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Comparative genomic hybridization and karyotyping of human embryonic stem cells reveals the occurrence of an isodicentric X chromosome after long-term cultivation

J.Inzunza[1,3], S.Sahlén[2], K.Holmberg[2], A.-M.Strömberg[1], H.Teerijoki[1], E.Blennow[2], O.Hovatta[1] and H.Malmgren[2]

[1]Department of Clinical Sciences, Division of Obstetrics and Gynaecology, Karolinska Institutet, Karolinska University Hospital, Huddinge, S-141 86 Stockholm and [2]Department of Molecular Medicine, Karolinska Institutet, Karolinska University Hospital, Solna, S-171 76 Stockholm, Sweden

[3]To whom correspondence should be addressed. E-mail: jose.inzunza@mednut.ki.se

Human embryonic stem (hES) cells are important research tools in studies of the physiology of early tissue differentiation. In addition, prospects are high regarding the use of these cells for successful cell transplantation. However, one concern has been that cultivation of these cells over many passages might induce chromosomal changes. It is thus important to investigate these cell lines, and check that a normal chromosomal content is retained even during long-term *in vitro* culture. Comparative genomic hybridization (CGH) was used to analyse three hES cell lines derived in our laboratory and cultured continuously for 30–42 weeks, comprising 35–39 cell passages. CGH could be successfully performed in 48 out of a total of 50 isolated single cells (96%). All three lines (HS181, HS235 and HS237) were shown to have a normal chromosomal content when analysed by both single cell CGH and by karyotyping up to passages 39, 39 and 35 respectively. No aneuploidies or larger deletions or amplifications were detected, and they were female (46,XX). However, HS237 was reanalysed at passage 61, and at that point an aberrant X chromosome was detected by karyotyping. The aberration was confirmed and characterized by single cell CGH and fluorescence *in situ* hybridization analysis, 46,X,idic(X)(q21). Thus, chromosomal aberrations may occur over time in stem cell lines, and continuous analysis of these cells during cultivation is crucial. Single cell CGH is a method that can be used for continuous analysis of the hES cell lines during cultivation, in order to detect chromosome imbalance.

Key words: chromosome/comparative genomic hybridization/culture/human embryonic stem cells

Introduction

Since the first reports of permanent human embryonic stem (hES) cell lines (Thomson *et al*., 1998; Reubinoff *et al*., 2000) these cells have been widely used in studies regarding their early differentiation (Trounson, 2002; Carpenter *et al*., 2003; Passier and Mummery, 2003). In the future it may be possible to use these cells in cell transplantation as a treatment of severe diseases. One concern regarding the use of hES cells in cell transplantation has been the possible risk of chromosomal changes occurring in the cells during the long cultivation periods. It has been shown that mouse ES cells can develop chromosomal errors during cultivation (Liu *et al*., 1997; Longo *et al*., 1997). However, it is not known if other primary cells become chromosomally unstable during cultivation. The experience from cultivation of embryos shows that chromosomal aberrations can occur over time (Munné *et al*., 1997). It is important to control the hES cells for chromosomal normality before transplantation, as there appears to be a risk of tumour development when using these cells (Ringdén *et al*., 2003).

Previous attempts to perform karyotyping of our hES cell lines have sometimes proven difficult, resulting in poor number and quality of metaphases. This prompted us to consider alternative methods in order to continuously evaluate the stem cell lines. Comparative genomic hybridization (CGH) is a method that gives an overview of the whole genome and allows the detection of DNA copy number changes (Kallioniemi *et al.*, 1992, 1994). The method has been successfully applied in the characterization of the chromosomal constitution of several cell types, including cancer cells. The CGH method was further refined as it was shown that the sample size to investigate could be a single cell (Klein *et al*., 1999; Voullaire *et al*., 1999; Wells *et al*., 1999). We have previously performed single cell CGH analysis of blastomeres from human preimplantation embryos from patients going through preimplantation genetic diagnosis (PGD) for inherited structural chromosome aberrations. The aim was to verify the PGD results for the specific translocation, to reveal the overall genetic balance in each cell and to visualize the degree of mosaicism regarding all the chromosomes within the embryo. We could detect the unbalanced translocations, and estimated the resolution to be 10–20 Mb (Malmgren *et al*., 2002). We have now applied the same single cell CGH method to investigate the chromosomal constitution and stability of three hES cell lines, which have been established in our laboratory and cultured for long periods of time.

Materials and methods

hES cell lines

The three human ES cell lines characterized by single cell CGH were established in our laboratory and cultured for long periods. All three human ES cell lines (HS181, HS235 and HS237) were derived from supernumerary

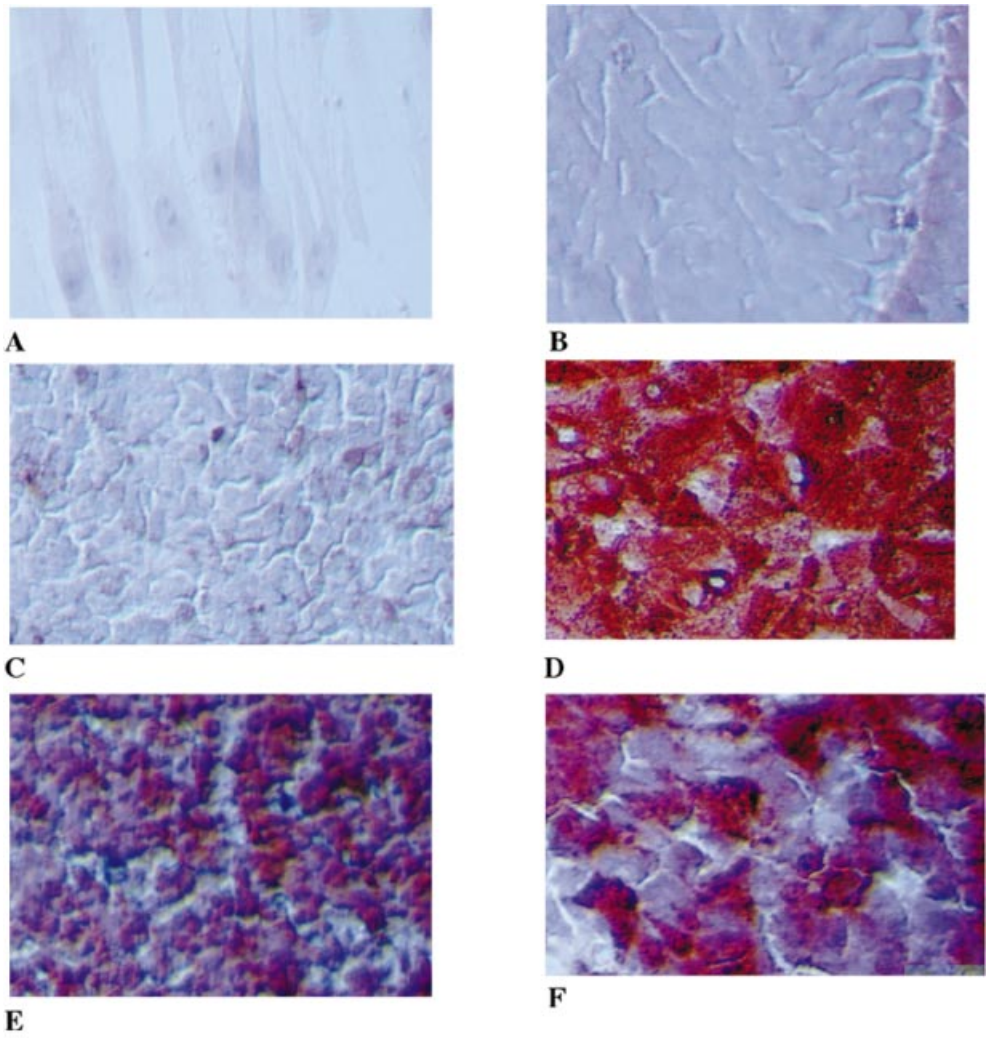

Figure 1. Immunohistochemistry of the hES cell line HS237. The following primary mouse antibodies were used: SSEA-4, SSEA-1, TRA-1–60, TRA-1–81 (Santa Cruz Biotechnology, Inc.). The secondary antibody was biotinylated rabbit anti-mouse IgG + A + M which was localized with horseradish peroxidase–streptavidin conjugate (Zymed laboratories Inc.). As negative controls the only-secondary antibody staining and the feeder cells were used. (**A**) Immunohistochemistry for SSEA-4, foreskin fibroblast feeder cell control, original magnification ×40. (**B**) hES cells, line HS237, preimmune serum instead of primary antibody, original magnification ×20. (**C**) hES cells, line HS237, primary antibody against SSEA-1. (**D**) hES cells, line HS237, primary antibody against TRA 1–60, original magnification ×40. (**E**) hES cells, line HS237, primary antibody against TRA-1–81, original magnification ×20. (**F**) hES cells, line 237, primary antibody against SSEA-4, original magnification ×40.

blastocysts donated for stem cell research by couples undergoing IVF treatment at our clinic. All couples gave their informed consent. We have approval from the Ethics Committee of the Karolinska Institutet to establish and characterize human ES cell lines from embryos considered unsuitable for use in infertility treatments. The hES cell lines were derived and cultured on feeder cells, human foreskin fibroblasts (CRL-2429; ATCC, USA) that were mitotically inactivated using irradiation (35 Gy). The characterization of cell line HS181 has recently been published (Hovatta *et al*., 2003), and the lines HS235 and HS237 have been characterized in a similar way. The hES cells of all these three cell lines express markers typical of hES cells—alkaline phosphatase, SSEA-4, TRA-1–60 and Oct-4—but are SSEA-1 negative. The lines HS235 and HS237 were also analysed for the expression of TRA-1–81, another marker specific for hES cells. The expression of the markers SSEA-4, SSEA-1, TRA-1–60 and TRA-1–81 in cell line HS237, are shown in Figure 1. The expression of Oct-4 was demonstrated using RT–PCR (Figure 2). Total RNA (50 ng) was reverse transcribed into first-strand cDNA and used as a template in PCR using primers derived from different exons in order to ensure that the PCR products represent the specific product of 245 bp from mRNA and not from genomic DNA. The PCR primers used: F: 5′-CGTGAAGCTGGAGAAGGAGAAGCTG-3′ and R: 5′-AAGGGCCGCAGCTTACACATGTTC-3′. The PCR reaction was performed with HotStart DNA polymerase (Qiagen) and reaction included

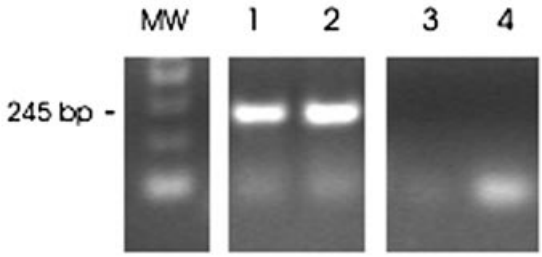

Figure 2. Oct-4 expression in hES cell lines determined by RT–PCR: (1) cell line HS235, (2) cell line HS237, (3) human foreskin fibroblast, (4) RT–PCR negative control.

denaturation at 95°C for 15 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s.

The cells from HS181 have been shown to form teratomas when injected into severely compromised immunodeficient (SCID) mice, as recently described by Hovatta *et al*. (2003). The same procedure has been applied to cell lines HS235

and HS237, and teratomas were formed. The pluripotency has also been demonstrated by *in vitro* differentiation of embryoid bodies cultured without feeder cells for 1 month (H.Koivisto *et al*. unpublished). The markers of tissue components characteristic of ectodermal (Sox-1), mesodermal (α-cardiac actin) and endodermal (α-fetoprotein) development were determined using RT–PCR primers and conditions reported by Henderson *et al*. (2002) and Amit *et al*. (2003).

The cells have been re-plated every 5–7 days, and spontaneously differentiated cells (5–20%) have been removed. Cell aggregates containing 1000–2000 cells have been mechanically dispersed after dispase treatment (10 mg/ml dispase; GibcoBRL, Life Technologies) and aggregates containing 100–200 cells have been transferred onto new feeder cells, which have been mitotically inactivated using irradiation (35 Gy). The culture medium consisted of Knockout™ Dulbecco's modified Eagle's medium ×1 (GibcoBRL) supplemented with 2 mmol/l L-glutamine (GibcoBRL), 20% FCS (R&D, Sweden) or 20% serum replacement (GibcoBRL), 0.1 mmol/l β-mercapto-ethanol (GibcoBRL) and 1% non-essential amino acids (GibcoBRL). The line HS235 has a tendency to differentiate easily, and 35–40% spontaneously differentiated areas were seen in 45% of the aggregates during the 5–7 day culture period.

Sex determination by multiplex PCR

The sex of the cell lines was determined by multiplex PCR analysis of two microsatellite markers on the X chromosome, DXS998 (Apessos *et al*., 2001) and p39 (Wehnert *et al*., 1993), and a sequence of the amelogenin gene (Nakahori *et al*., 1991). The amelogenin sequence is present on both the X and the Y chromosome with 88.9% homology. Amplification of a non-homologous sequence generates amplified fragments that differ in size as the sequence on the X chromosome has a deletion of 6 bp (Sullivan *et al*., 1993). This results in two fragments (104 and 110 bp) if the template is from a male, and only a 104 bp fragment if the template is from a female. Four cell samples (10–30 cells each) were isolated from each hES cell line and transferred to individual PCR tubes containing 2.5 µl alkaline lysis buffer 200 µmol/l NaOH (MERCK) and 50 mmol/l dithiotceitol (Amersham). The tubes were placed on dry ice for 1 h, and the cells were lysed by incubation at 65°C for 15 min. After cooling to 4°C, PCR amplification was carried out in the total volume of 25 µl by adding PCR mix with the final concentration of 10% dimethylsulphoxide, 1×Expand HF buffer 2 (Roche), 200 µmol/l dNTP, 2 IU of Expand HF enzyme (Roche), 20 mmol/l tricine pH 5.4, 0.5 µmol/l each of primers DXS998F and DXS998R (Apessos *et al*., 2001), 0.25 µmol/l each of primers p39F (9120) and p39R (9121) (Wehnert *et al*., 1993), AMELF and AMELR (Sullivan *et al*., 1993). The reactions were incubated at 95°C for 6 min followed by 45 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 2 min. This was followed by an incubation at 72°C for 10 min. Primer DXS998F and p39F were labelled with 6-Fam and AMELF was labelled with Hex, allowing the PCR products to be analysed on an ABI 310.

Single cell CGH

For each cell line, a number of colonies were isolated and cells were separated with dispase. Random single cells were isolated, washed in phosphate-buffered saline, and transferred to individual PCR tubes using a special capillary pipette. Each cell was analysed by single cell PCR amplification and subsequent CGH analysis using the protocol developed by Klein *et al*. (1999). In brief, the cells were lysed by proteinase K treatment, and the DNA was digested with MseI. Linkers were ligated to the generated restriction fragments and PCR was conducted using primers complementary to the linker sequence. The PCR products were reamplified and labelled with digoxigenin and detected with a fluorescein isothiocyanate (FITC) antibody. The reference DNA (normal female) was labelled with Spectrum Red using a ULS labelling kit (Qbiogene). The test DNA and the reference DNA were co-hybridized to male metaphase slides (Vysis, USA). After CGH hybridization in a moist chamber for 48 h, the slides were washed and analysed using Quips CGH karyotyping Imaging Software (Vysis). Ten to 15 metaphases were captured and evaluated for each cell sample. Red/green ratios of 1:>1.25 indicated amplified regions and ratios 0.75:<1 indicated deleted regions. Some regions were excluded from analysis (the centromeres, the telomeric regions, 1q, 16p, chromosomes 19 and 22), as these regions are difficult to interpret by CGH (Kallioniemi *et al*., 1994). An analysis was considered successful if the PCR products from the single cell was in large amounts and in the expected size range and that the CGH resulted in strong, even, red/green balanced hybridization to the metaphases.

We have previously used this single CGH method for the analysis of single blastomeres from human preimplantation embryos from patients going through PGD for inherited structural chromosome aberrations (Malmgren *et al*., 2002). We could detect unbalanced translocations, and estimated the resolution to be 10–20 Mb.

Karyotyping

Karyotyping of cell line HS181 was performed at passage 22 and the result has been published previously (Hovatta *et al*., 2003). Karyotyping of cell line HS235 was carried out at passage 59 and HS237 at passages 17 and 61. Samples of cells were treated with 10 mmol/l colcemid overnight. After washing, the cells were incubated in 0.4% trypsin solution (GIBCO) for 2–3 min. Cells were treated with 1400 IU/ml collagenase (Worthington) at 37°C for 20 min, and harvested using standard proceedures. The metaphases were analysed after Q-banding.

Results

The sex of the cell lines was determined by multiplex PCR analysis of cell samples (10–30 cells), using two microsatellite markers on the X-chromosome (DXS998 and p39) and a sequence of the amelogenin gene. All three ES cell lines were derived from female pre-embryos, all showing a single 104 bp fragment. HS181 was heterozygous for both polymorphic markers DXS998 and p39, HS235 was homozygous for both markers, and HS237 was heterozygous for marker p39 but homozygous for DXS998. Genotyping of the feeder cells was also carried out, and results showed a male genotype with one single allele for each of the markers DXS998 and p39, and these alleles were distinguishable from the alleles found in the hES cell lines.

The first ES cell line analysed by single cell CGH was HS181, which has been in continuous culture from the beginning of March 2002 and had, by the time of analysis in January 2003, been continuously cultured for 42 weeks, 39 passages. The two other lines, HS235 and HS237, have been continuously cultured from the beginning of October 2002, being at passage levels 39 and 35 respectively, when analysed by single cell CGH in May 2003.

A total of 24 cells from HS181 (passage 39), 12 cells from HS237 (passage 35) and 14 cells from HS235 (passage 39) were amplified, and 48 of these 50 cells (96%) were successfully analysed by CGH. All 12 cells from cell line HS237 as well as the 12 cells analysed from HS235 showed a balanced chromosomal content, 46,XX (Figure 3A, B). No aneuploidies or larger deletions or amplifications were detected within the cells, using the single cell CGH analysis.

The CGH results of the 24 cells from HS181 were balanced for all chromosomes with the karyotype 46,XX in 19 out of 24 cells (Figure 3c), but five out of 24 cells showed monosomy for X. This could be explained either by the presence of a clone of cells that had lost one X chromosome, or because some samples were feeder cells (male skin fibroblasts). In order to elucidate these results, the generated PCR products derived from the samples showing monosomy X were reamplified using the multiplex PCR assay described above. The Y-specific fragment was detected in all five samples, and the X-chromosomal markers DXS998 and p39 revealed the alleles specific for the feeder cells. Thus, these samples consisted of feeder cells (male skin fibroblasts). In an earlier chromosome analysis of cell line HS181, at passage level 22, G-banding showed normal karyotype 46,XX (Hovatta *et al*., 2003).

Karyotyping of cell line HS235 at passage 59 and cell line HS237 at passage 17 showed a normal female karyotype, 46,XX. However, karyotyping of HS237 in December 2003 at passage 61 revealed an aberrant X chromosome (Figure 4A). Single cell CGH analysis of cells from this passage showed an amplification of Xpter-Xq21 and deletion

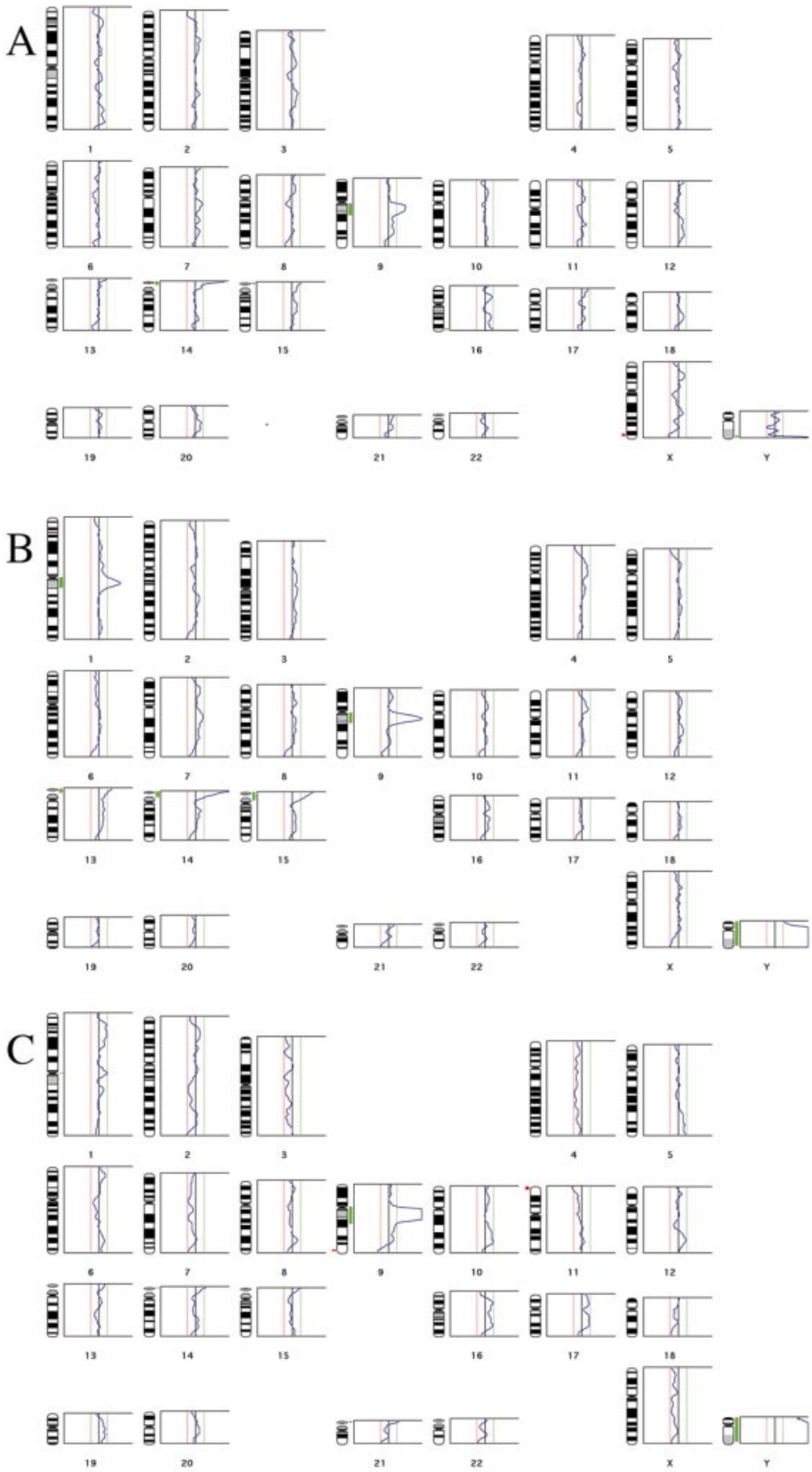

Figure 3. The comparative genomic hybridization (CGH) results of one cell from hES cell lines (**A**) HS235 passage 39, (**B**) HS237 passage 35 and (**C**) HS181 passage 39 respectively show a balanced chromosomal content regarding all chromosomes including the X chromosome, which indicates a female karyotype. The peaks observed at the centromeres of chromosomes 1 and 9 are due to an incomplete suppression of the heterochromatin by Cot-1 DNA. These regions are not included in the interpretation (see Materials and methods). As the tested sample, as well as the reference DNA, is female, the Y chromosome will remain unlabelled and thus the CGH curve of the Y chromosome will be uninterpretable.

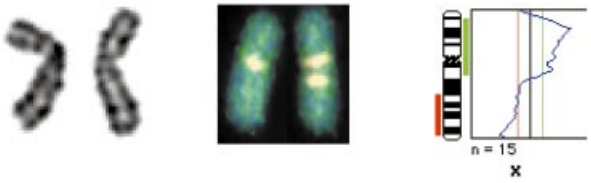

Figure 4. The cell line HS237 was reanalysed in December 2003 at passage 61. *Left*: Karyotyping (Q-banding) revealed an aberrant X chromosome. *Middle*: FISH analysis was performed using a standard protocol. An X centromeric probe (DXZ1) labelled with Spectrum Orange (SO) and an X chromosome-specific library (Total chromosome DNA, Chromosome X, green; Appligene Oncor) was used and the analysis identified a dicentric X chromosome consisting of purely X chromosome material. *Right*: Single cell comparative genomic hybridization showed an amplification of Xpter-Xq21 and a deletion of Xq21-Xqter, but no other amplifications or deletions. These results indicate the karyotype 46,X,idic(X)(q21) in HS237 cells at passage 61. This aberration has occurred in HS237 sometime between passage 35 and 61.

of Xq21-Xqter (Figure 4C). No other deletion or amplification was detected. FISH analysis of these cells using a probe specific for the X centromere, as well as an X chromosome-specific library, revealed a chromosome consisting of purely X chromosome material but with two centromeres (Figure 4B). These results indicate an isodicentric X chromosome composed of two p-arms and the q-arm material between the centromeres and the breakpoint in Xq21, 46,X,idic(X)(q21). This isodicentric X chromosome will be further characterized.

Discussion

CGH proved to be a feasible technique in order to characterize the chromosomal constitution of single hES cells. CGH is a method that gives an overview of the whole genome and allows the detection of DNA copy number changes. However, neither smaller deletions or amplifications, nor balanced rearrangements are detected. In a previous study we found a resolution limit of 10–20 Mb for this single cell CGH method, and smaller imbalances might be missed (Malmgren *et al*., 2002). This is in agreement with another report on single cell CGH analysis that estimates the resolution to be 10–40 Mb (Voullaire *et al*., 1999). The number of cells that may be analysed on each occasion is limited only by laboratory capacity, as CGH is a time-consuming method and all current single cell CGH protocols have a time requirement of ~4–5 days.

There are other possible strategies in order to investigate the chromosomal constitution within these cell lines. An obvious strategy is to perform karyotyping with G-banding or Q-banding. We have previously experienced difficulty in obtaining an even, high quality of the metaphases. Thus, the number of cells amenable to analysis will be limited, and clones of cells with chromosomal changes could easily be missed. Another possible approach is interphase FISH using a set of chromosome-specific probes. Using this method, a large number of cells may be screened for chromosomal aberrations in a relatively short time. However, due to technical limitations, only a limited part of the genome can be analysed this way. The more probes included, the more unreliable the interpretation of the results, thus only a few chromosomes may be included in the interphase FISH analysis.

All our three hES cell lines have been derived from 46,XX embryos as verified by the multiplex PCR microsatellite marker study. HS181, which had been continuously cultured for a very long time, showed the karyotype 46,XY in five out of 24 cells studied. The explanation was that some isolated cells were feeder cells (male foreskin fibroblasts), as these specific samples contained a Y chromosome and an X chromosome harbouring DXS998 and p39 alleles specific for the feeder cells. During chromosome analysis by karyotyping, only dividing cells are analysed, and the mitotically inactivated feeder cells are not a source of error, as appears to be the case in CGH. Therefore, it may be an advantage to culture the hES cells in a feeder cell-free culture system for a period before CGH analysis. An alternative way of identifying the cell identity of the collected samples is to carry out a haplotype analysis of each PCR-amplified sample, using a number of polymorphic markers.

It has been shown that mouse ES cells can develop chromosomal errors during proliferation (Liu *et al*., 1997; Longo *et al*., 1997). The experience from cultivation of embryos shows that chromosomal aberrations can occur over time (Munné *et al*., 1997). In one of the cell lines analysed in this study, HS237, an aberrant X chromosome was detected at passage 61, after 58 weeks of continuous cultivation. This isodicentric X chromosome, 46,X,idic(X)(q21), was not present in the cells analysed by CGH at passage 35, and must have occurred after that passage. The aberrant X chromosome will be further characterized, and investigations will be carried out in order to determine at what passage it first occurred.

The other ES cell lines analysed in this study (HS181 and HS235) appear stable through at least 39 and 59 passages respectively. However, the number of cells analysed for each cell line is still limited, and clones of aberrant cells might be missed regardless of using single cell CGH or karyotyping. Neither karyotyping nor single cell CGH would allow the screening of a large number of cells. Nevertheless, single cell CGH is still an alternative method to reveal unbalanced chromosomal changes that may occur in hES cells during long-term cultures, especially when it is difficult to obtain good quality metaphases. CGH may be used for continuous analysis of the hES cell lines during cultivation in order to give reassurance of the chromosomal stability/constitution, which is crucial when considering transplantation of these cells.

Acknowledgements

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