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The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimaerism

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Mouse pluripotent embryonic stem (ES) cells, once reintroduced into a mouse blastocyst, can contribute to the formation of all tissues, including the germline, of an organism referred to as a chimaeric. However, the reasons why this contribution often appears erratic are poorly understood. We have tested the notion that the chromosome make-up may be important in contributing both to somatic cell chimaerism and to germ line transmission. We found that the percentage of chimaerism of ES cell-embryo chimaeras, the absolute number of chimaeras and the ratio of chimaeras to total pups born all correlate closely with the percentage of euploid metaphases in the ES cell clones injected into the murine blastocyst. The majority of the ES cell clones that we tested, which were obtained from different gene targeting knockout experiments and harboured 50 to 100% euploid metaphases, did transmit to the germline; in contrast, none of the ES cell clones with more than 50% of chromosomally abnormal metaphases transmitted to the germline. Euploid ES cell clones cultured *in vitro* for more than 20 passages rapidly became severely aneuploid, and again this correlated closely with the percentage of chimaerism and with the number of ES cell–embryo chimaeras obtained per number of blastocysts injected. At the same time, the ability of these clones to contribute to the germline was lost when the proportion of euploid cells dropped below 50%. This study suggests that aneuploidy, rather than 'loss of totipotency', in ES cells, is the major cause of failure in obtaining contributions to all tissues of the adult chimaera, including the germline. Because euploidy is predictive of germline transmission, karyotype analysis is crucial and time/cost saving in any gene-targeting experiment.

Keywords: karyotype; mouse; ES cells

Introduction

Mouse embryonic stem (ES) cells are derived from the inner cell mass of a 3.5 day embryo (the blastocyst). ES cells remain totipotent after *in vitro* culture under appropriate conditions (Evans and Kaufman, 1981; Martin, 1981), and it is therefore possible to introduce exogenous genetic information or mutate and disrupt endogenous genes by homologous recombination, while still retaining the ability of the cells to contribute to all tissues of an organism, referred to as an ES cell–embryo chimaera (Gossler *et al*., 1986). Similarly, *in vitro* culture and manipulation of ES cells does not prevent their ability to colonize the germline of an ES cell–embryo chimaera. In this way, a desired mutation can be transmitted by an ES cell–embryo chimaera, eventually generating an entire organism carrying the mutation in 100% of its cells (for reviews, see Capecchi, 1989; Smithies, 1993).

Germline transmission (GLT) is central to this type of experiment, yet the factors influencing the ability of ES cells to contribute to the formation of all tissues including the germline are poorly understood. An empirical finding by all groups dealing with ES cell technology is that the number of culture passages

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correlates negatively with their ability to contribute to all tissues including the germline. This is often attribute to loss of totipotency, as an increasing number of ES cells commit towards terminal differentiation. At the same time, it is known that ES cells almost invariable display a modal distribution of chromosomes and that aneuploid cells tend to increase with increasing culture time (Robertson, 1987).

In order to assess the relative roles of chromosome make-up versus loss of totipotency in enabling ES cells to contribute to the germline, we analysed ES cell clones, obtained from various gene targeting experiments, by karyotyping them at the earliest possible passage and immediately prior to each microinjection session into blastocysts. Here we report that chromosome make-up correlates with the capacity of ES cell clones to contribute to the formation of all tissues, including the germline, of the adult chimaeras. Our data support the notion that karyological instability, and not loss of totipotency, is the major reason for the lack of contribution to chimaerism of individual ES cell clones, and that karyotype analysis is a predictor of GLT.

Materials and methods

Cell lines, targeting vectors, electroporation, selection and identification of recombined clones

AB1, CJ7 and CCE ES cells were routinely cultured on inactivated cellular feeder layer as described (McMahon and Bradley, 1990; Swiatek and Gridley, 1993; Robertson *et al*., 1986). CJ7 were also cultured in the presence of murine leukaemia inhibitory factor (LIF) at the concentration of 1×10^3 U ml^{-1} (Hilton *et al*., 1988). The cells were electroporated with linearized 'replacement' vectors for gene targeting knock out derived from isogenic DNA (Thomas and Capecchi, 1987; Mansour *et al*., 1988; te Riele *et al*., 1992; Hasty and Bradley, 1993; Pandolfi *et al*., 1995a). In all the vectors used the gene of interest was interrupted by the presence of the pMC1 NEO cassette and polyadenylation signal (Thomas and Capecchi, 1987; Pandolfi *et al*., 1995a; and our unpublished results) and a viral thymidine kinase gene driven by the same promoter as the *neo* gene was added outside the region of homology (Mansour *et al*., 1988; Pandolfi *et al*., 1995a). Therefore, a double selection strategy was applicable upon transfection (Mansour *et al*., 1988). Cells (1×10^7) were mixed with 30 μg of linearized targeting DNA in a volume of 900 μl. AB1 were electroporated performing two shocks in Hepes buffered saline (HPSS) using a Bio-Rad Gene-Pulser at 230, 240 V, 500 μF, electrode distance 0.4 cm. CJ7 and CCE cells were electroporated performing a single shock in PBS (PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) at 220 V, 960 μF, electrode distance 0.4 cm. Cells were reseeded on 10 cm tissue culture dishes at a density of about 3×10^6 cells per plate. Drug selection (G418, 350 μg ml^{-1}; Ganciclovir, 2 μM) was started after 24 h. After 8–12 days, colonies were randomly picked and expanded. Genomic DNA was prepared from each individual clone, digested with the appropriate restriction enzyme and probed with probes that do not cross-hybridize with the targeting vector (Laird *et al*., 1991; Hasty and Bradley, 1993). ES cell clones in which the inactivation of the gene of interest was successfully achieved by homologous recombination, as shown by Southern blot analysis, were subsequently injected into the blastocysts to generate chimaeric mice.

Cytogenetics

ES cell cultures were prepared by passaging a confluent culture from a 60 mm cell culture dish 1 to 2 or 1 to 3 to give a 50–70% confluence on day of sampling. After 2 h incubation with fresh medium, a Colcemid (Hybri-max) solution was added to a final concentration of 0.02 μg ml^{-1} for 1 h. The cells were then washed in PBS, trypsinized and spun down. The pellet was resuspended carefully in hypotonic solution (0.56% KC1 w/v), in order to obtain a single cell suspension, and left at room temperature (RT) for 6 min. After spinning and removing hypotonic solution, 5 ml of ice-cold fixative (3:1 methanol:acetic acid) was added dropwise to the suspension, left at RT for 5 min and then spun down. The fixing procedure was repeated a further three times. Finally, the pellet was resuspended in a final volume of 1 ml fixative. The cells were then dropped onto 5% acetic acid–ethanol (ice-cold) washed slides and stained with Giemsa. The modal distribution of chromosomes of each ES cell clone was assessed at the earliest possible passage after it was grown up under double selection (usually 5–6 passages after picking) and immediately prior to injection. For each analysis performed, at least 30–40 metaphases were examined. The number of chromosomes as well as the presence of structural chromosomal abnormalities (i.e. chromosome markers) was recorded according to the following criteria: metaphases were considered abnormal if their chromosome number exceeded 40 or if they exhibited marker chromosomes; polyploid and hypodiploid metaphases were taken into account only if they were found more than five times in the same clone. We refer to any clone as euploid if it has at least 75% euploid metaphases. Giemsa (G)-banding analysis was also performed in some cases to confirm the karyotypic characterization of the supposed euploid clones (Rudnicki and McBurney, 1987).

Generation and analysis of chimaeras and GLT assessment

Recombined ES cell clones were arbitrarily chosen and microinjected into the blastocysts (Bradley *et al*., 1984; Bradley, 1987). The injected blastocyts were transferred to the uterus of pseudopregnant females as described

(Bradley, 1987). Overt chimaerism was scored by simple observation, not earlier than 10 days from birth, based on the coat pigmentation differences between the Agouti 129sv strain, from which all the ES cell lines we have utilized originate, and the Black C57BL6 strain of the recipient blastocyst. Of course, the germline originates from a different region of the epiblast than other foetal tissues and therefore GLT could occur in mice that lack agouti coat colour altogether. However, the fact remains that everybody in the field tends to interbreed only animals that apppear chimaeric on the basis of simple coat colour observation. It would be too time-consuming and costly to do otherwise.

We divided chimaeric mice into four different categories: A, B, C and D based on < 25, 25–50, 50–75 and 75–100% of chimaerism, respectively. The simple observation of the coat colour was followed and corroborated by a Southern blot analysis assisted by Phosphor-Imager/Densitometer quantitation (Molecular Dynamics and Bio-Rad). DNA extracted from individual tissues (skin, brain, lung, heart, bone marrow, peripheral blood, liver, spleen, testis and ovary) was probed with probes external to the region of homology contained in the targeting vectors. The level of chimaerism was quantified by comparing the intensity of the wild-type band(s) and the intensity of the band(s) that recognize the recombined allele (data not shown). Chimaeras clinically affected by hydrocephalus were terminated by CO_2 exposure, fixed and sectioned as described (Smith and Bruton, 1977).

In order to identify the mice in which ES cells contributed to the germline, each male chimaera, irrespective of the percentage of chimaerism, was bred with MF1 or C57BL6 females to obtain a minimum of 50 offspring. GLT suggested by the presence of agouti pups in the litter was confirmed each time by Southern blot analysis carried out on DNA extracted from the tails of agouti progeny.

Analysis of embryos

Embryos, yolk sacs and placentas obtained from the injection of chromosomally abnormal ES clones into the blastocyst were scored for the presence of gross abnormalities at day 10, 12 and 14 of gestation. Chimaerism was assessed by Southern blotting as described in the previous paragraph.

Results

Euploid metaphases in ES cell recombined clones correlate with the yield of chimaerism

We analysed cytogenetically 32 different ES cell clones generated from five different gene targeting experiments (Pandolfi *et al*., 1995a; and our unpublished results) and derived from 3 distinct ES cell lines (AB1, CJ7 and CCE: see Materials and methods). The percentage of euploid metaphases ranged from 0 to 100% (Table 1 for 20 representative clones and Figs 1–2). Irrespective of the karyotype, cells from each clone were injected into blastocysts and re-implanted into pseudopregnant females as described. Fifty to 100 blastocysts were injected per ES cell clone (Table 1). The pups obtained were analysed 10 days after birth to score the number of chimaeras, their sex, and the level of chimaerism. We found that the

Table 1. Fate of 11 representative euploid and aneuploid ES cell recombinant clones

Clone	*Modal chromosome number*	*Euploid Cells* (%)	*Blastocysts injected*	*Pup yield*[‡] (%)	*Chimaeras among pups* (%)	*Contribution of recombinant ES cells to chimaeras*[§] (%)	*Main abnormalities*	*GLT*
P7	42	0	105	20	22	A	Hydroc.; Steril.	No
P13	42	16	93	20	21	A	Hydroc.; Poly	No
P80	41	30	52	24	21	A	n.o.a.	No
P16N	40	0 (m)	76	26	25	A	Micr. + C.Atr.	No
P113	41	20	56	18	19	A	Hydroc.; Steril.	No
A17	40	60	44	55	45	B	n.o.a	Yes
D22	40	60	75	47	40	B	n.o.a	Yes
P93	40	50	56	60	45	B	n.o.a	Yes
P5	40	100	151	54	10	A	n.o.a	No
S16C	40	100	145	68	65	D	n.o.a	Yes
S77C	40	100	96	70	62	D	n.o.a	Yes

Data shown in this table rely on karyotype analysis performed at the earliest possible passage of each ES cell clones.
[‡]Number of pups per number of blastocysts implanted.
[§]Chimaeras have been scored by inspection of the coat colour and assigned to one of four categories: A, B, C, and D (see Materials and methods).
m = marker chromosome; hydroc. = hydrocephalus; steril. = sterility; poly. = polydactylia; micr. = microphathalmia; c. atr. = corneal atrophy; n.o.a. = no obvious abnormalities.

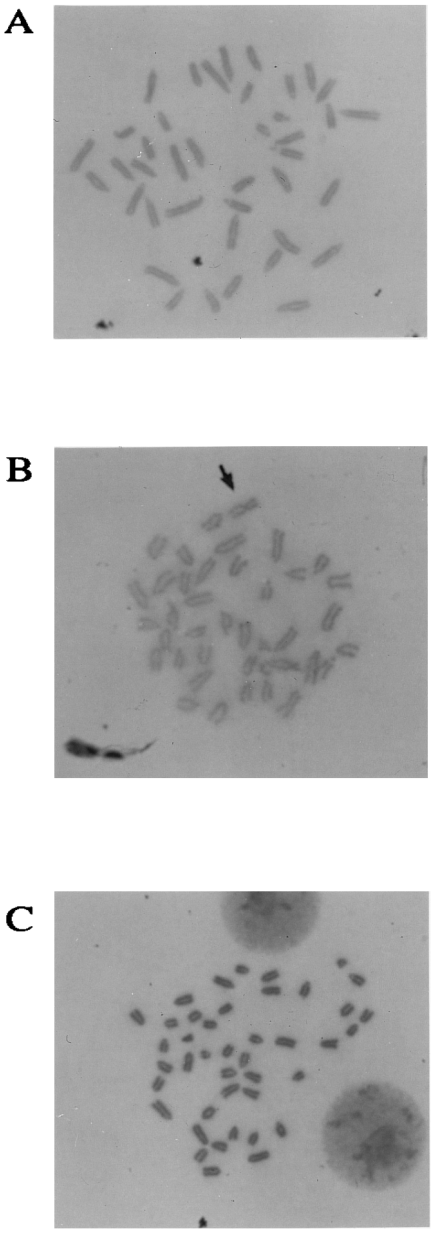

Fig. 1. Metaphase analysis in ES cell knockout clones. A. Euploid metaphase with 40 chromosomes. B. Abnormal metaphase with 39 chromosomes + 1 chromosome marker indicated by the arrow. C. Abnormal metaphase with 42 chromosomes.

number of pups born per number of blastocysts injected, the number of chimaeras obtained per pups born, and the level of chimaerism depended on the percentage of euploid cells (Tables 1 and 2, and Fig. 3).

In addition, chimaeric mice obtained injecting chromosomally abnormal clones had frequent abnormalities such as unilateral or bilateral polydactylia, hydrocephalus, microphthalmia, and unilateral or bilateral corneal atrophy and sterility (see Table 3 and Fig. 4).

We next needed to assess whether the low number of

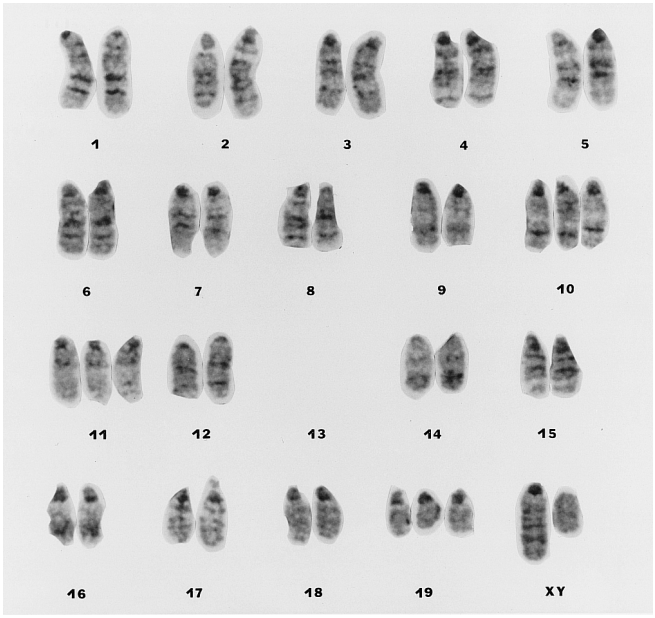

Fig. 2. G-banded karyotype of a cell from the ES clone P113 (Table 1). The karyotype is 41, XY, −13, −13, +10, +11, +19.

chimaeras and low level of contribution obtained from chromosomally abnormal clones was due to reduced capacity to contribute to the formation of all tissues, or to the fact that highly chimaeric embryos were dying *in utero*. For this purpose, we produced more embryos from those ES cell clones which gave few adult chimaeric mice with frequent congenital abnormalities (clones P7, P13 and P113, see Table 1). Ten-, 12- and 14-day embryos were scored for the presence of extra fingers and hydrocephalus. All the chimaeric embryos (60–80% of the total litter) as per Southern analysis, showed the aforementioned abnormalities (data not shown). The percentage of pup yield and the percentage of chimaeras among pups born from these clones is shown in Table 1. It is worth mentioning that the three ES clones we examined were almost 100% abnormal at the karyological analysis (Table 1), therefore contribution to chimaerism was almost certainly due to the aneuploid ES cells. In addition, the placenta and the yolk sac were frequently abnormally greenish when compared with the normal non-chimaeric embryo yolk sac and placenta, probably as a result of the accumulation of meconium or pigments. We have not been able to characterize these pigments although we could not detect accumulation of haem catabolites such as bilirubin (data not shown). These data suggest that, at least in some cases, chromosomally abnormal ES cells do not lose the capacity to contribute to the formation of the embryo. However, in these cases, a high level of contribution from chromosomally abnormal ES cells is not compatible with the proper development and/or the survival of the embryo.

Table 2. Euploidy is a major determinant of contribution to somatic cell and germ chimaerism

*Euploidy** (%)	*n*†	*Pup yield*‡ (%)	*Chimaeras yield* (%)	*Contribution*§ (%)	*Sex distortion*	*GLT*
0–25	226	23	24	A	No	No
25–50	108	42	33	B	No	No
50–75	156	58	45	B	No	Yes
75–100	438	65	68	D	Yes	Yes

Data shown in this table rely on karyotype analysis of each ES cell clone performed at the earliest possible passage and immediately prior to injection.
*Euploidy is represented as a percentage of euploid metaphases present in each clone.
†Total number of pups obtained.
‡Number of pups per number of blastocysts implanted.
§See legend to Table 1.

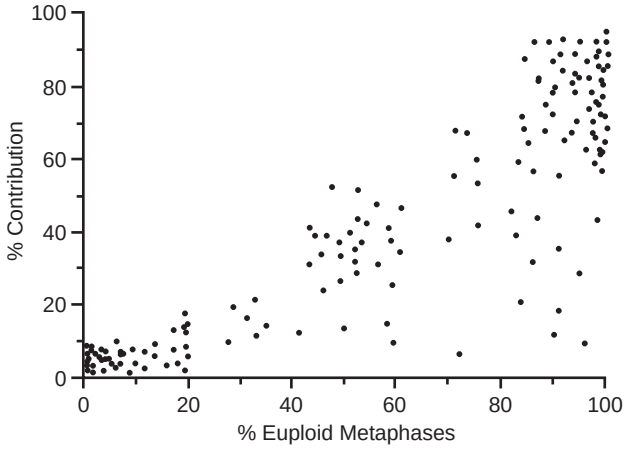

Fig. 3. The percentage of chimaerism is directly proportional to the number of euploid metaphases in an ES cell recombined clone. Each dot corresponds to an individual chimaeric mouse. On the abscissa the percentage of euploid metaphases of injected clones is shown. On the ordinate the percentage of contribution to chimaerism of each chimaeric mouse obtained is shown.

Table 3. Most frequent abnormalities found in adult chimaeras obtained from the injection of aneuploid clones

- Hydrocephalus (25%)
- Unilateral or bilateral polydactylia (25%)
- Unilateral or bilateral microphthalmia and corneal atrophy (25%)
- Sterility (20%)

Euploidy is predictive of GLT

Our injected ES cells were derived from a male 129sv mouse and sex distortion in favour of male mice was observed only when injecting clones with more than 75% euploid metaphases (Table 1), and was regularly associated with transmission to the germline. the capacity of each ES cell clone to contribute to the germline was tested by breeding each male chimaera as well as highly chimaeric females, into a C57BL6 or MF1 background, until 50 offspring were obtained. GLT was obtained exclusively with clones having more than 50% euploid

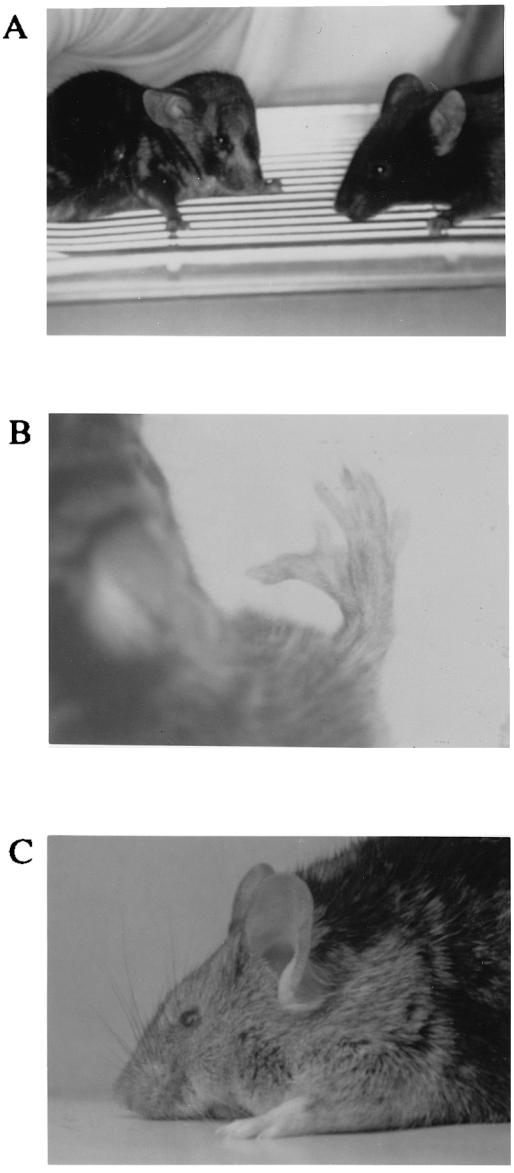

Fig. 4. Phenotype abnormalities in chimaeric mice obtained from chromosomally abnormal ES cell knockout clones. (A) On the left a chimaeric mouse with hydrocephalus and on the right a normal control. (B) Chimaeric mouse with polydactylia. (C) Chimaeric mouse with unilateral corneal atrophy.

metaphases (Tables 1 and 2). The reverse is not always true, since, as expected, aneuploidy and loss of totipotency are not mutually exclusive. In fact, not all the euploid clones were able to revert the sex of the chimaeras and to transmit to the germline, probably due, in some cases, to partial commitment and loss of totipotency (e.g. clone P5 in Table 1). Alternatively, these very rare euploid clones unable to properly contribute might be affected by more subtle genetic abnormalities not detectable by conventional cytogenetics analysis. In general, a modal euploid distribution of chromosomes with over 50% of euploid cells is predictive of transmission to the germline.

Aneuploidy increases with long-term ES cell culture and impairs the capacity of a clone to contribute to the germline

We could not determine from these experiments to what extent lack of GLT is specific to the properties of an individual ES cell clone or how far it is linked to aneuploidy *per se*. In order to address this point, four independent euploid ES cell knockout clones with proven capacity to transmit to the germline were selected for long-term culture and tested again at regular intervals. For this purpose, each clone was expanded to a 60 mm dish and the ES cells were passaged every three days, splitting the subconfluent dish at a ratio of 1:3 for a total of 30 passages from the moment in which the clone was picked after G418/Ganc double selection. ES cells were cytogenetically analysed at passage 5–6, 10, 15, 20 25 and prior to injection. In all four cases we found that: (a) the proportion of euploid metaphases dropped significantly, so that by passage 25 only 20% of metaphases were euploid (Fig. 5); (b) ES cells were able to contribute to the germline until passage 15 but not as passage 20 where the euploid metaphases were, on average, only 40% (Fig. 5).

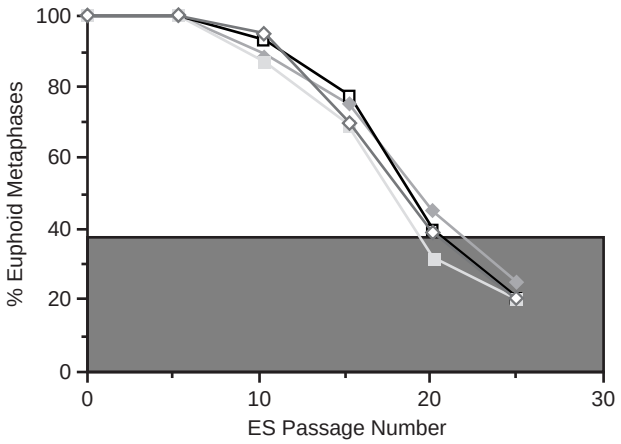

Fig. 5. Eupoidy is gradually lost with increasing number of culture passages. Squares, diamonds, circles and triangles correspond to 4 different ES cell clones. On the abscissa is the passage number of each individual clone. On the ordinate is the percentage of euploid metaphases. The hatched area represents the region within GLT was never obtained.

Discussion

The long-term aim of protocols involving ES cell manipulation is to obtain an ES cell–embryo chimaera capable of transmitting to its progeny a desired mutation introduced into the ES cell genome, usually by homologous recombination. ES cell lines, like any cell line kept in culture for a long period of time, are karyologically unstable and can drift towards an aneuploid modal distribution of chromosomes (Robertson, 1987).

It is not clear to which extent an aneuploid chromosome make-up of individual ES cell clones impairs the capacity to colonize the blastocyst. To address this question, we cytogenetically analysed each ES cell clone injected into the blastocyst at initial passages and immediately prior to injection.

Cytogenetic analysis and contribution potential of targeted ES cell clones in short-term culture

Analysis of our data (Table 1) shows that in ES cell clones obtained from transfection with targeting constructs and subjected to short-term culture (up to 10 passages), three situations may arise: (a) the majority of cells are still euploid; (b) the majority of cells are chromosomally abnormal. probably because they have been derived from an aneuploid cell, with few metaphases appearing euploid but may in fact be pseudo-euploid; (c) one-half or more of cells are euploid, but the rate of aneuploidy is increasing. In our experience, an ES cell clone with more than 50% abnormal metaphases was never able to contribute to the germline of the adult chimaera (Table 1 and 2). The only exception to this rule was an ES clone with a 39(X;0) karyotype which gave, among others, a highly chimaeric female mouse which transmitted to the germ line (data not shown). On the other hand, even though this is very rarely the case, if a clone is euploid, this does not necessarily imply ability to transmit to the germline and may only result in contribution at low levels to any tissue of the chimaera (Table 1 and Fig. 3). This could be due to a real 'loss of totipotency' of the ES cell from which a specific euploid ES clone arose; or, alternatively, it might be due to a more subtle genetic alteration not detectable by conventional cytogenetic analysis. However, injection of chromosomally abnormal ES cell clones (Tables 2–3 and Figs 3–4) consistently yields very few and poor chimaeras, that frequently exhibit congenital abnormalities. Many more chimaeras affected by similar abnormalities were observed when we analysed 10-, 12- and 14-day embryos originating from the injection of chromosomally abnormal clones. This suggests that the low yield of chimaeras obtained from chromosomally abnormal ES cells is due, at least in some cases, to intrauterine death of

the chimaeric embryos, or to cannibalization postpartum rather than to loss of totipotency. These findings are not surprising, since the presence of additional chromosomes could completely alter the differentiation programme of ES cells once they are reintroduced into the blastocyst, and the disorderly proliferations of those cells could have toxic effects.

In contrast, what we did not expect was to observe a linear relationship between the proportion of euploid metaphases of each individual ES cell clone and its ability to contribute to the formation of each tissue including the germline of the adult chimaera (Tables 1–2 and Fig. 3). Indeed, ES cell clones with less then 50% euploid metaphases were unable to contribute to the germline (Tables 1 and 2).

Cytogenetic analysis and GLT in long-term culture of recombined ES cell clones

The previous findings prompted us to investigate what would happen to a euploid clone capable of GLT if it was kept in culture for a long period of time (up to 25 passages, equivalent to more than 2 months of continuous culture). We found that the karyotype of the 4 clones thus studied remained reasonably stable until passage 15 (more than 70% euploid metaphases; see Fig. 5). After passage 15 the proportion of euploid metaphases dropped rapidly so that by passage 25 only 20–30% of cells were still euploid. At the same time, those clones at passage 20 that had only 30–50% of euploid cells were no longer able to contribute to the germline when injected into the blastocyst.

This study supports the idea that, in either short- or long-term ES cell culture, it is aneuploidy more than loss of totipotency that is detrimental for the achievement of GLT and of high level of chimaerism. Nagy *et al*. (1993) have indirectly analysed the impact of aneuploidy on the transmission to the germline in a paper which describes a different method of obtaining ES embryo/chimaeras (tetraploid embryo aggregation) and a new ES cell line (R_1). With this very specific methodology, unmanipulated subclones of R_1 ES cells in long-term culture, but also in some cases in short-term culture (before passage 14), appear to lose their capacity of contributing in a harmonious manner to the formation of embryos. Unfortunately, no karyotype analysis is provided for these subclones. On the other hand, the authors show that the karyotype of one unmanipulated and parental R_1 batch of ES cells after 11 and 33 passages is fairly stable, and suggest therefore that factors other than aneuploidy must cause the incapacity of R_1 cells to sustain an effective contribution in long-term culture in tetraploid aggregation. In others and our own experience neither unmanipulated AB1 nor unmanipulated CCE, E14, CJ7 and D3 ES cell lines are karyologically stable in long-term culture (Brown *et al*., 1992), especially after selection with G418, as previously reported for CCE (Robertson, 1987). Therefore, systematic screening of ES cell clones by cytogenetic analysis prior to injection into the murine blastocyst is extremely useful in predicting their ability to contribute to the germline. In addition, such a prediction can avoid time-consuming and expensive injection/breeding procedures of ES cell clones from which contribution will be low. Furthermore, these findings suggest that it is possible to 'rescue' an ES cell line which became partially chromosomally abnormal, due to extensive culturing. This can be achieved by cloning out diploid clones from the parental ES cell lines and testing individually of their ability to contribute to the germline.

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