An Atypical Human Induced Pluripotent Stem Cell Line With a Complex, Stable, and Balanced Genomic Rearrangement Including a Large De Novo 1q Uniparental Disomy

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Key Words. Induced pluripotent stem cells • Genomic integrity • Uniparental disomy • Abnormal karyotype • Teratoma formation • Directed differentiation

ABSTRACT

Human induced pluripotent stem cells (hiPSCs) hold great promise for cell therapy through their use as vital tools for regenerative and personalized medicine. However, the genomic integrity of hiPSCs still raises some concern and is one of the barriers limiting their use in clinical applications. Numerous articles have reported the occurrence of aneuploidies, copy number variations, or single point mutations in hiPSCs, and nonintegrative reprogramming strategies have been developed to minimize the impact of the reprogramming process on the hiPSC genome. Here, we report the characterization of an hiPSC line generated by daily transfections of modified messenger RNAs, displaying several genomic abnormalities. Karyotype analysis showed a complex genomic rearrangement, which remained stable during long-term culture. Fluorescent in situ hybridization analyses were performed on the hiPSC line showing that this karyotype is balanced. Interestingly, single-nucleotide polymorphism analysis revealed the presence of a large 1q region of uniparental disomy (UPD), demonstrating for the first time that UPD can occur in a noncompensatory context during nonintegrative reprogramming of normal fibroblasts.

INTRODUCTION

Aneuploidies, copy number variations (CNVs) [1, 2], and point mutations [3] have been reported as frequent genomic aberrations of human induced pluripotent stem cells (hiPSCs), including those generated using nonintegrative reprogramming strategies. Some of these mutations are pre-existing in the somatic cell population [4, 5], and others are acquired during the reprogramming process or during early passages [1, 6]. Apart from one article demonstrating that the level of genomic instability is positively correlated with hiPSC tumorigenic potential [7], the link between genomic integrity and tumorigenesis is poorly studied. Here we report the characterization of a modified messenger RNA (mmRNA)-derived hiPSC line exhibiting a complex but stable and balanced chromosomal rearrangement and a large de novo region of uniparental disomy on chromosome 1q. Interestingly, the genomic rearrangements do not impact the iPSC line characteristics in terms of stemness-marker expression and of in vitro spontaneous differentiation potential, but it impairs the induced pluripotent stem cell (iPSC) line ability to generate teratomas in vivo.

MATERIAL AND METHODS

hiPSC Generation and Characterization

The generation, characterization, and culture conditions of the mmRNA-derived hiPSC lines have been fully described [8]. Briefly, human foreskin fibroblasts (ATCC, CRL 2097) were transfected daily with a homemade mmRNA cocktail encoding OCT4, SOX2, KLF4, c-MYC, and LIN28 (stoichiometry 3:1:1:1:1) following a protocol adapted from Warren et al. [9].

Differentiation Potential

The protocols for embryoid bodies and teratoma formation, as well as directed differentiation into specialized cell types, are fully described in the supplemental online data.

Genomic Analyses

Karyotype and fluorescent in situ hybridization (FISH) were performed on the A1 iPSCs cultured on mouse embryonic fibroblast feeder cells (GlobalStem). The A1 iPSCs were cultured on Gel-trex for three passages before genomic DNA extraction for single-nucleotide polymorphism
RESULTS AND DISCUSSION

hiPSC Generation and Characterization

The clone A1 was picked, expanded, and characterized together with several other hiPSC clones. The morphology of hiPSC line A1 in culture was typical of pluripotent stem cells; small cells with high nucleocytoplasmic ratio (supplemental online Fig. 1A). The expression of the stemness was shown by reverse transcription (RT)-polymerase chain reaction (PCR) (supplemental online Fig. 1C), and the hiPSC line was positive for alkaline phosphatase activity (supplemental online Fig. 1D). Additionally, immunostaining confirmed the presence of the proteins OCT4, NANOG, SSEA4, and TRA-1-60 (supplemental online Fig. 1B), supporting the results obtained on a transcriptional level. Finally, flow cytometry analysis revealed that 90% and 98% of the cells expressed TRA-1-81 and TRA-1-60 (supplemental online Fig. 1D). Additionally, immunostaining confirmed the presence of the proteins OCT4, NANOG, SSEA4, and TRA-1-60 (supplemental online Fig. 1B), supporting the results obtained on a transcriptional level. Therefore, hiPSC line A1 was indistinguishable from the other hiPSC lines in terms of morphology, stemness phenotyping, and growth rate.

Differentiation Potential

The spontaneous differentiation capacity of the hiPSC line was validated in vitro by generating embryoid bodies (Fig. 1A). RT-PCR confirmed the presence of derivative cells of all three germ layers in these embryoid bodies (Fig. 1B). Subsequently, the pluripotency was analyzed in vivo through generating teratomas after the intramuscularly injection of the cells into immunodeficient mice (Fig. 1C). Surprisingly, in the first experiment, we observed that injected A1 hiPSCs failed to generate teratomas, whereas all the seven control iPSC lines formed teratomas composed of derivatives of the three germ layers (data not shown). Because A1 iPSCs did not generate teratomas in vivo, we proceeded to confirm the results obtained from embryoid body test by further in vitro directed differentiation of the cells into specialized cells from the three germ layers. As endoderm-derived cells, we differentiated A1 iPSCs into hepatoblasts expressing AFP (alpha fetoprotein gene) and HNF3b (hepatocyte nuclear factor 3b), two hepatic progenitor specific proteins (Fig. 2A). To investigate the ectoderm differentiation potential, we generated retinal pigmented epithelium (RPE) displaying the typical morphology of RPE cells (flaveolate cells) expressing the specific combination of MITF (microphthalmia-associated transcription factor) and ZO-1 (tight junction’s protein) proteins (Fig. 2B). Finally, we differentiated the A1 hiPSCs into mesenchymal cells expressing α-smooth muscle actin, Vimentin, and Calponin (Fig. 2C; supplemental online Fig. 2). These cells were CD105- , CD73-, and CD90-positive and CD31- and CD34-negative, as shown by flow cytometry (supplemental online Fig. 2). Altogether, these experiments confirmed that the hiPSC line A1 is pluripotent in vitro. Regarding in vivo differentiation potential, the absence of teratoma formation in our experiments could be explained by a partially reprogrammed state; however, we do not support this hypothesis because the iPSC line maintained the pluripotent stem cell characteristics and the differentiation capacity in vitro. Moreover, altering the conditions of cell injections may have resulted in the generation of teratomas, such as the addition of adjuvants or using more cells. However, these experiments were not performed for ethical reasons. Therefore, we cannot fully confirm that the iPSC line A1 is unable to form teratomas but rather that its capacity is impaired.

Complex, Balanced, and Stable Chromosomal Rearrangement

In order to assess the chromosomal stability of the hiPSC line, karyotypic analysis was carried out. In contrast to the other hiPSC lines generated in parallel displaying the parental normal karyotype, we found that the A1 hiPSCs exhibited a de novo complex and rare chromosomal rearrangement. The karyotype showed a chromosome 1 with an abnormal short arm, der(1); a derivative

| Cell line | Passage | # mice | # teratoma |
|-----------|---------|--------|------------|
| A1 (hiPSC) | P20 | 1 | 0 |
| H9 (hiESC) | P73 | 1 | 1 |
| A13 (iPSC) | P19 | 1 | 1 |
| P2 (hiPSC) | P16 | 1 | 1 |

| Exp 1 | A1 (hiPSC) | P33 | 2 | 0 |
| Exp 2 | A1 (hiPSC) | P33 | 2 | 0 |
| Exp 3 | A1 (hiPSC) | P19 | 2 | 0 |
| Exp 4 | A1 (hiPSC) | P21 | 1 | 0 |

Summary

| Cell line | Passage | # mice | # teratoma |
|-----------|---------|--------|------------|
| A1 (hiPSC) | 0/6 | 11/11 |

Control cell lines
Abnormal Karyotype and Uniparental Disomy in hiPSCs

The resulting karyotype formula was as follows: 46,XY, der(1), der(8), der(21), rec(11), +rec(11); ish.der(1)(CEH108/7+, D1Z5+, wcp1, D1Z5+, t(1qT610+), der(8)t [8;21;22](q11.1;11.1; q11.1)[D8S504+, wcp8+; D8Z2+, wcp22+; D8S670+, der(21)t [8;21; 22](q11.1p11.1, q11.1)p13.12; D21Z1+; wcp21+; VyuRM2029+], rec(11)[D11Z1+, wcp11+, D11S1037+], + rec (11)[D11S2071+, wcp11+, D11Z1+].

Interestingly, the karyotype of this cell line at early (passage 16), intermediate (passage 30), and late (passage 54) passages was found to be identical with no occurrence of additional chromosomal events. This showed that the rearrangement acquired during reprogramming or early passages, although complex, was stable throughout long-term propagation. Moreover, we previously demonstrated that A1 iPSCs were able to efficiently differentiate into specialized cells from all three germ layers, confirming that this large genomic rearrangement is not a barrier to differentiation in vitro. In the case of hepatocyte differentiation, this finding has also been shown recently with aneuploid human iPSCs [10]. Our data not only corroborate this finding but also broaden this result for three completely different cell types, using an hiPSC line with a particularly complex karyotype.

hiPSC Line A1 Displayed a Large Region of 1q Uniparental Disomy

Affymetrix SNP 6.0 microarray analysis was performed on both the parental fibroblasts and the hiPSC line A1. Interestingly, compared with the parental fibroblasts and using our criteria (≥100 kb, ≥20 markers), no additional CNVs were detected in the hiPSC line A1, indicating that this complex chromosomal rearrangement was balanced around the breakpoints underlined by the karyotype analysis. However, the hiPSC line A1 presented a region with 584 consecutive SNP loci showing a loss of heterozygosity on chromosome 1q when compared with parental fibroblasts, without a loss of genomic material as determined by CNV analysis (Fig. 4). These data indicate the presence of a large de novo uniparental disomy (UPD) of the long arm of chromosome 1 from position 144,988,936 to position 249,143,646 (i.e., 104 Mb including 1,087 genes) (OMIM database). Importantly, no UPD was detected in the seven other iPSC lines derived from the same fibroblasts that were analyzed in parallel (five mRNA-derived and two retroviral-derived hiPSC lines) [8]. In order to assess the presence of the UPD in the initial population of fibroblasts, three detection attempts were performed in two different samples of the fibroblasts that were analyzed in parallel (five mmRNA-derived and two retroviral-derived hiPSC lines) [8]. In order to assess the presence of the UPD in the initial population of fibroblasts, three detection attempts were performed in two different samples of the fibroblasts that were used for reprogramming (at passage 6). No UPD was detected based on our SNP genotyping platform, suggesting that these abnormalities may have been generated during the reprogramming process itself or at early passing stages of the cells. However, if UPD was present in a small percentage of the fibroblasts, it would go undetected because of the limited sensitivity of this technique. To accurately address this question, more robust techniques such as droplet digital PCR or deep sequencing would be necessary.

Interestingly, UPD has never been reported in hiPSCs until recently [11]. The authors used fibroblasts from patients affected with Miller-Dieker syndrome, with the presence of a ring chromosome 17, and reprogrammed them into hiPSCs using episomal vectors. They showed that multiple hiPSC lines generated do not have the ring chromosome, suggesting the participation of a compensatory UPD mechanism. Our data confirm that UPD can occur, even in a noncompensatory context, during
nonintegrative reprogramming of normal fibroblasts. The functional consequences of UPD are significant; UPD may lead to an imbalance of paternal versus maternal genetic information (which may trigger dysfunction in the case of the presence of imprinted genes in the implicated genome region) or may result in the acquisition of homozygosity for a recessive allele involved in a genetic disorder [12]. Therefore, the presence of UPD should be regarded as an important genomic defect. Because we have shown that UPD can occur during the reprogramming process or early iPSC colony expansion, further studies are required to determine its frequency and the subsequent functional impact on the cells, independent of any karyotype abnormality. These findings also underline the added value of SNP microarray analysis as being the only available approach enabling an accurate detection of regions with consecutive loss of heterozygosity. Regarding the impaired capacity of this iPSC line to form teratoma in vivo (while conserving a large differentiation potential in vitro), we support the hypothesis that there is a direct relationship between this phenotype in vivo and the genotype alterations we describe (especially the UPD because the karyotype

Figure 3. Cytogenetic analyses of the hiPSC line A1: karyotype and fluorescence in situ hybridization (FISH) analyses. (A): Conventional cytogenetic methods: representative standard karyotype (G-bands by trypsin using Giemsa) of A1 cell line at passage 30 showing a male abnormal karyotype with several structural abnormalities (arrows): a derivative chromosome 1 (der{1}), a derivative chromosome 8 (der{8}), two derivative chromosomes 11 (der{11}), a derivative chromosome 21 (der{21}), and the absence of one chromosome 22. (B–S): FISH analysis for derivatives of chromosomes 1 (B–E), 11 (short arm derivative [F–H]; long arm derivative [I–K]), 8/21 [L–O], and 8/22 [P–S] in A1 cell line at passages 19 and 30. (B): Whole painting probes (wpc) 1 (spectrum green). (C): Chromosome enumeration probe (CEP) 1 α-sat (spectrum red). (D): CEP1 satIII (spectrum red). (E): Subtelomere 1pter (spectrum green) and subtelomere 1qter (spectrum red). (F, I): wcp11 (spectrum red). (G): Subtelomere 11pter (spectrum green). (H, J): CEP11 (spectrum green). (K): Subtelomere 11qter (spectrum red). (L): wcp8 (spectrum red)/wcp21 (spectrum green). (M): CEP21 (spectrum red). (N): Subtelomere 8qter (spectrum red). (O): Subtelomere 21qter (spectrum red). (P): wcp8 (spectrum green)/wcp22 (spectrum red). (Q): CEP8 (spectrum green) R: Subtelomere 8pter (spectrum green). (S): Subtelomere 22qter (spectrum red).
is balanced), which may be due to a defect in cell migration or implantation pathways. However, exploring this relationship was beyond the scope of this work.

CONCLUSION

This work demonstrates that an hiPSC line, generated with a non-integrative reprogramming strategy, may harbor an abnormal, complex, and stable karyotype, which in turn does not impact the pluripotent stem cell in vitro characteristics and differentiation potential. The teratoma formation capacity is impaired in this cell line, but further investigations are required to establish the relationship between this feature and its genetic characteristics. This study also demonstrates that UPD can occur during the reprogramming process or at early passages, and further studies looking for UPDs are required to determine the frequency of occurrence in iPSCs with normal karyotypes and the subsequent functional importance. UPD detection could only be performed through SNP analysis. This highlights the importance of combining karyotyping with complementary genomic analyses and further enforcing the use of SNP analysis as a routine screening method for quality control of hiPSC lines suitable for clinical applications.

ACKNOWLEDGMENTS

We thank Angélique Terray and Olivier Goureau for their helpful advice on RPE differentiation, Martin Gaillard for his help in animal experiments, and Denis Clay for his expertise in flow cytometry. We are grateful to Catherine Guettier and Deborah Burks for interpretation of teratoma pictures. Anne Weber is warmly acknowledged for constant support, helpful discussions, and advice. C.S. and N.D. were supported by fellowships from Association Française contre les Myopathies (AFM) and Région Ile de France (DIM Stem Pôle), respectively. E.L. was supported by Grant 2011-Rare-006 “HEMO-iPS.” This work was supported by Agence Nationale pour la Recherche (ANR)-2010-RFCS-004 “Liv-iPS” and European Union Seventh Framework Program (FP7)-HEALTH.2011.1.4- “InnovaLiv.”

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.
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