



The Genomic Health of Human Pluripotent Stem Cells: Genomic Instability and the Consequences on Nuclear Organization

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Human pluripotent stem cells (hPSCs) are increasingly used for cell-based regenerative therapies worldwide, with embryonic and induced pluripotent stem cells as potential treatments for debilitating and chronic conditions, such as age-related macular degeneration, Parkinson's disease, spinal cord injuries, and type 1 diabetes. However, with the level of genomic anomalies stem cells generate in culture, their safety may be in question. Specifically, hPSCs frequently acquire chromosomal abnormalities, often with gains or losses of whole chromosomes. This review discusses how important it is to efficiently and sensitively detect hPSC aneuploidies, to understand how these aneuploidies arise, consider the consequences for the cell, and indeed the individual to whom aneuploid cells may be administered.

Keywords: aneuploidy, genome, stem cell, chromosome, nucleus (positioning)

INTRODUCTION

Stem cells are unspecialized cells that can give rise to a ranged of different cell types through self-renewal. Adult (mesenchymal) stem cells (MSCs) can be found throughout the body in various niches, such as the small intestine, colon or bone marrow (Barker et al., 2007; Héroult et al., 2017). Embryonic stem cells (ESCs) on the other hand are derived from the inner cell mass of an early preimplantation embryo or blastocyst and can differentiate to form all three germ cell layers. Such cells are known as pluripotent cells, since they give rise to every cell type of the body, excluding the extra-embryonic membrane and placental tissue. With such immense therapeutic potential, stem cells could be used for tissue repair and potentially replacement of whole organs through tissue engineering, circumventing the problem of a current lack of organ donors (Badylak et al., 2011). Due to their pluripotent properties, the treatment of many diseases such as age-related macular degeneration (Song et al., 2015), spinal cord injuries (Deshpande et al., 2006), type 1 diabetes (Farooq et al., 2018), and Parkinson's disease (Bjorklund et al., 2002; Takagi et al., 2005; Grealish et al., 2014; Barker et al., 2016) may soon become a reality.

Induced pluripotent stem cells (iPSCs) are pluripotent cells generated by the reprogramming of differentiated cells and can likewise give rise to a range of different cell types. iPSCs may be considered as the ideal therapeutic resource since an autologous stem cell transplant negates the need for human leukocyte antigen (HLA) matching and any immunosuppression required with allogenic transplants, as well as providing an endless supply of personalized therapeutic product if

required. It has been estimated that a relatively small number of iPSC lines need be generated to meet a demand that covers most of the world's population via the generation of HLA matched banks, making it both cost-effective and simpler for thorough characterization from a regulatory perspective (Taylor et al., 2012; Turner et al., 2013; Solomon et al., 2015). iPSCs are created from differentiated cells and can be reprogrammed to become pluripotent mainly through three genes: *OCT4*, *SOX2*, and *NANOG*, which induce and maintain the upregulation of pluripotency genes whilst repressing lineage-associated genes.

Both ESCs and iPSCs are noted for their accumulation of chromosomal aneuploidies, especially after prolonged *in vitro* culturing (Amps et al., 2011). Similarly, cells of the blastocyst also exhibit a high rate of mitotic aneuploidy (Taylor et al., 2014) and thus it is possible that the chromosomes of pluripotent cells are inherently unstable. Interestingly, in the blastocyst, more chromosome losses than gains are observed (Chung et al., 2013; Yao et al., 2016), in contrast to hESCs having more gains, which may lead to these affected hESCs having a greater selective advantage in cell culture (Amps et al., 2011). Typically hESC chromosome aneuploidies include chromosomes 1, 12, 17, 20, and X (Draper et al., 2004; Maitra et al., 2005; Baker et al., 2007) (**Figure 1**). This is in contrast to live births, where the most common aneuploidies are for chromosomes containing fewer genes i.e., autosomes 13, 18, and 21 (Caine et al., 2005) along with the sex chromosomes (Munné et al., 1998), and spontaneous abortions, where common aneuploidies include chromosomes 4, 7, 13, 15, 16, 21, and 22 (Fritz et al., 2001) (**Table 1**). Seemingly the aneuploidies accumulating in the hPSC culture are incompatible with life and are strikingly similar to the aneuploidies found in human embryonal carcinoma cells (hECCs), with respect to the types of karyotypic changes observed (Summersgill et al., 2001; Reuter, 2005; Harrison et al., 2007) and in their gene expression profiles (Sperger et al., 2003), suggesting a tumorigenic potential. Furthermore, stem cells with these recurrent gains or losses display a growth advantage in culture (Amps et al., 2011; Avery et al., 2013; Peterson and Loring, 2014), signifying that these chromosomes contain critical genes needed for cell growth, pluripotency and possibly tumorigenesis. This poses a serious threat to the therapeutic use of hPSCs, as the effects of using genomically abnormal or unstable stem cells in patients is unknown (Brimble et al., 2004; Draper et al., 2004; Peterson and Loring, 2014). Those chromosomal rearrangements common to hESCs and hECCs are candidates as drivers of tumorigenesis. Gene sequence and copy-number mutations affecting known oncogenes may also drive tumorigenesis. Screening oncogenes for mutations in hESCs might therefore become a necessity in providing a risk analysis of hESC lines prior to use in cell therapies. Indeed, in a study of 140 hESC lines, 5 were found to contain mutations in the oncogene *TP53* (Merkle et al., 2017), highlighting the risk of employing hPSCs for cellular therapies.

What effect(s) the hPSC aneuploidies may have, if cells containing them are administered to patients, needs to be addressed. An issue that is particularly important to address is the risk of transplanting hPSCs into individuals without being able to control their self-renewal capacity (Kanemura et al., 2014). The possibility of a malignant transformation of the cells

followed by unregulated proliferation could limit stem cells use for future therapies (Blum and Benvenisty, 2008; Herberts et al., 2011; Ben-David et al., 2014). Worryingly, it has already been demonstrated that the transplantation of aneuploid cultured murine MSCs leads to malignant transformation *in vivo* (Miura et al., 2006). This could lead to devastating consequences if patients were recipients of genomically unstable hPSCs. Tumor development from non-host origin has been reported after the injection of karyotypically normal neural stem cells into an Ataxia Telangiectasia patient (Amariglio et al., 2009). Whilst many details of the procedure were not disclosed, it is thought that sufficient genomic characterization of the donor cells was not performed prior to transplantation (Baker, 2009). This case, along with the supporting studies presenting mosaicism (Amps et al., 2011; Merkle et al., 2017) and recurrent chromosomal abnormalities (Brimble et al., 2004; Draper et al., 2004; Baker et al., 2007; Amps et al., 2011) giving rise to growth advantage in culture, highlights the importance of vigorous characterization of the hPSCs before transplantation if such cells were to be used regularly in therapies, and also the need for the development of novel analytics for such characterization.

Additionally, it has been reported that somatic cells with pre-existing chromosomal mutations limited the reprogramming of the cells to iPSCs (Yang C. et al., 2008). However, recent *in vitro* studies, generating hESCs with trisomies of either chromosomes 6, 8, 11, 12, or 15, demonstrate that proliferation may not be the issue, but the ability of stem cells containing aneuploidies to be able to differentiate efficiently and in a timely fashion is (Zhang et al., 2016). These experimentally induced aneuploidies also gave rise to global changes in gene expression profiles, evident in the differentiated somatic cells whereby gene expression alterations were found throughout the genome (Dürrbaum and Storchová, 2016). These technical issues once again demonstrate the inefficiency and potential malignancy of using aneuploid hPSCs in therapies.

It is concerning that aneuploid hPSCs may have a growth advantage *in vivo*, due to the selection of specific gene gains or losses, driving the concomitant gain or loss of part or whole chromosomes e.g., the gain of chromosome 20 in hPSCs driven by the *BCL2L1* gene (Enver et al., 2005; Baker et al., 2007). This gene is associated with anti-apoptotic properties (Boise et al., 1993; Amps et al., 2011; Avery et al., 2013; Na et al., 2014) and is a hallmark of cancer (Herszfeld et al., 2006; Yang S. et al., 2008; Avery et al., 2013). Knock-down of *BCL2L1* diminished the growth advantage effect and thus, this gene is likely to be the driver of chromosome 20 accumulation in hESC cultures (Avery et al., 2013). Following the event that creates aneuploid cells, selection is then required to increase the proportion of aneuploid cells relative to the normal diploid cell population. There are several points during hESC culture at which selection could operate, but evidence points to the mechanism used for disaggregating cells for passaging. For example, aneuploidies were gained when employing enzymatic and non-enzymatic methods of cell dissociation, rather than manual colony cutting in hESC cultures (Mitalipova et al., 2005). Furthermore, aneuploid cells showed an increase in the expression of pluripotency genes and early differentiation genes, implying that the cell

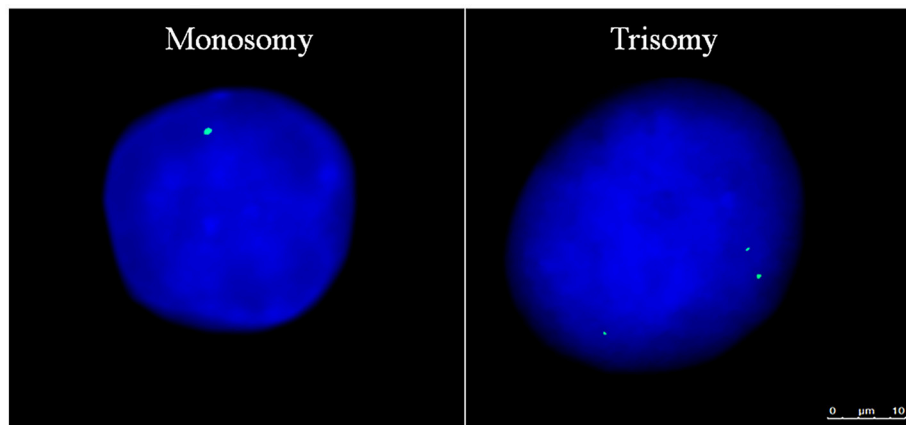


FIGURE 1 | Aneuploid Gene Loci within Human Embryonic Stem Cells. Aneuploid pluripotent stem cell nuclei subjected to fluorescence *in-situ* hybridization displaying *AMELX* gene loci in green and nuclear DNA stained with DAPI in blue. Scale bar is 10 μm .

disaggregation method may induce widespread changes in the phenotype of the cell culture. Candidate genes suggested to infer a growth advantage include the pluripotency-related genes *NANOG*, *DPPA3*, and *GDF3*, oncogene *KRAS*, and cell cycle regulator *CCND2* on chromosome 12, and *BIRC5* (*SURVIVIN*) on chromosome 17 (Na et al., 2014). It is also possible that mutation-bearing cells with no selective advantage may become present at significant levels to chance-effects in the bottleneck created by colony-cutting and poor cell survival rates upon passage. However, with the limitations of current analytics, it is difficult to discern the precise levels of aneuploidies appearing in culture.

In this article, we will review the mechanisms by which aneuploidies may arise in hPSCs, and the potential impact on genome organization and stability, concluding with an analysis on the current tools available to measure genomic aberrations toward ensuring safe therapeutic application.

HOW ANEUPLOIDIES ARISE

In order to maintain genomic integrity, it is essential that with each cell division the distribution of chromosomes in each daughter cell is matched. Unfortunately, how exactly aneuploidies arise in human pluripotent stem cells is not yet entirely known. We discuss here a number of mechanisms that could lead to the formation of aneuploidies and discuss the genomic abnormalities that may contribute to aneuploidy status.

Mitotic Segregation Defects

Telomeres are repetitive nucleotide sequences found at the end of chromosomes to prevent chromosome end-to-end fusions, which can result in chromosome instability. Normally, telomeres shorten as a result of each cell division, although in stem cells telomerase is active to ensure the maintenance of telomere length (Greider and Blackburn, 1989; Feng et al., 1995; Nakamura and Cech, 1998). In hESCs, the telomerase enzyme is continually active in order to maintain the extended length of telomeres

and in iPSCs, telomerase is re-activated after reprogramming and the process of telomere lengthening begins (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Marión et al., 2009). When two end-to-end fused chromosomes are being pulled apart by opposing mitotic spindle tubules, anaphase bridges or chromatin bridges can occur which create a link between the two daughter cells. Although the formation of anaphase bridges does occur in normal cells (Baumann et al., 2007; Chan et al., 2007), it is strongly associated with the erosion of telomeres (Tusell et al., 2010). The inability of the fused chromosomes to part leads to one daughter cell gaining a chromosome and the other losing a chromosome. Further, end-to-end fusion of chromosomes can cause breakage-fusion-bridge (BRB) cycles to be established, resulting in genomic instability (DePinho, 2000; Gisselsson et al., 2001; Hackett et al., 2001) and in turn causing the shearing of ultra-fine bridges also generating aneuploidy.

Telomeric sequences are associated with a group of proteins; TRF1, TRF2, RAP1, POT1, TIN1, and TIN2, collectively known as the shelterin complex (Liu et al., 2004). Disruption of these proteins can cause fragile sites in the genome, contributing to DNA replication defects (Sfeir et al., 2009), anaphase bridges (Bunch et al., 2005; Nera et al., 2015), chromosome fusions (Pardo and Marcand, 2005) and the activation of DNA damage responses (Palm and de Lange, 2008). A recent study has revealed that overexpression of the telomere repeat-binding factor 1 (TRF1) in mouse ESCs can indeed cause anaphase bridges to form (Lisaingo et al., 2014), thus indicating the importance of telomere protection in hESCs. Most interestingly, in ESCs with short telomeres (Huang et al., 2011) and in the full knockout of a subunit of telomerase, *Tert*^{-/-} ESCs (Pucci et al., 2013), reduced levels of pluripotency have been observed. Indeed, long telomeres and high TRF1 levels have been proposed as additional stem cell markers (Flores et al., 2008; Huang et al., 2011; Schneider et al., 2013). However, although the overexpression of telomerase did improve the self-renewal and proliferation rate, it increased resistance to apoptosis and caused a suppression in the differentiation capacity of ESCs (Armstrong et al., 2005; Yang C.

TABLE 1 | Chromosomal abnormalities in specific cell types or in live births and spontaneous abortions.

Cell type	Chromosomal abnormalities
Embryonic stem cells	1, 12, 17, 20, X
Induced pluripotent stem cells	1, 9, 12, 20, X
Human embryonal carcinoma cells	1, 12, 17, 20, X
Live births	13, 18, 21, X, Y
Spontaneous abortions	4, 7, 13, 15, 16, 21, 22

Specific chromosome gains and/or losses that occur most commonly in the different cell types, and in live births and spontaneous abortions.

et al., 2008). These findings suggest a potential range for optimal telomere length in the hPSCs, which could be used as a screening method, in the cells intended for clinical use.

On occasion, the sister chromatids are not resolved correctly during mitosis, due to the lack of kinetochore attachment to the mitotic spindle, with one daughter cell receiving both chromosomes, and an aneuploid status in both cells. How the mitotic spindles assemble in hPSCs is not well investigated, however, spindle defects such as asymmetric orientation have been linked with carcinogenesis in *Drosophila melanogaster* (Causinus and Gonzalez, 2005; Castellanos et al., 2008) and in human gut epithelial stem cells (Quyn et al., 2010). A balance of symmetric or asymmetric cell divisions are necessary for normal development and tissue homeostasis, however this can lead to abnormal proliferation (Noatynska et al., 2012). Alternatively, lagging chromosomes derived from mitotic spindle detachment or the bipolar orientation of chromatids (Cimini et al., 2002) can instead form a separate compartment of chromatin away from nuclei. Atelometric and acentric, whole or fragmented chromosomes, can become micronuclei (Cimini et al., 1999; Minissi et al., 1999; Norppa and Falck, 2003) or double-minute (DM) chromatin, where small fragments of amplified genes occur extra-chromosomally (Haaf and Schmid, 1988; Itoh and Shimizu, 1998). Although nuclear contents may be lost in this manner, they can also be engulfed into nuclei (Minissi et al., 1999). Micronuclei or DMs can appear as a result of replicative stress and sometimes still remain transcriptionally active, albeit at reduced levels (Hoffelder et al., 2004; Utani et al., 2007). These micronuclei can also contain nucleoskeletal structural components such as nuclear lamins and thus are not totally inert (Tanaka and Shimizu, 2000). Both pluripotent and differentiating ESCs seem to have a propensity to form micronuclei: in mouse ESCs, an increase in micronuclei formation and apoptosis was observed with the downregulation of the pluripotency marker *OCT4* (Zhao et al., 2014), additionally differentiation of murine ESCs to neural progenitor cells causes a nearly 2-fold increase in micronuclei formation and an increase in chromosome instability (Sartore et al., 2011). Indeed, the high rate of proliferation of hESC in itself could promote the formation of micronuclei and thus be a factor contributing to their genomic instability (Stopper et al., 2003).

The apoptosis inhibitor protein, survivin, normally protects against polyploidy through its function in the control of

the spindle assembly checkpoint and cytokinesis. Impairment of survivin expression has been associated with polyploidy development in human cells (Li et al., 1999). Survivin is highly expressed in ESCs (Adida et al., 1998) and has been shown to be fundamental in maintaining pluripotency (Mull et al., 2014; Kapinas et al., 2015) by being involved, with its splice variants, in the upregulation of *NANOG* and *OCT4* (Mull et al., 2014). Thus, there is a case for survivin expression to be tested for as part of a genomic health screen for clinical-grade stem cells.

DNA Damage

During development, blastocyst cells may have to compromise their DNA proof-reading capability in order to achieve a rapid rate of cell division. This postulation is supported by the shortened G₁ phase of interphase in ESCs in culture (Becker et al., 2006; Ghule et al., 2008), exposing them to potentially higher replicative errors. Furthermore, studies of the TP53-p21 pathways in hESCs have revealed that during stress stimuli, the p21 mRNA is upregulated in hESCs, however no p21 protein is detected (Dolezalova et al., 2012). This could imply that although the cell has responded to stress, it has not been able to achieve p21 function, allowing replication errors to remain. During DNA damage in hESCs, TP53 binds directly to *NANOG*'s promoter, suppressing it and promoting hESC differentiation (Lin et al., 2005). If p53 levels are reduced, the levels of spontaneous differentiation are also reduced (Kawamura et al., 2009). It seems that in hiPSCs, DNA damage does not give rise to single-stranded DNA regions, checkpoints are not activated, and thus DNA repair does not occur (Desmarais et al., 2012), despite there being elevated expression levels of DNA repair genes (Momcilovic et al., 2010). This is echoed in studies of mouse cells, whereby iPSCs were less able to perform double-strand break repair, especially by homologous recombination repair, compared with both primary cells and ESCs (Zhang et al., 2018). Furthermore, hiPSCs have been found to be deficient in intra-S checkpoints and also in G₂/M decatenation or chromatin dis-entanglement, preventing delayed entry of inappropriately condensed chromosomes into mitosis and permitting the formation of anaphase bridges (Damelin et al., 2005; Filion et al., 2009; Weissbein et al., 2014; Lamm et al., 2016). Topoisomerase II permits chromatin decatenation to occur in G₂ to delay mitosis and allow smooth sister chromatid segregation (Uemura et al., 1987; Holm et al., 1989). When the decatenation checkpoint is disrupted, entangled chromosomes segregate and then form new cells with aneuploidy (Gorbsky, 1994; Andoh and Ishida, 1998). Chromosome decatenation deficiency has also been reported in mouse ESCs and human multipotent progenitor cells, however improved decatenation was observed later with cell differentiation (Damelin et al., 2005). The reason behind such entanglement of ESC chromatin may be due to the lack of higher chromatin organization in the nucleus, such as heterochromatin. hESC nuclei lack chromatin silencing markers, such as methylation on H3K9 and H3K27. The plasticity of the chromatin, causes the DNA to be a highly open structure and coupled with the dispersed presence of the DNA damage marker, γ -H2AX in hESCs (Meshorer et al., 2006), in stark comparison to more localized foci in somatic cells (Mariotti et al., 2013), suggests

a more exposed, and therefore a more easily damaged chromatin. The plasticity of the more-open chromatin state in stem cells could be one of the reasons for the increased genomic instability of hPSCs when cultured *in vitro*. Increased levels of γ -H2AX were also noted in hiPSCs compared with their source primary line (Vallabhaneni et al., 2018), suggesting a similar scenario in these cells. Although, this may be debatable since no additional protection of heterochromatin, in comparison to euchromatin, has been observed from the reactive oxygen species (ROS)-induction of double-stranded breaks (Woodbine et al., 2011). But, lower levels of Ataxia-telangiectasia mutated kinase (ATM) phosphorylation in iPSCs has been previously reported in cells treated with low levels of radiation, alongside hypersensitivity to apoptosis (Nagaria et al., 2016). ATM phosphorylates a number of proteins, related to apoptosis, cell cycle checkpoints, and DNA repair (Lee and Paull, 2007), therefore its potentially reduced role in hPSCs should be carefully considered. The exact role of ATM in DNA damage in heterochromatin is still unknown, but it has been suggested to be preferentially required in the DNA damage repair of heterochromatin (Goodarzi et al., 2008). As hPSCs lack the presence of heterochromatin (Francastel et al., 2000; Meshorer and Misteli, 2006), the reduced levels of ATM phosphorylation (Nagaria et al., 2016) probably would not have a significant effect on the genomic integrity of the cell. However, ATM-deficient cells were less efficient in reprogramming to iPSC, which influenced the appearance of genomic variation (Marión et al., 2009; Kinoshita et al., 2011; Lu et al., 2016). Similarly, Artemis, an endonuclease associated with non-homologous end-joining, is required for the maintenance of genomic stability (Woodbine et al., 2011), but its absence from stem cells did not impair myeloid differentiation, reprogramming or show any signs of significant genomic instability (Felgentreff et al., 2014).

Despite the susceptibility of hPSCs to DNA damage *in vitro*, steps may be taken to alleviate this by the modification of culture conditions, including freeze-thaw techniques, passaging (Mitalipova et al., 2005), and media composition: a reduction in MEK inhibition (involved in the regulation of DNA damage/repair and cell cycle) was observed to maintain naive hESCs, accelerate proliferation, and reduce the accumulation of chromosomal abnormalities in culture (Di Stefano et al., 2018).

Bystander Effect?

Another putative mechanism for the process of aneuploidy accumulation is that cells acquire an aneuploidy and then via a bystander effect further aneuploidies accumulate in neighboring cells. Such mechanisms have been observed with radiation-treated cells causing cell senescence in neighboring cells (Nelson et al., 2012), increased sister chromatid exchange (Nagasawa and Little, 1992; Deshpande et al., 1996), increased *TP53* expression (Hickman et al., 1994; Azzam et al., 1998), and most importantly chromosomal instability (Lorimore et al., 1998; Sawant et al., 2001). This instability in the irradiated cells is probably observed due to the ROS produced from the radiation (Yamamori et al., 2012) causing DNA damage to occur (Yermilov et al., 1996; Balasubramanian et al., 1998). Most interestingly, a bacterium species has been shown to induce aneuploidy, amongst other hallmarks of genomic instability, in human cells, through a

bystander effect. *Enterococcus faecalis*, an intestinal bacterium, where the production of ROS molecules induced chromosome instability in cells with defects in mismatch repair genes (Huycke et al., 2001, 2002; Wang et al., 2008). Although this theory needs to be investigated further, it is well established that ROS and nitrogen species from both radiation and metabolism can cause oxidative stress that can lead to DNA damage and senescence in cells (Lindahl, 1993; Suh et al., 1999; Geiszt et al., 2000). Moreover, it may be the case with hPSCs that if one event triggers an aneuploidy to occur, a bystander effect could then cause neighboring cells to also acquire aneuploidies, through transmission of substances through the culture media or delivered in exosomes. For example, if mitomycin C, a commonly used growth inhibitor of feeder cells, were to negatively affect the hPSC basement membrane, then we theorize that this might affect the neighboring stem cells. This event can then cause or promote the generation of further aneuploidies in the hPSC culture. As more hESC lines are developed on, or adapted to other alternative matrices, it should become more apparent if there are any effects and whether it is the stem cells or the feeder cells that potentially instigate aneuploidy.

It has been previously proposed that the increased age of cells and the amount of ROS are linked (Finkel and Holbrook, 2000). As human pluripotent stem cells are metabolically very active and can be maintained in cultures for long periods of time, the increased age and the fast metabolism required in these cells could also be an aspect that factors in the genomic instability often observed. In contrast, it has been reported that both high and low levels of ROS can impair the reprogramming ability of cells into iPSCs (Zhou et al., 2016) and elevated levels can impair their differentiation ability as well (Rönn et al., 2017). These studies suggest that optimal levels of ROS may be required for the cells to grow stably in culture. With the effect of ROS established above, very precise growth conditions must be maintained in the hPSC culture to ensure genomic integrity. We hypothesize that the use of reagents, such as mitomycin C, could potentially affect the neighboring hPSCs and should be carefully considered before the assumption of no effect.

Nuclear Lamin Depletion

Lamins are a meshwork of proteins found at the nuclear periphery with intimate associations with the inner nuclear membrane and co-located proteins (Gruenbaum et al., 2000; Zastrow et al., 2004). Nuclear lamins, which play an important role in the maintenance of nuclear morphology and chromosome organization (Aebi et al., 1986; Bridger et al., 2007; Dechat et al., 2008; Bickmore and van Steensel, 2013), have also been suggested to be involved in many other processes within the nucleus, such as DNA replication and repair, transcription and RNA processing (Cai, 2001; Laguri et al., 2001; Wolff et al., 2001; Spann et al., 2002).

In humans, A-type lamins, such as lamin A and C, are encoded by *LMNA*, whereas B-type lamins, such as lamins B1 and B2 are encoded by *LMNB1* and *LMNB2*, respectively (Wydner et al., 1996). Unlike A-type lamins, lamins B1 and B2 are endogenously expressed in both somatic and embryonic cells (Höger et al., 1990;

Pollard et al., 1990; Lin and Worman, 1995). The presence of A-type lamins in embryonic cells is still debated, as some reports show that A-type lamins are expressed only in somatic cells (Lehner et al., 1987; Stewart and Burke, 1987; Höger et al., 1990; Hutchison, 2002), and are completely absent from the nuclei in both ESCs (Constantinescu et al., 2006) and iPSCs (Mattout et al., 2011), whereas more recent reports suggest that A-type lamins are expressed at low levels in ESCs (Kim et al., 2011; Eckersley-Maslin et al., 2013). In early embryos, A-type lamins can be observed (Foster et al., 2005), but these are thought to be gamete-derived and soon disappear.

A-type lamins are found to accumulate with the down-regulation of *OCT4*, a hallmark of cell differentiation, and this is thought to contribute to the ESC nuclear plasticity (Constantinescu et al., 2006; Meshorer et al., 2006; Pajeroski et al., 2007). Lamin A then associates with and anchors, forming heterochromatin at the nuclear periphery, helping to organize the genome, regulating it for lineage commitment (Solovei et al., 2013); the accumulation of A-type lamins during differentiation have been associated with the loss of nuclear plasticity (Constantinescu et al., 2006; Meshorer et al., 2006; Pajeroski et al., 2007). Mutations in the A-type lamins give rise to a family of diseases commonly referred to as laminopathies, often associated with tissues derived from the mesenchyme, such as skeletal muscle, skin, cardiac muscle, tendons, adipose, and neurons (Worman and Bonne, 2007). Indeed, *LMNA* mutations cause impaired differentiation of adult mesenchymal stem cells (Gotzmann and Foisner, 2006; Pekovic and Hutchison, 2008; Scaffidi and Misteli, 2008), alterations in *Notch* and *Wnt* signaling pathways required for early development (Espada et al., 2008; Meshorer and Gruenbaum, 2008; Scaffidi and Misteli, 2008; Hernandez et al., 2010) and MSC death (Halaschek-Wiener and Brooks-Wilson, 2007; Meshorer and Gruenbaum, 2008; Prokocimer et al., 2009). Additionally, lamin A knockdown affects the serum response factor (SRF) pathway that promotes expression of abundant actin-myosin cytoskeletal components involved in the differentiation of cells (Swift and Discher, 2014). The SRF pathway is partially regulated by nuclear actin (Olson and Nordheim, 2010; Baarlink et al., 2013), which binds to lamin A (Simon et al., 2010) and other proteins associated with lamin A, such as emerin (Simon and Wilson, 2011). In contrast, Lamin B1 and B2 knockout does not affect the differentiation of blastocysts, but does affect organogenesis in mice (Coffinier et al., 2010; Kim et al., 2011), as well as mitotic spindle orientation and formation (Tsai et al., 2006; Ma et al., 2009; Kim et al., 2011). This suggests that B-type lamins have a functional role in ensuring chromosomes are efficiently segregated during mitosis. This correlates with findings of lamin B2 depletion being associated with aneuploidy formation, prolonged mitosis and formation of anaphase bridges in cancerous cells (Kuga et al., 2014; Ranade et al., 2017). Additionally, the depletion of lamin B2 caused the mislocalization of chromosome territories (CTs) in aneuploid cells (Ranade et al., 2017). In contrast, in mouse ESCs the knock-out of B-type lamins and the mutation of *Lmna* did not cause any effect on the proliferation and differentiation of mouse ESCs, nor did it change the total number of chromosomes in nuclei (Kim et al., 2013). It has been suggested that lamin B2, alongside

the inner nuclear membrane protein SUN1 (Malone et al., 2003; Razafsky and Hodzic, 2009), supports the spindle pole during mitotic spindle formation (Kuga et al., 2014). Indeed, SUN1 is required for telomere binding to the nuclear envelope and disruption of SUN1 affects meiotic division (Ding et al., 2007). We hypothesize that nuclear proteins, especially lamins, have a key role in the maintenance of genomic stability of hPSCs. Further work is required to establish whether B-type lamin loss causes aneuploidies or aneuploidies induce the loss of B-type lamins.

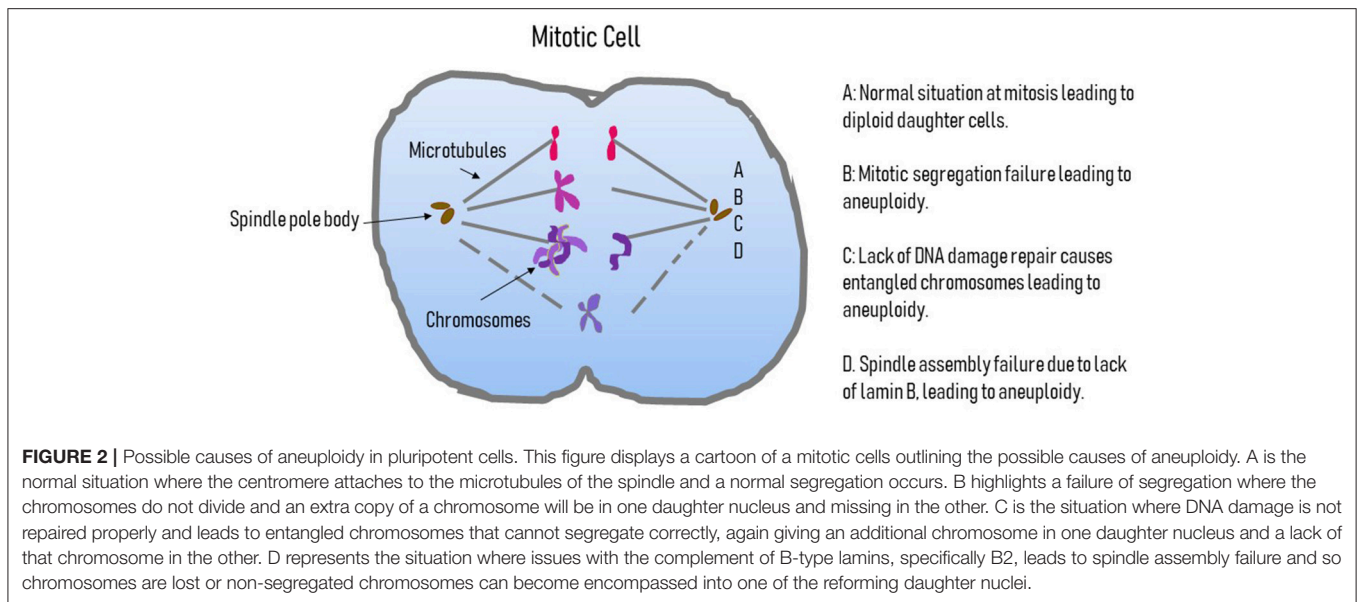
Chromosome Integrity Checkpoints

With all the scenarios that can go wrong in a cell with respect to genomic instability, chromosome integrity and DNA damage it is important that cells have adequate and well-functioning checkpoints, to assess the health of the genome (Sperka et al., 2012). For correct chromosome segregation there are two critical checkpoints, known as the spindle assembly checkpoint and the decatenation checkpoint. The G1 tetraploidy checkpoint also assesses for chromosome aberration, especially additional chromosomes (Brown and Geiger, 2018). Very interestingly in murine ESCs the spindle assembly checkpoint was not activated as it would be in somatic cells, leading to apoptosis and so the possibility of a higher numbers of cells with aneuploidy (Rohrbaugh et al., 2008). Furthermore, the decatenation checkpoint which verifies for entanglement of chromosomes that can happen with inadequate DNA damage repair, has been revealed to not be activated in murine ESCs, although it is activated once cells have committed to a lineage (Damelin et al., 2005; Suvorova et al., 2016). Thus, the lack of checkpoint function in embryonic stem cells is perhaps a process to maintain stemness and openness of chromatin, allowing aneuploidy and instability to arise in a population but which can be overcome later, removing individual cells that are too compromised. A further checkpoint that monitors the numbers of centrosomes, a building block of the spindle pole bodies has not yet been studied in stem cells; such a screening test to assess centrosome number by antibody staining probably should be included in a panel of assessments and parameters to be tested prior to stem cell use in the clinic.

Cyclin D1 levels are low in ESCs as compared to somatic differentiated cells. Cyclin D1 is a pivotal component of the G1/S transition in interphase. Interestingly, it is the presence of specific microRNAs regulated by *OCT4* and *SOX2* that prevent the expression of cyclin D1 (Card et al., 2008). For iPSCs, reprogramming back to a less controlled cell cycle, with “looser” checkpoints and shorter G1 and G2 phases is thwarted by cyclin D1 (Chen et al., 2014). **Figure 2** gives an overview of the causes discussed that may permit aneuploidy to arise.

Genome Organization Is Different in Stem Cells

Earlier studies have analyzed the genome in somatic and indeed stem cells with specific chromosome probes in fluorescence *in situ* hybridization (FISH) visualized by high resolution



microscopy (Clements et al., 2016). The genome is highly organized in somatic, differentiated cells (Bridger and Bickmore, 1998; Parada and Misteli, 2002; Tanabe et al., 2002; Foster et al., 2012), with interphase chromosomes organized into individual territories (Cremer and Cremer, 2001), called chromosome territories in similar nuclear locations between different cell types, with a few specific tissue related differences (Kuroda et al., 2004; Parada et al., 2004; Foster et al., 2012; Robson et al., 2016). On the whole, in proliferating cells a gene-density distribution is observed with gene-rich chromosomes found toward the nuclear interior and gene-poor toward the nuclear periphery (Bridger et al., 2014). A re-positioning occurs when cells leave the proliferative cell cycle to quiescence or senescence (Bridger et al., 2000; Mehta et al., 2010; Criscione et al., 2016). Here, we review how chromosomes are arranged in hPSCs compared with somatic cells and discuss whether the type of strict genome organization and chromosome positioning found in differentiated cells is pertinent and relevant to stem cells.

A gene-density radial distribution of CTs has been observed in hESCs (Wiblin et al., 2005; Bártová et al., 2008), as it has been in human somatic cells (Croft et al., 1999; Boyle et al., 2001) and in human blastomeres (Finch et al., 2008). These data were corroborated for stem cells by studies in pig cells whereby there was very little difference in chromosome positioning between mesenchymal stem cells from bone marrow and cells within differentiated tissues (Foster et al., 2012). However, gene-rich human chromosomes 17 and 19 were positioned more centrally in granulocytes when compared to hESC (Bártová et al., 2001), even though chromosome 12 and its centromere positioning in pluripotent and somatic cells were reportedly the same (Bártová et al., 2008). These data indicate that CT positioning in ESCs is not as it will be once the cells have differentiated. This would suggest that embryonic nuclei have mechanisms in place to re-position interphase chromosomes. Further, in cloned bovine embryos, CTs also do not relocate upon development but the

pluripotency genes are relocated to more transcriptionally active regions of the territories (Orszynowicz et al., 2017). Genes looping away from CTs has been reported previously to be associated with dependent transcription in specific cell types (Volpi et al., 2000; Mahy et al., 2002). Indeed, the 12p region that contains a group of clustered pluripotency genes, including *NANOG*, was found to be located more centrally in hESCs than in somatic cells (Wiblin et al., 2005). In contrast, chromosome 6p, containing the pluripotency marker *OCT4*, did not show any difference in its nuclear position, whilst the *OCT4* locus was reported to move to outside its CT in ESCs (Wiblin et al., 2005).

Reports of a less rigid chromatin state, due in part to the lack and/or absence of chromatin remodeling markers, in undifferentiated cells has been reported (Keohane et al., 1996; Francastel et al., 2000; Lee et al., 2004; Meshorer et al., 2006). In normal somatic cells, centromeres are mostly found nearer to the nuclear periphery or around nucleoli, and also often by the CT periphery (Weierich et al., 2003; Gilchrist et al., 2004), although this may depend on the stage of the cell cycle (Ferguson et al., 1992; Weimer et al., 1992; Hulspas et al., 1994). Previous reports have found that in human cells during differentiation, centromeres tend to move nearer to the nuclear periphery (Salníková et al., 2000; Bártová et al., 2001; Galiová et al., 2004; Horáková et al., 2010), or relocate more centrally (Bártová et al., 2008) to the heterochromatin surrounding nucleoli, and cluster together in chromo-centromeres (Alcobia et al., 2000; Beil et al., 2002). Movement of the centromeres toward the nuclear periphery was also observed in early rabbit embryos, once they had passed the 4-cell stage (Bonnet-Garnier et al., 2018). Such heterochromatic chromosomal regions may be more likely to be positioned toward the nuclear periphery which is supported by the findings of an increased association of chromatin silencing markers with perinuclear centromeres (Bártová et al., 2008) and with the under-acetylation of centromeres in both mouse and human undifferentiated cells (O'Neill and Turner, 1995; Keohane

et al., 1996). Immaturely developed centromeres, lacking specific markers of heterochromatin, in embryos and stem cells might be less able to attach to the mitotic spindle, resulting in aneuploidy. Indeed, interfering with centromere structure does lead to mitotic catastrophe in mice (Howman et al., 2000; Artus et al., 2006).

More recently global genome organization has been analyzed by a range of chromosome conformation capture (3C) experiments. Based on forming cross-links between pieces of chromatin that sit adjacent to each other, fragmenting, ligating and sequencing the new ligated DNA pieces reveals which parts of the genome sit together in three-dimensional space within nuclei. These studies have revealed that the genome is folded and organized into topologically associated domains (TADs) which have two sub-types A and B (Lieberman-Aiden et al., 2009; Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012). Type A TADs contain active open chromatin whereas B-type TADs are comprised of inactive more heterochromatic regions of the genome (Figure 3). These TADs have been found not only in somatic cells but in ESCs too, revealing similar types of organization of the genome present before differentiation. However, in ESCs the number of TADs are increased and the size is reduced, suggesting that there is in fact a less organized genome organization (Glinsky et al., 2018). However, closer study with 3C combined with chromatin factor binding data reveal that inactive chromatin in PSCs is not organized as would be expected in somatic cells (de Wit et al., 2013) and there is noticeably less heterochromatin. Whereas, active regions of the genome bound by pluripotency factors such as NANOG and OCT4 bring specific clusters of genes together (de Wit et al., 2013) to maintain pluripotency. Indeed, at the NANOG locus, specific proteins interact to regulate NANOG expression being bound together in an “interactome” containing mediator, a transcriptional coactivator and a chromosomal architectural protein with cohesin with the other key players in pluripotency SOX2, c-MYC, and OCT4 (Apostolou et al., 2013). Others have shown that OCT4 behaves in a similar way in mouse and humans iPSC construction (Wei et al., 2013; Zhang et al., 2013). Phillips-Cremins discusses the differences in ESC nuclei with respect to gene association with the different TAD sub-types and how this can switch upon differentiation (Phillips-Cremins, 2014). Indeed, pluripotency genes move from associating with A TADs to B TADs (Lin et al., 2012). The association of the genome with the nuclear periphery is also massively altered in mouse ESCs with genes required to maintain pluripotency away from the repressive environment of the nuclear edge (Peric-Hupkes et al., 2010). Figure 3 gives an overview of the differences between ESCs, iPSCs, and somatic cells, with respect to genome organization.

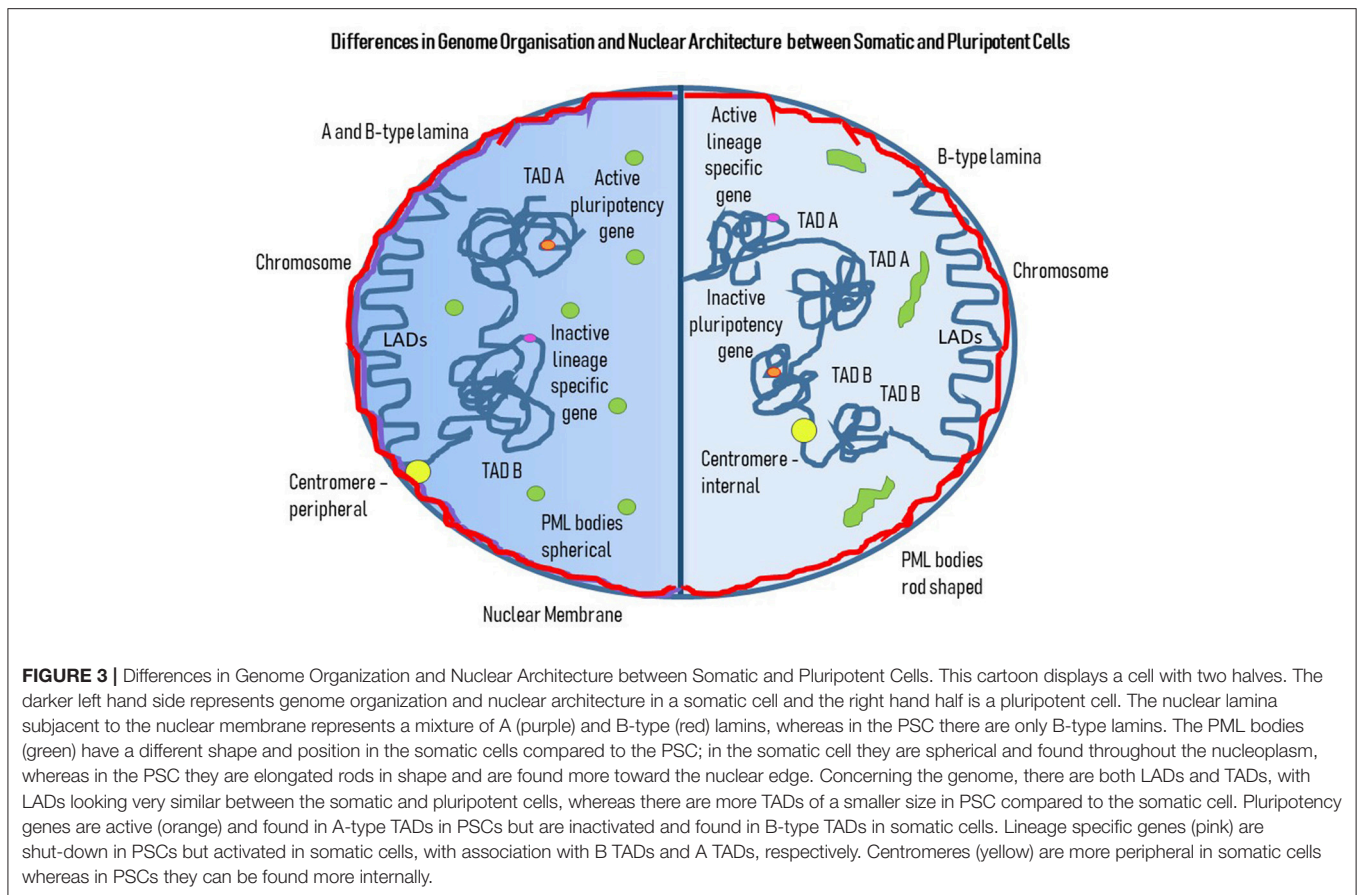
It is as yet not clear the effect that aneuploidy could have on genome organization, with extra genomic regions needing space at the nuclear envelope or elsewhere. Indeed, although reports show that extra chromosomes are located in the correct nuclear compartment in somatic cells, the same is not as clear for pluripotent cells that lack A-type lamins and have other altered nuclear architecture. Gene expression can be changed on a large scale when there are extra chromosomes, and this could be a

more important issue than more simply having extra copies of some genes. Thus, the real impact of extra chromosomes on genome organization into TADs and indeed lamina-associated domains (LADs, see below) and genome function as a whole remains to be elucidated.

Nuclear Architecture and Sub-Components

The nuclear lamina is located at the nuclear envelope and is comprised of A and B-type lamins, combined with a plethora of nuclear envelope transmembrane proteins (Czapiewski et al., 2016) with many of these proteins having chromatin binding abilities. Indeed, the nuclear lamins are chromatin-binder and anchoring specific regions of the genome through LADs (van Steensel and Belmont, 2017). LADs are regions of the genome that on the whole are comprised of heterochromatin and repressed sequences. This is not the case for genes that are more proximal to nuclear pore complexes that can be active. In mouse and human iPSCs, LADs have a higher mutation rate than in non-LADs which could be due to oxidative stress generated during the reprogramming process (Yoshihara et al., 2017) (Figure 3).

In human and mouse ES cells, the presence of lamins B1 and B2 was observed with lamin A/C absent (Constantinescu et al., 2006). Removal of lamin B1 in murine ESCs appeared in one study to be essential for heterochromatin to be located at the nuclear periphery (Zheng et al., 2015) but in another study, the lack of all nuclear lamins, both A-type and B-type did not have any effect on genome organization and LAD positioning, implying that other proteins are responsible for the positioning and anchorage of chromatin through LADs at the nuclear envelope, for example the integral membrane protein emerin (Amendola and van Steensel, 2014). In another study, Robson et al. demonstrated how nuclear envelope transmembrane proteins NET39, TMEM38A, and WFS1 anchor myogenic specific genes to the nuclear periphery for repression in stem cells prior to differentiation (Robson et al., 2016). Despite some studies (Eckersley-Maslin et al., 2013), the A-type lamins do not appear to be expressed or required by undifferentiated embryonic stem cells (Rober et al., 1989; Smith et al., 2017) and also have been observed to completely disappear with successful reprogramming of iPSCs (Mattout et al., 2011; Zuo et al., 2012). Indeed, it seems that A-type lamin upregulation is concomitant with or even responsible for the start of lineage commitment. The incorporation of A-type lamins and emerin into the nuclear lamina induces size and morphology changes in nuclei (Butler et al., 2009), and correspondingly, nuclei lacking A-type lamins and emerin fail to change their morphology, with compromised ability to undergo endoderm differentiation, along with changes in gene expression (Smith et al., 2017). A-type lamins were also found to accumulate with the downregulation of OCT4, a hallmark of differentiation. The absence of lamins A/C has been suggested to contribute to the ESC nuclei plasticity compared to the more rigid state of somatic cell nuclei, with hESC lacking heterochromatin at the nuclear periphery (Smith et al., 2017) and a global remodeling of the genome organization during lineage commitment (Peric-Hupkes and van Steensel, 2010). Mutations in the lamin A gene, *LMNA*, that cause muscular



dystrophy, interfered with the formation of typical LADs at the nuclear envelope, altering their heterochromatic status which as a consequence changed the repression of the *SOX2* locus, allowing them to be upregulated (Perovanovic et al., 2016). Lamin A knockdown affects the SRF pathway that promotes expression of abundant actin-myosin cytoskeletal components involved in the differentiation of cells (Swift and Discher, 2014). The SRF pathway is partially regulated by nuclear actin (Olson and Nordheim, 2010; Baarlink et al., 2013), which binds to lamin A (Simon et al., 2010) and other proteins associated with lamin A, such as emerin. This would suggest a functional role of lamin A in the indirect regulation of the differentiation of cells via an inhibitory effect on nuclear actin and myosins. Nuclear actin and myosin have been shown to work in concert to move regions of the genome around nuclei (Fedorova and Zink, 2008; Mehta et al., 2010; Bridger and Mehta, 2011; Kulashreshtha et al., 2016), but they are also involved in gene expression and processing. With the significant changes at the nuclear lamina between the pluripotent state and the somatic/lineage situation it seems unlikely that there are not changes with respect to LADs associating with the nuclear lamina, even though they have not been revealed. Indeed, LADs can also be internally located near A-type lamins (Briand et al., 2018) and so genome organization would be expected to change substantially after the A-type lamins arrive (Figure 3).

Promyelocytic Leukemia Bodies

There exists an emerging role for promyelocytic myeloid (PML) bodies in stem cell pluripotency and reprogramming, with their presence required to maintain pluripotency and reprogramming of cells to iPSCs (Hadjimichael et al., 2017). Some regard PML bodies in hESCs as comparable structures to those in somatic cells (Wiblin et al., 2005; Meshorer and Misteli, 2006), with their spherical unmistakable morphology. Alternatively, one study argues that PML bodies in stem cells and somatic cells are long linear structures or “rods and rosettes” in the embryonic stem cell nuclei. The study suggested that the unique PML bodies appear in the early stages of the cell life before any epigenetic imprinting may occur. Unlike in somatic cells, the PML bodies would often associate with the nuclear edge and appear less frequently, independent of different cell line, feeder/matrix, passaging method and the stage of cell-cycle (Butler et al., 2009). Additionally, the “rods and rosettes” were often found to appear near the edge of the undifferentiated ESC colonies. Additionally, Lawrence and colleagues (Butler et al., 2009) found that the composition of the PML bodies is different to that found in somatic cells. hESC PML bodies were found to not contain SUMO, SP100, or DAXX, which are usually present in those of somatic cells. These findings have been supported by Tokunaga et al. (2014), who have also found similar “rod” structures in their reprogrammed iPSCs. Additionally, it was suggested that

the round “rosettes” found in their reprogrammed cells that failed to produce successful iPSCs was a sign of a transitional stage from somatic cell to iPSC (Tokunaga et al., 2014). Salsman et al. revealed PML body loss upon differentiation of myoblasts and the relocation of DAXX protein (Salsman et al., 2017) (**Figure 3**).

The question concerning the differences in genome organization in ESCs and iPSCs is whether it is important to assess with respect to risk in a whole organism? It seems that genome organization is more dis-organized and plastic and possibly more random. But whether this is detrimental is debatable since there is evidence that once cells have initiated their lineage journey these aspects are corrected. However, there may be more genome instability evident and the consequences that follow such a situation i.e., chromosomal aberrations. This may be the downside of maintaining a plastic open genome and the question as to whether an adult, possibly of advanced age, has the same capacity to tolerate genomically compromised cells remains.

Epigenetic Modifications in Pluripotent Cells

How exactly specific chromatin conformation in ESC nuclei influences differentiation is unknown, however there has to be a certain openness of the chromatin (Meshorer and Misteli, 2006), with markers such as H3K4me3 (Harikumar and Meshorer, 2015). Presumably, this flexibility permits a normal global gene activity in the cells, whilst cells remain pluripotent and maintain their self-renewal capacity. This theory is supported by findings of an increased accumulation of heterochromatin upon differentiation (Francastel et al., 2000), implying that with a reduced need of certain genes in specific cell types, transcription can be silenced (Jiménez et al., 1992; Hu et al., 1997). The chromatin state of terminally differentiated cell types is more “rigid,” in comparison to cells with differentiation capability (Meshorer et al., 2006). This would be an efficient way to establish tissue-specific gene expression and has been found to be true for the differentiation of mammalian hemopoietic cells and in *Caenorhabditis elegans*; with more terminally differentiated cells having more heterochromatin accumulation (Reviewed in Francastel et al., 2000). Indeed, differentiation-dependent chromatin modifications are observed with an increase of silencing chromatin markers, such as H3K9me3 and global cytosine methylation (Lee et al., 2004; Meshorer et al., 2006), decreased active chromatin markers, such as H3K4me3 (Guenther et al., 2010) and increased H4 deacetylation in centromere heterochromatin as cells differentiate (O’Neill and Turner, 1995; Keohane et al., 1996). Interestingly, in hESCs many genes show both chromatin marks; for repression H3K27me3 and for expression H3K27ac and H3K4me3, indicating genes are poised ready for expression once differentiation is initiated (Harikumar and Meshorer, 2015; Theunissen and Jaenisch, 2017; Godini and Fallahi, 2018). More specifically in ESCs, genes have the active chromatin mark at their promoters and the repressive chromatin marks within the body of the gene, known as bivalency (Harikumar and Meshorer, 2015). These genes seem to fall into the category of genes that are required for future development of

the embryo and differentiation. This bivalency was revealed using chromatin immunoprecipitation ChIP (Bernstein et al., 2006).

Although, the epigenome of any cell can be altered by the cell itself and by various drugs applied through the medium, it remains that ATP-chromatin modeling, histone modification and DNA methylation are critical in tightly regulating the journey of a stem cell, whether it be embryonic, an induced pluripotent or otherwise. Interestingly, a stem cell may have a different epigenetic code to its parent cell, allowing them to be flexible in becoming which ever lineage they are signaled to become. In iPSCs reprogramming with the transcription factors (*OCT4*, *SOX2*, *KLF4*, and *c-MYC*) leads to the resetting of the epigenome (Papp and Plath, 2011), with DNA demethylation leading to the active transcription of pluripotency genes (He et al., 2017). There is concern and evidence that there is an epigenetic memory in iPSCs that could remain in the genomes (Papp and Plath, 2011; Godini and Fallahi, 2018), with the possibility that this leads to instability later in their differentiation journeys. Indeed, in low methylated regions this epigenetic memory lasts for many passages. Whereas, in hypomethylated and hypermethylated genomic memories are located at conserved sites for active gene expression (Luu et al., 2018). With respect to DNA cytosine methylation in preimplantation embryos, DNA is hypomethylated, allowing for a poised/active gene state, with a global remethylation commencing at implantation (Guo et al., 2014; Okae et al., 2014). Indeed, DNA methylation is critical in cell fate, being directly involved in gene expression in pluripotency (Singer et al., 2014).

Studies have been performed to compare the epigenetic landscape of iPSCs with ESCs, to determine their similarity. Indeed, there are a number of differences (Bilic and Belmonte, 2012). These differences may be due to variations within populations since when ESCs and iPSCs were derived from the same origin there were no differences (Mallon et al., 2014). Thus, it could be argued that to be of clinical use iPSCs should be screened for specific histone marks and DNA methylation status of a selected panel of genes prior to being used.

CURRENT METHODS FOR ANEUPLOIDY DETECTION

Preimplantation genetic screening is commonly performed on human IVF embryos for an increased likelihood of a healthy birth (Munné et al., 1995), as it has been estimated that over 70% of normally developing human preimplantation embryos have chromosomal abnormalities (van Echten-Arends et al., 2011; Mertzaniidou et al., 2013). As previously mentioned, the effects of low-level of aneuploidies in hPSCs are unknown and pose a serious threat to their therapeutic use because of their growth advantage in culture and tumorigenic potential, therefore is vital that they are well-characterized before use. For hPSCs to become a future treatment option for patients, especially for cell and gene therapies with a short shelf life, fast and robust methods for the sensitive detection of chromosomal abnormalities must be used. Currently, a number of different methods are available

TABLE 2 | Current methods used for aneuploidy detection and their individual sensitivities.

Method	Sensitivity of aneuploidy detection
qPCR	10% (D'Hulst et al., 2013)
G-Banding	5–10% (Baker et al., 2007)
FISH	1–5% (Downie et al., 1997; Baker et al., 2007)
CGH	10–25% (Lu et al., 2007; Xiang et al., 2008; Manning et al., 2010; Novik et al., 2014)
dPCR	≤5% (El Khattabi et al., 2016)
NGS	<1% (Sachdeva et al., 2017)

for such screening, each with their advantages and disadvantages with regards to sensitivity, resolution, turnover time, cost and staff requirement. Commonly used assays to detect chromosomal abnormalities are listed in **Table 2**.

The most common method utilized for aneuploidy detection is G-banding of metaphase chromosome spreads. The traditional technique that uses a dye to stain and observe specific banding patterns in condensed chromosomes is highly labor-intensive and requires trained cytogenetics for analysis. Typically, only 10–30 metaphase spreads are analyzed to assign a karyotype for the whole population, limiting the sensitivity of such a method. In addition, this creates difficulty in the detection of low-level mosaicism in culture; it has been estimated that only up to 5–10% mosaic aneuploidy detection is possible using G-banding (Baker et al., 2007) (**Table 1**), and can often fail to observe genomic imbalances less than 10 Mbs (Miller et al., 2010). In addition, G-banding results can be interpreted differently by different cytogeneticists resulting in inconsistent outcomes; it is also known for high turnaround times. Despite these drawbacks, G-banding results in a single-cell analysis, and examines every chromosome in each cell analyzed, unlike other cytogenetic-based methods, can detect balanced translocations and is relatively cost-efficient.

Another common cytogenetic method, fluorescence *in-situ* hybridization (FISH), employing a DNA specific probe in metaphase or interphase cells, also works at the single-cell level, with the option of multiplexing using different colored labels for each chromosome (mFISH), which can greatly aid the interpretation of complex translocations. FISH has been estimated to be approximately 1 to 5% sensitive for the detection of specific aneuploidies (Downie et al., 1997; Baker et al., 2007), can be carried out and analyzed within a few days and is once again, relatively cost-effective. However, the technique is still labor-intensive and requires the use of targeted probes for known abnormalities. FISH, could be successfully utilized as a sensitive screening method before the therapeutic use of hPSCs if designed for common aneuploid chromosomes.

The technique of multiplex ligation-dependent probe amplification (MLPA), originally designed to measure gene-copy number variations, can also be applied to detect aneuploidies of specific chromosomes. MLPA is designed to work by the detection of gene dosage abnormalities by utilizing up to 45 different DNA sequences. Rather than amplify the nucleic

acids in the sample, the technique amplifies the probes that are added to the sample; the amplification depends on the presence of specific sequences in the sample. The probe intensities are quantified and the whole experiment typically takes 2–3 days (Sellner and Taylor, 2004; Shaffer, 2007). While MLPA is high-throughput and cost-effective, its sensitivity for detection of mosaicism is unclear, but likely does not exceed 10% (van Veghel-Plandsoen et al., 2011; Yan et al., 2011) and cannot detect structural aberrations.

Quantitative PCR (QPCR) may also be employed for the measurement of gains and losses of specific sequences; multiple short tandem repeats of the common aneuploidies in live births, such as trisomy 13, 18, and 21 and the sex chromosome aneuploidies can be used to amplify the regions of interest. The method allows the multiplexing of different fluorescence intensities produced from the PCR, resulting in a fast method for chromosome copy number detection. Studies have demonstrated 99.2% accuracy for whole chromosome aneuploidy detection in prenatal diagnosis (Cirigliano et al., 2004; Ogilvie et al., 2005), however, a level of sensitivity of 10% has been claimed for aneuploidy detection in mESCs (D'Hulst et al., 2013). Furthermore, QPCR has been demonstrated to be able to detect the presence of 20–30% mosaicism (Donaghue et al., 2005) and has been reported to be a much cheaper and faster alternative to other assays and many laboratories have now replaced traditional FISH with QF-PCR (Shaffer, 2007). Unfortunately, the limitation of QF-PCR is the inability to detect balanced chromosomal translocations and the assay's sensitivity is limited by the measurement of cycle-threshold differences. Digital PCR (dPCR), a more novel and sensitive technique, employs the same chemistry and amplification process as QPCR, therefore its potential for detecting mosaicism and future potential is much greater (Uchiyama et al., 2016). In the massively parallel partitioning of single-target molecule PCR reactions, dPCR has much greater power than QPCR to detect subtle difference in copy-number. In an analysis of trisomy 21 DNA samples, mosaicism for chromosome 21 was detected as low as 5% sensitivity (El Khattabi et al., 2016). With a greater number of replicate samples and a battery of assays for each chromosome arm, it is feasible that dPCR could be capable of detecting genome-wide aneuploidy to a level close to 1%.

Alternatively, chromosomal microarray methods, such as array-based comparative genomic hybridization (aCGH), KaryoLite-Bac on Beads[®] (KaryoLite BoBs[®]) and single-nucleotide polymorphism techniques can also be used to detect aneuploidies. DNA microarrays use a panel of DNA sequences that compare the copy number of each area of interest to a control to then calculate the gene copy number of the sample (Shaffer, 2007). The advantage of using a DNA microarray is the ability to construct the target molecules, although most commonly the pre-designed, commercially-available microarray platforms for aCGH are used. The technique can detect DNA dosage imbalances, such as aneuploidies, deletions and duplications with a high resolution, dependent on the target of interest and is a much faster approach than methods, such as FISH, due to the high-throughput data produced. In addition, these techniques provide a significantly higher resolution of up

to 50–500 kbs (Coe et al., 2007; Askree et al., 2013; WiCell, 2017), but in contrast to FISH and G-banding, the detection sensitivity of mosaicism is only about 10–25% (Lu et al., 2007; Xiang et al., 2008; Manning et al., 2010; Novik et al., 2014) but has been reported to be capable of detecting aneuploidy mosaicism as low as 5% (Menten et al., 2006), although such high levels of sensitivity are uncommon.

The evolution of next-generation sequencing (NGS) based methodologies extends the possible breadth of data which may be collected on molecular-level changes including at the single cell level. Whole genome sequencing may allow capture of the entire DNA sequence, whilst whole exome sequencing may offer a more affordable approach; both are challenged by some sequence variables including mononucleotide repeats, translocations, inversions, and large copy number variations. Targeted-panels, particularly for cancer-associated variants (such as those routinely used in cancer diagnostics) may provide focused data on known-impact genomic changes and also enable, through a higher number of reads per base pair sequenced, the detection of sub-clonal mutations down to a level of ~10% of cells. In a study analysing cells from hundreds of pre-implantation embryos with whole genome NGS very high sensitivity and specificity for aneuploidy of all chromosomes was reached (Sachdev et al., 2017), which could be described as a detection sensitivity of < 1%. NGS is also useful to assess the genomic health of PSCs by being employed in RNA-seq and ChIP-seq (Kidder et al., 2011; Zhang et al., 2013). Interestingly, RNA-Seq of PSCs with additional chromosomes reveals that transcription is affected across the whole genome, even for chromosomes and genes that have a normal copy number (Zhang et al., 2013). This consequence of aneuploidy is potentially dramatic if these cells survive in a body.

Additionally, newer karyotyping methods have been developed to use the changes in global gene expression changes to monitor chromosomal aberrations (Mayshar et al., 2010; Weissbein et al., 2016). Such methods could be used in the future to determine the cell karyotype, however further work is required to detect the method's sensitivity in detecting chromosomal abnormalities. In addition, testing of different cell culture conditions would be required, as changes in gene expression would be detected with changes in the stem cell growth condition.

A challenge lies, even in the advent of highly sensitive aneuploidy-detection methods, in determining what confers an unacceptable level of genomic instability in hPSCs. Much data may be collected on genomic alterations in *in vitro* studies, but until there is a consensus on what safe limits may be, there is a risk of being overly cautious or hasty in realizing their therapeutic potential.

CONCLUSION

Chromosomal aneuploidies in hPSCs can impair differentiation potential (Zhang et al., 2016) and potentially lead to

tumorigenicity (Blum and Benvenisty, 2008; Ben-David and Benvenisty, 2011), which could limit their future therapeutic use. Studies on the genomic instability of hPSCs in culture are ongoing to optimize protocols for best practice. However, the ability of aneuploid cells to revert to diploid status over time in culture should not be overlooked, as observed with trisomy 18 hiPSCs (Li et al., 2017). Furthermore, some studies have demonstrated that an aberrant karyotype may not affect the quality of human preimplantation embryos (Mertzanidou et al., 2013), and indeed using mosaic embryos may still result in newborns with a normal karyotype (Greco et al., 2015). Although these studies are encouraging for the employment of embryos for preimplantation, their use must still be questionable, due to the possibility of future malignancy (Amariglio et al., 2009) and findings may not be transferable to using hPSCs in a similar state.

The high rate of aneuploidies observed in PSCs arises from a number of possible mechanisms and we have highlighted impaired mechanisms that affect mitotic segregation of chromosomes such as DNA damage, lamin B depletion, DNA damage repair, spindle assembly and checkpoint function. There are also important differences in the way the genome is organized and interacted with in interphase nuclei. The epigenome is also significantly different between PSCs and differentiated cells, seeming much more “malleable” prior to differentiation. The impact of aneuploidy on the epigenome is not clear and needs further exploration.

The prevalence of aneuploidies in PSCs in culture appears to be driven by the selection of genes which promote survival during periods of cell stress or offer a growth advantage. To move forward in the use of embryonic or induced pluripotent stem cells as therapeutics, methods that can easily be established in the clinic need should be considered for the high-throughput and sensitive detection of aneuploidies, such as population and single cell NGS, Hi-C, ChIP-seq, and RNA-Seq. However, much more research is required to determine any long-term detrimental effects using heterogenous stem cell cultures with respect to genomic content and behavior traits, nuclear architecture and content, and the epigenome. This will create the knowledge for the field to agree what constitutes a safe, acceptable limit of genomic instability in pluripotent cells.

AUTHOR CONTRIBUTIONS

JB and JMB are both corresponding authors, added to the review and oversaw the completion of the manuscript. MH has done most of the writing as primary author. JH wrote parts of the review and also was involved in the final versions of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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