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Germline potential of parthenogenetic haploid mouse embryonic stem cells

Martin Leeb*, Rachael Walker, Bill Mansfield, Jenny Nichols, Austin Smith* and Anton Wutz*

SUMMARY

Haploid embryonic stem cells (ESCs) have recently been derived from parthenogenetic mouse embryos and offer new possibilities for genetic screens. The ability of haploid ESCs to give rise to a wide range of differentiated cell types in the embryo and in vitro has been demonstrated. However, it has remained unclear whether haploid ESCs can contribute to the germline. Here, we show that parthenogenetic haploid ESCs at high passage have robust germline competence enabling the production of transgenic mouse strains from genetically modified haploid ESCs. We also show that differentiation of haploid ESCs in the embryo correlates with the gain of a diploid karyotype and that diploidisation is the result of endoreduplication and not cell fusion. By contrast, we find that a haploid karyotype is maintained when differentiation to an extra-embryonic fate is forced by induction of Gata6.

KEY WORDS: Haploid embryonic stem cells, Germline, Transgenic mice, Parthenogenesis

INTRODUCTION

Haploid embryonic stem cells (ESCs) have recently been established following chemical activation of unfertilised mouse eggs (Elling et al., 2011; Leeb and Wutz, 2011). Haploid parthenogenetic ESCs can be maintained as lines through flow cytometric purification of a haploid genome complement. Notably, haploid ESCs exhibit many aspects of the biology of normal diploid ESCs in culture despite their unusual karyotype. In addition, largely overlapping stem cell marker and genome-wide gene expression profiles suggested that haploid ESCs resemble pluripotent mouse ESCs to a large extent. They therefore afford the potential for performing genetic screens for elucidating developmental pathways. Haploid ESCs have the ability to give rise to a wide range of differentiated cell types in vitro and in chimeric embryos following blastocyst injection. This is accompanied by the gain of a diploid karyotype (Leeb and Wutz, 2011). A defining property of mouse ESCs is their competence for contributing to the germline of chimeric mice (Bradley et al., 1984). The ability to transmit genetic modifications through the germline has important implications for developing mouse models. Here, we investigate further the developmental competence of haploid ESCs and, in particular, their capacity for genetic modification and functional germline colonisation.

MATERIALS AND METHODS

Derivation and maintenance of haploid ESCs and comparative genomic hybridisation

Haploid ESCs were established and maintained as previously reported (Leeb and Wutz, 2011). Cell sorting for DNA content was performed after staining with 15 μg/ml Hoechst 33342 (Invitrogen) on a MoFlo flow sorter (Beckman Coulter) equipped with a Co-Lase tower (Propel Labs). The haploid 1n peak was purified and replated. Haploid HOct4^{Gip} ESCs were derived from a mouse strain that carries a GFP-IRES-puro transgene under the control of the *Oct4* (*Pou5f1*) promoter (Ying et al., 2002). Haploid HRex1 ESCs were derived from mice carrying a destabilised GFPd2 reporter targeted into the *Rex1* gene (Wray et al., 2011). For GFP and dsRed marking of ESCs, piggyBac vectors carrying a CAG promoter-driven GFP or dsRed transgene were introduced by co-lipofection with a piggyBac transposase-encoding plasmid (Lipofectamine 2000, Invitrogen). Pure populations of marked cells were established after sorting. DNA for comparative genomic hybridisation (CGH) experiments was extracted from ESCs using the Gentra Puregene gDNA Purification Kit (Qiagen) and sent to Source Biosciences for CGH analysis using NimbleGen 3×720K mouse whole-genome tiling arrays with an average probe spacing of 3.5 kb. CGH datasets were deposited in the GEO repository under accession number GSE30749.

In vitro differentiation of haploid ESCs

For generation of Gata6GR transgenic ESCs, a Gata6GR-IRES-puro construct was electroporated into haploid H129-1 ESCs using a Bio-Rad Gene Pulser Xcell (230V, 500 μF) and selected with puromycin (Shimosato et al., 2007). A pool of expressing cells was maintained under continuous puromycin selection and extra-embryonic endoderm (ExEn) differentiation was induced by the addition of dexamethasone (Dex, 0.1 μM final) in standard ESC medium. After the first passage, ExEn-like cells were cultured with continuous addition of Dex but without LIF.

Determination of the developmental potential of haploid ESCs in vivo

Chimeras were generated by injection of haploid ESCs into C57BL/6 host blastocysts. The purity of the injected haploid population was confirmed by recording a cell cycle profile on the day of blastocyst injection. Resulting female chimeras were mated to C57BL/6 males for assessing germline transmission. For tetraploid complementation experiments, GFP- and dsRed-marked ESCs were aggregated with two tetraploid B6CBA F1 hybrid 4-cell embryos as described previously (Nagy et al., 1990) or injected into tetraploid blastocysts. Embryonic day (E) 7.5 embryos were dissociated using 0.05% trypsin and fixed in 70% ethanol prior to propidium iodide staining. Cell cycle profiles were recorded on a CyAn ADP analyser (Beckman Coulter).

RESULTS AND DISCUSSION

Germline competence of haploid ESCs

Haploid ESCs have opened new possibilities for genetic manipulation of the mouse genome in vitro. A further consideration is the suitability of these cells for the production of mouse models for understanding gene function in development. Initial reports have shown a wide differentiation potential of haploid ESCs but

Centre for Stem Cell Research, Wellcome Trust and Medical Research Council Stem Cell Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK.

*Authors for correspondence (ml553@cam.ac.uk; austin.smith@cscr.cam.ac.uk; aw512@cam.ac.uk)

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germline transmission from chimeras was not evaluated (Elling et al., 2011; Leeb and Wutz, 2011). To investigate the feasibility of generating mice from haploid ESCs we produced a series of chimeras by blastocyst injection. Each of eight independent haploid ESC lines from various genetic backgrounds yielded chimeric mice (Table 1, supplementary material Fig. S1). These included a cell line at passage 29 that had been genetically modified in culture by stable transfection with a piggyBac transposon vector for expression of GFP. A total of 57 male and female chimeras showing overt coat colour chimerism developed normally to adulthood (Table 1, Fig. 1A, Fig. 2A). Notably, we obtained very high contribution chimeras from injection of haploid ESCs derived from the inbred 129/Sv mouse strain (Fig. 2A,B, supplementary material Fig. S1A), which is widely used for ESC-based genetic manipulation.

Since haploid ESCs are derived from parthenogenetic embryos and lack Y-chromosomal genes, transmission through the male germline is excluded. We therefore selected 15 chimeric females for testing germline transmission by mating with C57BL/6 males. Eight of these females produced agouti offspring in the course of the experiment, indicative of transmission of the haploid ESC genome (Fig. 1A, Table 1). In all, we observed germline transmission of five out of seven independent haploid ESC lines tested including different genetic backgrounds. Notably, four GFP-expressing pups were obtained from a chimera generated from genetically modified haploid ESCs (Fig. 1B). These developed into healthy adults and maintained GFP expression (Fig. 1B), demonstrating the production of transgenic mice from haploid ESCs manipulated in vitro.

These findings show that germline competence is a general feature of haploid ESCs and is maintained at high passage and after genetic manipulation. Our observations are consistent with reports that diploid parthenogenetic ESCs can contribute to the germline (Allen et al., 1994; Jiang et al., 2007; Liu et al., 2011). We attribute the high rate of germline competence of haploid ESC lines to our derivation and culture conditions under chemical inhibition of GSK3 and MEK kinase activity (Ying et al., 2008; Nichols et al., 2009; Leeb and Wutz, 2011), which suppresses heterogeneity and maintains a ground state of pluripotency (Wray et al., 2010; Marks et al., 2012).

To further assess whether haploid ESCs can contribute to development and to exclude the possibility that chimeras were formed from rare diploid ESCs, we aggregated flow cytometrically purified haploid GFP-labelled H129-1 ESCs at passage 26 with 8-cell embryos (Fig. 2C). We observed integration of haploid GFP-positive cells in E7.5 chimeric embryos (Fig. 2D-F). Chimera formation was comparable to that of diploid ESCs, indicating that haploid ESCs can efficiently contribute to the developing epiblast before diploidisation.

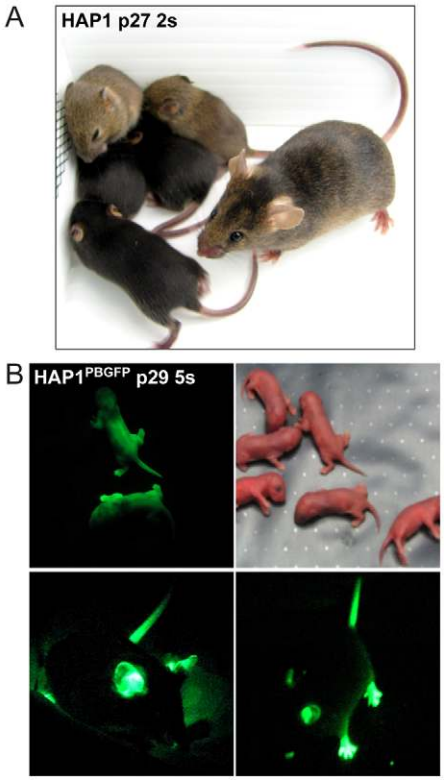

Fig. 1. Transgenic mice produced from germline-competent haploid ESCs. (**A**) Female chimera from injection of haploid HAP1 ESCs (agouti) at passage 27 into C57BL/6 blastocysts (black coat colour) and litter from mating to C57BL/6 male showing germline transmission of coat colour (two agouti pups). (**B**) GFP-expressing transgenic newborn offspring from a female chimera from injection of genetically modified HAP1 ESCs at passage 29. Expression of the GFP transgene is maintained into adulthood (bottom row).

Developmental potential of haploid ESCs

The developmental potential of mouse parthenogenetic embryos and diploid ESCs has been assessed in a number of studies. Owing to the absence of paternal imprinting the contribution of parthenogenetic cells to development may be compromised. Whereas work involving in vitro reconstituted uniparental embryos

Table 1. Germline transmission of chimeras from haploid ESCs

Haploid ESC line		Chimera			Germline transmission	
Name*	Genetic background	♂	♀	Total pups (% chimera)	Germline chimera (chimera tested)	Germline pups/first litter size
HAP-1 p27 2s	CBAB6 F1	1	3	6 (66)	1 (1)	2/5
HAP1^{PBGFP} p29 5s	CBAB6 F1	1	2	4 (75)	1 (2)	4/6
HAP-2^{PBGFP} p25 3s	CBAB6 F1	6	5	16 (69)	0 (3‡)	n.d.
H129-1 p20 3s	129/Sv	3	3	12 (50)	2 (2)	4/7, 4/6
H129-2 p20 3s	129/Sv	4	2	8 (75)	1 (3)	3/3
HOct4^{GiP}-1 p8 2s	MF1×129 F1	8	6	17 (82)	3 (3)	3/9, 1/3, 2/10
HTG-2^{PBGFP} p22 3s	Mixed	3	1	11 (36)	0 (1‡)	n.d.
HRex1^{GFPd2} p14 2s	129/Sv	4	3	9 (78)	n.d.	n.d.
HRex1^{GFPd2} p16 2s	129/Sv	1	1	4 (50)	n.d.	n.d.

*The number of total passages (p) and the number of purifications of the haploid 1n population by cell sorting (s) are given.
‡Only chimeras with low contribution were obtained.
n.d., no data obtained.

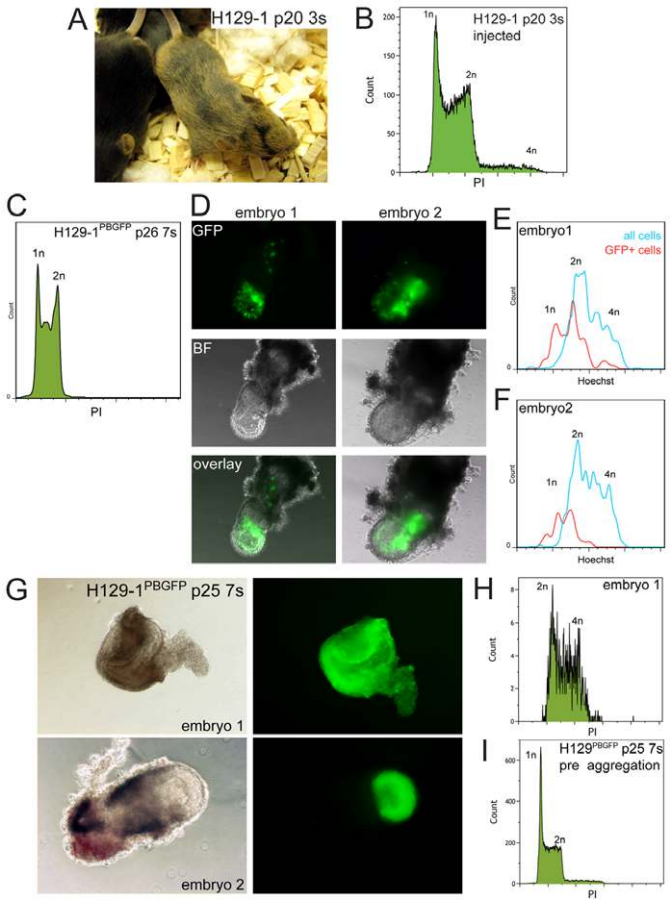

Fig. 2. Developmental potential of haploid ESCs from the 129/Sv mouse strain. (**A**) A high contribution male chimera from injection of H129-1 haploid ESCs into C57BL/6 blastocysts at passage 20 after three rounds of flow sorting. Agouti coat colour represents ESC contribution; host coat colour is black. (**B**) Flow analysis of propidium iodide (PI)-stained cells used for injection confirms a haploid genome content. (**C**) A pure haploid population of H129-1 ESCs at passage 26 was aggregated with diploid host morulae 48 hours after flow cytometric purification of the haploid 1n peak. (**D**) Embryos formed after morula aggregation show a clear epiblast contribution of GFP-labelled cells. (**E**,**F**) Cell cycle profiles showing DNA content of all cells (blue) and GFP-positive cells (red). (**G**) Brightfield and fluorescence images from E7.5 embryos obtained from aggregation of GFP-marked H129-1 ESCs with B6CBF1 tetraploid embryos. Contribution of haploid-derived cells to the embryo (above) and epiblast (below) identified by the green fluorescence. (**H**,**I**) Flow analysis of cells from embryo 1 after PI staining indicates a mainly diploid karyotype (H) relative to the haploid genome of the cells used for injection (I). BF, brightfield.

concluded that the tissue distribution of parthenogenetic cells is restricted (Fundele et al., 1990), other studies using ESCs have indicated that a wide range of tissues can be populated in chimeric mice (Allen et al., 1994; Jiang et al., 2007; Chen et al., 2009; Liu et al., 2011). One study, which used tetraploid complementation, generated functional extra-embryonic tissues from tetraploid host cells and obtained one live mouse composed substantially from parthenogenetic ESCs (Chen et al., 2009).

We therefore sought to address the developmental potential of haploid parthenogenetic ESCs by tetraploid complementation. For this, we aggregated tetraploid B6CBA F1 4-cell embryos with H129-1 haploid ESCs transfected with the GFP reporter. We observed efficient integration of GFP-positive cells into the inner cell mass of the developing blastocyst. After implantation we also found extensive contribution to the developing epiblast at E7.5 after diploidisation in vivo (Fig. 2G-I). In one case, GFP-positive cells were observed in a head-fold stage embryo, in which they appeared distributed over a wide range of embryonic tissues. We interpret these results as indicative of a high degree of autonomous developmental potential. In additional experiments using dsRed-marked HRex1 haploid ESCs, we also observed different degrees of contribution to E7.5 embryos (supplementary material Fig. S2A-F). We investigated the genomic status of these chimeras and found in some embryos a significant fraction of cells that maintained a haploid karyotype. However, our data suggest that the extent of haploid cell contribution correlates with aberrant development. Taken together, our data suggest that haploid ESCs have a wide developmental potential after diploidisation but that a haploid karyotype appears incompatible with postgastrulation development in high contribution chimeric embryos.

To further assess whether the developmental potential is increased after diploidisation we injected purified diploid GFP-marked H129-1 ESCs into tetraploid blastocysts and followed development at E9.5, E12.5 and E14.5 (supplementary material Fig. S3). At E9.5, three out of three embryos contained GFP-positive cells but only one showed clear somite development. One out of 12 implantation sites at E12.5 contained embryonic structures derived from GFP-positive cells. However, development was aberrant. From 22 implantation sites at E14.5, only three contained GFP-positive yolk sac tissue. These observations suggest that, using tetraploid complementation, embryo development beyond E9.5 from parthenogenetic cells is inefficient even after diploidisation.

Stability of a haploid karyotype in ESC differentiation

Previous studies have reported that haploid ESCs gain a diploid karyotype when they differentiate (Elling et al., 2011; Leeb and Wutz, 2011). This prompted us to investigate possible mechanisms for diploidisation. Experiments with mixed cultures of GFP-marked haploid H129-1 ESCs and dsRed-marked haploid H129-2 ESCs clearly indicate that diploidisation is primarily the result of endoreduplication of the haploid genome (supplementary material Fig. S4). This observation shows that cell fusion makes no, or negligible, contribution in culture. However, at present we cannot fully rule out the possibility that in certain developmental settings mechanisms other than endoreduplication contribute to diploidisation.

We then investigated the stability of a haploid karyotype during differentiation. We followed the differentiation into neural progenitors (Pollard et al., 2006) of haploid HRex1 ESCs derived from mice carrying a destabilised GFPd2 reporter targeted to the *Rex1* (*Zfp42* – Mouse Genome Informatics) gene locus (Wray et al., 2011). For inducing neural differentiation we shifted haploid HRex1 ESCs to N2B27 medium without 2i inhibitors and LIF (Fig. 3A). *Rex1* was rapidly downregulated, indicating exit from ground state pluripotency (Marks et al., 2012). We observed that a prominent haploid population was maintained after 5 days in N2B27 culture. When we subsequently aggregated the cells in suspension culture we observed a rapid increase in diploid cells (Fig. 3A). After replating aggregates the fraction of haploid cells further diminished, indicating that a haploid karyotype is lost rapidly during neural differentiation.

To investigate the capacity to maintain a haploid genome in the early stages of differentiation we investigated the conversion into postimplantation epiblast stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007). We shifted haploid HRex1 ESCs to medium containing Fgf and activin and observed a loss of Rex1-GFPd2 reporter expression after 48 hours, indicating exit from naïve pluripotency (Guo et al., 2009). A predominant haploid DNA content was observed at this time point (Fig. 3B). We subsequently sorted the haploid G1 fraction of these cultures and followed them through further differentiation. After 3 days, an increase in the number of diploid cells was already apparent and after one passage the fraction of haploid cells had been lost (Fig. 3B, middle and right panels, respectively). This indicates that a haploid genome content might be incompatible with maintaining an EpiSC, or primed pluripotent, state. These observations are consistent with the requirement for dosage compensation in differentiating female ESCs and postimplantation epiblast development (Penny et al., 1996; Marahrens et al., 1997; Stavropoulos et al., 2001; Lee, 2005).

By contrast, extra-embryonic development has been observed to be remarkably stable in the absence of dosage compensation before implantation (Marahrens et al., 1997). This prompted us to investigate extra-embryonic differentiation of haploid ESCs. Mouse ESCs are normally excluded from forming extra-embryonic fates but can be induced to extra-embryonic differentiation by expression of dominant fate-determining transcription factors such as Gata6 or Cdx2 (Murakami et al., 2011). We transfected haploid ESCs with a vector for expression of a glucocorticoid receptor fusion of Gata6 (Gata6GR). In this way, Gata6 translocation to the cell nucleus can be induced by addition of dexamethasone (Dex) to the culture medium. We observed characteristic changes in cell morphology 48 hours after addition of Dex and followed the DNA content of the cell population over three passages (Fig. 3C). In these experiments a predominant haploid karyotype was maintained and only a minor fraction of diploid cells emerged (Fig. 3C). These observations suggest that Gata6-induced cell fates might be compatible with a haploid genome. This would be consistent with earlier observations of implantation of haploid embryos that implied that functional extra-embryonic lineages can be formed (Kaufman et al., 1983; Latham et al., 2002). From these data we conclude that extra-embryonic differentiation may be compatible with a haploid karyotype.

Conversely, our data suggest that differentiation into embryonic cell lineages correlates with selection for a loss of haploid and gain of diploid genome state as early as the postimplantation epiblast. EpiSCs are known to be dosage compensated, as observed by *Xist* expression and a heterochromatic inactive X chromosome (Guo et al., 2009), although it is not clear whether X inactivation is obligatory in these cells. Our data could suggest that the transition from naïve to primed pluripotency during in vitro differentiation or in the embryo presents a bottleneck, possibly owing to the requirement for a precise balance of X chromosomal to autosomal genes (X:A=1:2) that cannot be achieved with a haploid karyotype (X:A=1:1). This notion is consistent with the increased stability of a haploid karyotype in ESC cultures held in the naïve ground state using 2i and LIF as compared with serum and LIF (supplementary material Fig. S5). Interestingly, the proliferation of near-haploid human tumour cells might suggest that this requirement could be overcome in certain differentiated lineages through oncogenic signals (Kotecki et al., 1999; Carette et al., 2009; Carette et al., 2011). In the future, it will be interesting to further define the requirements for maintaining a haploid genome in differentiated cells.

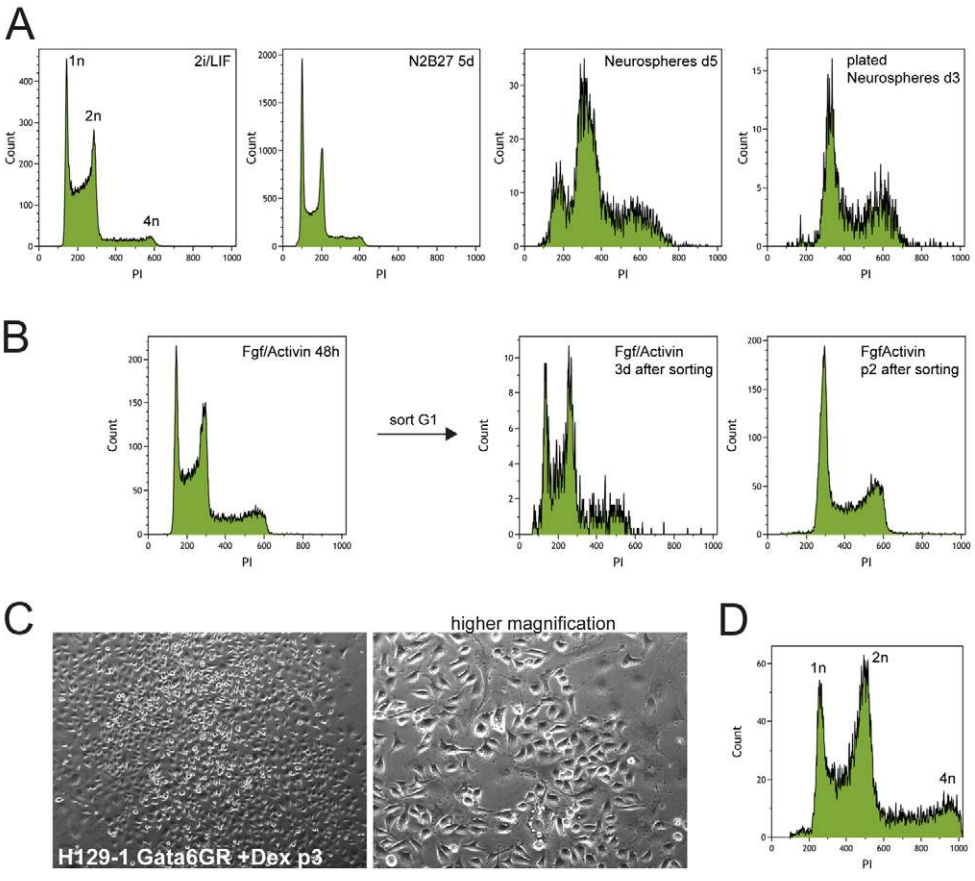

Fig. 3. Differential stability of the haploid karyotype during in vitro differentiation. (**A**) Haploid HRex1 ESCs were differentiated along the neural lineage using an in vitro differentiation protocol (see text). The DNA content was analysed by flow cytometry after PI staining. (**B**) Differentiation of haploid HRex1 ESCs in EpiSC conditions results in the rapid gain of a diploid karyotype (see text). (**C**) Induction of Gata6GR expression in transgenic haploid H129-1 ESCs induces extra-embryonic differentiation as observed by changes in cell morphology. (**D**) Cells with a haploid genome content were observed after three passages as shown by flow analysis after PI staining.

In summary, germline potential is considered a defining hallmark of mouse ESCs. A recent report has used androgenetic haploid mouse ESCs for mouse production by semicloning, thereby opening up the possibility for transferring genetic modifications into mice (Yang et al., 2012). In contrast to androgenetic haploid ESCs, which may lack the capacity to produce germline chimeras (Yang et al., 2012), our results establish that parthenogenetic haploid ESCs are fully competent for functional colonisation of the mouse germline. This confirms their identity as authentic pluripotent ESCs and additionally creates the potential for extending in vitro genetic screens and manipulations directly into mouse models.

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Competing interests statement
The authors declare no competing financial interests related to the results reported in this paper but wish to disclose that the establishment and germline transmission of haploid mouse ESCs has been submitted as part of a patent application.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.083675/-/DC1

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