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SHORT REPORT

The *in vitro* survival of human monosomies and trisomies as embryonic stem cells

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Abstract Chromosomal aneuploidies are responsible for severe human genetic diseases. Aiming at creating models for such disorders, we have generated human embryonic stem cell (hESC) lines from pre-implantation genetic screened (PGS) embryos. The overall analysis of more than 400 aneuploid PGS embryos showed a similar risk of occurrence of monosomy or trisomy for any specific chromosome. However, the generation of hESCs from these embryos revealed a clear bias against monosomies in autosomes. Moreover, only specific trisomies showed a high chance of survival as hESC lines, enabling us to present another categorization of human aneuploidies. Our data suggest that chromosomal haploinsufficiency leads to lethality at very early stages of human development.

Introduction

The occurrence of chromosome numerical disorders in humans is a common phenomenon during early embryonic development (Delhanty, 2005), and is responsible for as much as 65% of clinically recognized miscarriages (Wilton, 2002). Whole-chromosome imbalances can be frequently detected in *in vitro* fertilization (IVF) procedures by preimplantation genetic screening (PGS) (Mir et al., 2010; Munne et al., 2010; Li et al., 2005; Baart et al., 2006; Rubio et al., 2007). These aberrations can originate from meiotic errors in the gametes, resulting in homogeneous aneuploid embryos, or from post-zygotic errors produced during the first mitotic divisions, leading to mosaic embryos composed of euploid and aneuploid cells, or cells carrying different aneuploidies (Li et al., 2005; Colls et al., 2007; Vanneste et al., 2009).

Aneuploid embryos can be used to derive aneuploid hESC lines for modeling genetic disorders. We have previously reported that about 2/3 of the cell lines derived from aneuploid embryos resulted euploid following expansion in culture, and about 1/3 remained aneuploid (Biancotti et

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al., 2010; Narwani et al., 2010; Lavon et al., 2008). This occurrence may be explained by the high incidence of mosaicism within cleavage-stage embryos, and a selection in favor of euploid cells in culture. Such bias against aneuploid cells also takes place *in vivo,* and is evidenced as a significantly higher rate of aneuploidy in preimplantation blastocysts when compared to embryos at post-implantation stages (Rubio et al., 2007; Fragouli et al., 2008).

In the present work, we report the analysis of more than 400 aneuploid PGS embryos, and show that despite the similar occurrence of trisomy and monosomy on any specific chromosome, hESC lines derived from these embryos display a categorical bias toward trisomies of autosomes.

Materials and methods

Derivation of hESC lines from PGS-analyzed blastocyst

Derivation of hESCs was performed under the approval of the Stem Cell Research Oversight Committee at Cedars-Sinai Medical Center, according-to-protocol # 9647: "Establishment of hESCs from Genetically Abnormal and Spare IVF-Derived Embryos."

The trophectoderm (TE) was manually removed by dissection utilizing ultra sharp splitting blades (Bioniche Animal Health USA, Athens, GA). The use of manual cut increased the success of derivation of hESC lines. The subsequent steps of derivation were performed as described before (Lavon et al., 2008).

Karyotype analysis

Five wells of a 6 well plate of rather confluent hESCs in log growth phase were fed with fresh media the night before the procedure. Next morning, cells were treated with 100 ng/ml of KaryoMAX colcemid (Invitrogen, Carlsbad, CA) for 30 min in incubator at 5% CO_2. Then, cells were collected by trypsinization, treated with hypotonic solution and fixed with methanol:acetic acid 3:1 and kept at −20 °C until analysis. Chromosomes were classified according to the International System for Human Cytogenetic Nomenclature (ISCN), using the standard G banding technique.

Staining for markers of undifferentiation

To reveal alkaline phosphatase enzymatic activity, hESCs grown on 12 well plates were fixed with a solution of citrate-acetone-formaldehyde, and stained using the 86R-1KT kit (Sigma-Aldrich, St. Louis, MO) following manufacture's protocol. For immunocytochemistry, hESCs grown on coverslips were incubated with a mixture of mouse IgG anti-human Oct 3/4 (1:20) and mouse IgM anti-human TRA-1-60 (1:100) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and revealed with a combination of either goat anti-mouse IgG-PE (1:300) and goat anti-mouse IgM-FITC (1:200) or goat anti-mouse IgG-FITC (1:300) and goat anti-mouse IgM-PE (1:200). Bis-benzimide (Hoechst 33258; Sigma-Aldrich, St. Louis, MO) was used for nucleus staining. Flow cytometry analysis was carried out by incubating cells with either mouse IgG anti-human SSEA4 (1:50) or mouse IgM anti-human TRA-1-60 (1:50) and then goat anti-mouse IgG-FITC (1:200) or goat anti-mouse IgM-FITC (1:200) (all from Santa Cruz Biotechnology, Santa Cruz, CA) on ice for 20 min each. Following washes, cells were analyzed immediately in a FACSCalibur analyzer (Becton-Dickinson, Franklin Lakes, NJ). Data analysis was performed by CellQuest software (Becton-Dickinson, Franklin Lakes, NJ).

In vitro and *in vivo* differentiation of hESC

Embryoid bodies (EBs) were generated by aggregation of hESCs and maintained in suspension as described before (Itskovitz-Eldor et al., 2000) for 20 days. To produce teratomas, $2–3\times10^6$ hESCs were injected under the kidney capsule of 6–8 weeks-old nude mice (Robertson, 1987). One month later, mice were euthanized and teratomas collected, fixed with 10% buffered formalin and embedded in paraffin. Four microns sections were stained with hematoxilin and eosin (H&E). Care of animals was in accordance with institutional guidelines as approved by the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee, according-to-protocol # 2182.

Statistics

Binomial test was used to determine frequencies of monosomy and trisomy. Generalized estimation equations (GEE) were applied to model the risk of monosomy or trisomy (in separate models) in each chomosome while controlling for the repeated measures within each embryo (SAS PROC GENMOD), using a link-logit function and an unstructured correlation structure. Contrasts of risk between each chromosome were estimated and differences were considered significant where $p<0.05$.

Results and discussion

In order to derive hESCs with congenital aneuploidies, we have analyzed 417 blastocyst-stage human embryos from couples undergoing PGS, using probe panels covering up to 12 chromosomes (8, 13, 14, 15, 16, 17, 18, 20, 21, 22, X, Y chromosomes). The total number of either monosomic or trisomic events was 341 and 361, respectively. Analysis of the number of events per chromosome indicates an overall similar occurrence of monosomy and trisomy in each specific chromosome in the cleavage-stage embryos, with the exception of chromosome 22 that has significantly more trisomy than monosomy events ($p=0.03$) (Fig. 1A). However, the incidence of numerical defects is different between chromosomes; for example, chromosomes 16 is significantly more likely to present with a monosomy than chromosomes 8, 13, 15, 17, 20, 22, and X, while chromosome 21 will more likely present with a trisomy than chromosomes 8, 15, 17, 20, and X (Fig. 1A).

From 417 aneuploid embryos, we were able to derive 47 hESC lines; 25 of these cell lines were characterized before (Biancotti et al., 2010), and 22 are described here (Supplementary Table 1). In agreement to what we reported earlier (Biancotti et al., 2010), about 2/3 of the new cell lines developed into normal euploid cells, while the remnant 1/3 remained aneuploid, carrying trisomy of chromosome 21 (Down syndrome, 2 lines), chromosome 20 (3 lines) or chromosome 12

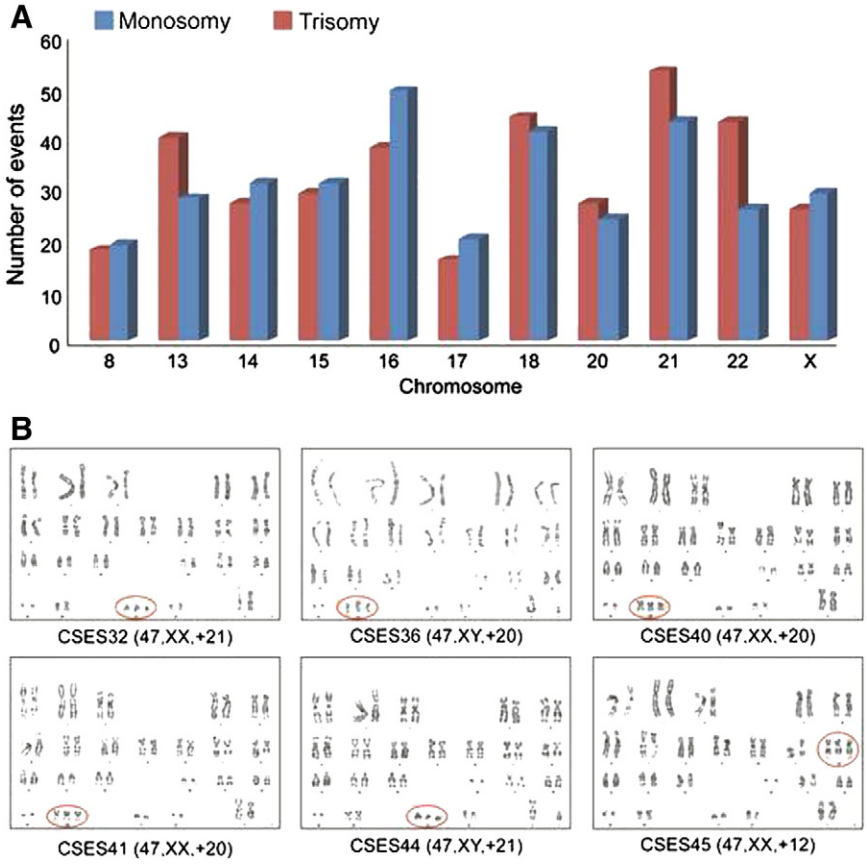
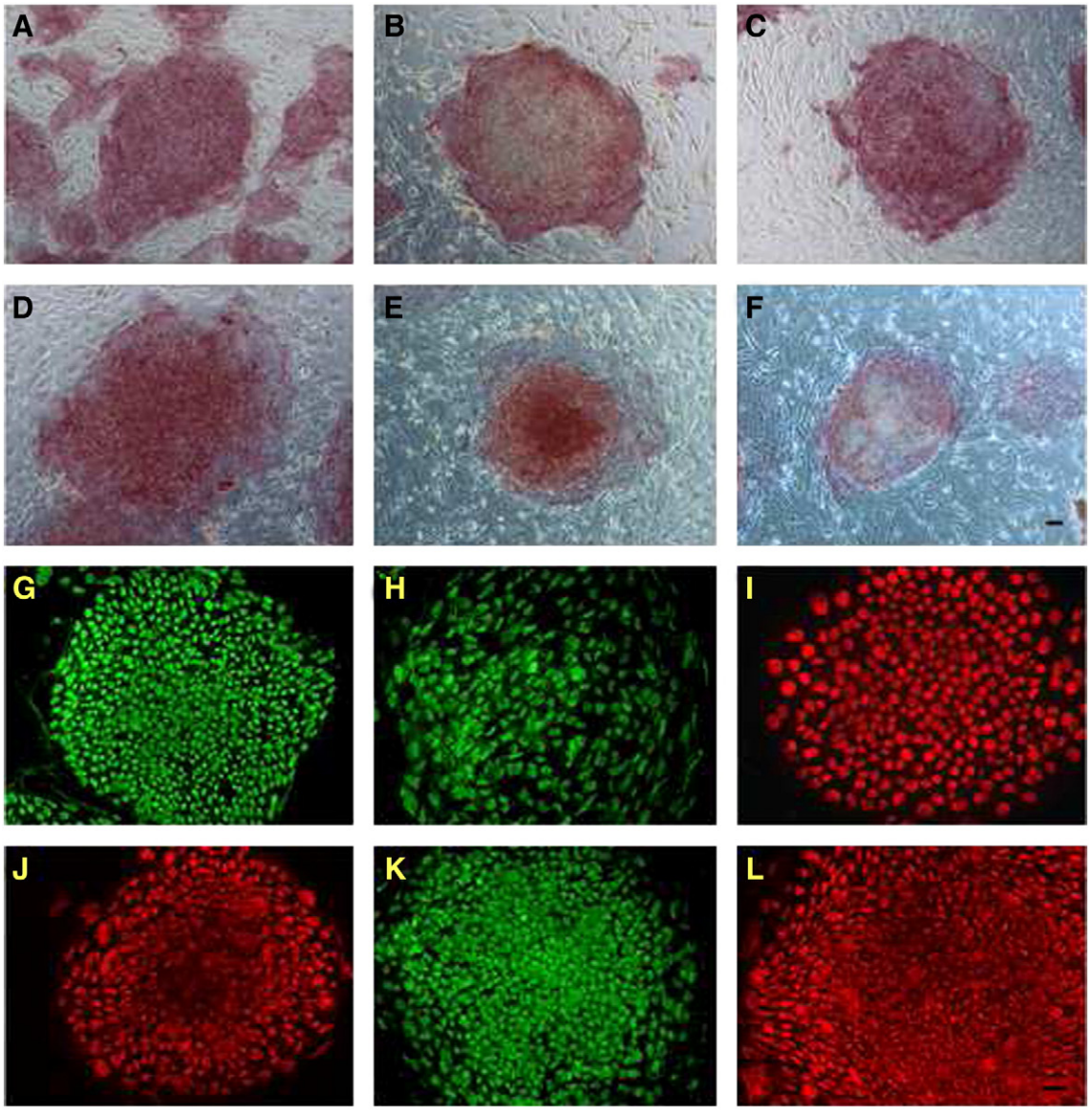


Figure 1 Analysis of monosomies and trisomies in PGS embryos and karyotype of aneuploid hESCs. A. Total number of monosomies or trisomies detected by PGS, arranged by chromosome. Only events correspondent to monosomies or trisomies were considered in the quantification. Only chromosome 22 was found to present significantly more trisomy events than monosomy events ($p=0.03$). B. Karyograms corresponding to the aneuploid hESC lines. Karyotype analysis was performed between passages 11 and 20. For most of the cell lines, the karyotype was consistent in the 20 metaphases analyzed, with the exception of CSES41 (see Supplementary Table 1).

(1 line) as determined by karyotype analysis (Fig. 1B). All the aneuploid hESC lines exhibit self-renewal capacity, express typical markers of undifferentiated cells and have the ability to differentiate into derivatives of the three embryonic germ layers. In Fig. 2 we show alkaline phosphatase activity and expression of Oct4 by immunocytochemistry for the aneuploid cell lines. Analysis by flow cytometry revealed that on average 85% of the cells stained positive for SSEA4 and TRA-1-60 cell surface antigens (Fig. 2). Pluripotency was determined by the ability of the cells to differentiate *in vitro* into embryoid bodies and *in vivo* into teratomas following injection under the kidney capsule of immunocompromised mice (Fig. 3).

Next, we analyzed the ratio between the number of events for each chromosome, and the number of aneuploid hESC lines derived from these embryos. Fig. 4A shows that blastomeres carrying an extra copy of either chromosomes 13, 16, 17, 20, 21 and X survived derivation and expansion in culture and generated established hESC lines. However, almost all human embryos with monosomies did not survive the *in vitro* growth as ESC, with the exception of only one hESC line with monosomy of chromosome X being generated (Fig. 4B). Our inability to generate monosomic hESCs persists whether we use immunologic or manual techniques to derive the cells (see Materials and methods). Monosomy X (Turner syndrome) is the only monosomy found in live humans, and the one generated as hESC line. Although most X0 embryos are spontaneously aborted during the first trimester of pregnancy, females with Turner syndrome that survive develop almost normally with minor phenotypic features (Saenger, 1996). In addition, inactivation of an X chromosome in females parallels to some extent the lack of an X chromosome, except for the fact that X inactivation is not complete leaving pseudoautosomal genes transcriptionally active. Haploinsufficiency of pseudoautosomal genes involved in development, was suggested to be the cause for both, early lethality of monosomic embryos and phenotype of surviving individuals (Zinn and Ross, 1998; Urbach and Benvenisty, 2009). The bias against autosomal monosomies indicates that the lack of an autosomal chromosome is critical for cell survival and development.

Based on the ability to derive hESC lines with an extra copy of a particular chromosome, the different trisomies can be divided in two groups. One group comprises trisomies for those chromosomes found to be present in blastomeres by PGS, but unlikely to survive as trisomic ESC lines. This is the case of trisomies 8, 14, 15, 18 and 22. The second group clusters trisomies of chromosomes that are present in blastomeres and survive as ESC lines, such as trisomies 12, 13, 16,



M	CSES 32	CSES 36	CSES 40	CSES 41	CSES 44	CSES 45
SSEA4	93%	92%	81%	79%	81%	92%
TRA-1-60	92%	92%	73%	80%	84%	85%

Figure 2 Aneuploid hESC lines express markers of undifferentiated cells. Panels A-F correspond to staining for alkaline phosphatase activity, and panels G-L in green or red, to immunostaining for Oct4. The six aneuploid cell lines are depicted as follows: CSES32 (A, G), CSES36 (B, H), CSES40 (C, I), CSES41 (D, J), CSES44 (E, K), and CSES45 (F, L). All cell lines were grown on feeder layer composed of mouse embryonic fibroblasts (MEF) as seen in the background of panels A–F. (M) Results of flow cytometry analysis displaying the percentage of SSEA3/4 and TRA-1-60 single-staining positive cells. Bars in A–F = 100 μm and in G–L = 50 μm.

17, 20, 21 and X, although with different frequency of success in the derivation. Particularly, trisomies 17 and 20 seem to have a better chance to survive as ESC lines. Interestingly, these chromosomal alterations also frequently occur during cellular adaptation of embryonic stem cells (Amps et al., 2011; Ben-David et al., 2011; Baker et al., 2007; Mayshar et al., 2010). There are cases in which the chromosomal abnormality detected by karyotype of the hESC line does not match the result of PGS, *i.e.* CSES36 and CSES41 have a PGS with +22 and a karyotype for the hESC line with no +22 but +20 instead (Supplementary Table 1). This lack of correspondence between PGS and karyotype may be due in part to the limitations of the PGS analysis, the occurrence of erroneous results, and to the high incidence of mosaicism in early-stage embryos. In our analysis of euploid hESCs derived from aneuploid embryos, we did not detect the occurrence of

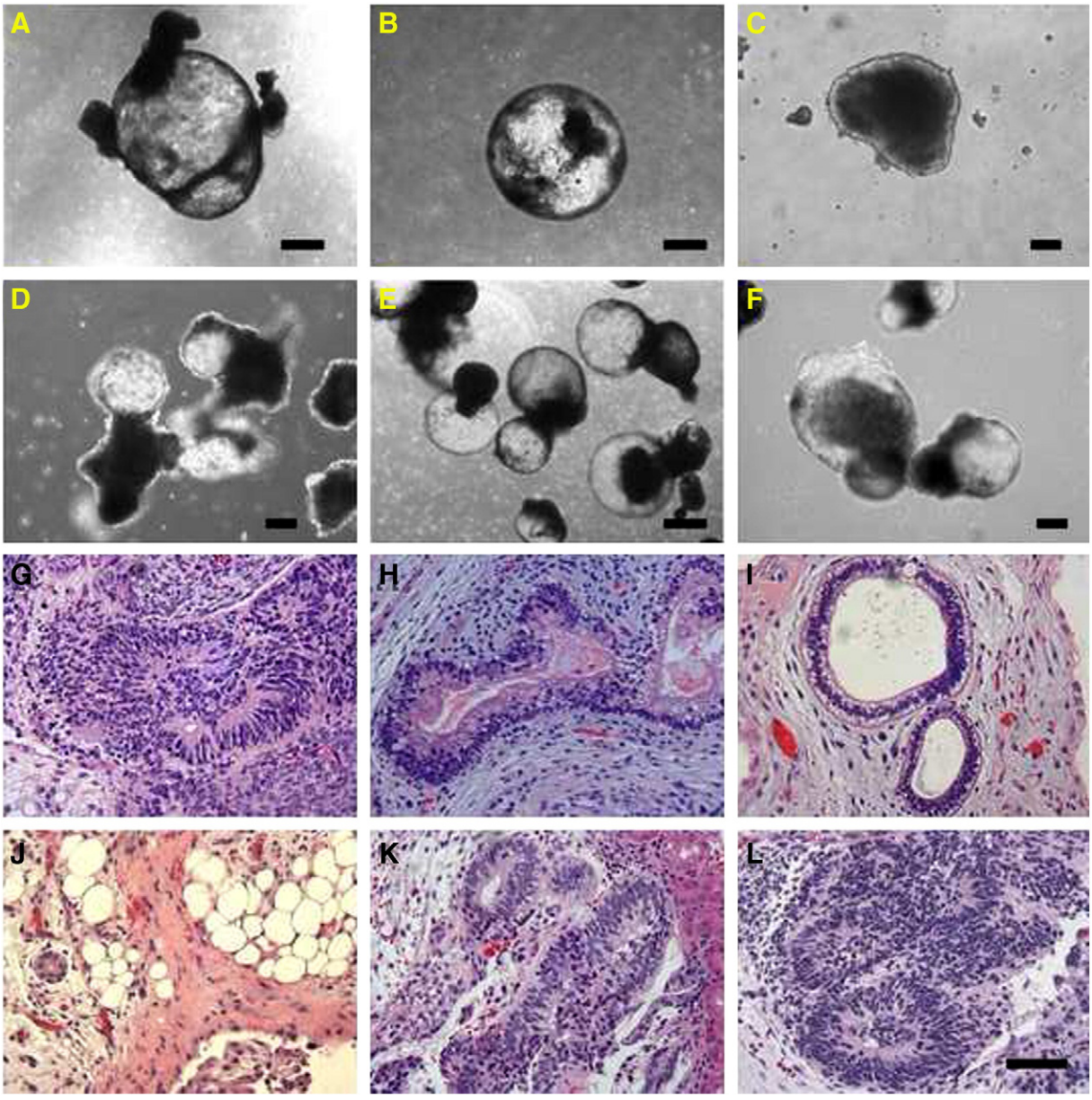


Figure 3 *In vitro* (embryoid bodies) and *in vivo* (teratomas) differentiation of aneuploid human embryonic stem cells. Panels A–F correspond to 20 days embryoid bodies and panels G–L to H&E-stained sections of 30 days teratomas for the six aneuploid cell lines. (A, G) CSES32; (B, H) CSES36; (C, I) CSES40; (D, J) CSES41; (E, K) CSES44; (F, L) CSES45. Bars in A, B, E = 200 μm, in C, D, F = 100 μm, and in G–L = 100 μm.

uniparental disomy as consequence of duplication or loss of a whole chromosome (Lavon et al., 2008), supporting the notion of high incidence of mosaicism in human embryos.

ESCs appear to be very sensitive to haploinsufficiency. In mice it has been demonstrated that monosomic embryos die *in utero* (Magnuson et al., 1982; Baranov, 1983a; Magnuson et al., 1985). Our results suggest that human monosomic embryonic cells at early stages of their development cannot survive even in culture. Furthermore, trisomies are the most frequent type of aneuploidy found in human spontaneous miscarriages, usually occurring during the first trimester of pregnancy (Fritz et al., 2001; Jobanputra et al., 2002; Stephenson et al., 2002; Vorsanova et al., 2005). Considering that trisomies and monosomies occur at approximately the same rate in embryos at the blastocyst stage, the above mentioned evidence suggests that cells with monosomies of autosomal chromosomes are not viable and die early during the peri-implantation period. One possible explanation for this lack of viability is the loss of imprinted genes due to the missing chromosome. However, human parthenogenetic ES and induced pluripotent stem (iPS) cells survive and differentiate in culture (Stelzer et al., 2011; Turovets et al., 2011; Brevini et al., 2009), arguing against imprinting as the barrier in survival of the monosomic ESC lines. Another possibility is that 50% reduction in gene expression of critical loci scattered over all the autosomes is responsible for the non-viability of the embryos. In support of this hypothesis stand the facts that dosage alteration of gene expression also appears to be the

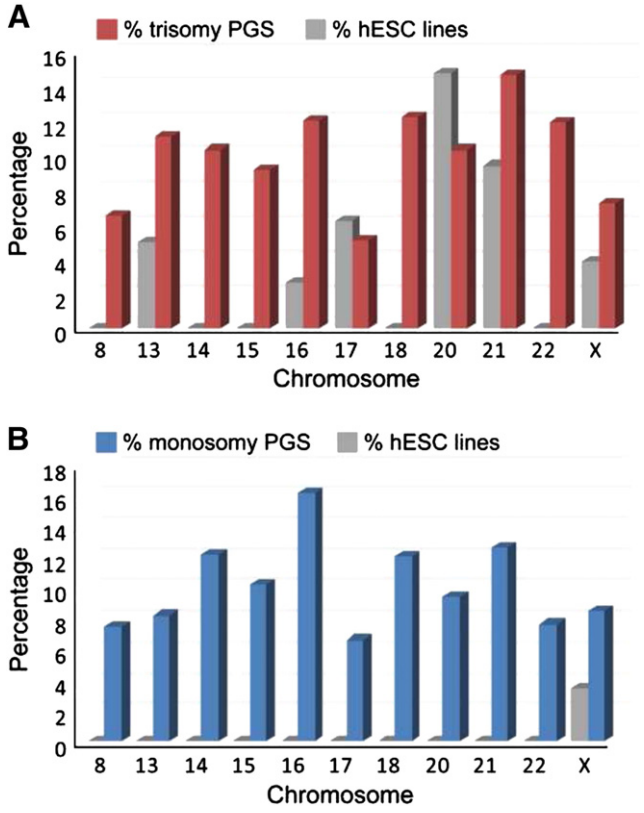


Figure 4 Aneuploid human ES cell lines derived from PGS embryos. A) Red bars represent the percentage of events for each specific trisomy as diagnosed by PGS (*% events for a trisomy = number of events for a trisomy / number of events for all trisomies × 100*), and grey bars the percentage of trisomic human ES cell lines derived from PGS embryos (*% hESC lines = number of hESC lines with a trisomy / number of events for that trisomy × 100*). B) Blue bars stand for the percentage of monosomic events for each chromosome (*% events for a monosomy = number of events for a monosomy / number of events for all monosomies × 100*), and grey bars the percentage of monosomic human ES cell lines derived from PGS embryos (*% hESC lines = number of hESC lines with a monosomy / number of events for that monosomy × 100*).

cause of the phenotype observed in trisomic ESCs (Biancotti et al., 2010), and that chromosomal haploinsufficiency has already been suggested to play a role in early lethality in mice (Baranov, 1983b; Schimenti et al., 2000). There is, however, occurrence of partial deletions in autosomal chromosomes, which originates partial monosomies that are responsible for a variety of diseases in humans.

Unlike monosomies, an extra copy of an autosomal chromosome seems to less dramatically affect the development of the embryo. This is supported by the fact that full trisomies of chromosomes 13, 18 and 21 can be seen in live births, although with major phenotype, and usually early death. We have demonstrated that trisomic hESC lines can be derived from PGS embryos and maintained in culture. However, there is a subgroup of trisomies detected by PGS that failed to generate ESC lines. Most unexpected is the case of trisomy 18, in view of the potential to engender live offspring (Edwards syndrome). Yet, most of trisomy 18 conceptions spontaneously abort during the first trimester (Hook, 1983). More importantly, although chromosome 18 abnormalities have been found in hESC lines during cellular adaptation, these are deletions rather than duplications, suggesting that a loss and not a gain in chromosome 18 confers growth advantage in culture (Amps et al., 2011). What causes that certain trisomic cells survive and develop in culture whereas others die, is not fully understood. Another question that arises is how a chromosomal duplication, such as trisomy 21, can have so distinct phenotypes, from early death and miscarriage to full development with mild alterations. These questions remain unanswered, although the study of multiple hESC lines with the same trisomy may shed some light on the existence of genetic and/or epigenetic differences between phenotypes.

Author contribution

Juan Carlos Biancotti: Conception and design, collection and assembly of data, data analysis and interpretation, preparation of figures, manuscript writing.

Kavita Narwani: Provision of study material, collection and assembly of data, data analysis and interpretation.

Berhan Mandefro: Provision of study material, collection and assembly of data, data analysis and interpretation.

Tamar Golan-Lev: Collection of data, data analysis and interpretation, preparation of figures.

Nicole Buehler: Provision of study material, collection and analysis of data.

David Hill: Provision of study material, data analysis and interpretation.

Clive Svendsen: Manuscript editing and data analysis

Nissim Benvenisty: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2012.07.002.

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