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# Chromosome Instability in mouse Embryonic Stem Cells

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**Embryonic Stem Cells (ESCs) are expected to show a stable euploid karyotype, but in the last decade (sub)chromosomal aberrations have been systematically described in these cell lines when maintained *in vitro*. Culture conditions and long-term culture have been traditionally proposed as possible factors involved in the acquisition of chromosomal abnormalities. Thus, we analyzed the chromosome constitution, the undifferentiated state and the functional pluripotency of three different mouse ESCs grown under the same culture conditions. Two cell lines were unstable from early passages, whereas the third one retained its chromosome integrity after long-term culture despite using enzymatic methods for cell disaggregation. Trisomy 8 and 11 were clonally selected in both unstable cell lines, which also showed a higher growth rate than our normal cell line and suffered morphological changes in colony shape with increasing passage number. Regardless of the length of culture or the chromosome instability, all cell lines preserved their differentiation potential. These results confirm that double trisomy 8 and 11 confers a growth advantage to the abnormal cells, but not at the expense of cell differentiation. The presence of chromosome instability, widely related to tumor development and cancer disease, highlights the risk of using pluripotent cells in regenerative medicine.**

ESCs were first isolated from the Inner Cell Mass (ICM) of mouse preimplantation blastocysts in the early 80 s[1,2] and were defined as pluripotent, self-renewal cell lines with a normal and stable karyotype[1,2]. The characterization of mouse ESCs (mESCs) as euploid cell lines was crucial, because until then, only one type of pluripotent cells had been successfully established *in vitro*, the Embryonal Carcinoma Cells, with the disadvantage that most of them are chromosomally unstable[1].

Initially, mESCs lines were considered a good model for *in vitro* studies of early development[3,4]. Soon after and linked to the improvement of embryo micromanipulation methods, research was focused on germ-line transmission and knock-out mouse generation[5,6]. However, cell-therapy and regenerative medicine based on *in vitro* ESC differentiation became one of the most important goals of this field when human ESCs (hESCs) were successfully established[7].

The first studies describing chromosomal abnormalities in mESC lines were published in the 90 s. The authors suggested a correlation between chromosomal abnormalities, long-term culture and inefficiency in contributing to the germ-line in adult chimaeras[8–10]. Unfortunately, most of these results went unnoticed, and researchers continued culturing ESCs without checking their chromosome integrity, in part, because the ESCs had been defined as cell lines with a normal karyotype.

The presence of chromosomal abnormalities in mouse and human ESCs started to be systematically described in the mid 2000s, and most of them appear in a recurrent manner. Trisomy 8 and trisomy 11 are frequent in mESCs[9,11–13] whereas trisomy 12, 17 (which is partially syntenic with mouse chromosome 11[14]) and amplifications of 20 p are typical of hESCs[15–22]. All these changes have been reported to confer a growth advantage[15–19,21–23] at the expense of cell differentiation[16,18,19,23], as a result of the unbalanced dosage of key genes involved in self-renewal, differentiation or cell-cycle regulation. Extended culture of ESCs has also been related to mitochondrial DNA mutations and epigenetic changes[17]. In this sense, the epigenetic state of different mES cell lines and among different subclones derived from the same mESC has been described as extremely unstable[24].

The causes promoting the acquisition of chromosomal abnormalities have been traditionally associated with the *in vitro* culture methods, such as i) long-term culture[15–17,20], ii) *in vitro* oxygen tension[25], iii) adaptive pressure to culture conditions[19,23], or iv) mechanical/enzymatic methods for cell detaching[18,19,22,26]. However, many pluripotent stem cells (PSCs) preserve their chromosome integrity under these conditions. Cowan and colleagues[15] derived 17 hESCs reporting chromosomal abnormalities in only two cell lines after extended culture despite using enzymatic dissociation. Adaptation to feeder-free systems do not always yield chromosomal abnormalities[19], and chromosome alterations using mechanical methods as early as passage (p)19 have been reported, when other cell

lines are normal after more than 100 passages in the same conditions[26]. Therefore, it seems that culture methods do not fully explain the presence of chromosome abnormalities in ESCs, and actually, it has been recently proposed that some or even all PSC lines are prone to accumulate genomic damage as an inherent property[19,27,28].

Thus, the aim of this work was to analyze the chromosomal integrity of three different mESCs during their maintenance *in vitro* and to assess the correlation between chromosomal abnormalities, long-term culture and a reduced pluripotency. All cell lines were grown under the same culture conditions using enzymatic passage methods, and pluripotency was analyzed both at the undifferentiated state as during the spontaneous differentiation into derivatives of the three germ layers.

## Results

In this study three different mESC lines, CMT, J1 and E14 (provided at p9, p13 and p26, respectively) were analyzed at different time points. To simplify the results, we have established three groups depending on the culture time: early (less than p22), middle (between p23–p33) and late (more than p34).

The CMT cell line retained a euploid modal karyotype after extended culturing. The percentage of cells with normal constitution (40 acrocentric chromosomes) at early (p16), middle (p24) and late passages (p40) was 72, 52.1 and 58.3%, respectively (Fig. 1A and Table 1). In all time points, the second population most represented had 39 chromosomes with a frequency ranging from 14–25% of the cells analyzed. In addition, a normal morphology was observed regardless of the culture time. At early and late passages, the CMT colonies showed the typical rounded and homogeneous shape with well-defined edges (left column of Fig. 2A) and the spliting ratio (1 : 5 twice a week) did not change throughout the study.

In contrast, J1 was an aneuploid cell line with 55% of the cells showing 41 chromosomes at the first passage (p11) analyzed (Fig. 1C and Table 1). Since this result was unexpected, additional passages were analyzed in all groups (early, middle and late) to accurately monitor the chromosome constitution of this cell line. The modal number of 41 chromosomes was maintained up to passage 30, although the percentage of these cells was decreasing over time (48.9, 51, 46.9 and 39.2%, at p17, p20, p25 and p30, respectively). A transition phase characterized by an almost trimodal distribution was observed at p35, where metaphases with 40 chromosomes were the most frequent (26.4%), followed very closely by cells with 41 and 43 chromosomes (24.5% each one). Finally, at p40, the modal number increased to 43 chromosomes (30.9% of the cells), while metaphases with either 40 or 41 chromosomes decreased to less than 20% of the cell population. Morphologically, the colony shape of J1 mESCs was abnormal at late passages, and its occurrence coincided with the increment of chromosomal abnormalities. At early passages, the cells formed thick and rounded-shaped colonies, but became flattened with an irregular shape and diffuse edges at late passages (middle column of Fig. 2A). Despite this abnormal shape, no proliferating changes were detected. Cells were always subcultured 1 : 10, twice a week.

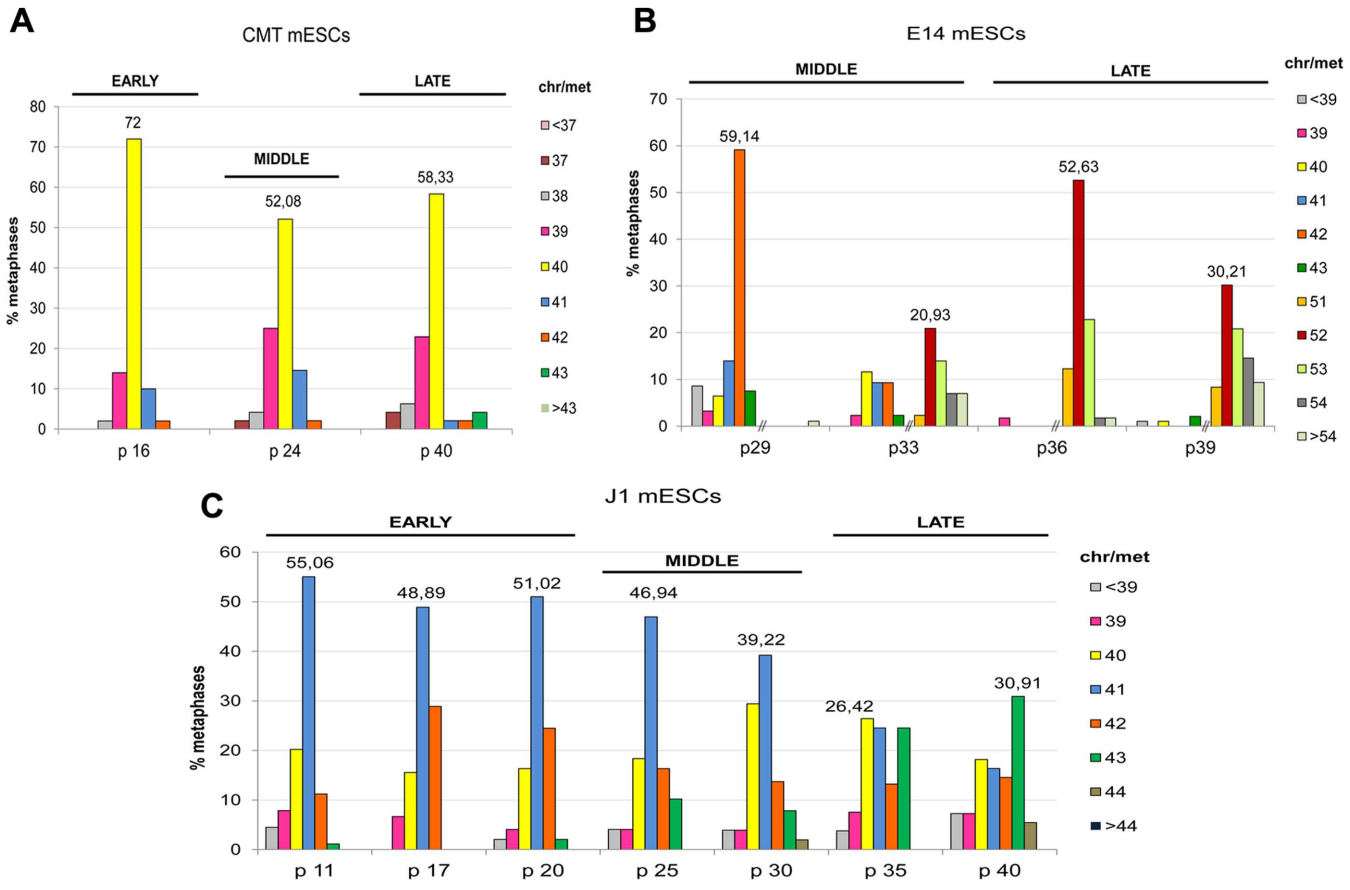


**Figure 1 | Modal karyotype of CMT, E14 and J1 mESCs.** Chromosome counting of three mESCs at early (<22 p), middle (23–33 p) and late passages (>34 p). (A) More than 50% of the CMT cells had a normal chromosome number of 40 acrocentric chromosomes at all different time points. (B) E14 mESCs were an aneuploid and unstable cell line. The modal karyotype increased from 42 (p29) to 52 chromosomes in just four passages (from p29 to p33) leveling off in this number at late passages. Populations with a modal karyotype ranging from 44 to 50 chromosomes are not represented in the figure. This gap is shown as//in the graph. (C) J1 mESCs were also an aneuploid cell line (55% of the cells had 41 chromosomes at p11) and retained this modal karyotype until late passages, when the modal number increased up to 43 chromosomes.

Table 1 | Chromosome counts of CMT, E14 and J1 mESCs at different time points

| | | chromosome counting | | | | |
|---|---|---|---|---|---|---|
| cell line | stage | passage number | modal number | n | modal cells n (%) | euploid cells n (%) |
| CMT | E | p16 | 40 | 50 | 36 (72) | 36 (72) |
| | M | p24 | 40 | 48 | 25 (52) | 25 (52) |
| | L | p40 | 40 | 48 | 28 (58.3) | 28 (58.3) |
| J1 | E | p11 | 41 | 89 | 49 (55) | 18 (20.2) |
| | | p17 | 41 | 45 | 22 (48.9) | 7 (15.6) |
| | | p20 | 41 | 49 | 25 (51) | 8 (16.3) |
| | M | p25 | 41 | 49 | 23 (46.9) | 9 (18.4) |
| | | p30 | 41 | 51 | 20 (39.2) | 15 (29.4) |
| | L | p35 | 40 | 53 | 14 (26.4) | 14 (26.4) |
| | | p40 | 43 | 55 | 17 (30.9) | 10 (18.2) |
| E14 | M | p29 | 42 | 93 | 55 (59.1) | 6 (6.5) |
| | | p33 | 52 | 43 | 9 (20.9) | 5 (11.6) |
| | L | p36 | 52 | 57 | 30 (52.6) | 0 (0) |
| | | p39 | 52 | 96 | 29 (30.2) | 1 (1) |

Abbreviations: E, early passages (less than p22); M, middle passages (between p23–p33); L, late passages (more than 34 p); n = number of cells.

E14 mESC was also an aneuploid cell line; almost 60% of the metaphase spreads had 42 chromosomes at the first analysis (p29) whereas the euploid population represented less than 7% of the cells (Fig. 1B and Table 1). Therefore, and as it has been explained for the J1 cell line, we closely analyzed the chromosome constitution of E14 mESCs. Only four passages later (p33), the modal number increased from 42 to 52 chromosomes (20.9% of the cells), remaining stable at 52 chromosomes at late passages (52.6 and 30.2% at p36 and p39, respectively). The morphological changes in this cell line were more remarkable. During the first passages, colonies remained compact and thick despite presenting irregular edges, but most of them became totally flat and almost undistinguishable from feeder layers at late passages (right column of Fig. 2A). In addition, these morphological changes appeared together with a higher proliferation ability, since subcultures (1 : 10) increased from twice to three times a week.

Statistical analysis showed that there were no significant differences in the number of cells with more than 40 chromosomes in CMT mESCs when early, middle and late passages were compared,

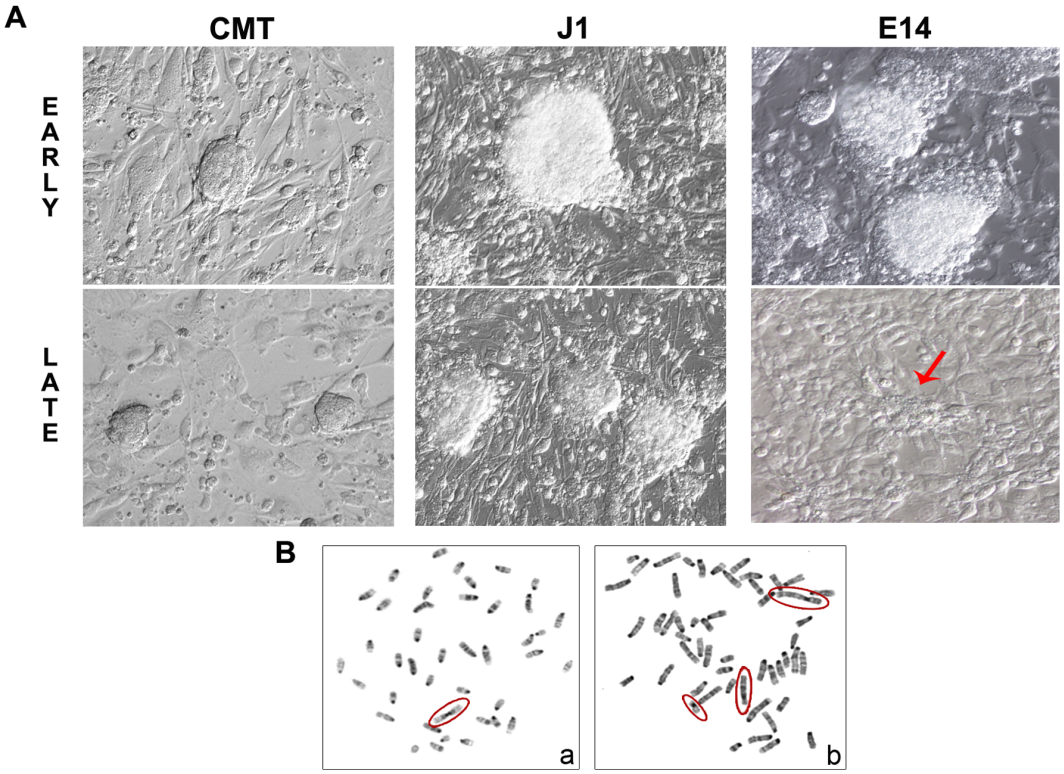


**Figure 2 | Morphological changes in colony shape and chromosome abnormalities in mESCs.** (A) Colonies from the CMT cell line showed a normal shape throughout the study, whereas J1 colonies presented irregular edges at late passages. In E14 mESCs, the colony shape was irregular during the first passages and became almost invisible in later passages (red arrow). Magnification: 10×. (B) Metaphase spreads showing (in red) a Robertsonian translocation in J1 mESC (a) and different chromosomes with structural reorganizations in the E14 cell line (b).

Table 2 | Karyotypes from J1 mESCs at early (p11) and middle (p25) passages by mFISH

| nº cells (n = 12) | Karyotype p11 | nº cells (n = 15) | Karyotype p25 |
|---|---|---|---|
| 1 | 38,XY,−5,+8,−9,+Rb(11.11),−16 | 1 | 39,OY,Del(4),+Rb(11.11) |
| 1 | 39,XY,−3,+Rb(11.11) | 1 | 39,OY,−3,+Rb(6.6),+8,+10,+Rb(11.11),+12,−14,−15 |
| 1 | 39,XY,+8,Rb(8;12),+Rb(11.11),−16 | 1 | 40,XO,+8,+Rb(11.11) |
| 1 | 40,XY,+8,Rb(11.11) | 1 | 40,OYY,Rb(Y.Y),+6,+8,+Rb(11.11),+12,−13,Del(14)−17[2] |
| 1 | 40,XO,+8,+Rb(11.11) | 3 | 41,XY,+8,+Rb(11.11) |
| 2 | 41,XY,+8,+Rb(11.11) | 1 | 41,XY,Del(3)+6,+Rb(11.11) |
| 1 | 41,XO,+8,+Rb(11.11),+18 | 1 | 41,XY,+4,+8,Rb(11.11),Rb(15.15),+19 |
| 1 | 41,XY,+8,+9,+Rb(11.11),−17 | 1 | 41,XY,Del(4),+8,+Rb(11.11),+15,−16,−16,+18 |
| 1 | 41,XY,+Y,−3+8,+Rb(11.11)[1] | 1 | 42,XY,+8,+Rb(11.11),+15 |
| 1 | 41,XY,−3,+8,+Rb(11.11),+13,+16−18 | 1 | 42,XY,+8,+Rb(11.11),+18 |
| 1 | 42,XY,+Y,−6,+8,+Rb(11.11),−14,+15 | 1 | 42,XYY,Rb(Y.Y),+1,−6,+Rb(11.11),+15,+18 |
| | | 1 | 42,XY,−5,+8,+10,+Rb(11.11),+15 |
| | | 1 | 43,XY,−6,+8,Rb(11.11),+14,+14,+15,+17 |

Nomenclature based in the **MGI**= Mouse Genome Informatics http://www.informatics.jax.org/mgihome/nomen/anomalies.shtml
[1]Fig. 3A.
[2]Fig. 3B.

whereas the J1 and E14 cell lines significantly incremented the number of chromosomes with increasing the culture time (Table 1 of Supplementary Information).

Chromosome counts not only revealed numerical abnormalities, but also structural aberrations in both J1 and E14 mES cell lines. Most of the metaphase spreads of J1 mESCs systematically showed one or two Robertsonian (Rb) translocations (centric fusions of acrocentric chromosomes), and different reorganizations were also observed in the E14 mES cell line (Fig. 2B). However, CMT cells did not show any structural rearrangement after prolonged culture. To identify the chromosomes involved in the recurrent Rb translocations observed in J1 mESCs, mFISH was used to karyotype this cell line at early and middle passages (p11 and p25, respectively). The results showed that J1 mESCs were highly unstable from early passages (Table 2), since only 2 out of the 12 cells analyzed at p11 shared the same karyotype, specifically, 41,XY,+8,+Rb(11.11). Homologous Rb translocation of chromosome 11 (Rb(11.11)) was detected in 100% of the metaphases, forming an unbalanced structure (trisomy 11) in 91.7% of the cells (+Rb(11.11)). Trisomy 8 and the double trisomy 8 and 11 were also found in a high percentage (83.3 and 75% of the cells, respectively) and other different trisomies and monosomies were observed, but in a low frequency (Fig. 3A).

The results were similar at passage 25 (Fig. 3B, Table 2); only 3 out of the 15 metaphases analyzed showed the karyotype 41,XY,

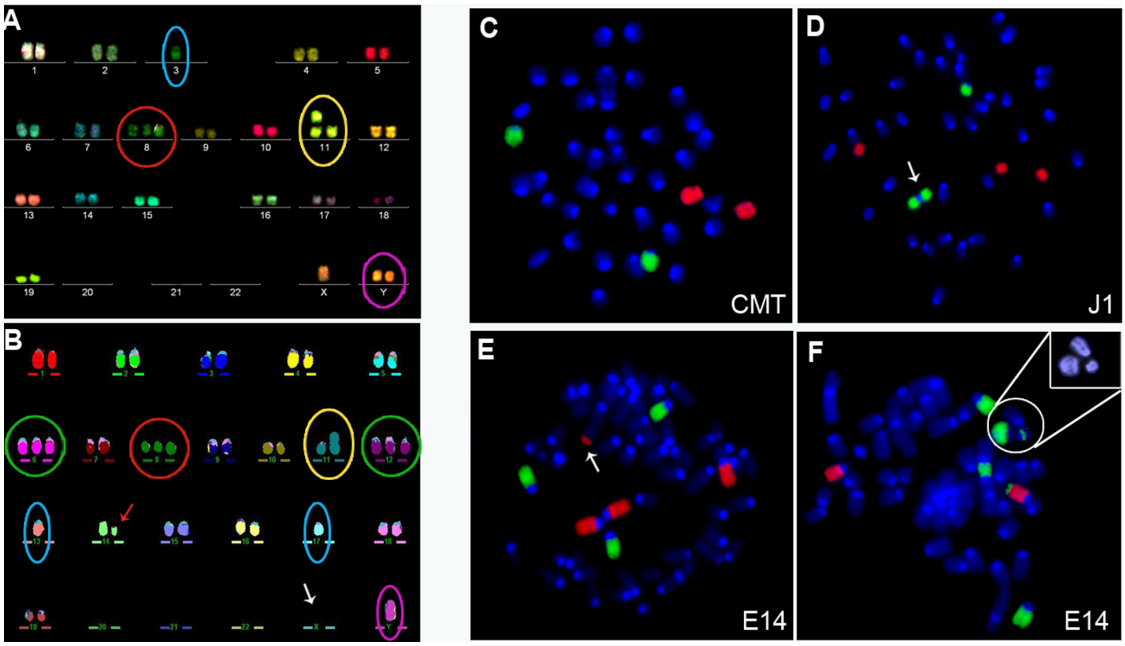


**Figure 3 | mFISH and chromosome painting analysis.** (A), (B) mFISH showing numerical and structural chromosomal abnormalities in J1 mESCs at p11 and p25, respectively. Yellow circle; trisomy 11 formed by one chromosome 11 and a homologous Robertsonian (Rb) translocation of chromosome 11. Red circle; trisomy 8. Green circle; other trisomies. Blue circle; monosomies. Purple circle; two copies of chromosome Y as single chromosome (A) or forming a Rb translocation (B). Red arrow; partial deletion. White arrow; loss of X chromosome. (C–F) Chromosome painting at late passages. Chromosome 8 in red, chromosome 11 in green, and DAPI counterstain in blue. (C) Normal euploid metaphase from CMT line with two copies of chromosomes 8 and 11. (D) Double trisomy 8 and 11 in J1 mESCs. Arrow: Rb translocation of chromosome 11. (E) Double trisomy 8 and 11 from E14 cell line with a short duplication of chromosome 8 (arrow). (F) Metaphase spread from the E14 cell line with a complex karyotype: a Rb translocation of chromosome 11 forming a ring chromosome, a derivative chromosome 8 (unbalanced translocation 8;11) and extra fragments of chromosome 11.

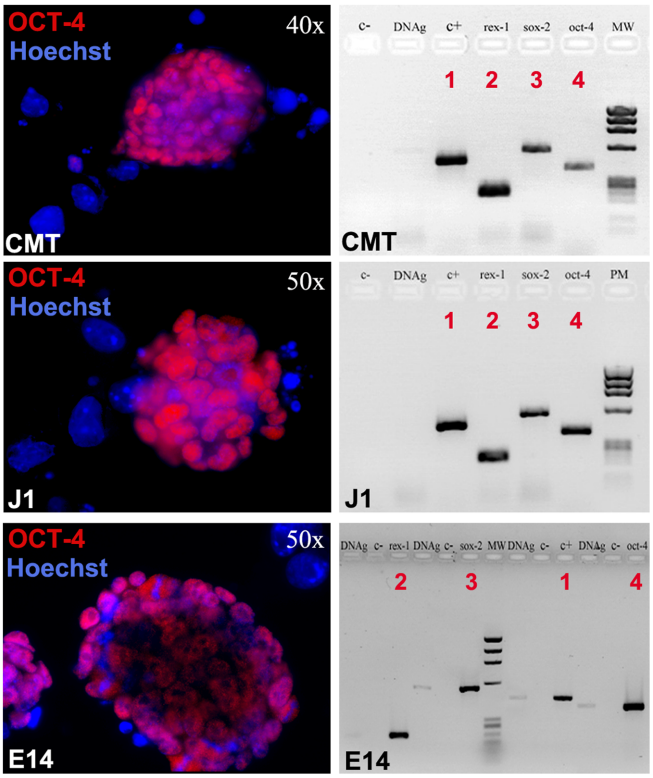


**Figure 4 | Expression pattern of stemness markers in undifferentiated cells at late passages by immunofluorescence and RT-PCR.** All three mES cell lines expressed the nuclear transcription factor OCT-4 (left column) and the stemness markers *Oct-4, Sox-2* and *Rex-1* at late passages (right column). **1:** *Gapdh.* **2:** *Rex-1.* **3:** *Sox-2.* **4:** *Oct-4.* **MW:** ØX174. Negative controls (C-: non template control. DNAg: DNA contamination control).

+8,+Rb(11.11). Homologous Rb translocation of chromosome 11, trisomy 11 and trisomy 8 were again the most frequent anomalies detected, with percentages of 100, 86.7 and 80%, respectively. More than 66% of the cells presented the double trisomy 8 and 11, and other structural aberrations such as Rb translocations of chromosomes 6, 15 or Y, or chromosomal arm deletions were detected.

Considering that only trisomy 8 and 11 appeared in a high percentage when the J1 mES cell line was karyotyped by mFISH, we exclusively focused our work on studying the copy number of these particular chromosomes in all cell lines using specific painting probes. In this sense, 100% of the CMT mESCs had two copies of each chromosome at early and late passages, and no rearrangements were detected at any time (Fig. 3C). In J1 mESCs, the double trisomy 8 and 11 had been finally fixed in the whole population (Fig. 3D), since 100% of the cells had three copies of both chromosomes at late passages (chromosome 11 was always involved in a homologous Rb translocation). In E14 mESCs, trisomy 8 was found in 95% of the cells, of which 21% also showed an extra short fragment of this chromosome forming an unbalanced translocation (Fig. 3E). Partial or total duplications of chromosome 11 were detected in 100% of the cells (Figs. 3E and 3F), specifically trisomy 11 (20%), gains of at least one extra fragment of chromosome 11 (50%), or both abnormalities together in the same metaphase spread (30%).

The next purpose was to assess if chromosome instability or long-term culture could affect the mESCs pluripotency. The expression of the *Oct-4, Sox-2* and *Rex-1* stemness markers by RT-PCR at early and late passages (right column of Fig. 4) demonstrated that all mES cell lines maintained their undifferentiated and self-renewal condition during long-term culture. Immunofluorescence detection of the OCT-4 protein confirmed these results. In all three mESC lines, the OCT-4 labeling was restricted to ES cells, while feeder cells were only counterstained with Hoechst (left column of Fig. 4). However, the expression of stemness markers only reflects the undifferentiated state, but does not demonstrate a functional pluripotency. Therefore, we induced the formation of Embryoid Bodies (EBs) (*in vitro* cell clumps recapitulating the early events of embryogenesis) by culturing the cells in non-adherent dishes without feeder cells. As shown in Figure 5A, all cell lines were able to grow in suspension and to form rounded cell clusters. To verify that ESCs could generate differentiated cells from the three germ layers, the expression pattern of ecto, meso and endodermal markers (nestin, SMA and AFP, respectively) was assessed in spontaneously differentiated EB-derived cells from J1 (as unstable cell line) and CMT (as a control) at early and late passages. These results (Fig. 5B) demonstrated that both cell lines were pluripotent, regardless of the length of culture or the chromosome instability.

## Discussion

PSCs have an extraordinary potential to be used in cell-therapy and regenerative medicine but, as any other cell type in culture, they may suffer genomic alterations when maintained in culture. Thus, it is essential that these cells retain their genetic and epigenetic integrity for clinical applications, but it has been widely reported that PSCs acquire different abnormalities *in vitro*, becoming in some cases, unstable cell lines[29]. Chromosomal instability, which includes numerical (aneuploidy) and/or structural chromosome changes, is a common characteristic of tumor cells[30], and occurs when the mutational burden accumulated by the cells increases over time at a higher rate than in normal cells[31]. This is noteworthy because there is a tendency to talk about chromosomal instability after a punctual assessment without i) monitoring the karyotype over time, and ii) taking into account that some alterations are stable, for instance Down syndrome.

Therefore, we analyzed the chromosome constitution of three mESCs at different time points during their long-term culture. The first passage analyzed in all three mESC lines showed that the CMT cell line had a normal karyotype of 40 acrocentric chromosomes, while J1 and E14 mESCs were aneuploid lines with a modal karyotype of 41 and 42 chromosomes, respectively. The CMT cell line, despite showing a high percentage of aneuploid cells at middle passages, maintained its normal modal karyotype over time whereas unexpectedly, both aneuploid mESCs were also chromosomally unstable.

Chromosome number characterization was sufficient to detect the chromosomal instability of E14 mESCs, as the modal karyotype increased drastically in just two weeks of culture (up to 52 chromosomes between p29–p33). In contrast, using the same method, the J1 mESC instability was overlooked at early and middle passages, and it was only suspected when the modal number increased to 43 chromosomes at late passages. Although the chromosome constitution seemed to change above p30, mFISH analysis revealed a strong heteroploid pattern in this cell line as early as p11, reflecting the persistent generation of new chromosomal variants and, therefore, demonstrating that it was chromosomally unstable from early passages. This result suggests that: i) there is not a safe passage number preserving the chromosome integrity and ii) chromosome counting is not an appropriate method to test aneuploidy, since different cells can suffer distinct numerical alterations maintaining the total number of chromosomes.

Trisomies 8 and 11 were the most frequent abnormalities detected in J1 mESCs by mFISH, and the mosaicism for double trisomy 8 and 11 increased over time, reaching 100% at late passages. When the copy number of these chromosomes was analyzed in E14 mESCs by chromosome painting, trisomy 8 together with total or partial trisomy of chromosome 11 was detected in 95% of the cells. These results suggest a clonal selection of the double trisomy 8 and 11 conferring a growth advantage to these cells, and in fact, the passaging frequency

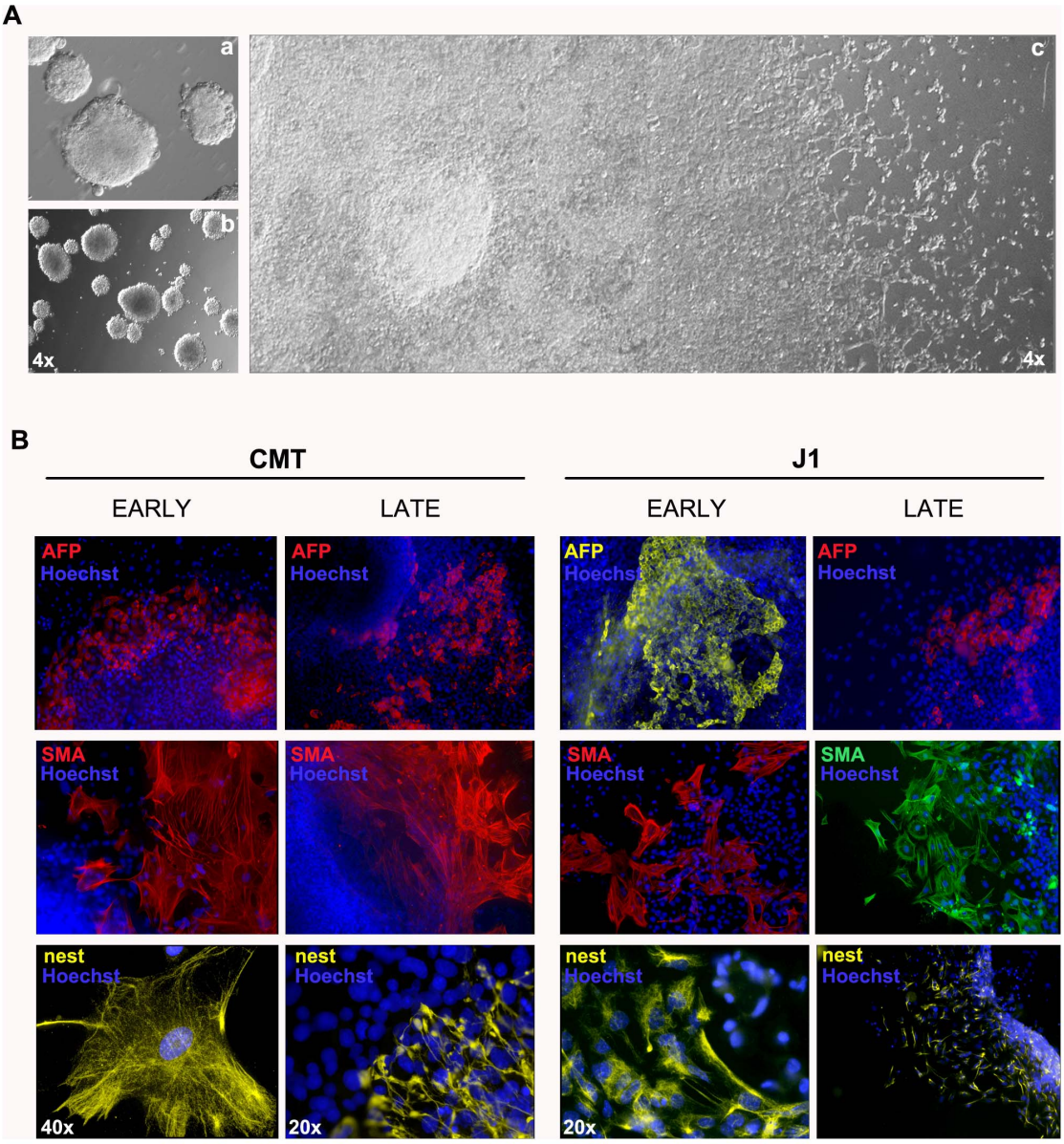


**Figure 5** | **Spontaneous differentiation of mESCs.** EBs from all cell lines (A) and inmunofluorescence assays of differentiation markers in J1 and CMT cell lines at early and late passages (B). (a), (b) EBs in bacteriological dishes from J1 and E14 cell lines, respectively. (c) Cell migration and differentiation from attached EBs obtained from the CMT cell line (two images overlapped at 4× magnification). (B) CMT and J1 cell lines (chromosomally stable and unstable, respectively) maintained a functional pluripotency throughout the study. Both mESCs expressed markers of the three germ layers after at least 10 days of spontaneous differentiation. **AFP**: Alpha Fetoprotein, endodermal marker. **SMA**: alpha Smooth Muscle Actin, mesodermal marker. **Nest**: Nestin, ectodermal marker. A and B magnification:10×. Other magnifications are shown on the pictures. Images obtained from independent experiments.

of E14 mESCs increased from twice to three times a week at late passages and, the splitting ratio of J1 mESCs was always two-fold higher than the CMT cell line. These findings are consistent with those reported previously. Trisomies 8 and 11 have been reiteratedly described in different mESC cell lines[9,11–13,32] (including J1 and E14 sublines[9,32]) and promote cell proliferation, leading to a faster clonal selection when cells simultaneously carry the double trisomy 8 and 11[11].

But, in contrast to other works reporting a reduced pluripotency of mESCs as a result of the presence of aneuploidy with increasing the culture time[8–10], all three mESCs retained their undifferentiated state (expressed *Oct-4, Sox-2* and *Rex-1*) and were able to form EBs at each time point, suggesting that all cell lines were pluripotent. The expression of ectoderm, mesoderm and endoderm markers in spontaneously differentiated cells confirmed that J1 and CMT cell lines maintained a functional pluripotency over time, regardless of the chromosome instability or the length of the culture, but we cannot rule out differences in the expression levels of these genes.

J1 colonies showed less-defined edges with increasing culture time, and most of the E14 mESC colonies became flat and virtually invisible at late passages. Lack of well-defined edges and loss of 3D structure are related to spontaneous cell differentiation and loss of pluripotency[28,33]. Our cell lines were maintained without the addition of Leukemia Inhibitory Factor (LIF) to the culture medium, thus, it is possible that spontaneous differentiation foci emerged at the edges of the colonies. However, the CMT cell line did not suffer morphological changes under the same culture conditions, and we did not detect a decrease in the stemness hallmark genes, nor in the OCT 4 expression. Therefore, the absence of LIF does not fully explain the abnormal colony shape which could be also attributable to

chromosome instability, as these changes appeared only in unstable cell lines at late passages.

We have no data of the chromosome constitution of J1 subline before our first analysis at p11, but the E14 subline used in this work had a normal karyotype once derived[5]. Therefore, E14 mESCs became unstable at early passages (before it was acquired), whereas the CMT cell line retained an euploid chromosomal constitution after extended culture despite using enzymatic dissociation. This suggests that culture methods are not the only factor promoting chromosomal abnormalities, and some authors have proposed that certain ESC lines could be more prone to acquire chromosomal abnormalities than others[19,34]. It is likely that this predisposition to accumulate genomic damage is an inherent property of all PSCs in culture, not only a characteristic of certain cell lines. A recent study has shown that normal PSC lines exhibit a pervasive mosaic non-clonal aneuploidy in a higher rate than other cell types in culture[27]. This particularity has also been reported in pre-implantation embryos and neural progenitor cells[27,35], and we could include in this group the CMT cell line which, in fact, is a mosaic of euploid/aneuploid cells. One explanation of this mosaicism could be the particular cell-cycle regulation of PSCs[28,36–38] which generates an unusual tolerance to genetic abnormalities, and thus, increases the risk of genome instability in vitro. The deep analysis of the abnormal chromosome regions will be essential to better understand the mechanisms of the PSCs' genomic integrity maintenance and the functional consequences of these changes on cell differentiation and malignant potential of the PSCs.

In conclusion, trisomies 8 and 11 are recurrent anomalies in mESCs conferring a growth advantage to the cells but not at the expense of pluripotency. Culture conditions contribute to the selection of fast-growing cells, which may overtake the euploid population in long-term cultures, but the particular ES cell-cycle regulation could be another factor involved in chromosomal abnormalities that should be considered. We have demonstrated that chromosome counting is insufficient to detect chromosome gains and losses within a cell population sharing the same total number of chromosomes, being more suitable to identify each chromosome pair. In addition, morphological changes in colony shape could be a good indicator of chromosomal instability, not only a sign of spontaneous differentiation. The presence of chromosomal instability, largely related to tumor formation, seriously limits the use of PSCs in cell-therapy and regenerative medicine. Therefore, it is crucial to accurately check the genomic integrity of these cell lines because at present, it is impossible to establish a safe passage number threshold.

## Methods

**Cell lines and cell cultures.** Three mouse ES cell sublines, E14TG2a (HPTR-deficient, named in this work E14[5];), J1 (ATCC®SCRC-1010, constitutively expressing GFP. ATCC; Manassas, Virginia, USA) and CMTI-1 mESC (Millipore; Billerica, Massachusetts, USA, and referred to this work as CMT), established from 129/Ola, 129/terSv and 129/SVEV mouse strains, were provided by different laboratories at p 26, 9 and 13, respectively. All mESCs lines were co-cultured onto 0.2% gelatin-coated Petri dishes with 10 μg/ml of mitomycin C (Sigma-Aldrich; Spain) inactivated STO mouse fibroblasts (ECACC; UK) and dissociated enzymatically (trypsin-EDTA) twice a week. The splitting ratio for E14 and J1 mESCs was 1 : 10, whereas the CMT cell line was subcultured in a 1 : 5 ratio to maintain the same replanting frequency in all cell lines. A high glucose DMEMGlutaMAX™-I culture medium supplemented with 15% fetal bovine serum (FBS), 1% β-mercaptoethanol (0.07%) and 1% non-essential amino acids (100×) was used (all from Gibco/Invitrogen; Spain). Cells from all three cell lines were cryopreserved at different passages.

To induce EB formation, cells were detached and cultured in non-adherent bacteriological dishes with mESC medium for 5 days. Then, EBs were transferred onto Petri dishes and once attached, cells were maintained in mESC medium for at least 10 days to allow further spontaneous differentiation. The medium was changed every two days.

**Gene expression by RT-PCR.** Total RNA from mESCs was isolated and purified at early (middle in E14 mESC) and late passages using the RNAgents® Total RNA Isolation System (Promega; Spain) according to the manufacturer's instructions. RNA extraction was performed on ice using RNase-free material to preserve RNA integrity, and once isolated, RNA samples (100 ng/μl) were kept at −20°C until used. cDNA was obtained from 200 ng of RNA in a total volume of 50 μl using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Invitrogen; Spain), which includes random primers, dNTPs and reverse transcriptase. For the polymerase chain reaction (PCR), 2.5 μl of cDNA was used in a final volume of 50 μl containing 200 μM dNTPs, 1.5 Mm $MgCl_2$, 0.025 U/μl of EcoTaq polymerase (all from Ecogen; Spain) and 0.4 μM of specific primers (Roche; Spain). Four genes were amplified using a standard protocol: *Oct-4, Sox-2* and *Rex-1* as pluripotency markers (specific primers[39]) and the housekeeping gene *Gapdh* (specific primers[40]) as a positive control. Each PCR reaction consisted of 35 cycles with the following conditions: 30 s at 94°C for denaturation, 30 s at 57°C (*Sox-2, Rex-1* and *Gapdh*) or 30 s at 68°C (*Oct-4*) for annealing, and 1 min at 72°C for extension. After the last cycle, samples were incubated for 10 min at 72°C and the products obtained were evaluated in 1.5% agarose gels.

**Metaphase spreads and staining.** Subconfluent cultures were arrested in metaphase by the addition of KaryoMAX colcemid (Gibco) at different conditions (0.08 μg/ml for 3 h, 0.015 μg/ml for 1 h 30 min, and 0.1 μg/ml for 3 h), depending on the cell line (E14, J1 and CMT, respectively). Cells were then washed in HBSS, dissociated with trypsin-EDTA, and centrifuged at 300 g for 5 min. The pellet was carefully resuspended and incubated for 15–20 min in 0.075 M KCl hypotonic solution at 37°C. After removing the hypotonic solution by centrifugation, a fixative solution (3 : 1, methanol : glacial acetic acid) was added drop-wise to the cell suspension. Cells were collected, rinsed with fixative solution at least twice, and finally spread onto −20°C cold glass slides. After 24 h at room temperature (RT), slides were stained, or kept at −20°C for further analysis. For modal characterization, a total of 826 metaphase spreads were examined. The number of cells analyzed per passage and cell line is summarized in Table 1. As it is technically difficult to obtain optimal chromosome metaphase spreads, the passages analyzed in each cell line were not always identical at each time point.

**Multicolor fluorescence *in situ* hybridization (mFISH).** The 21XMouse Chromosome mFISH Probe Kit containing specific painting probes for each of the 21 mouse chromosomes was used to karyotype the J1 mESCs at early and middle passages according to the manufacturer's instructions (Metasystems; Germany). Briefly, slides were pre-treated with 100 μg/ml RNase A for 1 h at 37°C and with pepsin solution (0.1 mg/ml of pepsin in 0.01 N HCl) for 7 min at 37°C prior to a post-fixation step with formaldehyde-$MgCl_2$ solution (1% formaldehyde in 1× PBS +50 mM $MgCl_2$). Denaturation of chromosomes and probes were performed separately. Chromosomes were stabilized in 2× SSC at 70°C for 30 min, cooled down to 37°C and incubated consecutively in 0.1× SSC for 1 min at room temperature (RT), in 0.07 N NaOH for 1 min at RT, in 0.1× SSC and in 2× SSC both for 1 min at 4°C and dehydrated with an increasing ethanol series. Probes were denatured at 75°C for 5 min, placed on ice for a few seconds and incubated at 37°C for 30 min. Denatured cocktail probes (3 μl) were added onto the slides and after covering and sealing the samples, hybridization was carried out in a humidified chamber for 48–72 h at 37°C. The rubber cement and coverslips were then removed, and slides were rinsed in 1× SSC for 5 min at 75°C and in 4× SSC-Tween20 at RT for 5 min. Finally, chromosomes were counter-stained with DAPI after detection of biotin-labeled probes. Twenty-seven metaphase spreads were karyotyped (12 from early passages and 15 from middle passages).

**Chromosome painting.** Chromosomes 8 and 11 were simultaneously examined using the XCyting Mouse Chromosome Painting Probes (Metasystems). Painting probes, labeled with Texas Red (chromosome 8) or FITC (chromosome 11) were mixed in a ratio of 2 : 1, respectively, and added to the dehydrated slides. After covering and sealing the samples, co-denaturation of slides and probes were carried out in a HYBrite™ hybridization plate (Vysis/Abbott Molecular; Des Plaines, Illinois, USA) for 2 min at 75°C, followed by an overnight hybridization in a humidified chamber at 37°C. Slides were washed once in 0.4× SSC at 72°C for 2 min, in 2× SSC-Tween20 at RT for 30 s and then were briefly rinsed in distilled water. Finally, slides were air dried and counterstained with DAPI. A total of 80 cells were analyzed at late passages in all three mESCs (n = 21, 20 and 18 in J1, E14 and CMT cell lines, respectively), and also at early passages in the CMT cell line (n = 18 cells).

**Immunofluorescence (IF) analysis.** Immunofluorescence detection was used to analyze the undifferentiated state in all cell lines, and also to assess the differentiation potential of J1 and CMT mESCs after EB induction. All the experiments were performed at early (middle in E14 mESC) and late passages. Undifferentiated mESCs colonies were seeded onto 22 × 22 sterile glass coverslips in 60-mm Petri dishes. When cultures reached a confluence of 60–70%, cells were fixed with 4% paraformaldehyde in PBS for 15 min, washed three times with 1× PBS for 5 min and incubated in blocking solution (consisting of 0.2% sodium azide, 3% goat serum and 0.5% triton-X-100 in 1XPBS) for 30 min at RT. Incubation with a mouse monoclonal anti-OCT-4 primary antibody (1 : 50, Santa Cruz Biotechnology; Dallas, Texas, USA) was carried out overnight in a humidified chamber at 4°C. Cells were then rinsed three times in 1× PBS and incubated for 2 h at RT with an anti-mouse Alexa fluor 594-conjugated secondary antibody (1 : 500, Molecular Probes/Invitrogen, Spain). After three washes with 1× PBS, the coverslips were air dried, mounted onto slides and counterstained with 2 μg/ml Hoescht 33258 (Molecular Probes/Invitrogen) in Vectashield antifade (Atom S.A, Spain).

EBs were also seeded onto 22 × 22 sterile glass coverslips in Petri dishes, and cells were fixed after 10 days of spontaneous differentiation. Applying the same protocol

described for undifferentiated cells, the following primary antibodies were used to evaluate the differentiation potential: rabbit polyclonal anti-Nestin (1 : 250, Abcam; UK), rabbit polyclonal anti-Alpha Fetoprotein (AFP, 1 : 400, Dako; Denmark) and mouse monoclonal anti-Smooth Muscle Actin (SMA, 1 : 400, Sigma-Aldrich) as ectodermal, endodermal and mesodermal markers, respectively. Anti-mouse Alexa fluor 594-conjugated, anti-rabbit Alexa fluor 532-conjugated (2 : 664) or anti-rabbit Alexa fluor 448-conjugated (2 : 664), all from Molecular Probes/Invitrogen, were used as secondary antibodies.

The results obtained by fluorescence methods (IF, mFISH and chromosome painting) were all analyzed with the Isis software (Metasystems) coupled to a conventional fluorescence microscope (BX60 with U-CMAD-2 camera; Olympus; Spain).

**Statistical analysis.** For statistical analysis, two distinct groups were established for each cell line to compare the total chromosome gains at the different time points. One group was formed by cells with a chromosome number less than or equal to the modal number of the first analysis, and the other one by cells with more chromosomes than the modal number. That is, $\leq 40$ and $>40$ chromosomes for CMT cell line, $\leq 41$ and $\leq 41$ for J1 and, $\leq 42$, and $>42$ chromosomes for E14.

The two-tailed Fisher's exact test, applying the Bonferroni's correction for multiple comparisons, was used to assess statistical differences between these two groups established in each cell line at the different time points (Supplementary Table S1). The alpha level in all tests was 0.05.


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## Author contributions

N.G. has contributed with the conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. C.N. has contributed with the conception and design, financial support, provision of study material, data analysis and interpretation and final approval of manuscript.

## Additional information

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