Temporal Analysis of Genome Alterations Induced by Single-Cell Passaging in Human Embryonic Stem Cells

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Simplified culture conditions are essential for large-scale drug screening and medical applications of human pluripotent stem cells (hPSCs). However, hPSCs [ie, human embryonic stem cells (hESCs), and human induced pluripotent stem cells (iPSCs)] are prone to genomic instability, a phenomenon that is highly influenced by the culture conditions. Enzymatic dissociation, a cornerstone of large-scale hPSC culture systems, has been reported to be deleterious, but the extent and the timeline of the genomic alterations induced by this passaging technique are still unclear. We prospectively monitored three hESC lines that were initially derived and cultured on human feeders and passaged mechanically before switching to enzymatic single-cell passaging. We show that karyotype abnormalities and copy number variations are not restricted to long-term culture, but can occur very rapidly, within five passages after switching hESCs to enzymatic dissociation. Subchromosomal abnormalities preceded or accompanied karyotype abnormalities and were associated with increased occurrence of DNA double-strand breaks. Our results indicate that enzymatic single-cell passaging can be highly deleterious to the hPSC genome, even when used only for a limited period of time. Moreover, hPSC culture techniques should be reappraised by complementing the routine karyotype analysis with more sensitive techniques, such as microarrays, to detect subchromosomal abnormalities.

Introduction

HUMAN EMBRYONIC STEM CELLS (hESCs) and induced pluripotent stem cells (iPSCs) are pluripotent stem cells (PSCs) that can be indefinitely maintained in culture in adequate conditions. At any time point, PSCs can be induced to differentiate for research or clinical objectives. Moreover, they proliferate constantly in vitro, providing means for massive cell expansion, a unique feature for normal cells. However, the long-term genomic integrity of PSCs in culture is a major concern [1–4]. Indeed, hESCs generally display a normal karyotype at derivation; however, karyotypic abnormalities, particularly gain of chromosome 1, 12, 17, and 20, may arise after prolonged in vitro culture. Subchromosomal changes were also reported suggesting that PSC genomic instability may be underestimated [5]. These genomic abnormalities are a serious concern not only for PSC medical applications, but also for their use in biomedical research [1,6].

PSC genomic instability during in vitro culture has raised numerous concerns about the effects of culture conditions. Indeed, the currently used human PSC (hPSC) culture methods are based on various culture media, culture feeders/matrices, or passaging techniques that may differently affect genetic stability or impart different selective pressure on cells. However, the effect of these variables on the incidence of PSC genomic defects has never been systematically assessed. The question of the optimal passaging technique is representative of this issue. Several reports suggest that enzymatic passaging (EP) [3,5,7–9] or feeder-free culture conditions [5,10] might lead to more frequent cytogenetic aberrations, but the impact of culture conditions on the genetic integrity of hPSCs remains debated [11,12]. Conversely, other works show that mechanical passaging might favor the appearance of cytogenetic abnormalities, such as amplification of the 20q11.21 segment [4,13]. Indeed, mechanical passaging may lead to a selection bias based on the

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colony morphology and some recurrent aneuploidies could result in the accentuation of pluripotency-associated features, such as reduction of spontaneous differentiation and more robust colony expansion [14].

While the resolution of the genomic abnormality detection methods and the number of studied hPSC lines increase, several crucial questions remain still unanswered, such as the rate at which such abnormalities accumulate and the contribution of the different culture conditions. Therefore, we precisely monitored genomic abnormalities in hESC lines, cultured in parallel, using mechanical and single-cell EP techniques up to 45 passages. Our results demonstrate that the use of single-cell passaging can be deleterious to PSC genome integrity and that subchromosomal and karyotype abnormalities can be detected just after five passages.

Materials and Methods

Cell culture

The human HD129, HD291, and HS306 ESC lines were derived as previously reported [15,16]. Cells were maintained in standard KO culture medium: 80% KO-DMEM, 20% KOSR, 2 mM L-glutamine, 1% nonessential amino acids, 0.5 mM β -mercaptoethanol (all from Gibco Invitrogen), supplemented with 10 ng/mL bFGF (Abcys). Cells were grown in 35-mm dishes that were precoated with irradiated (40 Gy) human foreskin fibroblast feeders (40,000 cells/cm²) and were either enzymatically or mechanically passaged every week.

Mechanical passaging (MP) was carried out under an inverted microscope in a hood using scalpels [17,18]. Undifferentiated cell clumps were excised and plated on freshly prepared (as above) feeders.

For EP, cells were pretreated with the Rho-associated protein kinase (ROCK) inhibitor Y-27632 for 1 h and dissociated with TrypLE™ Select (Invitrogen) at 37°C for 10 min. The reaction was stopped by adding KO culture medium and single cells were released by manual pipetting. Cell suspensions were passed through a Ø40- μ m strainer to eliminate feeder cells and hESC clumps [19]. Cells were then counted and seeded on freshly prepared feeders. To prevent apoptosis, during the initial EP, cells were plated at medium to high density (15,000–30,000 cells/cm²) at the beginning of the adaptation, which was then progressively reduced. Cell concentration was progressively reduced to 3,000 cells/cm² whereas cells adapted to EP.

Experiments were initiated using HD291 cells at passage (P) 13, HD129 cells at P16, and HS306 cells at P25, respectively (called initial passage) and maintained independently using MP or EP. Cell cultures were regularly checked for mycoplasma contamination and remained negative throughout the experiments.

DNA extraction

MP hESC colonies were excised carefully using a scalpel and EP cells were dissociated into single cells as described above. Cells were then purified and enriched using magnetic Dynabeads® SSEA-4 (Invitrogen) according to the manufacturer's instructions. Purified cells were then centrifuged and pellets incubated in the lysis buffer of the QIAamp DNA Mini Kit (Qiagen). Genomic DNA was obtained fol-

lowing the manufacturer's instructions with the addition of an RNase A digestion step.

Flow cytometry analysis

Polychromatic flow cytometry was carried out following the procedures we previously described [15]. Briefly, 50,000 cells were incubated with FITC-conjugated mouse anti-human TRA-1-60 or PE-conjugated mouse anti-human SSEA3 antibody (BD Pharmingen) for 15 min and saturated in 2% bovine serum albumin/phosphate buffered saline (PBS). After a wash with PBS, acquisition was carried out with a CyAn™ ADP Analyzer (Beckman Coulter) and data were analyzed using the FlowJo software (Tree Star, Inc.).

Alkaline phosphatase staining

The Red Alkaline Phosphatase Substrate kit (Vector Laboratories) was used for alkaline phosphatase staining according to the manufacturer's instructions.

Evaluation of cell population expansion speed and modeling of the cumulative cell number of the different populations

For EP cells, the population expansion speed was evaluated by comparing the number of seeded cells and the number of cells at harvesting after EP. Cells were counted with a hematology cell counter, plotted on a logarithmic scale and normalized to the culture time to obtain the doubling speed (doubling per day).

For MP cells, the surface area of five random colonies was monitored with a CCD camera during the interpassage interval at the indicated passages and quantified with ImageJ. The surface change was plotted on a logarithmic scale and normalized to the culture time to obtain the doubling per day.

To model the cumulative cell number of nonmutant (NM) and mutant (M) EP populations, their cell population doubling rate per passage (D), noted as D_{NM} and D_M , respectively, were calculated. D_{NM} is the mean D of the first five EPs (the adaptation phase) (EP1 to EP5, starting from P13 for HD291 and from P25 for HS306 cells), whereas D_M is the mean D of five EPs during the plateau phase of growth (P13+EP30 to P13+EP34 for HD291 and P25+EP10 to P25+EP14 for HS306 cells). For each hESC clone, we computed the theoretical cell number N before the next passage using the formula $N_{NM}(P) = N_{NM}(P-1)D_{NM}$ and $N_M(P) = N_M(P-1)D_M$ from the starting population $N_{NM}(0)$ and $N_M(0)$. As EP was initiated in HD291 and HS306 cells with 300,000 and 150,000 cells, respectively, $N_{NM}(0) = 300,000$ for HD291 and 150,000 for HS306 cells. $N_M(0)$ was hypothesized to be 1.

Karyotyping

At the indicated passage, cells were dissociated with TryPLE Select (Life Technologies) and grown for 3 days to reach mid-exponential phase. Then, single cells were incubated with the 1/10,000 KaryoMAX® Colcemid™ (Life Technologies) for 90 min for metaphase arrest before hypotonic swelling with 0.075 M KCl solution at 37°C for 20 min and three successive fixations in ice-cold methanol/glacial acetic acid (3:1, vol/vol). Twenty microliters of nuclei suspension were dropped on glass slides, air dried at

18.4°C and 60% humidity, and rehydrated in water for 5 min before denaturation in EARLE orange or 10× EBSS at 87°C for 55 min. Slides were then rinsed in cold water and stained with 3% GIESSA for 3 min, rinsed five times, and air dried. Spectral microscopy and analysis were carried out using the Metafer Slide Scanning Platform (MetaSystem).

Microarray analysis

For the Affymetrix SNP array 6.0 analysis, genomic DNA handling and hybridization were performed as described previously [20]. Data were analyzed with Genotype Console (Affymetrix) and passed the quality controls (QC Call Rate >95 and MAPD <0.4). To detect copy number variations (CNVs), a filter of minimum 50 markers per segment with a minimum length of 100 kb was applied to obtain stringent results.

Affymetrix microarrays were processed at the Microarray Core Facility of the Institute of Research on Biotherapy, CHRU-INSERM-UM1 Montpellier (<http://irb.chu-montpellier.fr/>).

Quantification of DNA double-strand break foci

To quantify DNA double-strand break (DSB) foci, cells were incubated with the rabbit anti-53BP1 antibody and then with anti-rabbit FITC-coupled IgGs, as described above. The 53BP1 signal was captured in random culture fields with a LSM Duo confocal microscope (Carl Zeiss) every 1 µm, covering a Z-axis depth of 20–30 µm to include all cell nuclei (from highest to lowest: see Fig. 4C, cells with a red nucleus). All the 53BP1-positive DSB foci from different depths were stacked on the Z-axis to one image (Fig. 4C).

For both MP and EP cultures, 21 nuclei were selected randomly in each stacked image. In total, the number of DSB foci in 105 nuclei (from five stacked images) from two independent experiments was counted using the ImageJ software. The nonparametric *t*-test was used for statistical analysis.

Results

Temporal analysis of karyotypic rearrangements in enzymatically passaged hESCs

To study the nature and the chronology of the genomic changes associated with EP, we compared cells passaged by EP and cells maintained by MP. Indeed, MP is considered a cytogenetically safe technique [21] and was thus used in this study as a reference. We first studied the HD291 hESC line and then extended the analysis to two additional hESC lines (HS306 and HD129). All three hESC lines were used at early passage (initial passage: HD291 cells: P13; HS306 cells: P25; and HD129 cells: P16), were derived and maintained by MP until the start of the study and were karyotypically normal at the beginning of the experiment. See Material and Methods for a detailed description of the two passaging methods.

With both passaging methods, hESC retained their normal morphology (Fig. 1A) and the expression of surface pluripotency markers. Moreover, transcriptome analysis demonstrated the conservation of a typical pluripotency signature (not shown) in all subclones of the three hESC lines used for this study. It must be noted that among the monitored pluripotency membrane markers, expression of TRA1-60, which is one the most sensitive to differentiation [15,22], increased

steadily after repeated EPs, whereas it remained relatively stable after MP (Fig. 1B). Analysis of SSEA3, another commonly used marker, gave similar results (data not shown). During the first EPs, in agreement with other reports [19], hESCs showed a significant mortality rate that resulted in cell loss and low population doubling rate, which then rapidly increased.

We analyzed the three hESC lines by conventional R-band karyotyping. MP hESCs had normal karyotypes at all studied time points after the beginning of the experiment (Fig. 1C and Table 1), whereas EP cultures showed several karyotypic abnormalities. Specifically, we detected a trisomy 12 in all analyzed metaphases in HD291 cells after 30 EPs; a trisomy 20 mosaicism (8/56 metaphases) in HD129 cells after 10 EPs; and an isochromosome with deletion of the long arm of chromosome 7 i(7)(q10) in 18 out of 47 mitoses in HS306 cells after only five EPs (Table 1). Overall, these data indicate that in hESC cultures, major genomic alterations can be detected after only five EPs, strongly suggesting that EP can have very rapid deleterious and irreversible consequences on cultured PSCs.

Rapid occurrence of subchromosomal rearrangements in hESCs that are enzymatically passaged

We used the Affymetrix SNP 6.0 microarrays to assess the occurrence of genomic alterations over time at sub-chromosomal resolution. First, we analyzed the HD291 hESC cell line at the beginning of the experiment (at P13=P0) and after 15 and 30 passages, in both MP and EP conditions. In EP conditions, the number of segments for which CNVs were detected was already increased 3.07-fold after 15 passages. Conversely, it remained nearly stable in MP cells (Fig. 2A). This observation suggests that genomic alterations might rapidly occur in EP hESCs. Indeed, the analysis of the other two hESC lines at earlier time points (ie, P5 and P10 after the start of the experiment) showed that the number of segments with CNV was already increased after five EPs (Fig. 2A, B). Overall, in EP cultures, 2.38 ± 1.92 new CNVs/passage (mean $\pm$ SEM of the three hESC lines) were observed. Moreover, the increase of the number of segments with CNVs and their total length was significantly correlated with the passage number in EP ($P < 0.01$, $\rho = 0.823$, Spearman's correlation), but not in MP cultures (-0.48 ± 0.85 CNVs/passage, mean $\pm$ SEM, $P = 0.35$, $\rho = -0.357$, Spearman's correlation). As EP was associated with the use of Y27632, we checked that the CNV increase was not due to a direct effect of this ROCK inhibitor by examining the genomic integrity of MP hESCs grown with and without Y27632. We did not observe any change in the CNV number in the presence of Y27632, suggesting that CNV increase in EP cultures is not due to a direct effect of Y27632 (Fig. 2C). We also evaluated CNV occurrence after passaging method switching. The CNV number remained stable after moving from EP to MP, whereas it sharply increased after switching from MP to EP (Fig. 2D). These results support our findings that subchromosomal abnormalities accumulate very rapidly, specifically in EP cultures.

Overall, these karyotypic abnormalities were detected also by microarray analysis (Fig. 3), with the exception of the trisomy 20 mosaicism in HD129 cells, possibly due to the low microarray sensitivity for mosaic gain or loss of

FIG. 1. Phenotype and karyotype modifications associated with single-cell enzymatic passing (EP). (A) Colony morphology of the three hESC lines (initial passage, P—at the beginning of the study: HD291 cells: P13; HS306 cells: P25; and HD129 cells: P16) cultured with single-cell enzymatic (EP) or mechanical passing (MP). Colonies at the indicated passage number were stained in red with the Alkaline Phosphatase Substrate kit. Scale bar, 1 mm. (B) TRA1-60 expression variation in HD291 cells during culture with MP (in blue) or EP (in red). The feeder cell signal was eliminated using a CD44-negative gate. The percentage of TRA1-60-positive cells is shown at the indicated passage number. (C) Representative abnormal karyotypes in the three hESC lines after the indicated number of EPs. hESC, human embryonic stem cell; MP, mechanical passing. Color images available online at www.liebertpub.com/scd

DNA. Conversely, a group of CNV gains in chromosome 20 was observed by microarray in HS306 at EP10, but not at EP5, suggesting the emergence of a trisomy 20 in a subclone that kept a low prevalence in the culture.

Modeling the nonmutant and mutant cell fractions during hESC culture

One central question concerning the genetic abnormalities promoted by EP is whether they pre-exist and EP just favors their selection, or whether EP promotes their occurrence and their subsequent selection. We observed that the cell population doubling rate (D) of hESCs cultured in EP conditions reached a plateau after about 5 to 10 passages (Fig. 4A). We thus reasoned that to be selected during EP, a genetically abnormal clone (mutant, M) should have had a cell population doubling rate (D_M) higher than that of the wild-type population (nonmutant, D_{NM}) and comparable to the doubling rate observed at the plateau phase in EP conditions (when the abnormal clone was detected and took over the culture). By using a mathematical model, we found that a single mutant cell within a culture of normal

TABLE 1. KARYOTYPING RESULTS

Cell line	Passage	Karyotype [no. of metaphases]
HD129	P16+MP5	46, XY [13]
	P16+MP9	46, XY [14]
	P16+EP9	mos 47, XY, +20 [8]/46, XY, -13, +20 [1]/46, XY [47]
HD291	P13+MP15	46, XY [11]
	P13+MP30	46, XY [15]
	P13+MP44	46, XY [20]
	P13+EP34	47, XY, +12 [14]
	P13+EP30MP17	47, XY, +12 [15]
HS306	P25+MP10	46, XX [17]
	P25+EP5	mos 46, XX, i(7)(q10) [12]/47, XX, i(7)(q10), +3q, del(10q)? [1]/47, XX, dble i(7)(q10), +7 [3]/46, XX, i(7)(q10), del(9q) [2]/46, XX, i(7)(q10), del(9ter), del(10q) [2]/47, XX, i(7)(q10), +20 [1]
	P25+EP11	46, XX, i(7)(q10) [17]

FIG. 2. Rapid acquisition of genomic abnormalities in single-cell EP culture conditions. **(A)** Incidence of copy number variation (CNV) during hESC culture with EP or MP. Total number (*upper panels*) and length (*lower panels*) of segments with CNV in the three hESC lines cultured with EP (*brown triangles*) or MP (*blue squares*) at the indicated passage number. **(B)** Representative examples of CNV detection: a segment gain (indicated by the *blue* mark; the probe signal is highlighted in *red*) on chromosome 15 (*upper panels*), a copy gain in the short arm (in *blue*), and a segment loss on the long arm (in *red*) of chromosome 7 (*lower panels*) in HS306 cells (P25+EP10) (Ordinate axis: log2 ratio of the probe signal). **(C)** Number (*solid bold lines*) and total length (*hashed bold lines*) of segments with CNV in HD129 (*left panel*) and HD306 (*right panel*) cells cultured with MP in the presence of Y-27632. The *gray lines* show the data for MP and EP hESCs reported in **(A)**. **(D)** Number (*solid lines*) and total length (*hashed lines*) of segments with CNV in HD291 cells at passage 15 after switching from EP (at EP30) to MP, or from MP (at MP30) to EP (highlighted in *gray*). The EP30 and MP30 points are the same experimental points as in **(A)**. Color images available online at www.liebertpub.com/scd

HD291 cells would represent 50% of the culture (ie, well above the detection sensitivity of classical karyotyping) about four EPs after its appearance (Fig. 4B). Therefore, based on this model, the detection in HD291 cultures of an abnormal clone with trisomy 12 only after EP30, whereas it was absent at EP15, strongly suggests that this chromosomal abnormality

did not pre-exist and appeared during EP. For HS306 cells, the mathematical model predicted that a mutant clone should be detectable after five EP (Fig. 4B) and indeed, a mosaic karyotype showing an isochromosome with deletion of the long arm of chromosome 7 was observed at passage EP5 and EP9. Overall, the careful measure of the changes of cell

FIG. 3. Summary of CNV positions in all analyzed samples. Segments of copy gain are indicated with a *blue arrowhead* and segments of copy loss as *red arrowheads*. Continuous segments are highlighted in *blue* (for copy gain) or in *red* (for copy loss). Color images available online at www.liebertpub.com/scd

population doubling rates gives hints about the origin of the mutant clones during EP and for these two hESC lines, the abnormal clone might have originated after the start of single-cell passaging, either around the passage method switch or later during the cell culture. In the case of HD129 cells, the presence of only 20% of abnormal mitoses at EP9 precluded the computing of D_M .

Single-cell EP is associated with increased DNA DSB incidence

These observations suggest that the identified karyotypic abnormalities did not pre-exist, but were instead induced by the passaging method. Therefore, we decided to investigate the origin of the genomic abnormalities associated with EP. As one of the main causes of CNV and some other karyotypic abnormalities is imperfect DNA DSB repair, we investigated whether DNA DSB occurrence was different in the two culture conditions by quantifying 53BP1 foci (a DNA DSB marker) in EP and MP hESCs by confocal microscopy (Fig. 4C). The number of 53BP1 foci was significantly higher in EP (mean: 4.38 foci/nucleus) than in MP cultures (mean: 2.26 foci/nucleus) (Fig. 4D, E), whereas 53BP1-negative nuclei were more numerous in MP (45.4%) than in EP hESC cultures (21.4%). These results suggest that DNA DSB occurrence is higher in suboptimal passaging conditions and this

could, at least partly, explain the increased CNV and karyotype abnormalities incidence in EP hESC cultures.

Discussion

The occurrence of genomic abnormalities in hPSC lines is well recognized, but, surprisingly, very few studies have evaluated prospectively and quantitatively their extent and occurrence. So far, most efforts have focused on the description and classification of such genomic alterations. Several reports suggest that EP [3,5,7,9] might lead to more frequent cytogenetic aberrations. However, EP is a generic term encompassing very different passaging methods, such as large clump passaging using collagenase IV or dispase, or dissociated cell passaging using EDTA, Accutase, TryPLE, or trypsin at high or low (clonal) density. Such heterogeneity may explain the discrepancies between our study and previous reports that did not find any correlation between passage method and number of aberrations, even for samples collected from the same cell line [11]. In this study, we chose to use single-cell EP because the routine implementation of this method could facilitate large-scale drug screening and medical applications of hPSCs. The use of the three different hESC lines that were derived and expanded by MP in two independent laboratories and were then cultured by using mechanical or EP methods for this study,

FIG. 4. Modeling of the expansion of mutant populations and quantification of DNA double-strand breaks (DSBs) in HS306 cells in different culture conditions. **(A)** Expansion rate of the three hESC lines in MP and EP conditions. Population doubling was calculated depending on the passaging technique (colony surface or cell population doubling per day) and was normalized to the culture time. Trend lines were calculated from the values at the different time points (passage number). **(B)** Modeling of putative nonmutant and mutant populations in the EP culture condition. We hypothesized that the starting cell population contains two types of undifferentiated cells [nonmutant (N_M) and mutant (M)], each with a different cell population doubling rate per passage (D), noted as D_{NM} and D_M , respectively. D_{NM} is the mean D of the first five EPs (EP1 to EP5 for both HD291 and HS306 lines), whereas D_M is the mean D of five EPs during the plateau phase of growth (EP30 to EP34 for HD291 and EP10 to EP14 for HS306 cells). For each hESC clone, we computed the theoretical cell number before the next passage following the formula $N_{NM}(P) = N(P-1)D_{NM}$ and $N_M(P) = N(P-1)D_M$ from a starting population of $N_{NM} = 300,000$ for HD291 and 150,000 for HS306 cells. N_M was hypothesized to be 1 ($N_M = 1$). When the two lines intersect, the two cell populations are predicted to be equally present in the culture. **(C)** Illustration of the foci counting strategy (see Materials and Methods for details). **(D)** Stacked images showing DNA DSB foci visualized as 53BP1-positive signals by immunofluorescence in HS306 cells cultured with EP (left), or MP (middle), and with MP after treatment with 0.5 M Melfhalan for 3 days to induce DNA DSBs (right). Scale bar, 50 μ m. **(E)** Quantification of 53BP1-positive foci in HS306 cells cultured with MP (P30 + MP4, blue dots) or EP (P30 + EP4, red triangles). Each dot represents the number of 53BP1-positive foci per nucleus, and the lines, the mean value for each condition. The P value was calculated with the nonparametric t -test ($n = 105$ cells, from two independent experiments). Color images available online at www.liebertpub.com/scd

allowed us to demonstrate that hESC genomic integrity can be dramatically altered by just changing the passaging method. Particularly, both karyotypic and subchromosomal abnormalities appeared very early after switching from MP to EP. Since the identification of the culture conditions that allow hESC maintenance [23], various culture methods have been developed with the aim of sustaining hPSC expansion, while maintaining pluripotency. A further concern has been to find a passaging technique, such as EP, that might be amenable to large-scale PSC culture. As single-cell EP is a debated technique [7], the most popular passaging techniques are collagenase- or EDTA-based clump passaging methods. Nonetheless, EP with trypsin, TryPLE, or Accutase is used to expand cells in long-term culture or before

differentiation [24–33]. Our work demonstrates that enzymatic cell dissociation can have deleterious effects on the genome of hPSCs and also highlights that the rapidity of the genomic consequences of such culture conditions may have been underestimated before. As some authors did not detect chromosomal aberrations upon long-term culture with single-cell passaging [26,28–30,33,34], it is now important to determine which part of the culture methodologies accounts for the reported increase in genetic abnormalities. As in our study, only the passaging method was different between the MP and EP conditions, the medium composition and feeder cells can be ruled out as a cause of genetic instability. On the other hand, the action of TryPLE on cell surface proteins of hPSC may have deleterious consequences that ultimately

result in genetic abnormalities. Indeed, it has been suggested that EDTA-based passaging procedures, differently from EP, favor the survival of dissociated hPSC and allows long-term expansion, whereas preventing karyotype abnormalities [35]. Another important parameter could be the cellular concentration after EP (we used an average cell density during the first passages: $\sim 200,000$ cells/ 35 mm^3 well). Indeed, low cell concentrations favor 100% clonality and are associated with very high mortality. On the other hand, high cell concentrations ($> 1,000,000$ cells/ 35 mm^3 well) might reduce stress in dissociated hPSC by promoting cell-cell contacts [36] and may explain the absence of genetic abnormalities in some studies using enzymatically passaged hESC [34]. Finally, different states of pluripotency have been observed in mouse and hPSC. hPSC with epigenetic characteristics similar to those of mouse ESCs (ie, ground-state, naive PSCs) survive better after single-cell passage and are less prone to genetic alterations after EP [29,30,33]. Transcriptome analysis of the three hESC lines we used in this study (HD291, HD129, and HS306) after EP and after MP did not show any marked upregulation or downregulation (fold change < 0.5 and > 2) of genes associated with the naive pluripotency state, such as *KLF4*, *DPPA3*/*STELLA*, *TFCP2L1*, *KLF2*, or *TBX3* (data not shown), suggesting that the naive/primed state of pluripotency of MP and EP cells is not significantly different.

Our results also highlight that most DNA abnormalities are subchromosomal. Therefore, they could go undetected if culture testing is done only by karyotyping. Hence, culture conditions that are considered as safe based on the results of conventional karyotyping [35,37,38] should be carefully reappraised by using more sensitive techniques, such as microarrays, to detect subchromosomal abnormalities. It is worth noting that, as already reported by others, some CNV tend to disappear, in particular between the initial passage and the subsequent time points during MP (Fig. 2A) [11,20,39]. In our setting, one possible explanation of this phenomenon could be that the clonal population dynamics of hESC shortly after thawing and during medium/long-term MP culture are different and this might favor the counter-selection of specific CNV.

It has been suggested that the incidence of genomic abnormalities precedes the adaptation to culture because the genetically abnormal clone displays an increased survival after single-cell passaging resulting in its rapid selection by EP [40,41], a selection process that could be accelerated by a population size effect [42]. Indeed, it is well established that cell dissociation induces apoptosis in hPSCs through the Rho/ROCK/MLC2 signaling cascade [19,43,44]. Specific genetic abnormalities can improve hPSC survival, for instance by increasing the expression of the antiapoptotic BCL2L1/Bcl-XL protein. This suggests that a pre-existing clone with such an abnormality could overcome the culture in conditions, such as cell dissociation passaging, in which normal cells would not survive [45]. Indeed, selection does occur, and only such a mechanism is likely to explain how a single genetic abnormality can become predominant. Our data on HD291 cells show that this cell line is characterized by a biphasic increase of population doubling per day (Fig. 1C), suggesting that the second phase, observed after 30 passages, is caused by the trisomy 12 that was detected at EP30, but not at EP15. The systematic occurrence of genetic

abnormalities during EP raises the issue of the baseline mutation rate in hPSC in culture, which may be higher in single-cell passaged culture. Our finding that the incidence of DNA DSBs in hESC cultures increases early after switching from MP to EP is consistent with such hypothesis. However, the underlying molecular pathways are still speculative. The high rate of aneuploidy and CNVs suggests that defects in the spindle checkpoint, the centrosome copy number, or DNA replication may play a role.

Future work should aim at unravelling the molecular cascade that underlies PSC modifications induced by single-cell passaging. This knowledge will provide a framework to explore new passaging methods that are compatible with industrial scale culture of hPSCs without resulting in genetic instability. These findings are also important for the optimal generation of hiPSCs. Indeed, it is not possible to infer the contribution of a reprogramming technique to DNA damage if the contribution of the culture conditions is not known.

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Author Disclosure Statement

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