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—Original Article—

Higher Copy Number Variation and Diverse X Chromosome Inactivation in Parthenote-derived Human Embryonic Stem Cells

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Abstract. Parthenote-derived human embryonic stem cells (phESCs) have many advantages over conventionally derived human embryonic stem cells (hESCs), but a more thorough investigation of these cells is needed before they can be implemented in cell therapies. In this work, we used a Cytogenetics Whole-Genome Array to study the copy number variation (CNV) status in phESCs and hESCs. We also investigated X chromosome inactivation (XCI) and expression levels of marker genes in these cells. More CNVs were found in phESCs than in hESCs in the present study, and gene expression appeared to be associated with the gain or loss of CNVs. In addition, a variable XCI status and different expression pattern of paternally expressed imprinted gene were also found in phESCs. In conclusion, although phESCs had a similar pluripotent profile to conventionally derived hESCs, these cells differed in imprinted gene expression, XCI status and number of CNVs. Our work highlights the need for a deeper investigation to elucidate the genetic and epigenetic characteristics of these cells.

Key words: Copy number variation, Gene imprinting, Parthenote-derived human embryonic stem cells, X chromosome inactivation

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Histocompatible human embryonic stem cells (hESCs) have great potential for clinical applications because cell-based therapies have less of a risk of triggering an immune response. There are three main methods for generating genetically matched cells: somatic cell nuclear transfer (SCNT) [1], parthenogenesis [2, 3] and induced pluripotency [4]. To date, successful derivation of donor-matched hESCs using SCNT has not been achieved, and the use of human induced pluripotent stem cells is still questionable, given the unknown risks associated with epigenetic memory [5] and aberrant reprogramming of DNA methylation [6], which will influence the applications of those cells for disease treatment.

As a potential resource for autologous transplantation and regenerative medicine, parthenote-derived human embryonic stem cells (phESCs) have been successfully derived [2, 3, 7–9]. PhESCs can be generated by the activation of metaphase II oocytes [2, 3] or from haploid oocytes after routine *in vitro* fertilization [7]. Although biological characterizations of phESCs have been published [2, 3, 7–9], investigation of phESCs has been limited to an analysis of global pluripotency and expression of differentiation markers. The genetic and epigenetic behaviors, such as copy number variation (CNV), gene imprinting and X chromosome inactivation (XCI), of these cells require further study.

CNV, amplified or deleted regions of the chromosome that range in size from 1 kilobase (Kb) up to 1 megabase (Mb) or more [10], has been recognized as a major source of human genome variability. Recently, CNV analysis has been shown to be a powerful tool for studying genome variability in hESCs: 843 CNVs ranging in size from 50 kb to 3 Mb have been identified in 17 hESC lines maintained in different laboratories [11]. Gene expression increases and decreases have been shown to be associated with the gain or loss, respectively, of CNVs in hESCs [11], and specific recurrent CNVs are always associated with disease [12]. Although variation has been reported in chromosomal and epigenetic behaviors of phESCs [7, 9, 13], it is not yet clear whether duplication of the entire maternal genome in phESCs will causes less or more genetic instabilities, such as CNV, than occur in normal hESCs and whether specific CNVs containing functional genes can influence the cell's gene expression profile and epigenetic status.

Epigenetic stability in phESCs is crucial to ensure the function and safety of these cells in regenerative medicine. XCI and gene imprinting are principal examples of epigenetic regulation. To date, research on XCI in phESCs has been very limited, and whether phESCs have diverse XCI similar to that in hESCs [14, 15] is fully unknown. Meanwhile, investigation of gene imprinting is also critical to ensure the safe use of phESCs in regenerative medicine because inappropriate expression of imprinted genes can cause several human pathologies and cancer [16, 17]. Therefore, in this study, we investigated and compared the copy number status, gene expression and XCI stability in two well-established phESC lines (FY-phES-018 and *ch*HES-32) [7, 9] as well as two female conventionally derived hESCs (FY-hES-7 and FY-hES-8) [18].

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Materials and Methods

Undifferentiated cell culture and spontaneous differentiation

This research was approved by the Ethics Committee of Guangzhou Medical Uniwersity. In this study, one phESC line (FY-phES-018)

and two hESC lines (FY-hES-7 and FY-hES-8) were derived and propagated in our laboratory [9, 18], and *ch*HES-32, another phESC line, was provided by the China National Engineering and Research Center of Human Stem Cell [7]. Methods for culturing, differentiating into embryoid bodies (EBs) and characterizing hESCs and phESCs were performed as previously described [7, 9, 18].

Extraction of total RNA and genomic DNA

Undifferentiated cells at different passages and differentiated EB cells at day 7 were collected for total RNA and genomic DNA isolation. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and DNaseI(Qiagen) digestion was performed to eliminate DNA from the RNA sample. DNase-treated RNA (0.5 μg) was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Isolation and purification of genomic DNA was performed with a Qiagen DNeasy Tissue Kit, according to the manufacturer's instructions (Qiagen).

Human embryonic stem cell PCR array

To analyze the differences in gene expression between phESCs and hESCs, FY-phES-018, *ch*HES-32 and FY-hES-7 cells at an undifferentiated passage (passage 38) were derived. For quantitative real-time PCR, the Human Embryonic Stem Cell RT^2 Profiler™ PCR Array (PAHS-081, SABiosciences, Frederick, MD, USA) was performed to assay marker genes for pluripotency according to the manufacturer's recommendations (Suppl e-Table 1: available at www.jstage.jst.go.jp/browse/jrd). Amplification was carried out with an ABI PRISM 7900 system (Applied Biosystems, Foster City, CA, USA). Data were collected and analyzed with the online SABiosciences PCR array data analysis web portal (http:// pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). GAPDH, B2M, RPL13A, HPRT1 and ACTB were used as housekeeping genes (HKG). Threshold cycle numbers (Ct) of the target genes were determined and standardized to the average HKG value (ΔCt = average of target genes' Ct – average of HKGs' Ct). Then, the fold change was calculated for each gene in the compared groups using the 2-ΔΔCt method.

Epigenetic status of phESCs

To assess the epigenetic status of phESCs, we analyzed the imprinted gene expression, DNA methylation and XCI status of FY-phES-018 and *ch*HES-32 at the undifferentiated stage and EB cells at day 7. For imprinting, insulin-like growth factor 2 (*IGF2*), small nuclear ribonucleoprotein polypeptide N (*SNRPN*) and *H19* genes were investigated by quantitative real-time PCR (SYBR® Premix Ex Taq™ kit, Takara, Otsu, Shiga, Japan). Each gene was analyzed in triplicate using GAPDH as the internal control. For XCI analysis, quantitative real-time PCR of *XIST* expression and DNA methylation of *XIST* and *HUMARA* genes were performed according to previously reported methods [19].

Copy number variation analysis

Genomic DNA of undifferentiated cells at passage 38 of FY-phES-018 (45, XO) and *ch*HES-32 (46, XX) were collected to elucidate the copy number status of the cells. FY-hES-7 and FY-hES-8, two karyotype-normal (46, XX) female cell lines, were used for comparison. As we previously demonstrated the X chromosome variation in FY-phES-018 [9], we analyzed the copy number status of this cell line at passage 38, 50 and 70 to confirm our previous finding that this cell line really has processing clone selection. For CNV analysis, a Cytogenetics Whole-Genome 2.7 M Array containing 2.7 million probes for CNV and 400,000 probes for SNPs was used according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Briefly, 100 ng of gDNA for each sample was processed in 5 steps: the first step was whole genome amplification, followed by purification and quantification, fragmentation and labeling, hybridization and then finally washing, staining and scanning. The data were analyzed using the Affymetrix Chromosome Analysis Suite (ChAS, Affymetrix). A marker count of 50 and size of 50 kb were set up as the segment filter for CNV analysis.

Data quality control and validation by TaqMan quantitative PCR

To confirm whether the data were credible in this study, we used the ChAS software to check the data quality of all samples. Because the phESCs have a duplicated genome, loss of heterozygosis (LOH) is a sensitive indicator to validate the results. For CNV validation, TaqMan Gene Copy Number Assay was used in this study (See Suppl e-Materials and Methods: available at ww.jstage.jst.go.jp/browse/jrd).

Results

Characterization of phESCs

We found that the undifferentiated phESCs are similar to those conventionally derived hESCs. For example, phESCs strongly express tumor-rejection antigen (TRA)-1-60, TRA-1-81 and stage-specific embryonic antigen (SSEA)-4, and they negatively regulate the expression of SSEA-1 (Suppl e-Fig. 1A: available at ww.jstage.jst.go.jp/browse/jrd). Classical markers for pluripotency and self-renewal, including *POU5F1, SOX2, NANOG, THY-1, FGF4, TDGF1* and *REX-1*, were all found to be expressed in FY-phES-018 and *ch*HES32 (Suppl e-Fig. 1B). The differentiation markers for endoderm (*AFP*), ectoderm (*NEUROD1*), and mesoderm (*HBZ*) were also expressed in those cells (Suppl e-Fig. 1C).

Gene expression profiles of phESCs and hESCs

The human embryonic stem cell PCR array showed no differences between *ch*HES32 and FY-hES-7 in the expression of the marker genes for pluripotency and self-renewal (Fig. 1A). Although the expression of some genes was found to be different among FY-phES-018, *ch*HES32 and FY-hES-7, for example, *LEFTY1, LEFTY2, LIFR, IL6ST* and *FOXD3,* which were downregulated in FY-phES-018 by more than 10-fold relative to FY-hES-7 (Fig. 1B) and *ch*HES32 (Fig. 1C), most of the studied markers were found to have similar expression patterns among the three cell lines.

Differences in imprinted gene expression between phESCs and hESCs

In FY-hES-7, all three imprinted genes, *H19, SNRPN* and *IGF2*, were expressed at both undifferentiated and differentiated stages. Compared with the expression of the maternally expressed gene *H19*, FY-phES-018 and *ch*HES-32 have more than two-fold differences compared with FY-hES-7 at both undifferentiated and differentiated

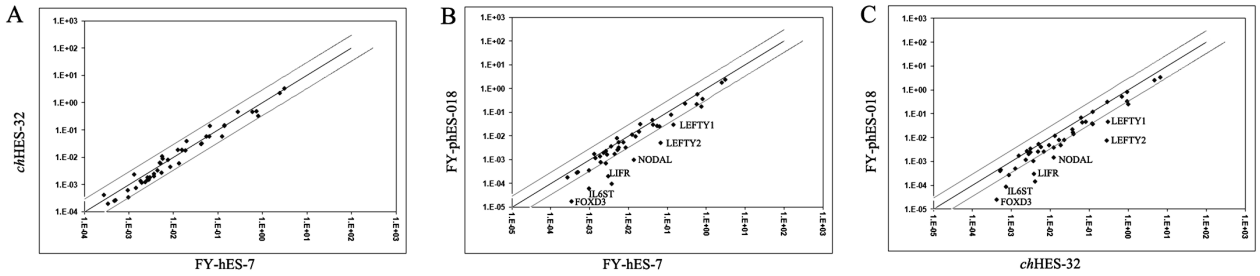

Fig. 1. Pluripotency marker gene expression in phESCs and hESCs. Markers for pluripotency and self-renewal were detected by PCR arrays, and no differences were found between *ch*HES32 and FY-hES-7 (A). Most of the studied markers had similar expression patterns among FY-phES-018, FY-hES-7 and *ch*HES32, although some genes were found to be downregulated in FY-phES-018; for example, *LEFTY2, LIFR, IL6ST* and *FOXD3* had more than 10- fold lower levels in FY-phES-018 than in FY-hES-7 and *ch*HES32 (B, C). Up- and downregulation were considered to be a > 2-fold change or a < 0.5-fold change, respectively.

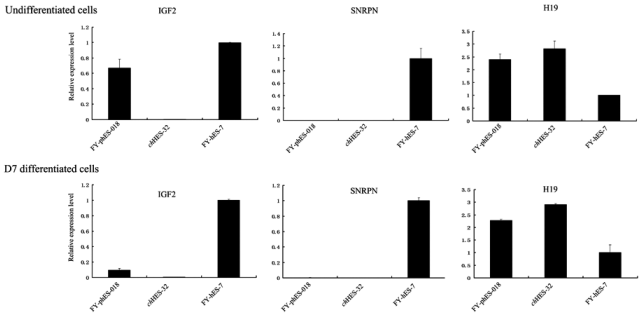

Fig. 2. Imprinted gene expression in phESCs and hESCs. The expression level for imprinted gene in FY-hES-7 was used as a reference (expression level set to 1.0) for the analysis of relative gene expression. Relative gene expression levels were normalized to *GAPDH*. Two-fold changes were observed in the maternally expressed gene *H19,* and no expression of the paternally expressed gene *SNRPN* was observed in FY-phES-018 and *ch*HES-32, suggesting that the normal imprinting status of these two genes is maintained in phESCs. Expression of the paternally expressed gene *IGF2* demonstrated a different and aberrant imprinting between the phESC lines.

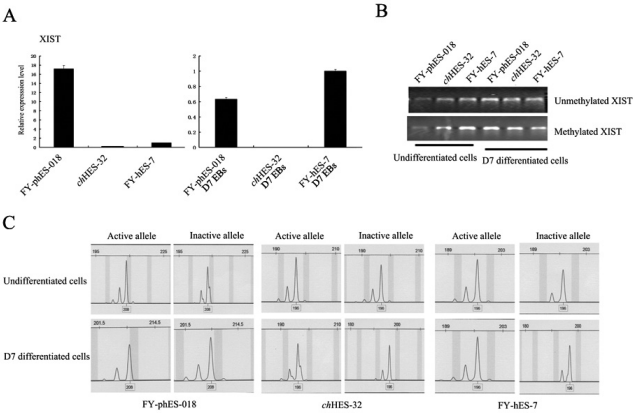

Fig. 3. XCI status in phESCs. FY-phES-018 and *ch*HES32 have different *XIST* expression patterns. The expression levels are detectable in both undifferentiated and differentiated FY-phES-018, whereas it is very weak in the undifferentiated *ch*HES32 cells and absolutely undetectable in the differentiated *ch*HES32 cells (A). The similar *XIST* methylation patterns (B) and *HUMARA* methylation patterns (C) in all 3 undifferentiated and differentiated cells demonstrated that similar XCI procession was initiated in these cells.

stages. The paternally expressed gene *SNRPN* was undetectable at both stages in FY-phES-018 and *ch*HES-32, whereas *IGF2* (another paternally expressed gene) showed different levels of expression in the two phESC lines. In FY-phES-018, the expression of *IGF2* was detectable at both undifferentiated and differentiated stages, whereas it was kept silent in *ch*HES-32 at undifferentiated stages and was expressed only weakly after 7 days of differentiation (Fig. 2).

X chromosome inactivation in phESCs and hESCs

Compared with the relative amount of *XIST* expressed in FY-hES-7, FY-phES-018 had a significantly upregulated *XIST* level at undifferentiated stages, whereas its expression in *ch*HES-32 was very weak (Fig. 3A). After 7 days of differentiation, *XIST* RNA levels in FY-phES-018 and FY-hES-7 were found to be similar but were fully undetectable in *ch*HES-32 (Fig. 3A).

To further elucidate whether XCI was initiated in *ch*HES-32, we examined the methylation status of the *XIST* and *HUMARA* genes. At undifferentiated and differentiated stages, both unmethylated and methylated *XIST* and *HUMARA* were detected in *ch*HES-32, and this was similar to the situation in FY-phES-018 and FY-hES-7 (Fig. 3B, C). These results demonstrated that although *XIST* expression varies across the three cell lines, XCI was initiated similarly in these cells.

CNV status in phESCs and hESCs

The CNV status was found to be different between phESCs and hESCs. In FY-phES-018, a total of 2941 CNVs, with a range of sizes all larger than 50 kb, were identified, and 326 CNVs of the same size scale were identified in *ch*HES-32. The numbers of CNVs that were identified in karyotype-normal FY-hES-7 and FY-hES-8 were 132 and 60, respectively, which were significantly fewer than those found in the phESCs (Fig. 4). The loss ratio of CNVs was higher than the gain ratio of CNVs in phESCs, whereas more gain than loss of CNVs was found in FY-hES-7 and FY-hES-8. The distribution

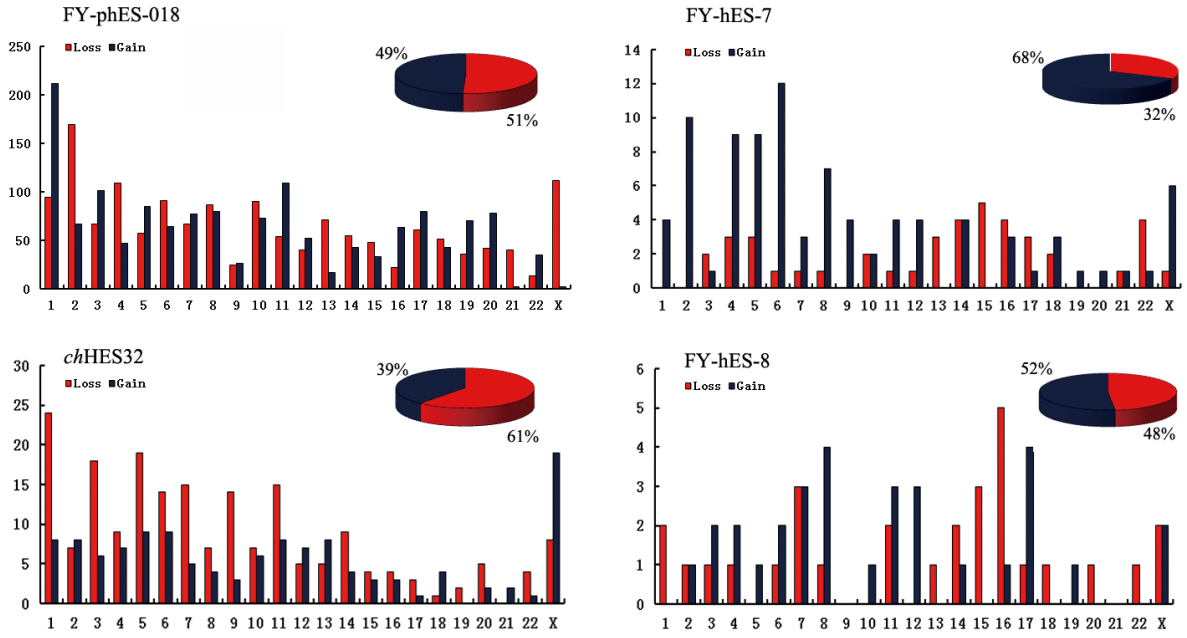

Fig. 4. CNVs status in phESCs and hESCs. The numbers of total CNVs in FY-phES-018 and *ch*HES32 are higher than those in FY-hES-7 and FY-hES-8, and the ratios of gain or loss of CNVs differ between phESCs and hESCs. CNVs were identified with a range of sizes larger than 50 kb. Differences in the chromosomal distributions of CNVs were most prominent in chromosomes 1 and X. The x-axis corresponds to the name of each chromosome, and the y-axis corresponds to the number of CNVs in each cell type.

of CNVs was also found to be prone to be located in chromosomes 1 and X (Fig. 4).

When 200 kb was used as the size scale to analyze the CNVs, the numbers of CNVs in FY-phES-018, *ch*HES-32, FY-hES-7 and FY-hES-8 were significantly reduced to 170, 27, 2 and 3, respectively, indicating that most of the CNVs in the phESCs and hESCs were smaller than 200 kb, and the number of CNVs that were more than 200 kb in the phESCs was still higher than in the hESCs (Suppl e-Fig. 2A: available at ww.jstage.jst.go.jp/browse/jrd).

Three different passages of FY-phES-018 cells were used to analyze whether prolonged culture would influence the stability of CNVs in these cells. At passage 38, the number of CNVs in FY-phES-018 was 170 with the size scale 200 kb, and the loss of CNVs distributed in the whole X chromosome clearly showed that these cells contained only one copy of the X chromosome (red arrow, Suppl e-Fig. 2B). At passages 50 and 70, the numbers of CNVs in FY-phES-018 were 34 and 23, significantly fewer than those at passage 38; furthermore, two copies of the X chromosome were present, demonstrating that the X chromosome was recovered after long-term culture and clone selection and that clones with fewer CNVs seem to be selected in

Fig. 5. Genes affected by loss or gain of CNVs. Changes in gene expression were found to be associated with a loss or gain of CNVs containing those genes. In FY-phES-018, the downregulated pluripotency marker gene, *LIFR* was associated with the 121 kb loss of CNV (A). The gain of *IGF2* in FY-phES-018 suggested that genomic changes may affect imprinted gene expression (B). The red bar with a triangle indicates the loss of CNVs, and the blue bar with a triangle indicates the gain of CNVs. The pink line with vertical markers indicated the gene distribution position.

this processing (Suppl e-Fig. 2B).

Effect of CNVs on gene expression

To investigate whether gene expression was affected by CNVs, we used the ChAS software to find genes contained in CNVs. We found the gain or loss of CNVs to be associated with certain changes in gene expression. For example, *LIFR*, a marker for pluripotency and self-renewal, was affected by a 121 kb loss of CNV in FY-phES-018, and its expression level was decreased by 14-fold relative to that in FY-hES-7, which had no loss of CNVs for this gene (Fig. 5A). Similar results were obtained for *IL6ST*, *LEFTY1* and *LEFTY2*: the decreased expression pattern was associated with 70 kb and 147 kb losses, respectively, of CNVs containing those genes (Suppl e-Fig. 3A, B: available at ww.jstage.jst.go.jp/browse/jrd). Interestingly, the change in CNVs was also associated with the aberrant epigenetic status. The abnormal expression of *IGF2* gene in FY-phES-018 was found to be accompanied by an 80 kb gain of CNVs, which contained the *IGF2* gene (Fig. 5B).

We also investigated the copy number status of mitotic spindle checkpoint genes and DNA mismatch repair system genes in phESCs to find the possible reason why FY-phES-018 cells have more CNVs than other cells. In the present data, we found an 87 kb loss of *MAD1L1,* 73 kb loss of *MSH2* and 43 kb gain of *MLH1* in FY-phES-018, respectively (Suppl e-Fig. 3C–E), and the expressions of these genes were found to be up- or downregulated compared with those in copy number normal FY-hES-7 cells (data not shown).

Data affirmation and CNV validation

The ChAS software showed that all cells were of sufficient quality. The ratios of LOH in phESCs and hESCs were 95% and 11%, respectively (Suppl e-Fig. 4A: available at ww.jstage.jst.go.jp/browse/jrd), suggesting that the data were firmly in accord with the origin of the phESCs. For CNV validation, three copies of *IGF2* (gain) in FY-phES-018 and two copies of *IGF2* (normal) in *ch*HES32 were validated by TaqMan Gene Copy Number Assay (Suppl e-Fig. 4B). The results demonstrated that the data derived from the whole genome array were credible.

Discussion

PhESCs are not involved in the destruction of viable embryos, do not use transgenes for genetic transformation, and most importantly are more histocompatible than conventionally derived hESCs. Thus, they hold tremendous promise for autologous transplantation and regenerative medicine. Although different activation techniques can generate differences in heterozygous or homozygous HLA typing cells, all of these cells have proliferation and multi-differentiation properties similar to those displayed by hESCs [2, 3, 7–9]. As they have a special genetic and epigenetic background, however, many questions about the biology of these cells remain. For example, more genetic instability and abnormally imprinted gene expression have been found in mouse and human parthenote-derived embryonic stem cells [2, 13, 20]. Therefore, a deeper investigation into the cellular properties and genetic and epigenetic variations of phESCs is required.

Gene expression profiles of various pluripotent cells have revealed similarities and differences [21–24]. Marker genes for undifferentiated hESCs, such as *NANOG* and *POU5F1*, are known to be closely associated with the pluripotent state [25, 26]. In the present study, marker genes for pluripotency and self-renewal were examined using a real-time PCR array, and no differences between undifferentiated samples of *ch*HES32 and FY-hES-7 were found. Although some of the pluripotency markers were found to be downregulated in FY-phES-018, the expression of all of these genes was detectable. The data in this study are in agreement with previous reports [7–9, 22, 27], confirming that pluripotent marker expression patterns in phESCs and hESCs are similar.

Epigenetic stability in hESCs is crucial to ensuring the function and safety of these cells in regenerative medicine. The most important epigenetic mechanisms are gene imprinting, DNA methylation and XCI. In hESCs, the available data on the pattern of XCI is diverse [14, 15, 19, 28]. It is unknown whether phESCs have a similar diverse XCI pattern to hESCs. We found previously that the XCI in FY-phES-018 is unstable [9], but there is still very little information available on the pattern of XCI in phESCs. With two copies of the same maternal X chromosome and loss of an XCI marker in *ch*HES32 cells, the data presented here showed that XCI was initiated in the undifferentiated phESCs cells, without taking into account the expression pattern of XCI markers. For the first time, we demonstrated that the XCI pattern in phESCs can also be categorized into three classes that are similar to those reported in hESCs [14, 15, 28]. Class I involves no inactive X in early passages of FY-phES-018 cells [9]. Class II includes the display of an inactive X and *XIST* expression in the undifferentiated latter passages of FY-phES-018. Class III in *ch*HES32 is the cells that exhibit XCI but lack *XIST* expression. Our data suggest that environmental influences and epigenetic regulation *in vitro*, and not the genetic background of the cells themselves, underlie the diversity in the XCI pattern in hESCs and phESCs [29].

Although imprinted genes are only a very small part of the entire genome, they play important roles in fetal growth and development [30]. The deletion of imprinted genes will cause developmental abnormalities, e.g., Prader-Willi syndrome [17, 31], and aberrant expression of imprinted genes has also been associated with cancer [16]. In phESCs, the entire genetic material is duplicated from the maternal genome; therefore, the paternally expressed imprinted genes should theoretically remain silenced. It is still unknown whether the inactivation of these genes negatively affects development in phESCs and their derivatives. Li *et al.* found that the paternally expressed imprinted gene *Igf2* is aberrantly expressed in mouse parthenote-derived stem cells; interestingly, this gene was also found to be inactivated in parthenogenetic embryonic fetuses and parthenote-derived stem cells [32], and artificial reduction of the maternally expressed *igf2r* gene reverses the growth inhibition of parthenote-derived cells [33, 34], suggesting that imprinted genes are critical for fetal development. In the present study, expression of *IGF2* was found in FY-phES-018 and in differentiated *ch*HES32 [7], and aberrant imprinted gene expression in FY-phES-018 cells has been reported previously by our group [9]. It is unknown why imprinted genes are aberrantly expressed in phESCs. One possible explanation is that long-term culture *in vitro* alters the epigenetic state of phESCs. In our previous report, we did not find *IGF2* to be expressed in early passages of FY-phES-018 cells, but after long-term culture, expression of this gene was detectable and maintained in

differentiated cells. It also seems possible that the copy number variation of imprinted genes may affect their expression pattern (see below).

The genomic instability of hESCs, e.g., the gain of chromosomes 12, 17 and X, suggests that hESCs would undergo adaptive changes during prolonged passage *in vitro* [35, 36]. With the limited resolution of 3 Mb to 20 Mb, the genomic aberrations that are less than 3 Mb are undetectable by the G-band method. The results presented here indicate that numerous small genomic aberrations are contained in the karyotype-normal FY-hES-7, FY-hES-8 and *ch*HES32 lines as well as in the karyotype-abnormal FY-phES-018 cells, suggesting that CNVs really provide a sensitive measure of genomic stability in hESCs and phESCs [11, 37].

One particularly interesting finding of this study is that the number of CNVs in phESCs is higher than in hESCs. Why phESCs have so many more subchromosomal genetic changes than hESCs is unknown. In humans, CNVs have been shown to have a meiotic origin [38] and can occur during somatic development [39]. The DNA mismatch repair system, which is a part of the mitotic spindle assembly checkpoint, was shown to have an important contribution to the genomic instability of hESCs [40]. In phESCs, it has been shown that there is an aberrant centriole distribution and altered expression levels of mitotic spindle checkpoint transcripts [41]. In our study, we found the copy number abnormality in some critical mitotic spindle checkpoint and DNA mismatch repair system genes in FY-phES-018. These differences may influence the function of the abnormal DNA repair system and could be one possible reason explaining why more CNVs exist in phESCs than in hESCs.

In recent studies, researchers have demonstrated that CNVs can affect the cellular phenotype by altering coding and regulatory sequences or by amplifying or deleting gene copies [11]. Many of the genes in the duplicated region on chromosome 12 were found to be more highly expressed in human pluripotent cells [37]. A significant increase or decrease in gene expression associated with the gain or loss of CNVs was also found [11], suggesting that duplication or deletion of CNVs does result in increased or decreased gene expression and that those regions contributing to the influence of CNVs can be either at the site of duplication/deletion or at distant sites [37]. In this study, we found the pluripotency markers that are decreased in FY-phES-018 can be associated with the loss of CNVs. In addition, we also found that a gain of CNV containing the *IGF2* gene is possibly associated with the abnormal expression of this gene, suggesting that the CNVs in phESCs can not only affect gene expression but also have the potential to influence imprinting.

In conclusion, the data presented here highlighted the diverse XCI status, aberrant imprinted gene expression, and most importantly, more unstable and considerably higher number of CNVs in phESCs, which demonstrate that more work is needed to characterize the genetics and epigenetics of these cells.

Acknowledgments

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