CDC25 and not via the p53–p21 pathway (Bárta et al., 2010). In another study, CDK2 inhibition by siRNA arrested 97% of the transfected hESCs in G1 phase within 4 d. CDK2 inhibition also resulted in morphological changes, differentiation to extra-embryonic lineages, and down-regulation of pluripotency factors, emphasizing the importance of CDK2 in cell cycle regulation and maintenance of the pluripotent state (Neganova et al., 2009).

Replication stress during S phase is sensed by the ATR kinase, which recognizes the single-strand DNA at the stressed replication fork. ATR and its partner CHK1 reduce the level of CDK1 and prevent entry into mitosis (Flynn and Zou, 2011). In contrast to somatic cells, upon treatment with the replication inhibitors thymidine and cisplatin, hESCs fail to activate S-phase checkpoint pathways and instead commit to apoptosis (Desmarais et al., 2012). Although some more details are known with regard to the regulation of CDK proteins in PSCs (Kapinas et al., 2013), a thorough mechanistic understanding of checkpoint enforcement in PSCs is currently lacking. Together, current data suggest that the unique cell cycle and checkpoint activation of PSCs may render them more susceptible than other cell types to genomic abnormalities (Fig. 1): rapid proliferation provides more opportunities for the acquisition of aberrations, whereas weak checkpoints allow the progression through the cell cycle even in the presence of replication defects (such as defective chromosomal segregation).

DNA damage response and apoptosis. Maintaining the DNA integrity of PSCs is essential because every change in the DNA content will be inherited to the cell progeny. Hence, PSCs are expected to activate a robust DNA damage response. In line with this notion, it has been shown that hESCs have the capacity to repair a variety of DNA lesions created by various agents (H_2O_2, UV-C, IR, and psoralen) more efficiently than somatic cells (Maynard et al., 2008). In this study it was also found that hESCs overexpress genes important for multiple DNA repair pathways, compared with differentiated cells after stress (Maynard et al., 2008). However, a failure to properly repair UV-induced DNA damage could lead to the accumulation of point mutations in hESCs (Hyka-Nouspikel et al., 2012), suggesting that increased activity of the repair machinery does not necessarily result in accurate DNA repair, and can introduce genomic aberrations into the cells.

The most dangerous form of DNA damage is DSBs that can arise from multiple sources such as IR, replication stress, reactive oxygen species (ROS), and others. To repair DSBs, cells use two main pathways: homologous recombination (HR) and nonhomologous end joining (NHEJ). NHEJ is considered a less accurate and error-prone form of repair. HR, on the other hand, utilizes a template—either a sister chromatid, a homologous chromosome, or repeated sequences—in order to achieve high-fidelity DNA repair. Studies have confirmed that HR is the predominant DSB repair pathway both in hESCs and in mESCs, in contrast to differentiated cells (Adams et al., 2010a; Tichy et al., 2010). Unlike mESCs, however, hESCs are also capable of performing efficient NHEJ that is independent of the canonical NHEJ proteins DNA-PKc and ATM (Adams et al., 2010b). Consistent with this finding, several studies have shown that hESCs more highly express genes from both repair pathways (Maynard et al., 2008; Fan et al., 2011).

An alternative mechanism to prevent the inheritance of genomic aberrations is to eliminate aberrant cells from the cell population. PSCs are extremely sensitive to DNA damage and readily undergo apoptosis or differentiation after genomic insults (Aladjem et al., 1998; Lin et al., 2005; Qin et al., 2007). Similar to other types of stem cells (Inomata et al., 2009; Wang et al., 2012; Schneider et al., 2013), the self-renewal of PSCs is limited in response to DNA damage (Qin et al., 2007): in response to such damage, mESCs activate p53, which leads to the reduction in levels of the key pluripotency transcription factor Nanog, and consequently to differentiation of the cells (Lin et al., 2005). Similarly, induction of p53 in hESCs can also lead to spontaneous differentiation (Jain et al., 2012); however, differentiation is only one of the two potential mechanisms to eliminate self-renewing PSCs in response to DNA damage, and apoptosis seems to be the more common response. DNA damage–induced differentiation was reported to be followed by apoptosis of the differentiated cells (Lin et al., 2005). Moreover, the undifferentiated cells themselves can undergo DNA damage–induced apoptosis: unlike in mouse embryonic fibroblasts, p53 translocation into the nucleus in response to DNA damage

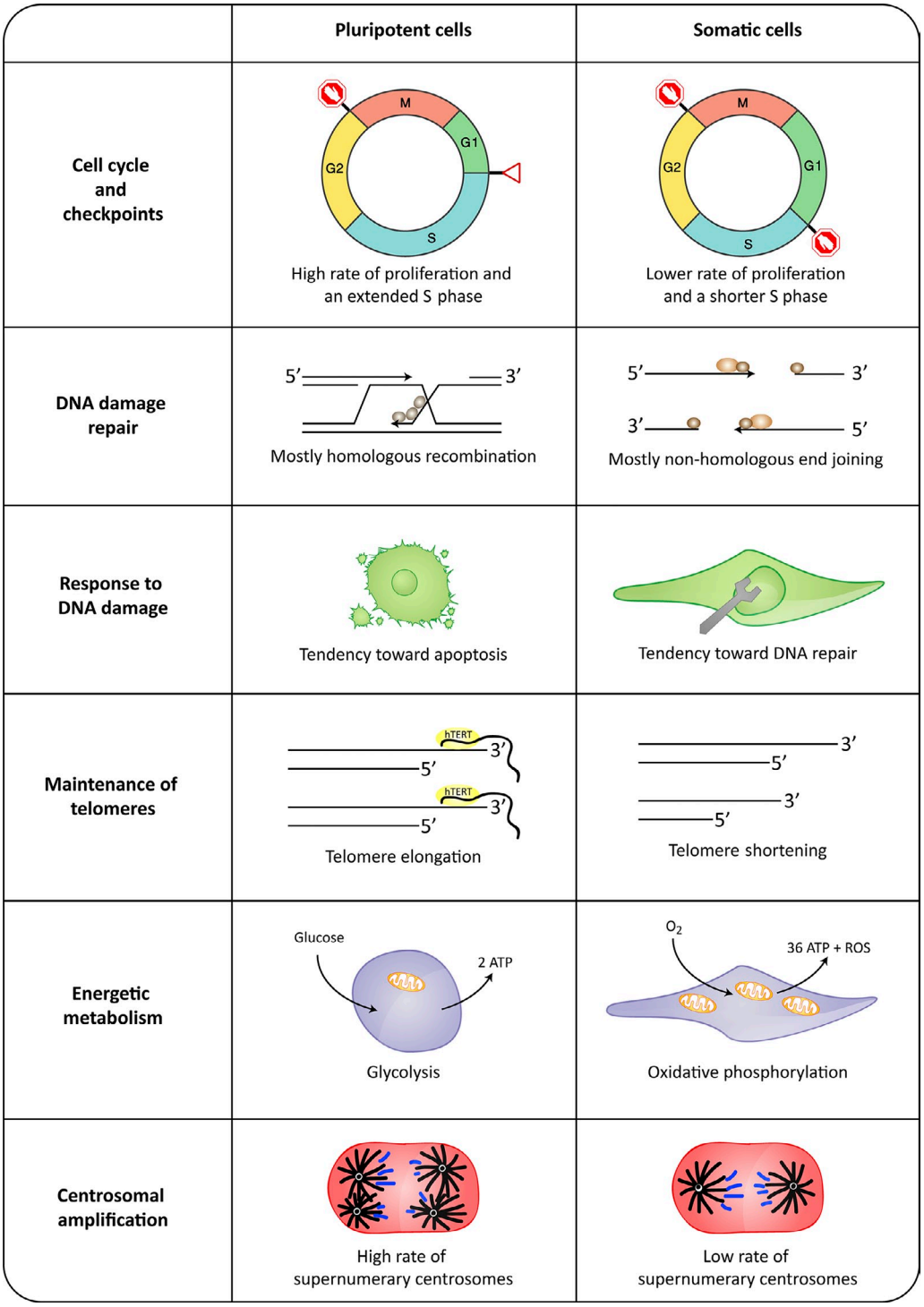

Figure 1. **Main challenges in the maintenance of PSC genomic integrity.** Mouse and human PSCs face inherent and environmental challenges that affect how they maintain their genomic integrity. Presented are key differences between PSCs and somatic cells, which contribute to the formation of these challenges and to the way PSCs cope with them. See the text for elaboration on each of these topics.

is inefficient in mESCs, leading to cell arrest only at the G2/M checkpoint and to p53-independent apoptosis (Aladjem et al., 1998). In hESCs, *NANOG* expression has also been shown to decrease as a result of DNA damage (Song et al., 2010). Unlike mESCs, however, hESCs respond to IR by increasing p53 activity, leading to up-regulation of p53 targets and to p53-dependent apoptosis, a major difference from the mouse model (Filion et al., 2009). In both species, therefore, widespread apoptosis of PSCs is induced in culture by the activation of the DNA damage response, through species-specific molecular mechanisms. Recently, two studies have revealed that the lower apoptotic threshold of hESCs is mediated by skewed balance between pro- and anti-apoptotic genes, which "primes" hESCs to rapid apoptosis (Dumitru et al., 2012; Liu et al., 2013).

The fact that PSCs readily undergo apoptosis despite their increased capacity to repair DNA damage is somewhat counterintuitive. However, given the importance of genome integrity maintenance in PSCs, and the destructive consequences of its failure, these two mechanisms seem to be complementary rather than contradictory. Considered in that light, it seems that the main mechanism implemented by PSCs to prevent genomic aberrations is rapid apoptosis, whereas the increased yet error-prone DNA repair capabilities remain a second line of defense (Fig. 1).

Telomere maintenance. The 5′ end of the lagging strand becomes shorter in each DNA replication due to the "end replication problem". Without a proper mechanism to maintain their telomere length, the telomeres of PSCs would shorten with each cell division. Such telomere shortening would soon result in loss of important genomic information. To cope with that problem, PSCs express the enzyme telomerase (Hiyama and Hiyama, 2007), which is responsible for elongating telomere ends by synthesizing additional telomeric repeats. Telomerase is a ribonucleoprotein comprised of telomerase reverse transcriptase (*TERT*) and telomerase RNA component (*TERC*). Telomerase expression and activity are restricted to PSCs and to adult stem cells, and are not detected in differentiated somatic cells. As expected, it has been shown that reprogramming of somatic cells into iPSCs is accompanied by the induction of telomerase expression and activity (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007; Agarwal et al., 2010) and the acquisition of telomeric heterochromatin features similar to those found in ESCs (Marion et al., 2009).

Several studies have revealed that long telomeres are required for high-quality PSCs. The length of the telomeres in mESCs correlates well with their proliferation rate and with the size and weight of the tumor that they can form (Huang et al., 2011). In addition, the successfulness of tetraploid blastocyst complementation is reduced with the decrease in telomere length (Huang et al., 2011), further indicating that long telomeres are essential for pluripotency. Moreover, reprogramming efficiency was found to correlate with the telomere length both in mouse and in human (Marion et al., 2009; Agarwal et al., 2010), and shortened telomeres were reported to lead to unstable differentiation (Pucci et al., 2013).

In humans, at least seven different mutations can cause dyskeratosis congenita (DC) disorder, characterized by telomere maintenance defects and short telomeres (Nelson and Bertuch, 2012). Two studies that used cells from patients with DC reported decreased efficiency of reprogramming. Both studies demonstrated a surprising reprogramming-induced up-regulation of multiple telomere-related genes such as *TERC*, *TERT*, *DKC1*, and *TCAB1* (Agarwal et al., 2010; Batista et al., 2011). An important discrepancy between these studies appeared when examining the telomere dynamics of the hiPSC lines from patients with the same *DKC1* mutation. In one study, the hiPSCs could self-renew for up to 66 passages (Agarwal and Daley, 2011), and elongation of the telomere ends was detected. In contrast, the other study could not detect telomere elongation, and their cells could not be maintained for more than 36 passages (Batista et al., 2011). A possible explanation of this discrepancy is the cell-to-cell variability in telomerase activity, which could be emphasized due to the clonal nature of the reprogramming process (Suhr et al., 2009; Agarwal and Daley, 2011).

Chromosome ends of early cleavage embryos can be significantly elongated by another mechanism, independent of telomerase, which is known as telomere sister chromatid exchange (Liu et al., 2007). An intriguing study in mESCs showed that at any given time only ∼5% of the cells express *ZSCAN4*, a key gene in this pathway, but that most of the cells express it at least once during 9 passages. Knockdown of this gene resulted in telomere shortening, aneuploidy, decreased proliferation, and increased apoptosis (Zalzman et al., 2010). Further work revealed that *ZSCAN4* is important for maintaining normal telomere length by telomere sister chromatid exchange, and it was found to colocalize on telomeres together with meiosis-specific homologous recombination proteins, such as *SPO11* and *DMC1*. The authors suggested that *ZSCAN4* is thus essential for the long-term maintenance of intact karyotype by regulating telomere recombination (Zalzman et al., 2010). Interestingly, *ZSCAN4* was later shown to be up-regulated in *TERC*-null ESCs (Huang et al., 2011). In summary, telomere maintenance is a unique genomic integrity problem that PSCs need to confront, and they seem to do so by applying several cellular interrelated mechanisms (Fig. 1).

ROS production and metabolic dependencies. The mitochondrial respiratory chain produces ROS that are detrimental for the DNA, as well as for proteins and lipid structures. At the blastocyst stage, inner cell mass cells are exposed to low concentrations of oxygen, until the implantation and vascularization in the uterus (Fischer and Bavister, 1993; Burton and Jaunaiux, 2001). In this hypoxic environment, cells cannot produce enough ATP via mitochondrial oxidative phosphorylation, and therefore rely mainly on anaerobic metabolism. Studies have shown that ESCs have only few mitochondria, with immature morphology (Oh et al., 2005; St John et al., 2005; Cho et al., 2006; Facucho-Oliveira et al., 2007), and upon differentiation they acquire more mitochondria with mature features, such as more developed cristae, denser matrix, and increased oxidative capacity (St John et al., 2005; Facucho-Oliveira et al., 2007). In agreement with the mitochondrial composition, ESCs produce less ATP and ROS, and exhibit lower activity of antioxidant enzymes (Cho et al., 2006). Consequently, the energetic metabolism of ESCs is mainly based on glycolysis rather than on oxidative phosphorylation (Xu et al., 2013), and this could help ESCs defend themselves from ROS-induced genomic damages.

As with other cellular properties, iPSCs recapitulate the energetic metabolism of ESCs. During reprogramming, the mitochondria morphology of iPSCs reverts to an immature state, the mitochondrial DNA content is reduced, and genes related to mitochondria biogenesis are down-regulated (Prigione et al., 2010; Folmes et al., 2011). The ATP production in iPSCs is identical to that of ESCs, and is much lower than in differentiated cells. Conversely, the lactate production is much higher in pluripotent cells. Taken together, iPSCs experience a transition from mitochondrial respiration to anaerobic glycolysis during reprogramming (Prigione et al., 2010; Folmes et al., 2011). In

accordance with the low levels of ROS in pluripotent cells, quantification of oxidatively modified DNA, proteins, and lipids confirmed that both ESCs and iPSCs suffer from free radical–induced damages less than differentiated cells (Fig. 1; Prigione et al., 2010). It may also suggest, however, that PSCs are less equipped to cope with ROS damages, once such damages are formed.

Centrosomal amplification. One of the major functions of the centrosomes, the principal microtubule-organizing centers, is to mediate the segregation of chromosomes during cell division (Schatten, 2008). Chromosomal instability, frequently seen in PSCs, is directly linked to the presence of supernumerary centrosomes (Ganem et al., 2009; Silkworth et al., 2009). A study that analyzed 12 low-passage hESC lines from various origins found that 10–24% of the mitoses in each cell line exhibited supernumerary centrosomes, in comparison to 2–5% in nonpluripotent cells (Holubcová et al., 2011). Both excessive rounds of centrosomal duplication and cell division failures contribute to the generation of supernumerary centrosomes. Practically, blocking cell division and replication, by inhibiting *AURORA A* or *CDK2*, or by activating integrin signaling, diminished significantly the occurrence of multiple centrosomes (Holubcová et al., 2011). Intriguingly, the percentage of multicentrosomal mitoses decreased with passages until reaching ∼5% after 100–200 passages (Fig. 1). It is also important to note that supernumerary centrosomes were identified in mouse neural progenitor cells, suggesting that PSCs are not the only rapidly replicating cells that suffer from this problem (Yang et al., 2003). Fig. 1 summarizes the main challenges for genome integrity that PSCs face in culture.

Consequences, detection, and alleviation of genomic abnormalities in PSCs

Consequences. Human PSCs are expected to soon become an important tool for regenerative medicine. The possibility of in vitro differentiation of PSCs into any specific cell type, followed by cell transplantation, holds great promise for future therapies (Ben-David et al., 2012). The discovery of iPSCs may allow the transplantation of cells that will not be rejected by the immune system, raising the expectations from PSCs even higher. However, prolonged culturing of PSCs, as well as the stressful reprogramming process, place PSCs under artificial selection pressures that they usually do not experience in their natural environment. The selected clonal populations of cells are sometimes genetically altered with enhanced growing capacities that can form more aggressive tumors in immunodeficient mice (Herszfeld et al., 2006; Yang et al., 2008; Werbowetski-Ogilvie et al., 2009). Genetic changes can also alter the ability of PSCs to differentiate, to respond to growth factors, and to self-renew, and can lead to marked changes in their global gene expression profile (Lund et al., 2012). Such changes may negatively affect both the efficacy and the safety of hPSC-based therapies (Ben-David and Benvenisty, 2011; Goldring et al., 2011).

Apart from their clinical application, PSCs are extremely important for research purposes: PSCs are routinely used for development studies, disease modeling, and drug screens (Ben-David et al., 2012; Ben-David and Benvenisty, 2012a). It has been shown that genetically aberrant cells could dramatically distort experimental results, leading to wrong scientific conclusions (Mayshar et al., 2010; Ben-David et al., 2011; Ben-David and Benvenisty, 2012b). As both research and clinical usages of PSCs depend on mass production of differentiated, functional, karyotypically normal cells, it is important to develop efficient detection protocols and robust prevention methods that would minimize the risk for genomic instability and would enable its identification.

It is also important to note that mouse and human PSCs may correspond to different developmental stages: human PSCs seem to represent an epiblastic pluripotent state, whereas mouse PSCs are believed to represent the in vivo pluripotent state of the inner cell mass cells (Nichols and Smith, 2009). This could lead to many of the above-mentioned differences in genome instability and in the cellular mechanisms that underlie it. It will therefore be interesting to examine the various aspects of genome maintenance in the recently described "naive" human PSCs (Gafni et al., 2013), and compare them to the "primed" human PSCs that have been studied so far.

Detection. Available methods for inspecting the genomic content of cells vary in their resolution, sensitivity, cost, and time. Generally, they can be divided into cytogenetic methods, isolated DNA–based methods, and isolated RNA–based methods (Ben-David and Benvenisty, 2012a; Ben-David et al., 2013). The cytogenetic methods, i.e., G-band karyotyping and spectral karyotyping, are based on analyzing chromosomes at the metaphase stage of mitosis. Their resolution is relatively low but their sensitivity is high because the analysis is performed at the single-cell level. In addition, their cost is not very high, and they are therefore very popular. The isolated DNA–based methods, comprised of array-comparative genomic hybridization, SNP arrays, and whole-genome sequencing, are based on isolating DNA from cell populations, resulting in lower sensitivity. The resolution of these methods, however, is high, and can get up to single-nucleotide resolution with whole-genome sequencing. All the isolated DNA–based methods can take a few weeks to come to a conclusion, and are generally more expensive then the cytogenetic methods. A third method, called e-karyotyping, is based on isolated RNA and utilizes the gene expression profiles of the cells. This method predicts chromosomal aberrations from gene expression biases (e.g., a chromosomal gain can be identified by consistent overexpression of genes throughout the aberrant region); it thus provides an accurate estimation of chromosomal integrity in stem cells, with sensitivity comparable to that of DNA-based methods and resolution comparable to that of cytogenetic methods (Mayshar et al., 2010; Ben-David et al., 2013). Its main advantage is that it enables the simultaneous analysis of gene expression and genome integrity, using the exact same biological material.

Currently, when characterizing new PSC lines, standard G-banding is usually performed. However, even small genetic changes, which cannot be detected in karyotype analyses, can dramatically affect PSC behavior (Yang et al., 2008; Werbowetski-Ogilvie et al., 2009). Therefore, it is necessary to consider applying higher resolution methods for characterization of new PSC lines. As advanced DNA-based methods remain relatively

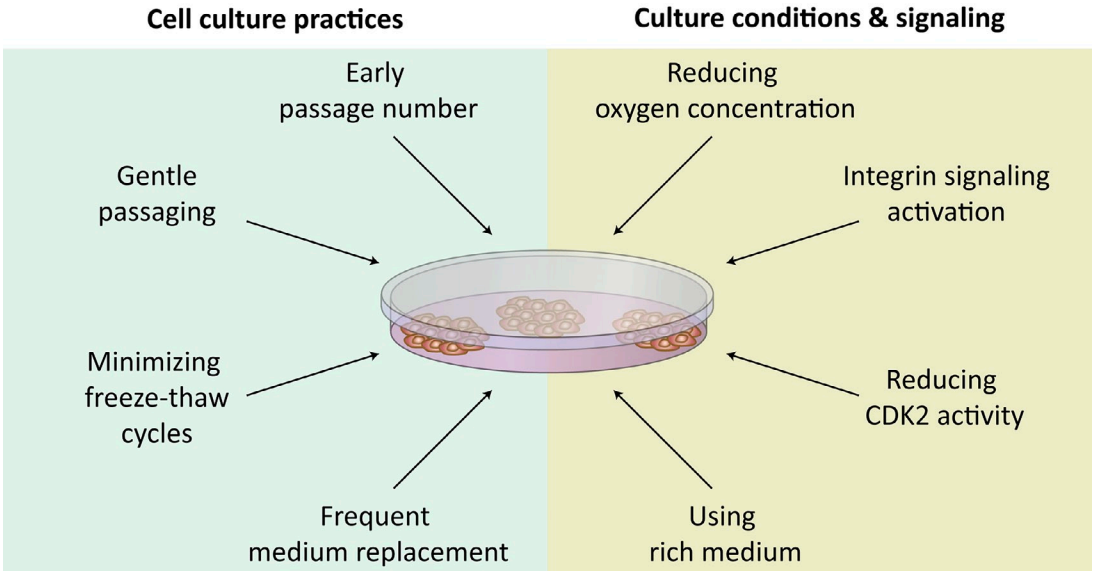

Figure 2. **Potential ways to minimize genomic insults in PSCs.** The genomic insults on PSCs in culture may be alleviated by adjusting their culture conditions (i.e., the signals to which they are exposed) or by executing cell culture practices that would reduce the selection for aberrant cells. Presented are main actions that may be taken to minimize the accumulation of genetic abnormalities in PSC cultures.

expensive and laborious, it might be advisable to combine standard karyotyping with direct examination (by FISH, for example) of common CNVs. Because gene expression profiling is usually performed as part of pluripotency characterization, it is recommended to use it for e-karyotyping as well. Combining these various assays would improve the effective detection of genomic aberrations without a significant increase in the required resources.

Of special concern with regard to detecting genomic aberrations in PSCs is the heterogeneity of PSC cultures (Stewart et al., 2006; Narsinh et al., 2011). This heterogeneity is manifested at the gene expression level, at the cellular differentiation capacity, and also at the DNA level (as discussed earlier). Therefore, at any time point, PSC cultures are expected to be heterogeneous in terms of genomic abnormalities. This highlights the importance of applying sensitive detection methods, as rare CNVs or point mutations could be easily missed due to detection limits. This heterogeneity also raises a need to define what should be done with aberrant cultures, based on the short and long term consequences expected of specific aberrations. Considering that every PSC culture contains some aberrant cells, it makes no sense to discard a culture as soon as a single aberration is observed, especially because most aberrations will be selected against; on the other hand, if common growth-promoting aberrations are detected, even at very few cells, it is advisable to discard the culture within few passages, as these aberrations are very likely to prevail. A catalog of common genomic abnormalities that emerge in PSC cultures, summarizing the available data regarding such aberrations and their known cellular consequences, will thus be a useful resource for the community.

Alleviation. The sources for genomic abnormalities that arise in PSC cultures are all related to the environmental conditions that they experience in vitro, which are different from the ones encountered in vivo. To reduce the risk of acquiring genomic aberrations, it is necessary to apply working practices and culture conditions that support the maintenance of normal diploid cells. Working with low-passage cells, applying gentle passaging techniques, and avoiding unnecessary freeze-thaw cycles, may reduce the accumulation of DNA damages (Fig. 2). Providing proper environmental conditions and stimulating or inhibiting some signaling pathways can further alleviate the risk for genomic insults. For example, low oxygen concentrations not only help to maintain the pluripotent state (Ezashi et al., 2005), but also push the PSCs toward anaerobic glycolysis, resulting in less ROS and DNA damage (Fig. 2).

The cell cycle of PSCs may also be amenable to manipulation: hESCs display high activity of CDK2, a key regulator of the G1/S transition and of centrosome metabolism (Holubcová et al., 2011). High CDK2 activity may uncouple the process of DNA replication and centrosome duplication, leading to the observed high frequency of supernumerary centrosomes. Inhibition of CDK2 using chemical inhibitors significantly reduced the prevalence of multicentrosomal mitoses (Holubcová et al., 2011), but also resulted in cell differentiation (Neganova et al., 2009); a mild inhibition of CDK2 that would reduce centrosomal amplification without inducing differentiation could thus be a potentially useful supplementation to the culture medium (Fig. 2). More broadly, compounds that increase cell cycle duration without impairing self-renewal could potentially increase genome stability.

Adhesion of hESCs onto the plate surface, and signaling from the culture substratum, can also affect genomic stability; activating the integrin signaling pathway was shown to reduce the frequency of multicentrosomal mitoses, and can thus potentially reduce karyotypic abnormalities (Fig. 2; Holubcová et al., 2011). Lastly, DNA breaks in rapidly proliferating cells are often coupled to replication stress, which can be ameliorated in some cases by exogenous supplementation of nucleosides (Fig. 2;

Bester et al., 2011). Therefore, it will be interesting to examine whether nucleoside supplementation would reduce replication stress, and consequently DNA damage, in PSCs. The potential ways to minimize genomic insults in PSCs are presented in Fig. 2.

Outlook

Genome maintenance is a demanding task for rapidly proliferating cells, such as self-renewing undifferentiated PSCs. With many of the recurrent culture-acquired abnormalities already known, mechanistic studies are now beginning to dissect the challenges faced by PSCs in their need to accurately preserve their genome integrity while maintaining their rapid proliferation and unique cell cycle characteristics. Understanding how PSCs execute this difficult task is important for several reasons. First, identification of the underlying mechanism for specific types of genomic aberrations can also shed light on the functional consequences of these aberrations. Second, as discussed in the previous section, it also enables the development of culture conditions and working procedures that will reduce the prevalence of these aberrations, and novel methods to detect aberrations once present. Third, PSCs make a unique system of rapidly proliferating noncancerous cells, and studying their genomic integrity can thus unravel basic principles of genome maintenance, which cannot be easily studied with post-mitotic cells and cannot be accurately mimicked with cancer cells. Lastly, due to the high similarity between PSCs and cancer cells, PSCs can also model some aspects of genomic instability in cancer. This field of research is therefore expected to yield many more exciting insights in the years to come.

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