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High-Resolution Analysis of the Subtelomeric Regions of Human Embryonic Stem Cells

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ABSTRACT

The use of human embryonic stem cells (hESCs) in most applications is dependent on their undifferentiated proliferation in vitro. Recent studies have illustrated the possibility that chromosomal changes may occur in hESCs during in vitro propagation of these cells. However, no studies so far have screened for chromosomal abnormalities in hESCs using high-resolution techniques that can detect alterations on a few base-pair levels. We have used the recently developed multiplex ligation-dependent probe amplification procedure to analyze the possible occurrence of deletions or duplications in the subtelomeric regions of hESCs in early and late passages. In this study we show that no subtelomeric anomalies were detected in any of the nine hESC lines investigated, supporting the conclusion that hESCs, under appropriate conditions, maintain genomic stability during in vitro propagation. STEM CELLS *2005;23:483–488*

INTRODUCTION

Human embryonic stem cells (hESCs) can be isolated from the inner cell mass of blastocysts and represent a population of pluripotent stem cells [1]. These cells have the potential to develop into cell types representing the three embryonic germ layers in vitro and in vivo, and they are capable of unlimited, undifferentiated proliferation in vitro. Based on these fundamental properties, hESCs hold great promise to prove useful for future cell replacement therapies, but they are also likely to revolutionize the drug discovery process and provide novel possibilities for in vitro toxicology [2, 3].

Several independent investigators have successfully established hESC lines using a variety of experimental protocols [1, 4–7]. In most studies, the hESC lines seemed to maintain their pluripotency and normal karyotypes during long-term culture in vitro. However, a recent study indicated that hESCs may be subject to genomic distortions [8]. In support of this observation, an isodicentric X chromosome was detected in another hESC line after long-term culture in vitro [9]. Furthermore, a moderate frequency of aneuploidy was observed in hESC lines subjected to long-term feeder-free culture, although none of these changes resulted in increased proliferation rate for the hESC, and no significant difference in aneuploidy frequency was observed between early- and late-passage cultures [10]. Contrary to these reports, a separate study performed using six other hESC lines demonstrated that these cells were indeed chromosomally stable during extended in vitro culture [11]. Based on the limited data available and the conflicting reports in the literature, it is at this

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point impossible to assess how common chromosomal alterations in hESCs are. Additional studies are needed to resolve this critical issue and also, more important, to establish the biological significance of any detected alteration.

The telomeric regions of the chromosomes consist of TG-rich repeats $(TTAGGG)_n$, and adjacent to these are complex families of repetitive chromosome-specific DNA sequences. Next to these, along the chromosome, are very gene rich areas with high transcriptional and recombinational activities [12]. The homology between subtelomeric regions of nonhomologous chromosomes may cause crosstalk between these regions during meiosis, which leads to subtelomeric rearrangements. Abnormalities in subtelomeric sequences in patients cause mental retardation and malformations [13], indicating that these aberrations have direct effects on the developmental potential of the affected cells. It is not yet understood whether deletions of all chromosome ends are associated with specific phenotypes besides mental retardation, but some end deletions cause recognizable syndromes [14].

In this study we used multiplex ligation-dependent probe amplification (MLPA) analysis to investigate the possible occurrence of subtelomeric deletions or duplications in eight different hESC lines and in one clonally derived hESC line. The recently developed MLPA method is based on the polymerase chain reaction (PCR) amplification of probe pairs that have been hybridized to genomic DNA and subsequently ligated. Presently 41 telomere regions can be screened in just one multiplex reaction [15]. The hESCs were analyzed in early and late passages and after freeze/thaw cycles to determine if in vitro manipulation of hESCs could result in subtelomeric deletions. We did not detect any deletions in any of the cell lines analyzed in this study. Taken together, these data support the conclusion that hESCs can be cultured in vitro for extended time periods while retaining genomic stability, which is a prerequisite for many downstream applications.

Materials and Methods

Cell Culture and DNA Extraction

The hESC lines used in this study (SA001, SA002, AS034, AS034.1 [clonally derived from AS034], AS038, SA121, SA181, SA240, and SA461) were established as described [7] using supernumerary human embryos created for the purpose of assisted reproduction and donated after informed consent from the patients and approval of the local ethics committees at Göteborg University and Uppsala University. The cell lines were established and maintained on mitotically inactivated mouse embryonic fibroblast (MEF) feeder layers using VitroHES medium (Vitrolife AB, Göteborg, Sweden), and low-resolution cytogenetic analysis of the hESCs was performed as described [7]. Established hESC lines were passaged every 4–5 days to plates with fresh medium and MEF by mechanical dissociation using a Stem Cell Tool (Swemed Lab International AB, Billdal, Sweden) without using any enzymatic or chemical methods of cell dissociation. For MLPA analysis, undifferentiated hESCs were harvested and frozen at –80°C. The DNA was prepared by adding 20 µl 2% fetal calf serum followed by extraction using the Puregene DNA isolation kit (Gentra Systems, Minneapolis).

Multiplex Ligation-Dependent Probe Amplification

MLPA was performed using the SALSA MLPA telomere P019/P020 kit and the SALSA MLPA telomere P036 kit (MRC-Holland Amsterdam, The Netherlands). Genomic DNA, 200–300 ng, was diluted in 5 µl Tris-EDTA buffer, and the MLPA analysis was performed according to the manufacturer's instructions with minor modifications. The hybridization time was 17–18 hours, and the number of PCR cycles was 36. The samples were analyzed using a capillary electrophoresis system (ABI 3100 Genetic Analyzer, Applied Biosystems), and Genescan software (Applied Biosystems) was used to quantify the peaks generated. Specific peaks corresponding to the different telomeric regions were identified according to their migration relative to a size standard and subsequently exported to a Microsoft Excel spreadsheet. The normalized peak area for each fragment for the hESC DNA samples was then compared with the normalized peak areas for control samples to give a dosage quotient for each amplicon. The MLPA analyses were performed in a laboratory independent from the laboratories where the hESCs were cultured and prepared. The analysts had no prior knowledge about the specific characteristics of any of the individual hESC lines included in this study.

Fluorescent In Situ Hybridization

Fluorescent in situ hybridization (FISH) analysis using 6p/6q subtelomere probes (Abbot/Vysis, Inc., Downers Grove, IL) was performed on chromosomes prepared from undifferentiated hESCs according to the manufacturer's instructions, and signals were visualized using an image analyzing system from MetaSystems GmbH, Altlussheim, Germany. Metaphase spreads were prepared from undifferentiated hESC cultures using standard cytogenetic techniques.

Results

All of the cell lines included in this study were established and cultured on MEF feeder layers using VitroHES medium, and the cells were dissociated and passaged by mechanical cutting every 4–5 days. The hESC lines have been extensively characterized, and they display the typical markers and properties of undifferentiated hESCs (Cellartis, unpublished results) [7] and have been subjected to freeze/thaw cycles. Importantly, data from karyotyping and FISH analyses of hESC lines SA002, AS034, AS034.1, AS038, SA121, and SA181 were recently published and demonstrated that the cell lines present with normal karyotypes, except for SA002, which display trisomy 13 [7]. The remaining

three cell lines included in this study (SA001, SA240, and SA461) have also been analyzed using the same techniques; the cells display normal stable karyotypes, and no large-scale genomic aberrations have been detected (Fig. 1, Table 1).

For the purpose of this study, undifferentiated hESCs were harvested in early and late passages, as indicated in Table 2, and analyzed using MLPA. With two exceptions (see below), the MLPA telomere P019/P020 analyses did not detect any subtelomeric abnormalities in the hESCs. Each MLPA P019/P020 analysis was performed in duplicate. In some cases (Table 2), these analyses were also confirmed using a more recently developed complementary MLPA telomere P036 assay. Except for one probe, all sequences detected by probes in the P019/P020 probe mixes are different from the probes in the P036 mix. The significant number of individual hESC lines analyzed and the long-term in vitro culture of several of these lines support the conclusion that undifferentiated hESCs can be propagated extensively while maintaining genomic stability.

As indicated above, one hESC line included in this study, SA002, displays trisomy 13 [7]. The MLPA analyses of this hESC line were performed using cells in passage 14 and 144. Without prior knowledge about the trisomy, the analyst detected this chromosome abnormality in hESC line SA002. Figure 2 illustrates the different peak patterns obtained from a normal XY hESC line (SA001, passage 49) (panel A) and from the trisomic XX hESC line SA002 (passage 144) (panel B) using the MLPA P020 probe combination that covers chromosomes 12-22 and Y. The peak area of each amplification product reflects the relative copy number of that target sequence, making the identification of deleted or duplicated sequences possible.

In another hESC line, SA121 (passages 73 and 117), an apparent 6p-deletion, was detected by MLPA analysis using the P019/P020 probe sets (Table 2, Fig. 3B). However, in subsequent analyses, this observed deletion could not be confirmed by FISH analysis using metaphase spreads from the same cell line (passage 81) using 6ptel and 6qtel probes (Fig. 4) nor by the MLPA telomere P036 assay using SA121 cells in passage 117 (Table 2). Taken together, these results indicate that the apparent 6p dele-

Table 1. Summary of the results from karyotyping and fluorescent in situ hybridization (FISH) analysis of human embryonic stem cell lines SA001, SA240, and SA461

Cell line	Karyotype (passage)	FISH (passage)
SA001	46, XY (p26, p27, p50, p52)	Diploid, XY (p32, p51)
SA240	46, XY (p9)	Diploid, XY (p4)
SA461	46, XY (p13, p24)	Diploid, XY (p12, p14)

Cells were cultured and processed as indicated in Materials and Methods. FISH analysis was performed using probes for chromosomes 13, 18, 21, X, and Y.

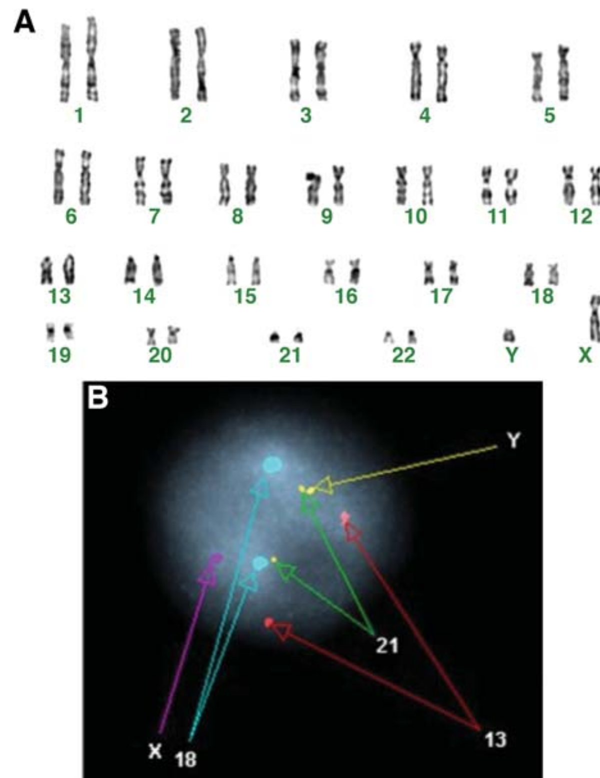

Figure 1. Karyotype of human embryonic stem cell (hESC) line SA001. **(A):** Representative normal karyotype from hESC line SA001 (passage 26). **(B):** Signals obtained from fluorescent in situ hybridization analysis of hESC line SA001 (passage 32) using probes for chromosomes 13, 18, 21, X, and Y.

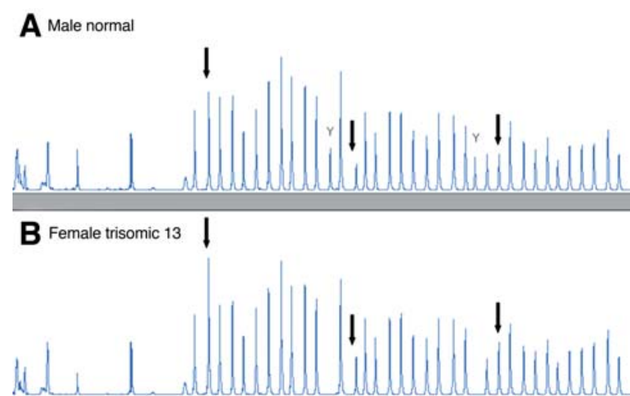

Figure 2. Representative peak patterns obtained from the multiplex ligation-dependent probe amplification telomere P020 analyses of undifferentiated human embryonic stem cells (hESCs). The P020 mix contains probes for chromosomes 12-22 and Y. **(A):** Normal XY hESC line (SA001, passage 49) with two specific Y peaks. **(B):** Trisomic XX hESC line SA002 (passage 144) in which all three chromosome 13–specific peaks show an approximate 1.5-fold increase and the Y specific peaks are absent. The arrows indicate the chromosome 13–specific peaks.

tion in SA121 most likely represents a polymorphism at the binding site of the probe, a finding also reported by others (personal communication, Dr. J.P. Schouten, MRC-Holland).

Discussion

Many hESC-based applications, including their potential future use in replacement therapies, depend on the possibility to culture and propagate the cells with sustained normal karyotypes. Although not experimentally proven yet, abnormalities in sensitive areas of the genome (e.g., the subtelomeric regions) are likely to cause significant functional and biological effects and render the hESC useless for certain applications. The results from the present study, in which several different hESC lines were evaluated after extensive in vitro manipulation, demonstrate that the cells retain genomic stability, at least under the culture conditions used here. This conclusion is in line with a previous report in which the investigators used manually passaged hESCs cultured on MEF and traditional low-resolution cytogenetic analysis [11]. The use of different culture conditions (e.g., feeder-free conditions) and different procedures for cell dissociation (e.g., enzymatic or chemical methods) has been attributed to potentially cause chromosomal instability in hESCs [11]. However, no experimental support has been presented for these statements at this point. It is clear that additional studies are needed to resolve these critical issues.

In this study we used the recently developed high-resolution MLPA technique to screen for the presence of one, two, or three subtelomeric regions [16]. Importantly, any abnormalities detected by this method should be verified by using another independent analysis, because a single base pair change at the binding site of the MLPA probes might inhibit the ligation reaction and subsequently indicate a false-positive result. In one hESC line (SA121), we obtained a false-positive result with the 6pter probe from the P019/P020 probe mix (Table 2, Fig. 3B). An independent FISH analysis (Fig. 4) and another MLPA analysis using a different probe set (P036) (Table 2) demonstrated that the 6p subtelomeric region was indeed unmodified in hESC line SA121. Furthermore, a previously known trisomy 13 in hESC line SA002 [7] was also verified using MLPA analysis (Table 2, Fig. 2B). These results showed that the reproducibility of relative signals obtained from the MLPA analysis enabled the detection of a single extra copy of a probe target sequence per diploid genome.

In previous studies, MLPA has been demonstrated to be suitable for the detection of submicroscopic subtelomeric abnormalities in patients with mental retardation [16]. The symptoms of subtelomeric defects in humans are often associated with growth and developmental defects such as congenital anomalies, prenatal and postnatal growth retardation, and dysmorphic features [14]. Studies have shown a higher rate of subtelomere deletions in children with more severe mental retardation compared with children with milder mental retardation [13], suggesting that there exists some type of dose-response relationship for these defects.

Table 2. Summary of the results from the multiplex ligation-dependent probe amplification (MLPA) telomere P019/P020 analyses of undifferentiated human embryonic stem cells (hESCs)

Cell line	Passage	Result P019/P020	Notes
SA001	26	No deletions	
SA001	49	No deletions	
SA002	14	Trisomy 13	
SA002	144	Trisomy 13	
AS034	11	No deletions	
AS034.1[a]	74 + 86[b]	No deletions	Confirmed by MLPA telomere P036 analysis
AS038	114	No deletions	Confirmed by MLPA telomere P036 analysis
SA121	73	6p deletion?	Deletion not verified by an independent FISH analysis
SA121	117	6p deletion?	Deletion not verified by MLPA telomere P036 analysis
SA181	59	No deletions	
SA240	47	No deletions	Confirmed by MLPA telomere P036 analysis
SA461	14	No deletions	Confirmed by MLPA telomere P036 analysis

Cells were cultured and processed as described in Materials and Methods. For independent verification of the results, some samples were also analyzed by fluorescent in situ hybridization or using the MLPA Telomere P036 kit.

[a]Clonally derived from hESC line AS034.

[b]The hESC line was clonally derived in passage 74 and then subsequently passaged 86 times before subjected to the MLPA analyses.

However, this is not surprising because of the gene richness and the high transcriptional activity of the subtelomeric regions, and any alterations in these regions are likely to result in phenotypic effects [12]. To date, approximately 2,500 patients with telomere deletions have been reported, and specific genotype-phenotype correlations have been described recently [14]. Many hESC-based applications depend on the pluripotency and developmental properties of these cells. Thus, it is important to assess whether in vitro manipulation of these cells could induce chromosomal rearrangements, such as subtelomeric deletions, which in turn cause significant adverse effects. In this study we have demonstrated that the MLPA technology readily can be used for this purpose.

The MLPA technique provides the possibility to discriminate single nucleotide differences in DNA samples, making it a very powerful tool for several applications [15], including the characterization of chromosomal aberrations in cell lines, as shown in the present study. However, to gain further insight into the genomic stability of hESCs, it is also important to use additional techniques to interrogate the pathways that are directly or indirectly responsible for causing genomic aberrations in hESCs. In particular, little is known about tumor suppressors, such as p53 and pRB, and their activities in hESCs. It would be very interesting to elucidate the role of these gatekeepers in maintaining the normal karyotype in hESCs. Knowledge about these processes in hESCs may prove useful for the preservation of genomic stability in hESCs during long-term in vitro culture.

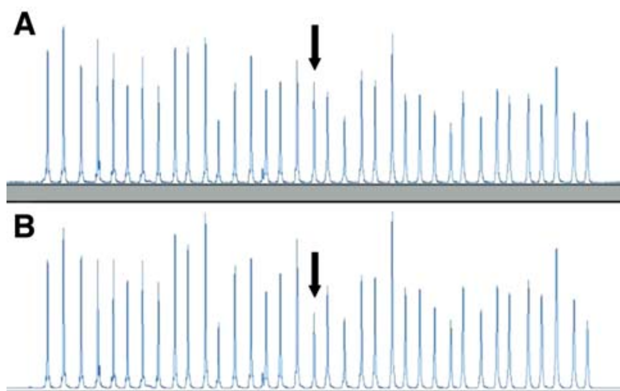

Figure 3. Representative peak patterns obtained from the multiplex ligation-dependent probe amplification telomere P019 analyses of undifferentiated human embryonic stem cells (hESCs). The P019 mix contains probes for chromosomes 1-11 and X. **(A):** Normal XY hESC line (AS034, passage 11). **(B):** Apparent 6p-deletion observed in SA121 (passage 73). The arrows indicate the 6p-specific peaks.

Conclusion

In this study, we have provided high-resolution experimental data supporting the conclusion that it is possible to maintain hESCs in culture for extended time periods without compromising their genomic integrity. Although these results are promising and provide confidence and support for the therapeutic potential of hESCs, additional studies are necessary to safely move hESC research from bench to bed.

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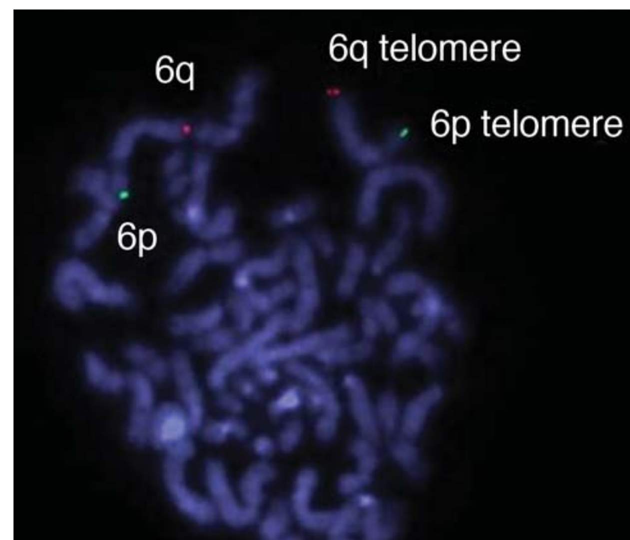

Figure 4. Representative illustration of a fluorescent in situ hybridization analysis using 6p/6q subtelomere probes performed on metaphase spreads prepared from undifferentiated human embryonic stem cells (SA121) in passage 81.

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