Cytogenetic analysis and *Dlk1-Dio3* locus epigenetic status of mouse embryonic stem cells during early passages

Aleksei Menzorov · Inna Pristyazhnyuk · Helen Kizilova ·
Anastasia Yunusova · Nariman Battulin · Antonina Zhelezova ·
Aleftina Golubitsa · Oleg Serov

Received: 15 October 2013 / Accepted: 7 June 2014 / Published online: 27 June 2014
© Springer Science+Business Media Dordrecht 2014

Abstract Mouse embryonic stem (ES) cells are widely used in early development studies and for transgenic animal production; however, a stable karyotype is a prerequisite for their use. We derived 32 ES cell lines of outbred mice (129 × BALB (1B), C57BL × 1B, and DD × 1B F1 hybrids). Pluripotency was assessed by utilizing stem-cell-marker gene expression, teratoma formation assays and the formation of chimeras. It was shown that only 21 of the 32 ES cell lines had a diploid modal number of chromosomes of 40. In these lines, the percentage of diploid cells varied from 30.3 to 78.9 %, and trisomy of chromosomes 1, 8 and 11 was observed in some cells in 16.7, 36.7 and 20.0 % of the diploid ES cell lines, respectively. Some cells had trisomy of chromosomes 6, 9, 12, 14, 18 and 19. In situ hybridization with an X chromosome paint probe revealed that 7 of the 11 XX-cell lines had X chromosome rearrangements in some cells. Analysis of the methylation status of the *Dlk1-Dio3* locus showed that imprinting was altered in 4 of

the 18 ES cell lines. Thus, mouse ES cell lines are prone to chromosome abnormalities even at early passages. Therefore, routine cytogenetic and imprinting analyses are necessary for ES cell characterization.

Keywords Mouse embryonic stem cells · Chromosome abnormalities · Aneuploidy · Chimerism · *Dlk1-Dio3*

Introduction

Mouse embryonic stem (ES) cells obtained from the blastocyst inner cell mass remain pluripotent during prolonged cell culture in vitro (Evans and Kaufman 1981; Martin 1981). ES cells participate in the development of chimeric mice (Hogan et al. 1994; Smith 2001) and have the ability to give rise to viable mice through tetraploid complementation (Nagy et al. 1990, 1993). These experimental procedures allow for the production of gametes with an ES cell genetic background and, thus, have progeny with the ES cell genotype. Therefore, ES cells provide a link between cell biology in vitro and experimental embryology in vivo. The ability of ES cells to colonize gonads is widely used in the production of transgenic animals with specific genome modifications using such approaches such as “gene targeting” and “gene knockout”.

Numerous studies suggest that prolonged culturing of ES cells leads to the accumulation of chromosome

A. Menzorov (✉) · I. Pristyazhnyuk ·
H. Kizilova · A. Yunusova · N. Battulin ·
A. Zhelezova · A. Golubitsa · O. Serov
Laboratory of Developmental Genetics, Institute of
Cytology and Genetics SB RAS, Prospekt Lavrentyeva
10, Novosibirsk 630090, Russia
e-mail: menzorov@bionet.nsc.ru

A. Menzorov · H. Kizilova · N. Battulin · O. Serov
Department of Natural Science, Novosibirsk State
University, Pirogova Str. 2, Novosibirsk 630090, Russia

abnormalities, such as trisomies. A recent study showed that, during early passages (9–11), three ES cell lines had 50–63 % diploid cells, but after 15 more passages, the number of euploid cells decreased two to three times (Rebuzzini et al. 2008a). Such trisomies and chromosomal rearrangements are considered as the main reason for inefficient chimerization by ES cells and particularly for the decrease in ES-cell genotype gametes (Nagy et al. 1993; Liu et al. 1997; Guo et al. 2005; Rebuzzini et al. 2008a). It is striking that ES cells have a high percentage of aneuploid cells even after early passages in culture upon derivation from blastocysts. Cytogenetic analysis of 15 ES cell lines upon 4–7 passages revealed only one cell line that lacked visible chromosome rearrangements (Nichols et al. 1990). It should be noted that to produce progeny with an ES cell genotype, at least 50 % of the cells should be diploid (Longo et al. 1997). Therefore, we assessed the karyotypes in de novo-produced ES cell lines.

The *Dlk1-Dio3* imprinted locus plays an important role in development, and its abnormal methylation leads to developmental arrest (da Rocha et al. 2008). Altered *Dlk1-Dio3* imprinting can decrease the pluripotency of induced pluripotent stem (iPS) cells (Stadtfield et al. 2010). iPS cell lines with altered methylation show a low efficiency for chimerism when cells are injected into diploid blastocysts and are not able to develop through tetraploid complementation. These findings suggest that monitoring of the *Dlk1-Dio3* imprinting status might be useful to eliminate ES cell lines with reduced pluripotency.

In the present study, we performed cytogenetic analysis and *Dlk1-Dio3* locus methylation status studies in ES cell lines during early passages to assess their prospects for chimeric mice generation.

Materials and methods

ES cell derivation and culture

To produce ES cell lines, we used 3.5-days post coitum (dpc) blastocysts of different genetic backgrounds. For the MA ES cell line series, we used outbred mice that were produced by crossing the 129 and BALB lines (hereafter, referred to as 1B), for the MC series—F₁ (1B × C57BL), and for MD—F₁ (1B × DD). We derived ES cells from hybrid

embryos because such ES cells have higher chimerization efficiency compared to a linear genetic background (Eggan et al. 2001, 2002; Li et al. 2005).

The ES cell lines MC01–MC04, MD01 and MD02 were produced using the Robertson (1987) protocol with minor modifications: culture medium contained 15 % KSR (knockout serum replacement, Invitrogen, Carlsbad, CA, USA) and 5 % ES cell-qualified FBS (PAA, Pasching, Austria). Addition of KSR was used to suppress the growth of trophoblast cells. The ES cell lines MA01–MA15 and MC05–MC15 were produced using the protocol developed by Bryja et al. (2006) with minor modifications. Briefly, 3.5-dpc blastocysts without zona pellucida were plated on mitomycin C-treated, ICR, 13.5-dpc fibroblasts (feeder) in ES culture medium supplemented with 20 % KSR. On day six, embryos were deaggregated with 2.5 % Trypsin–EDTA (Invitrogen) and plated on feeder cells in culture medium supplemented with 20 % FBS. The next day, the medium was changed to 20 % KSR, and this medium was used before passaging. Colonies were trypsinized using 0.05 % Trypsin–EDTA and plated in 20 % FBS medium for 1 day, and the next day, the medium was changed to 20 % KSR. After passage four, we used standard ES cell culture medium: DMEM (Invitrogen) supplemented with 15 % ES cell-qualified FBS, 1 × NEAA (Invitrogen), 1 × GlutaMAX (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO, USA), 1 × Penicillin–Streptomycin (Invitrogen) and 1,000 U/ml LIF ESGRO (Chemicon, Temecula, CA, USA). Cells were cultured in 6-well plates that were coated with 0.1 % gelatin on feeder cells. We used Mitomycin C inactivated 13.5 dpc strain ICR mouse embryonic fibroblasts as feeder cells.

ES cell cytogenetic analysis

Cytogenetic analysis for most ES cell lines was carried out during passages 6–7, with the exceptions of MD02 (passage 10); MC01, MC04 and MD01 (passage 11); and MC02 (passage 12). Preparation of metaphase chromosomes from the ES cells was performed as previously described with minor modifications: cells were treated with hypotonic solution (0.25 % KCl and 0.2 % sodium citrate) for 20 min (Pristiazhniuk et al. 2010). For each of the 32 ES cell lines, an average of 35 metaphase plates were counted. Karyotyping was performed on 5–10 metaphase plates for each cell line.

X chromosome identification was performed by fluorescent in situ hybridization (FISH) on metaphase plates with biotin-labeled, X-specific probe (CAM-BIO, Cambridge, England) as described elsewhere (Pristiazniuk et al. 2010). Metaphase plates were analyzed using a Carl Zeiss Axioscop 2 microscope (Jena, Germany) with VC-44 (PCO) CCD-camera, and digital images were analyzed using the ISIS 3 (In Situ Imaging System, MetaSystems GmbH, Altlussheim, Germany) software in the Public Center for Microscopy SB RAS, Novosibirsk.

Analysis of gene expression

Total ES cell RNA was isolated using Trizol Reagent (Life Technology, Carlsbad, CA, USA) according to the manufacturer's recommendations. After DNase I (Sigma Aldrich, St. Louis, MO, USA) treatment, cDNA was synthesized using the Reverse Transcription System (Promega, Madison, WI, USA) kit and random primers. PCR was performed with the following primers: NanogF 5'-AGG GTC TGC TAC TGA GAT GCT CTG-3', NanogR 5'-CAA CCA CTG GTT TTT CTG CCA CCG-3', Sox2F 5'-TAG AGC TAG ACT CCG GGC GAT GA-3', Sox2R 5'-TTG CCT TAA ACA AGA CCA CGA AA-3', Esg1F 5'-GAA GTC TGG TTC CTT GGC AGG ATG-3', Esg1R 5'-ACT CGA TAC ACT GGC CTA GC-3', Rex1F 5'-ACG AGT GGC AGT TTC TTC TTG GGA-3' and Rex1R 5'-TAT GAC TCA CTT CCA GGG GGC ACT-3' (Takahashi et al. 2007); and Oct4F 5'-CTC GAA CCA CAT CCT TCT CT-3' and Oct4R 5'-GGC GTT CTC TTT GGA AAG GTG TTC-3' (Schroeder et al. 2009). The PCR conditions included the initial denaturation of genomic DNA at 95 °C for 3 min with the following 30 cycles of subsequent amplification: denaturation at 94 °C for 15 s, annealing at 58 °C for 15 s and elongation at 72 °C for 30 s, with a final elongation for 3 min.

Bisulfite treatment and restriction analysis

We used combined bisulfite restriction analysis (COBRA) to analyze the imprinting status of the *Dlk1-Dio3* locus. Genomic DNA isolation and bisulfite treatment were performed using the EZ DNA Methylation-Direct kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's recommendations. Purified DNA was eluted into 10 µl of buffer and used as a matrix for two rounds of nested PCR. For the first

round, the primers IGC_out_F 5'-TTA AGG TAT TTT TTA TTG ATA AAA TAA TGT AGT TT-3' and IGC_out_R 5'-CCT ACT CTA TAA TAC CCT ATA TAA TTA TAC CAT AA-3' were used, and for the second round, IGC_in_F 5'-TTA GGA GTT AAG GAA AAG AAA GAA ATA GTA TAG T-3' and IGC_in_R 5'-TAT ACA CAA AAA TAT ATC TAT ATA ACA CCA TAC AA-3' were used. The PCR conditions included the initial denaturation of genomic DNA at 95 °C for 2 min with the following 30 cycles of subsequent amplification: denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, and elongation at 72 °C for 40 s. After the second round of amplification, the PCR products were purified using the MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). The PCR products were digested with *HinfI* (SibEnzyme, Novosibirsk, Russia) for 2 h at 37 °C according to manufacturer's recommendations, and the digestion products were analyzed in a 2 % agarose gel in TAE buffer.

Generation of chimeras

To generate chimeras, ES cells were injected into C57BL/6J blastocysts (Hogan et al. 1994). The injected blastocysts were then transferred to recipient F1 (C57BL/6J × CBA or CBA × C57BL/6J) females. Chimerism was evaluated by coat color, and chimeras of both sexes were bred to 1B and C57BL/6J mice.

Teratoma analysis

Teratomas were produced using a standard protocol (Hogan et al. 1994). From 3 to 7.5×10^6 ES cells were injected subcutaneously into immunodeficient mice (*nu/nu*). Dissection of teratomas was performed after 2–3 weeks, and the teratomas were fixed in Bouin solution. Paraffin sections were prepared according to a standard protocol and were stained with the histological stains Picro-Mallory trichrome (04-021822), Masson trichrome (04-011802), P.T.A.H.-hematoxyline (04-060802), Luxol fast blue Kreyer Barrera (04-200812), Azan trichrome (04-001802), Picrofuchsin Van Gizon (04-030802) (Bio-Optica Milano S.P.A., Italy) and hematoxylin-eosin. Images were analyzed using a Carl Zeiss Axioscop 2+ microscope with an Axio-Cam HRc CCD-camera, and digital images were taken using the AxioVision software from the Public Center for Microscopy SB RAS, Novosibirsk.

Microbiological test

To test for the presence of Mycoplasma DNA, we used PCR with Mycoplasma-specific primers: 5'-GGG AGC AAA CAG GAT TAG ATA CCC T-3' and 5'-TGC ACC ATC TGT CAC TCT GTT AAC CTC-3' (Choppa et al. 1998).

Results

ES cell derivation from mouse blastocysts

We produced 32 ES cell lines: 15 of the MA series, 15 of the MC series, and 2 of the MD series, in five experiments. The efficiency of ES cell colony formation was approximately 40 %, which is comparable with that of Bryja et al. (2006). ES cell lines were produced from disaggregated blastocysts that gave rise to multiple colonies with ES-cell morphology.

RT-PCR analysis of pluripotency marker gene expression

The results of *Nanog*, *Oct4*, *Sox2*, *Rex1* and *Esg1* RT-PCR analysis in 10 ES cell lines are shown in Fig. 3a. Transcripts of these genes were detected in all analyzed ES cell lines and were absent in mouse embryonic fibroblasts with the exception of *Sox2*. *Nanog*, *Oct4*, *Sox2*, *Rex1* and *Esg1* genes are currently considered pluripotency marker genes because they are active in the blastocyst inner cell mass and not expressed in the trophectoderm and primitive endoderm (Cockburn and Rossant 2010).

Cytogenetic analysis of ES cell lines

The results of the cytogenetic analysis are shown in Table 1. Of the 32 ES cell lines, 14 (43.7 %) were XY, and 16 (50.0 %) were XX. MA03 and MD02 had near-tetraploid karyotypes, XXX/XXX and XXX0, respectively, and the diploid cell line MA13 was X0.

One of the basic cytogenetic characteristics is the modal number of chromosomes. The modal number is the number of chromosomes that most of the analyzed metaphase plates contain. If percentage of cells that had different numbers of chromosomes was less than 10 %, then that cell line had more than one modal number. Of the 32 ES cell lines, 21 (65.6 %) had forty

chromosomes, which is a diploid modal number (Table 1). MA12 and MC01 had two modal numbers, 40 and 41, respectively, and in MA13 and MA14, a significant fraction of cells had 39 chromosomes.

For the ES cells with a diploid modal number, the percentage of cells with 40 chromosomes varied from 30.3 to 78.9 %, and, in 17 lines, the percentage of diploid cells exceeded 50 % (Table 1). As mentioned previously, an important condition of successful germ line colonization in chimeras is the absence of trisomies and chromosomal rearrangements. Detailed cytogenetic analysis of the 32 ES cell lines revealed that in 4 of the 17 above-mentioned lines, more than 20 % of the cells had chromosomal rearrangements. For instance, rearranged chromosome 1 was identified in a low percentage of cells in MA07 and MA09 and in 75 % of MC02 cells (Fig. 1e, f). Furthermore, many of the ES cell lines had chromosome fragments that could not be identified using G-banding.

Detailed karyotyping revealed aneuploidy of different chromosomes in many of the ES cell lines. As an example, metaphase plate and karyotype of a MC15 cell with trisomies of chromosomes 1, 8, 9 and 11 are shown on Fig. 1c, d. It should be noted that some diploid cell lines had “hidden aneuploidy” because some cells with 40 chromosomes had mono- or trisomies. In 7 of the 30 studied cell lines, we detected cells that lacked a sex-determining chromosome and that had a chromosome 8 trisomy.

Chromosome 8 trisomy is one of the most common ES cell karyotype abnormalities, and we detected it in 11 of the 30 near-diploid cell lines. We also observed additional copies of chromosomes 1 and 11 in 5 and 6 of the 30 cell lines, respectively (Fig. 1c, d). Most of the MC04 cells had an additional chromosome 1, and some cells were also trisomic for chromosome 13. Trisomies of chromosomes 6, 9, 13, 14, 18 and 19 were detected in some cells of many of the ES cell lines.

Cells with the X0 karyotype were noticed in 19 of the 30 diploid ES cell lines (Table 1). The MA13 cell line had predominantly X0 cells, and the results of the G-banding analysis were confirmed using X chromosome-specific-probe in situ hybridization on 11 cell lines (Table 2). X chromosome rearrangements were identified in 7 of the 11 studied ES cell lines, and an example is shown on Fig. 2. Such rearrangements included fragments, translocations and, rarely, deletions. In MA10, 60 % of the cells had one X

Table 1 Cytogenetic analysis of 32 ES cell lines

ES cell line	Sex chromosomes	Modal chromosome number	39 Chromosomes (%)	40 Chromosomes (%)	41–45 Chromosomes (%)	Tetraploid cells (%)	Chromosome rearrangements (%)	N
MA01	XY	40	7.5	77.5	15.0	0	2.0	40
MA02	XX	40	20.5	64.1	2.6	12.8	12.5	39
MA03	XXX/XXXX	78, 79	0	9.4	11.3	79.2	24.0	53
MA04	XX	40	5.6	52.8	13.9	27.8	20.0	36
MA05	XY	40	7.7	56.4	17.9	17.9	18.0	39
MA06	XX	40	0	62.1	20.7	17.2	0	29
MA07	XX	40	24.3	45.9	5.4	24.3	5.4	37
MA08	XX	40	14.3	68.6	5.7	11.4	13.8	35
MA09	XY	40	5.4	67.6	18.9	8.1	0	37
MA10	XX	40	27.6	48.3	17.2	6.9	17.0	29
MA11	XX	40	20.0	52.0	8.0	20.0	8.0	25
MA12	XY	40, 41	9.3	46.5	41.9	2.3	2.3	43
MA13	X0	39	65.7	20.0	5.7	8.6	46.0	35
MA14	XX	39, 40	39.4	42.2	12.1	6.0	9.0	33
MA15	XY	40	7.9	78.9	7.9	5.3	5.3	38
MC01	XX	40, 41	10.6	23.4	34.0	31.9	0	48
MC02	XY	40	2.3	62.8	18.9	16.3	75.0	43
MC03	XY	40	5.9	64.7	23.5	5.9	6.1	34
MC04	XY	42	0	2.5	82.5	15.0	15.0	40
MC05	XY	40	10.0	75.0	0	15.0	20.5	34
MC06	XX	40	12.1	30.3	6.1	51.5	12.0	33
MC07	XX	40	11.8	47.1	23.5	17.6	23.8	34
MC08	XY	40	20.6	35.3	20.6	20.6	32.4	34
MC09	XX	40	32.4	51.4	5.4	10.8	35.0	37
MC10	XY	40	8.8	56.0	8.8	17.6	11.8	34
MC11	XX	40	9.8	53.7	12.2	25.0	0	41
MC12	XY	40	10.3	53.8	7.7	7.7	6.0	39
MC13	XX	40	12.8	41.0	38.5	7.7	2.5	39
MC14	XY	40	15.6	53.1	28.1	9.0	3.0	32
MC15	XY	41, 42, 44	0	10.7	82.1	20	6.5	28
MD01	XX	40	4.0	34.0	36.0	26.0	7.5	50
MD02	XXX0	74, 76, 79, 80	0	2.5	2.5	95.0	26	40

N number of metaphase plates

chromosome and an X chromosome fragment translocated to chromosome 11.

A typical feature of the studied ES cell lines was an increased amount of tetraploid cells. Most of the publicly available cell lines have at least 5 % tetraploid cells. In 20 of the 32 lines, we observed more than 10 % tetraploid cells, and in MA03 and MD02, the majority of cells were tetraploid (Table 1).

These data indicate that spontaneous tetraploidization is common in early-passage ES cells.

Methylation status of the *Dlk1-Dio3* locus

The *Dlk1-Dio3* locus is important for development. It was shown that hypermethylation of the imprinting control region causes pluripotency reduction in iPS

Fig. 1 Metaphase plates and karyotypes of different ES cell lines: **a** and **b** MA02 ES cell line with diploid karyotype (40, XX); **c** and **d** MC15 ES cell line with chromosome 1, 8, 9 and 11 trisomies (44, XY); and **e** and **f** MC02 ES cell line with chromosome rearrangement that includes a chromosome 1 fragment

cells. Such methylation abnormalities have not currently been reported for ES cells. To assess whether abnormal imprinting of the *Dlk1-Dio3* locus occurs, we analyzed the methylation status of two CpG dinucleotides of the intergenic differentially methylated region (IG-DMR) within the *Dlk1-Dio3* locus. We had shown previously that methylation of these two dinucleotides correlates with IG-DMR methylation in the entire region (Battulin et al. 2012). We show here that among the 18 ES cell lines that were selected for this analysis, 14 had a correct methylation pattern, that is, one allele was methylated and another was unmethylated (Fig. 3b, c). In MA06, MA09,

MA11 and MC08, we, for the first time, observed *Dlk1-Dio3* locus hypomethylation in ES cells.

Generation and analysis of teratomas

The teratoma analysis data are shown in Table 3. We obtained 27 tumors, and histological analysis showed that all of the tumors were teratomas, except for the ones from MA06 and MC05 cells, which gave rise to teratocarcinomas. We also showed the presence of at least 30 cell morphotypes from three germ layers belonging to all basic tissue types and different types of epithelial cells types, such as secretory, glial,

Table 2 Results of in situ hybridization with X chromosome paint probe in 11 ES cell lines

ES cell line	Number of X chromosomes				Number of cells with chromosome rearrangements	Number of metaphase plates
	1	2	3	4		
MA02	8.3 %	70.8 %	8.3 %	4.2 %	8.3 %	24
MA03	–	22.2 %	44.4 %	33.3 %	–	27
MA04	7.7 %	80.8 %	3.8 %	7.4 %	–	26
MA06	13.0 %	52.2 %	4.3 %	17.4 %	13.0 %	30
MA07	23.7 %	47.4 %	2.6 %	7.9 %	18.4 %	38
MA10	31.2 %	–	–	–	68.8 %	16
MA11	9.1 %	72.7 %	3.0 %	6.1 %	3.0 %	33
MA13	96.0 %	4.0 %	–	–	–	25
MC01	14.3 %	76.2 %	–	9.5 %	–	21
MC11	5.7 %	45.7 %	5.7 %	40.0 %	2.9 %	35
MC13	27.3 %	60.6 %	–	–	12.1 %	33

Fig. 2 Metaphase plates after in situ hybridization with X-chromosome paint probe. *Arrows* X chromosomes, *arrow-heads* X chromosome translocations and fragments. **a** MA06 ES cell line with diploid karyotype (40, XX); **b** MA13 ES cell line

(39, X0); **c** MA02 ES cell line that contains X chromosome and its fragment (39, X0, FrX); **d** MA10 ES cell line with a translocation of an X chromosome fragment on chromosome 11 (40, X, T(X; 11))

Fig. 3 Molecular analysis of ES cell lines. **a** analysis of pluripotency marker gene expression. MEF—mouse embryonic fibroblasts, K—PCR negative control. **b** methylation status analysis of *Dkl1-Dio3* IG-DMR. L100—100 b.p. molecular marker. **c** Design of methylation status analysis by COBRA. Restriction sites for *HinfI* appear after bisulfite treatment of two CpG dinucleotides within the *Dkl1-Dio3* locus IG-DMR

connective, muscle, bone, adipose, cartilage, lymphogenic and hematopoietic. Many teratomas had extra-embryonic cell types, such as cytotrophoblast, syncytiotrophoblast and yolk sac. The MA06 and MC04 tumors showed a marked reduction in the differentiation spectrum, such as a lack of endoderm derivatives. We did not obtain teratomas from the MA09, MA13, MC07 and MC08 cell lines, possibly due to an insufficient number of experiments. We can conclude that ES cell lines are pluripotent *in vivo* and *ex situ*.

Generation of chimeras

We performed chimerism tests on three ES cell lines (Table 4). Most of the chimeras were alive at 21 days of postnatal development. At this time, chimerism was evaluated using the coat-color pattern. Two chimeras with MA01 cell contribution (male and female), nine (five males and four females) with MA02 cells and three (male and two females) with MA06 cells were found. Evaluation of coat-color chimerism ranged from 1 to 90 %, and the level of chimerism for the

Table 3 Cell types in ES cell-derived teratomas and teratocarcinomas

ES cell line	Number of experiments	Number of tumors	Number of embryonic cell types	Germ layers
MA01 ^a	28	13	12	ect, end, mes
MA02	3	2	11	ect, end, mes
MA04	3	2	5	ect, mes
MA06	3	2	5	ect, mes; car
MA09	2	–	–	–
MA13	1	–	–	–
MA15	2	2	10	ect, end, mes
MC03	1	1	8	ect, end, mes
MC05	4	1	2	car
MC07	1	–	–	–
MC08	1	–	–	–
MC11	2	2	12	ect, end, mes
MC12	3	2	11	ect, end, mes

ect ectoderm, end endoderm, mes mesoderm, car carcinoma

^a Data from teratoma analysis of five MA01 ES cell line subclones

MA02 cell chimeras was high (30, 50, 80, 90, 90, 90, 90, 90 and 90 % in the individual chimeras). Of the 14 chimeras, 6 had developmental defects, such as anomalies of the axial skeleton and/or skull, microphthalmia and hermaphroditism. However, most of the chimeras had a normal phenotype.

We tested germ line transmission for all of the chimeras. The chimeras were mated to the parental genotypes, C57BL/6J and 1B. Seven of the 14 chimeras had no progeny. One of the possible explanations for infertility is general physical underdevelopment with inherited abnormalities, and such chimeras die early because of kidney, lung or heart failures. A second reason is genuine hermaphroditism, gonadal dysgenesis and/or ovotestis, and such animals live long but are sterile. Four animals were produced from chimeras with MA01-cell contribution, 387 with MA02 and 131 with MA06. Therefore, we have not shown germ-line transmission.

Discussion

We have produced 32 ES cell lines with 40 % efficiency, which is comparable to the expected results (Bryja et al. 2006). Colonies had typical, ES cell

morphology and were positive for the following pluripotency marker genes: *Nanog*, *Oct4*, *Sox2*, *Rex1* and *Esg1*. We used strain ICR feeder cells for ES cell maintenance as primary fibroblasts from outbred mice are superior to those from inbred lines (Boheler 2003). Mycoplasma contamination could lead to chromosome abnormalities; however, all ES cell lines were negative for Mycoplasma DNA.

The cytogenetic analysis of early passages (less than 10) showed considerable variability in chromosome number. Of the 32 ES cell lines, 17 (53 %) had more than 50 % diploid cells. These results indicate that these lines are suitable for future use; however, 8 of them had from 12 to 75 % of cells with visible chromosomal rearrangements, making their contribution to germ lines unlikely. It was previously shown that ES cells should have at least 50 % normal diploid karyotypes to contribute to a chimeric animal germ line (Longo et al. 1997; Sugawara et al. 2006). Of our cell lines, 9 complied with this criterion (Table 1). Other researchers prefer to use ES cells with higher diploid cell content (Nichols et al. 1990). Moreover, to produce ES cell-derived mice using tetraploid complementation, more than 90 % of the cells should have a normal karyotype (Eggan et al. 2001; Wang and Jaenisch 2004).

Nearly one-third of the ES cell lines examined had chromosome 8 trisomy. Other researchers have also noted a high frequency of this karyotype abnormality (Liu et al. 1997; Park et al. 1998; Sugawara et al. 2006; Ben-David and Benvenisty 2012). Chromosome 8 trisomy does not decrease the differentiation potential of ES cells (Park et al. 1998) but blocks germ-line transmission in chimeras (Liu et al. 1997). Ben-David and Benvenisty (2012) have recently shown 4 chromosomal abnormality “hot spots”: chromosome 8 and 11 trisomy and chromosome 10 and 14 deletions in the 10qB and 14qC–14qE regions, respectively. As mentioned previously, we have also observed a high level of chromosome 11 and 14 trisomies. Interestingly, we observed chromosome 1 trisomy in many cells of the ES cell lines, and its high frequency has not been previously reported (Liu et al. 1997; Sugawara et al. 2006; Rebuzzini et al. 2008b).

Many of the cell lines had a high percentage of tetraploid cells. Twenty of the cell lines had more than 10 % tetraploid cells, and two of the cell lines were tetraploid (Table 1). Mantel et al. (2007) suggested that undifferentiated mouse ES cells in vitro are

Table 4 Chimerism

ES cell line	Number of experiments	Number of recipients	Number of blastocysts	Number of descendants [from number of blastocysts (%)]	Number of chimeras [from number of descendants (%)]
MA01	3	6	59	10 (16.9)	2 (20)
MA02	5	13	114	30 (26.3)	9 (30.0)
MA06	1	2	22	8 (36.4)	3 (37.5)

tolerant to polyploidy. After the mitotic-spindle checkpoint, aneuploid somatic cells initiate apoptosis, and pluripotent cells continue division. As a result, pluripotent cell culture accumulates polyploidy cells over time.

Imprinting alterations in the *Dlk1-Dio3* locus leads to developmental anomalies (da Rocha et al. 2008). The methylation status of the IG-DMR within the *Dlk1-Dio3* locus was correct in 14 of the ES cell lines. However, in four lines, this region was hypomethylated. Because the methylation status analysis was performed at early passages, we conclude that this alteration could have happened as early as at the blastocyst stage. Previously, hypermethylation of the *Dlk1-Dio3* IG-DMR in pluripotent cells was shown only in iPS cells and not in ES cells (Stadtfield et al. 2012). In a previous study, we showed that in another experimental model, fibroblast-ES hybrid cells, imprinting remains intact (Battulin et al. 2012). We could not find a correlation between imprinting status and ES cell line genotype or sex chromosome composition. In vivo pluripotency tests, teratoma assays and chimera formation were all successful in the MA06 ES cell line, which had a hypomethylated *Dlk1-Dio3* imprinting control region. Thus, we cannot conclude whether its hypomethylation influenced pluripotency or other ES cell properties.

We have produced 32 pluripotent ES cell lines, and cytogenetic analysis revealed karyotype instability in early passages. Our data show that to use ES cells in various experiments, routine cytogenetic analysis is a prerequisite, and, if necessary, subcloning should be performed. A significant number of ES cell lines had altered imprinting of the IG-DMR within the *Dlk1-Dio3* locus. Therefore, we suggest that *Dlk1-Dio3* methylation analysis should be included in basic ES cell pluripotency tests.

Acknowledgments This work was supported by state project N. VI.60.1.3 of Russian Academy of Sciences.

References

- Battulin NR, Khabarova AA, Boyarskikh UA, Menzorov AG, Filipenko ML, Serov OL (2012) Reprogramming somatic cells by fusion with embryonic stem cells does not cause silencing of the *Dlk1-Dio3* region in mice. *World J Stem Cells* 4:87–93
- Ben-David U, Benvenisty N (2012) High prevalence of evolutionarily conserved and species-specific genomic aberrations in mouse pluripotent stem cells. *Stem Cells* 30:612–622
- Boheler KR (2003) ES cell differentiation to the cardiac lineage. In: Wassarman PM, Keller GM (eds) *Differentiation of embryonic stem cells*, vol 365. Academic Press, USA, p 233
- Bryja V, Bonilla S, Cajánek L, Parish CL, Schwartz CM, Luo Y, Rao MS, Arenas E (2006) An efficient method for the derivation of mouse embryonic stem cells. *Stem Cells* 24:844–849
- Choppa PC, Vojdani A, Tagle C, Andrin R, Magtoto L (1998) Multiplex PCR for the detection of *Mycoplasma fermentans* *M. hominis* and *M. penetrans* in cell cultures and blood samples of patients with chronic fatigue syndrome. *Mol Cell Probes* 12:301–308
- Cockburn K, Rossant J (2010) Making the blastocyst: lessons from the mouse. *J Clin Invest* 120:995–1003
- da Rocha ST, Edwards CA, Ito M, Ogata T, Ferguson-Smith AC (2008) Genomic imprinting at the mammalian *Dlk1-Dio3* domain. *Trends Genet* 24:306–316
- Eggan K, Akutsu H, Loring J, Jackson-Grusby L, Klemm M, Rideout WM III, Yanagimachi R, Jaenisch R (2001) Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proc Natl Acad Sci USA* 98:6209–6214
- Eggan K, Rode A, Jentsch I, Samuel C, Hennek T, Tintrup H, Zevnik B, Erwin J, Loring J, Jackson-Grusby L, Speicher MR, Kuehn R, Jaenisch R (2002) Male and female mice derived from the same embryonic stem cell clone by tetraploid embryo complementation. *Nat Biotechnol* 20:455–459
- Evans M, Kaufman M (1981) Establishment in culture of pluripotent cells from mouse embryos. *Nature* 292:154–156
- Guo J, Jauch A, Heidi HG, Schoell B, Erz D, Schrank M, Janssen JW (2005) Multicolor karyotype analyses of mouse embryonic stem cells. *In Vitro Cell Dev Biol Anim* 41:278–283
- Hogan B, Beddington R, Constantini F, Lacey E (1994) *Manipulating the mouse embryo*, 2nd edn. Cold Spring Harbor Laboratory Press, New York, p 497
- Li X, Wei W, Yong J, Jia Q, Yu Y, Di K (2005) The genetic heterozygosity and fitness of tetraploid embryos and

- embryonic stem cells are crucial parameters influencing survival of mice derived from embryonic stem cells by tetraploid embryo aggregation. *Reproduction* 130:53–59
- Liu X, Wu H, Loring J, Hormuzdi S, Disteché CM, Bornstein P, Jaenisch R (1997) Trisomy eight in ES cells is a common potential problem in gene targeting and interferes with germ line transmission. *Dev Dyn* 209:85–91
- Longo L, Bygrave A, Grosveld FG, Pandolfi PP (1997) The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimaerism. *Transgenic Res* 6:321–328
- Mantel C, Guo Y, Lee MR, Kim MK, Han MK, Shibayama H, Fukuda S, Yoder MC, Pelus LM, Kim KS, Broxmeyer HE (2007) Checkpoint-apoptosis uncoupling in human and mouse embryonic stem cells: a source of karyotypic instability. *Blood* 109:4518–4527
- Martin G (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 78:7634–7638
- Nagy A, Gocza E, Diaz EM, Prideaux VR, Ivanyi E, Markkula M, Rossant J (1990) Embryonic stem cells alone are able to support fetal development in the mouse. *Development* 110:815–821
- Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci USA* 90:8424–8428
- Nichols J, Evans EP, Smith AG (1990) Establishment of germline-competent embryonic stem (ES) cells using differentiation inhibiting activity. *Development* 110:1341–1348
- Park JI, Yoshida I, Tada T, Takagi N, Takahashi Y, Kanagawa H (1998) Differentiative potential of a mouse parthenogenetic embryonic stem cell line revealed by embryoid body formation in vitro. *Jpn J Vet Res* 46:19–28
- Pristiazhniuk IE, Matveeva NM, Grafodatskii AS, Serdiukova NA, Serov OL (2010) Analysis of chromosome composition in interspecific embryonic stem hybrid cells of mice. *Tsitologiia* 52:136–143
- Rebuzzini P, Neri T, Mazzini G, Zuccotti M, Redi CA, Garagna S (2008a) Karyotype analysis of the euploid cell population of a mouse embryonic stem cell line revealed a high incidence of chromosome abnormalities that varied during culture. *Cytogenet Genome Res* 121:18–24
- Rebuzzini P, Neri T, Zuccotti M, Redi CA, Garagna S (2008b) Chromosome number variation in three mouse embryonic stem cell lines during cell culture. *Cytotechnology* 58:17–23
- Robertson EJ (1987) *Teratocarcinomas and embryonic stem cells: a practical approach*. IRL Press, Oxford, p 268
- Schroeder IS, Sulzbacher S, Truong TT, Blyszczuk P, Kania G, Wobus AM (2009) Insulin-producing cells from mouse embryonic stem cells. In: Lanza R, Klimanskaya I (eds) *Essential stem cell methods*, 1st edn. Academic Press, USA, p 490
- Smith AG (2001) Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol* 17:435–462
- Stadtfeld M, Apostolou E, Akutsu H, Fukuda A, Follett P, Natesan S, Kono T, Shioda T, Hochedlinger K (2010) Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. *Nature* 465:175–181
- Stadtfeld M, Apostolou E, Ferrari F, Choi J, Walsh RM, Chen T, Ooi SS, Kim SY, Bestor TH, Shioda T, Park PJ, Hochedlinger K (2012) Ascorbic acid prevents loss of Dlk1-Dio3 imprinting and facilitates generation of all-iPS cell mice from terminally differentiated B cells. *Nat Genet* 44:398–405
- Sugawara A, Goto K, Sotomaru Y, Sofuni T, Ito T (2006) Current status of chromosomal abnormalities in mouse embryonic stem cell lines used in Japan. *Comp Med* 56:31–34
- Takahashi K, Okita K, Nakagawa M, Yamanaka S (2007) Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc* 2:3081–3089
- Wang Z, Jaenisch R (2004) At most three ES cells contribute to the somatic lineages of chimeric mice and of mice produced by ES-tetraploid complementation. *Dev Biol* 275:192–201