LETTER

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Androgenetic haploid embryonic stem cells produce live transgenic mice

Wei Li[1,2]*, Ling Shuai[1,3]*, Haifeng Wan[1,2]*, Mingzhu Dong[1,2]*, Meng Wang[4], Lisi Sang[1,2], Chunjing Feng[1,2], Guan-Zheng Luo[4], Tianda Li[1,3], Xin Li[1,3], Libin Wang[1,2], Qin-Yuan Zheng[1,2], Chao Sheng[1,3], Hua-Jun Wu[2,4], Zhonghua Liu[3], Lei Liu[1], Liu Wang[1], Xiu-Jie Wang[4], Xiao-Yang Zhao[1] & Qi Zhou[1]

Haploids and double haploids are important resources for studying recessive traits and have large impacts on crop breeding[1], but natural haploids are rare in animals. Mammalian haploids are restricted to germline cells and are occasionally found in tumours with massive chromosome loss[2,3]. Recent success in establishing haploid embryonic stem (ES) cells in medaka fish[4] and mice[5,6] raised the possibility of using engineered mammalian haploid cells in genetic studies. However, the availability and functional characterization of mammalian haploid ES cells are still limited. Here we show that mouse androgenetic haploid ES (ahES) cell lines can be established by transferring sperm into an enucleated oocyte. The ahES cells maintain haploidy and stable growth over 30 passages, express pluripotent markers, possess the ability to differentiate into all three germ layers *in vitro* and *in vivo*, and contribute to germlines of chimaeras when injected into blastocysts. Although epigenetically distinct from sperm cells, the ahES cells can produce viable and fertile progenies after intracytoplasmic injection into mature oocytes. The oocyte-injection procedure can also produce viable transgenic mice from genetically engineered ahES cells. Our findings show the developmental pluripotency of androgenentic haploids and provide a new tool to quickly produce genetic models for recessive traits. They may also shed new light on assisted reproduction.

Haploid stem cells offer an easy-to-manipulate genetic system and therefore hold great value for studies of recessive phenotypes. Recently, haploid ES cells have been successfully derived from medaka fish[4] and mice[5,6], and this has provided opportunities for genetic manipulations at the cellular level in a haploid but pluripotent system. However, the feasibility of using mammalian haploid ES cells for animal-level assays is untested. In addition, several basic questions regarding mammalian haploid ES cells are still unsolved and deserve more investigations, including: first, whether the haploid nature of ES cells can be steadily maintained in further-differentiated cells; second, whether the haploid ES cells can function as haploid gametes to support fertilization and development; and third, whether genetic modifications in the haploid ES cells can bypass the germline barrier to be transmitted properly to offspring.

In an attempt to address the above questions, we established a protocol to generate mouse androgenetic haploid embryos by injecting sperm into enucleated MII phase oocytes[7,8] (Supplementary Fig. 1 and Supplementary Table 1). Using the 2i medium[9] with leukaemia inhibitory factor (LIF) and feeder cells, we generated a total of 27 androgenetic haploid embryonic stem cell (ahES) lines from 262 reconstructed embryos (Supplementary Table 2). The addition of commonly used additives for ES culture had no obvious effect on the ahES-cell-derivation efficiency (Supplementary Table 2). The transgenic chicken β-actin promoter-driven enhanced green fluorescent protein (eGFP) from the donor sperms was successfully inherited and expressed in the derived ahES-cell lines (Fig. 1a). We also established ahES-cell lines carrying an Oct4 (also known as Pou5f1) promoter-driven eGFP (Oct4–eGFP) reporter, by which eGFP expression was primarily restricted in pluripotent cells and germ cells[10].

Most of the originally obtained ahES-cell lines contained approximately 10–30% haploid cells. After three or more rounds of purification by separating Hoechst 33342-stained cells using fluorescence-activated cell sorting (FACS), haploid ahES cells with nearly 90% purity were obtained (Fig. 1b). The established ahES-cell lines all had a single X chromosome (Fig. 1c, d). They retained the haploid genome (19 autosomes and the X chromosome (19 + X)) after being passaged more than 25 times. Continuous haploid purification by FACS (more than 6 rounds) slowed down (with over 50% decrease) the autodiploidization rate of ahES cells (Supplementary Fig. 2). The lack of ahES cells containing the Y chromosome is in accordance with previous reports of the poor developmental ability of androgenetic embryos of YY chromosomes[11,12]. Comparative genomic hybridization (CGH) analysis revealed no consistent copy-number variation among the examined ahES-cell lines (AH129-5, AH129-N1 and AH129-NC1), except for a common deletion on the X chromosome (Fig. 1e and Supplementary Table 3). This deletion is a CGH artefact attributed to the probe design around the 1.75-Mb highly repetitive region (Supplementary Fig. 3). The same artefact was observed in previous reports of haploid ES cells[5,13] using the same NimbleGen whole-genome tiling array.

The ahES cells expressed typical pluripotent marker genes[14], including Oct4, Nanog and Sox2 (Supplementary Fig. 4). Global gene-expression analysis among 3 independently generated ahES-cell lines (AH129-5, AH129-NC1 and AH129-N1), 2 diploid ES-cell lines (R1 and CS 1-1), mouse round spermatids and embryonic fibroblast (MEF) cells revealed a very high correlation ($r = 0.98$) between ahES cells and ES cells, both with distinct gene-expression profiles from mouse round spermatids (Supplementary Fig. 4).

The ahES cells could form embryoid bodies and differentiate further into neural-lineage cells *in vitro*, but the majority of the differentiated cells had already undergone diploidization (Supplementary Fig. 5). Teratomas containing all 3 germ layers could be detected after subcutaneously injecting ahES cells (with nearly 90% purity) into severe-combined-immune-deficiency mice (Fig. 2a). We then injected freshly FACS-sorted G0- or G1-phase eGFP-positive ahES cells (from mice with black-coloured coats) into CD-1 blastocysts (from mice with white-coloured coats) to test their ability to form chimaeras. Chicken β-actin-driven eGFP and Oct4-eGFP expression were detected in both somatic and germline cells of the dissected chimaeric embryos at embryonic day 12.5 (E12.5), respectively (Fig. 2b, c, Supplementary Fig. 6 and Supplementary Table 4). Evidence for the

[1]State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China. [2]Graduate University of Chinese Academy of Sciences, Beijing 100049, China. [3]College of Life Science, Northeast Agricultural University of China, Harbin 150030, China. [4]Center for Molecular Systems Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China.

*These authors contributed equally to this work.

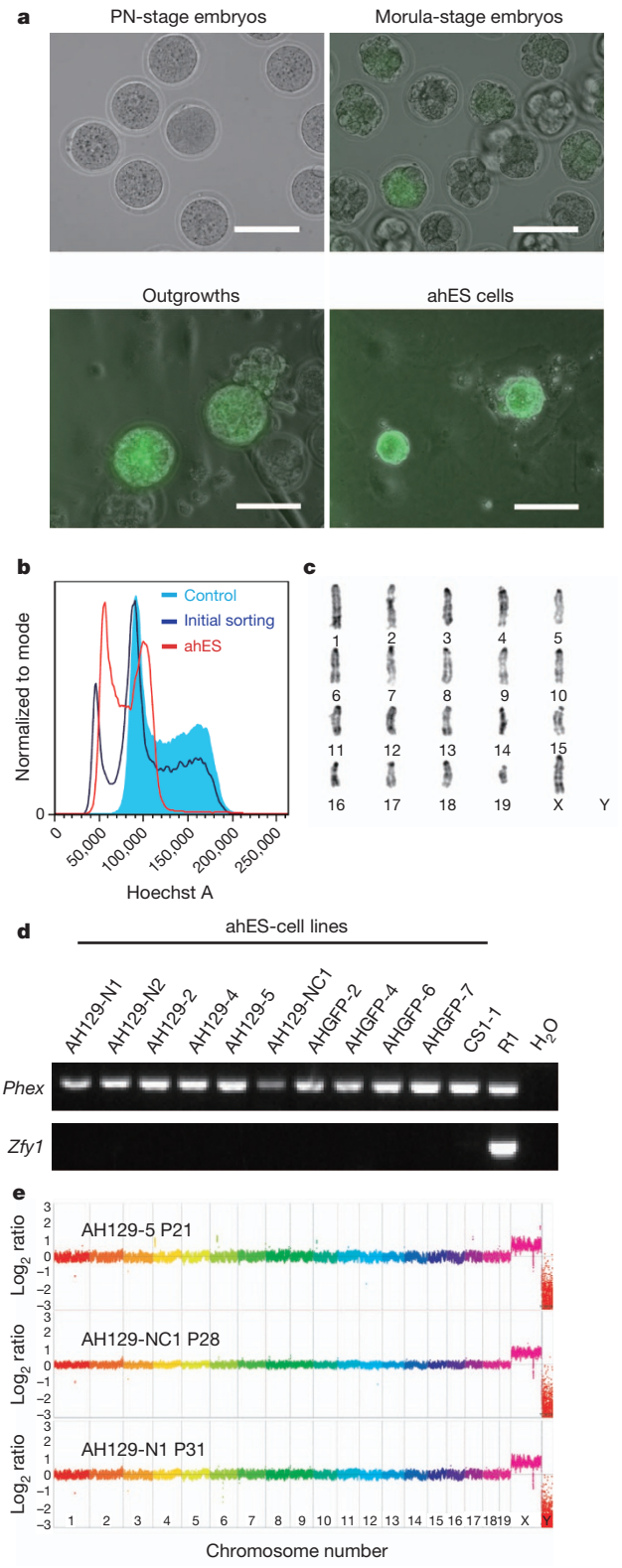

Figure 1 | Generation of androgenetic haploid ES cells. **a**, Schematic overview of the derivation process of ahES-cell lines carrying an eGFP marker. Scale bar, 100 μm. PN, pronuclear. **b**, FACS analysis of the DNA content of ahES-cell lines. Diploid ES-cell line with 2*n* chromosome sets is chosen as control (bright blue). The initial FACS sorting of the AHGFP-4 ahES-cell line at passage 5 resulted in 33.7% of haploid cells (dark blue). After purifying the G0- or G1-phase haploid cells for 3 times and followed by culturing the cells for 2 passages, 89.9% of haploid cells (passage 16) were obtained (red). **c**, Standard G-binding karyotype of an ahES cell line (passage 16), with 19 + X chromosomal set. **d**, Determination of the sex chromosome of ahES-cell lines by polymerase-chain-reaction (PCR) amplification of an X-chromosome-specific (*Phex*) or a Y-chromosome-specific (*Zfy1*) gene. ES-cell lines R1 (male) and CS 1-1 (female mouse ES-cell line.) were used as controls. **e**, CGH analysis of 3 ahES cell lines: AH129-5 (P21), AH129-NC1 (P28) and AH129-N1 (P31). Comparison of results between the genomic DNA of ahES cells and control 129Sv male mouse kidney is shown as the *y* axis, on a $\log_2$ base scale.

contribution of the ahES cells to adult chimaeric mice was provided by their coat-colour chimaerism (Fig. 2d) and by the presence of eGFP-positive cells within various tissues of dissected adult chimaeric mice, with $eGFP^+$ cells constituting 5.9%, 27.5%, 67.1% and 9.7% of the total number of cells analysed in the spleen, liver, kidney and heart, respectively (Supplementary Fig. 7). No haploid cells were detected among eGFP-positive cells (Supplementary Fig. 7), as they may have already undergone diploidization like observed in other studies[5]. Collectively, these results provide evidence of the pluripotent nature of the ahES cells.

We explored the feasibility of generating differentiated haploid cells from the ahES cells both *in vivo* and *in vitro*. Haploid G0- or G1-phase ahES cells freshly sorted by FACS were injected into diploid blastocysts to produce chimaeric embryos. In the E6.5 chimaeric embryos, approximately 6% of eGFP-positive cells were haploid by flow-cytometry analysis (Fig. 2e). However, no eGFP-positive haploid cells were identified from E12.5 embryos (Supplementary Fig. 6). After four or more rounds of FACS selection, we successfully established haploid epiblast-stem-cell-like cell lines with 87.8% of haploid cells (Fig. 2f, g and Supplementary Fig. 8), showing the feasibility of establishing haploid non-ES-cell lines in mammals. The cells expressed marker genes of epiblast stem cells (Oct4, Nanog, Gata4 and Fgf5), lost alkaline phosphatase activity and had a similar gene-expression profile to that of the diploid epiblast stem cells, but were distinct from the parental ahES cells (Fig. 2h and Supplementary Fig. 8c–e)[15–17]. The haploid epiblast-stem-cell-like cells maintained the haploid karyotype (19 + X) after being passaged for up to 15 generations. They had the ability to form embryoid bodies when being cultured without growth factors in low-attachment dishes (Supplementary Fig. 8f), and to differentiate further into haploid neural progenitor cells (with 42.3% haploid cells; Supplementary Fig. 9). These results show that the ahES cells could differentiate under the haploid state.

To test the genetic transmissibility of the ahES cells, we injected the FACS-selected G0- or G1-phase haploid ahES cells into pre-activated oocytes (intracytoplasmic ahES-cell injection (ICAI); Supplementary Fig. 10)[18,19]. The obtained embryos formed 'paternal' pseudo-pronuclei with a dynamic demethylation pattern typical of embryos produced by intracytoplasmic sperm injection[20,21] (Supplementary Fig. 11). A total of 599 2-cell embryos and 171 blastocysts reconstructed from 6 randomly selected ahES-cell lines were transplanted into 38 pseudopregnant mice. The derived embryos were normal at E13.5 with correctly inherited eGFP (Fig. 3a). Twenty-four female full-term pups were successfully obtained from four independent ahES cell lines, of which ten survived to adulthood (Table 1 and Supplementary Fig. 12a). The 14 pups that did not survive died within 30 min after birth, probably owing to developmental retardation, indicated by their abnormally small body size (Fig. 3b, c and Supplementary Fig. 12b).

Among the 10 surviving mice, 9 looked normal at adulthood and delivered healthy progeny with litter sizes of 8–11 pups when mated with CD-1 males at 8 weeks of age. The ICAI-produced mice were validated by the simple sequence-length polymorphism (SSLP) (Supplementary Fig. 12c), and their offspring showed Mendelian segregation of coat colour (Fig. 3d) and the inherited expression of β-actin eGFP (Supplementary Fig. 12d). The other mouse has a smaller body and has been unable to become pregnant so far. These results showed the ability of ahES cells to 'fertilize' oocytes and produce healthy and fertile animals, and thereby to transmit their genetic material to offspring.

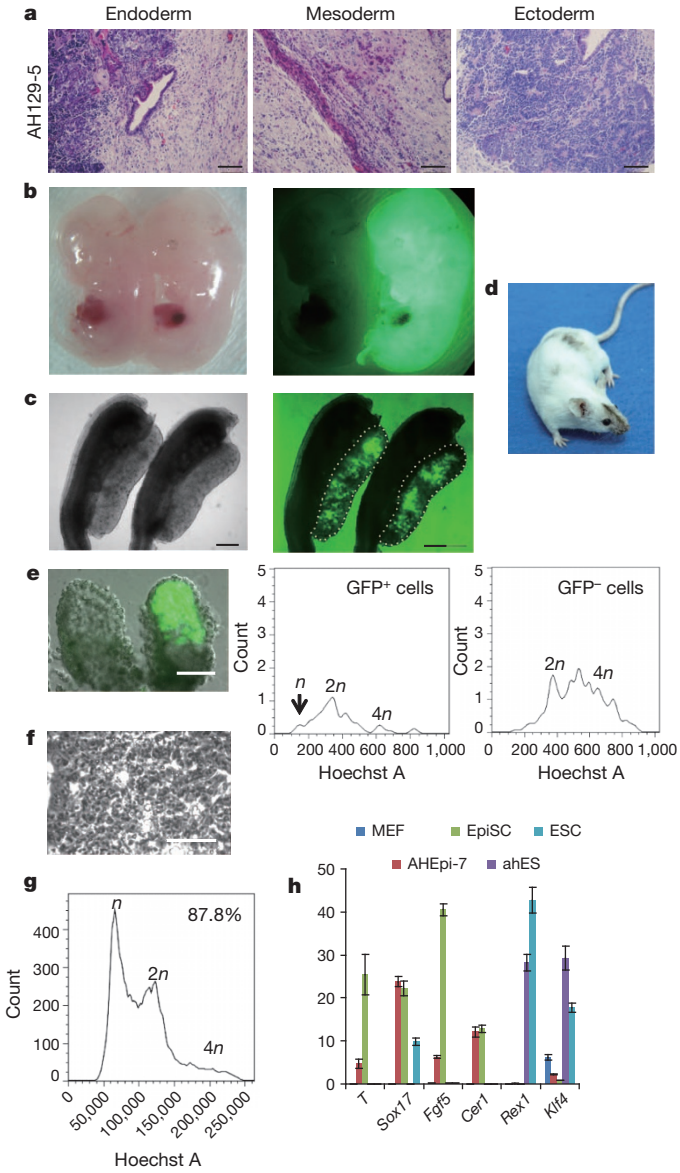

Figure 2 | The pluripotency of the ahES cells. **a**, Teratoma formation of ahES cells (from ahES-cell line AH129-5). Shown are the teratoma dissection slices representing all three germ layers identified by staining with haematoxylin and eosin in teratomas. Scale bar, 500 μm. **b**, E12.5 chimaeric embryos formed by blastocyst injection of G0- or G1-phase haploid AHGFP-4 cells into CD-1 diploid blastocysts. eGFP fluorescence indicated the chimaeric contribution of AHGFP-4 (right panel) and its non-chimaeric littermate without eGFP (left panel). **c**, Fluorescence detection of the gonads (female) of an E12.5 Oct4–GFP ahES cell derived chimaeric embryo. Scale bar, 2 mm. **d**, Adult chimaeric mouse produced by microinjection of G0- or G1-phase haploid AHGFP-4 cells into diploid blastocysts. **e**, Flow-cytometry analysis of DNA content of both the eGFP-positive and eGFP-negative cells in E6.5 chimaeric embryos. An E6.5 chimaeric embryo generated from ahES cells (AHGFP-4) is shown on the right of the image (left panel), and on the left of the image is a non-chimaeric embryo at the same stage as the control for eGFP detection. Scale bar, 100 μm. The arrow (middle panel) shows the peak of the haploid chromosome set among $eGFP^+$ cells. **f**, Brightfield of haploid epiblast-stem-cell-like cells (EpiSC-like cells; diploid EpiSCs derived from post-implantation embryos[17]). Shown is the AHEpi-7-cell line (haploid EpiSC-like cell line 7) at passage 8. **g**, Flow-cytometry analysis of DNA content of a haploid EpiSC-like cell line, AHEpi-7 (passage 6). **h**, Real-time PCR analysis of EpiSC-marker gene expression in different cell lines. Error bars, s.d. of triplicate reactions.

To demonstrate the feasibility of using ahES cells in transgenic manipulations, vectors carrying a neomycin-resistant gene (neo^r) were electroporated into 5×10^6 ahES cells (passage 12, FACS-sorted

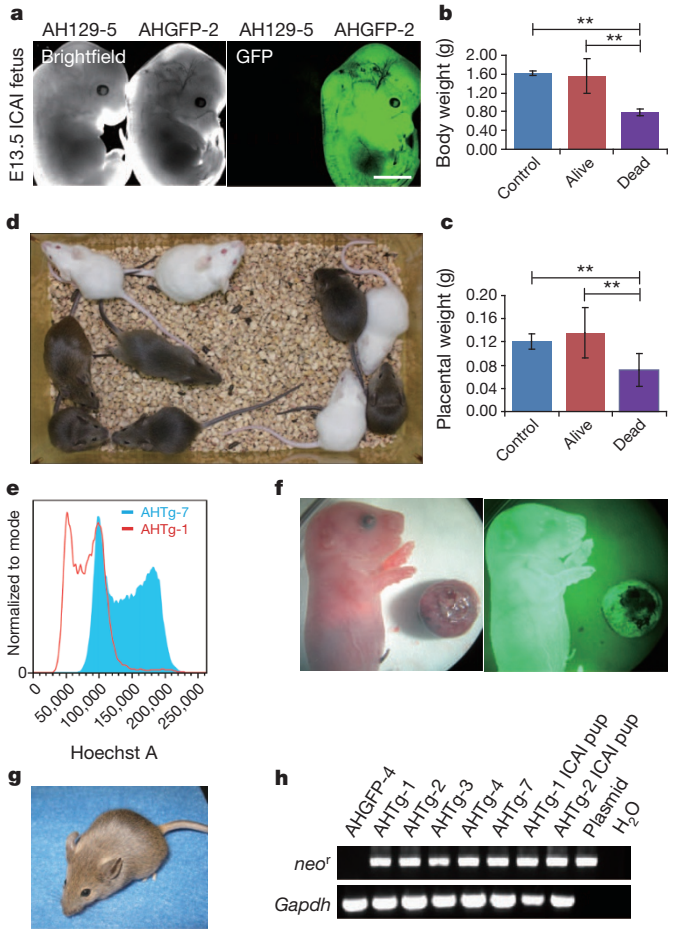

Figure 3 | Generation of ICAI offspring and transgenic mice. **a**, Live E13.5 ICAI embryos produced by 2 androgenetic haploid ahES-cell lines AH129-5 (left panel, eGFP negative) and AHGFP-2 (right panel, eGFP positive). Scale bar, 50 μm. **b, c**, Comparison of the body weights (**b**) and placenta weights (**c**) of the alive (1.56 ± 0.36 g; $n = 12$ (mean ± s.e.m.)) and dead (0.78 ± 0.07 g; $n = 17$) ICAI pups with the control group (wide-type mice, 1.63 ± 0.05 g; $n = 12$). $^{**}P < 0.001$ (student t-test). **d**, Coat-colour separation among the F2 generation of an ICAI-produced adult mouse (129/Sv X CD-1 background) after mating with a CD-1 male mouse. **e**, FACS analysis of the DNA content of 2 established genetically modified ahES-cell lines. AHTg-1 (red) maintained high proportions of haploid cells, whereas AHTg-7 (light blue) had no haploid cells. **f**, Live normal newborn mice produced from the transgenic ahES-cell line AHTg-1 through ICAI, with the expression of the eGFP transgene detected throughout the pup body and the placenta. **g**, A PGK–neo^r transgenic ICAI mouse that survived to adulthood. **h**, PCR analysis of the integration of the transgene neo^r in 5 transgenic cell lines, and the offspring of the AHTg-1 and AHTg-2 cell lines. Non-transgenic cell line AHGFP-4 is used as a negative control.

AHGFP-4 cells with the β-actin eGFP transgene). A total of 187 neomycin-resistant colonies appeared after 1 week of antibiotic (geneticin, G418) selection. We randomly picked and expanded 12 G418-resistant colonies into sub-lines (Supplementary Fig. 13a). Six lines (named AHTg-1 to AHTg-6) maintained the haploid chromosome set even after 17 passages (Fig. 3e and Supplementary Fig. 13b), whereas the other 6 cell lines (AHTg-7 to AHTg-12) were mostly converted to diploid cells (Fig. 3e and Supplementary Fig. 13c). After injecting these transgenic ahES cells (collected immediately after FACS sorting) from two sub-lines into pre-activated MII phase oocytes, we successfully obtained 143 2-cell embryos and transplanted them into pseudopregnant mice. Five full-term live pups derived from two transgenic cell lines were obtained from three foster mothers (Table 1) carrying the neo^r transgene and also inherited the eGFP transgene (Fig. 3f and Fig. 3h). Two pups were healthy and survived to adulthood (Fig. 3g). The others had lower body weights and died quickly after birth, probably owing to

Table 1 | Developmental efficiencies of ICAI embryos

Cell type	Donor cells	No. of injected oocytes	No. of cleavages (% injected)	No. of transferred embryos	No. of implantations (% ET)	No. of full-term pups (% ET)	No. of survived (% ET)
Control	Round spermatids	104	84 (80.8)	84	ND	7 (8.3)	7 (8.3)
ahES cells	AH129-NC1	257	200 (77.8)	48	21 (10.5)	0*†	0
	AH129-4	496	399 (80.4)	72	63 (15.8)	4 (1.0)*†	1 (0.3)
	AH129-5	337	240 (71.2)	51	24 (10.0)	2 (0.8)*†	1 (0.4)
	AHGFP-2	289	205 (70.9)	205	36 (17.6)	0*†	0
	AHGFP-4	241	170 (70.5)	170	ND	9 (5.3)	6 (3.5)
	AHGFP-6	317	224 (70.7)	224	51 (22.8)	9 (4.0)	2 (0.9)
Gene-modified ahES cells	AHTg-1	65	50 (76.9)	50	18 (36.0)	2 (4.0)	2 (4.0)
	AHTg-2	125	93 (74.4)	93	37 (39.8)	3 (3.2)	0

* $P < 0.05$ compared with control (two-tailed Fisher's exact test).
† $P < 0.05$ compared with the best ahES-cell AHGFP-4 (two-tailed Fisher's exact test).
ET, embryonic transfer. ND, not determined.

retarded growth (Supplementary Fig. 13d). During the revision process of this manuscript, a further 23 full-term transgenic pups were obtained, of which 6 have survived so far (Supplementary Table 5).

The production of normal progeny by ahES cells through 'fertilization' raises an intriguing question about the imprinting status of ahES cells, as the proper regulation of imprinted genes is essential for normal development of mammals[22–24]. We examined the differentially methylated regions (DMRs) of several genes in different cell lines by bisulphite sequencing. The DMRs of two maternally imprinted genes, *Snrpn* and *Airn*, kept their unmethylated status at both early and late cell passages, as in sperm (Supplementary Fig. 14a), whereas paternally imprinted genes (*H19* and *Gtl2*) showed inconsistent methylation status, with the potential to keep sperm-like methylated status in early cell passages and to partially lose methylation during passaging (Supplementary Fig. 14a). Whether this phenomenon was an intrinsic feature of ahES cells or caused by the culturing process requires further study.

The methylation changes of these genes were reflected well in their expression levels (Supplementary Fig. 14b). We analysed the expression of 90 imprinted genes in the mouse genome and detected reliable signals for 54 genes. Unexpectedly, the expression of these genes did not exhibit consistent parent-of-origin effects (Supplementary Fig. 14c). Four cell lines that produced live ICAI animals and two cell lines that failed to generate full-term ICAI pups were included in the study, and no group difference was found between them (Supplementary Fig. 14c).

We tested further the correlation of abnormal development of ICAI pups and the aberrant regulation of imprinted genes in the alive and early-dead (died within 30 minutes after birth) ICAI mice. The expression profiles of imprinted genes showed high concordance between healthy adult ICAI mice and age-matched wild-type controls, whereas there was more variation between the expression profiles of early-dead ICAI pups and controls (Supplementary Fig. 15a). The methylation level of *Gnas* was maintained well in all transgenic mice, but both early-dead mice lost imprinting at the *H19* DMR, and one of them showed slight hyper-methylation at the *Snrpn* DMR (Supplementary Fig. 15b), suggesting a potential link between the abnormal development of ICAI embryos and the altered regulation of imprinted genes[25,26].

Our data shows that laboratory-produced haploid pluripotent stem cells, although epigenetically distinct from germ cells, could function like gametes to form zygotes and produce viable progenies, which may provide an invaluable resource for assisted reproduction. The production of fertile adult mice from ahES cells shows that the genetic information in haploid ES cells is functionally complete and stable, thus enhances the merits of haploid ES cells in genetic studies. During the revision process of this work, an independent study[13] also reported the production of androgenic haploid ES cells and the feasibility of using them to produce healthy progenies, which confirmed part of our findings. The establishment of stable haploid epiblast-stem-cell-like cell lines proved that the haploid status of ahES cells can be transmitted and stably maintained in other cell types under certain circumstances. The work also provides a new approach for genetic manipulation in animal models without available germline-competent ES cells, including non-human primates[27], as modifications in such haploid stem cells could be transmitted to offspring through intracytoplasmic injection into mature oocytes, which may serve as a more efficient and simple strategy for gene-targeting studies.

METHODS SUMMARY

Androgenetic haploid embryos were reconstructed as described previously[7]. A total of 262 morula-stage embryos were plated in 4-well plates coated with mitomycin-C-treated mouse embryonic fibroblast (MEFs) as feeder cells and cultured with 2i medium[9], from which 27 ahES cell lines were successfully derived. ahES cells were purified and examined for their haploid and pluripotent characteristics using standard methods. Approximately 1×10^7 ahES cells were subcutaneously injected into the hind limbs of severe-combined-immune-deficiency mice to generate teratomas. Approximately 12–15 G0- or G1-phase ahES cells collected immediately after FACS sorting were microinjected into each blastocysts to produce chimaeric embryos. ICAI was carried out as described[18], by injecting ahES cells into oocytes. The embryo development was examined at E13.5 and at full term. PGK-*neo*^{r} vectors were transfected into the ahES cells by electroporation and ICAI was carried out to generate transgenic animals.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions Q.Z. and X.-Y.Z. designed the experiments;; W.L., L.S., H.W., M.D., M.W., L.S., C.F., T.L., X.L., L.W., Q.-Y.Z. and C.S. performed experiments; Q.Z, X.-Y.Z, X.-J.W., G.-Z.L., W.L., H.-J.W. and L.L. analysed data; Q.Z., X.-Y.Z., Z.L., and L.W..supervised experiments; W.L., L.S., H.W. and M.D. contributed to part of the Methods. Q.Z., X.-Y.Z., X.-J.W. and W.L. wrote the paper.

Author Information CGH data and gene-expression data are deposited at the Gene Expression Omnibus under accession numbers GSE39390 and GSE39391, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to Q.Z. (qzhou@ioz.ac.cn) or X.-Y.Z. (xyzhao@ioz.ac.cn).

METHODS

Mice. Specific pathogen-free (SPF)-grade mice were obtained from Beijing Vital River laboratory animal centre and housed in the animal facilities of the Institute of Zoology, Chinese Academy of Sciences. All studies were carried out in accordance with the guidelines for the Use of Animals in Research issued by the Institute of Zoology, Chinese Academy of Sciences. Male mice of 129S2/SvPasCrlVr, B6D2F1 (C57BL/6 × DBA/2), chicken β-actin–eGFP transgenic mice (gift from Q. Xu) and Oct4–eGFP transgenic mice backgrounds were used for sperm collection. Female mice of B6D2F1 (C57BL/6 × DBA/2) and CD-1 backgrounds were used to provide oocytes for micromanipulation.

Androgenetic haploid embryo reconstruction. Reconstruction of androgenetic haploid embryos was carried out according to the one-step micromanipulation (OSM)[7] method as described previously (Supplementary Fig. 1). In brief, MII oocytes were collected from 8-week-old B6D2F1 female mice by super-ovulation. The microinjection procedure was performed on a Piezo (PMAS-CT150, Prime Tech) drill micromanipulator (DMIRB, Leica). The sperm head was aspirated in the injection pipette (10 μm internal diameter) and injected into 1 oocyte, and then the spindle of the oocyte was removed by the injection pipette while withdrawing it from the oocyte. Reconstructed embryos were cultured in KSOM-AA medium supplemented with 5 μg ml^{-1} of cytochalasin B at 37 °C, 5% CO_2, for 5–6 h, then the cytochalasin B was removed and the embryos were cultured for a further 3 days to morula stage for ahES-cell derivation.

Derivation of ahES cells. Haploid morulas were cultured in 2i medium as described previously[9] for cell-line derivation. The 2i medium consists of N2B27 medium supplemented with 1 μM MEK inhibitor PD0325901 (Stemgent), 3 μM GSK3β inhibitor CHIR99021 (Stemgent) and mouse recombinant LIF (Millipore). Three culture systems were used for the derivation of haploid ES cells: 2i medium; 2i medium with 2 μM p53 inhibitor Piffithrin-α (Calbiochem); and 2i medium supplemented with 2 μM p53 inhibitor Piffithrin-α, vitamin C (Sigma), α-lipid acid (Sigma) and α-tocopherol (Sigma). ahES cells derived from chicken β-actin–eGFP transgenic mice were cultured in 2i medium with 2 μM piffithrin-α and 5% knockout serum replacement (GIBCO). For the derivation of haploid ES cells, morulas were seeded in four-well dishes. The outgrowths were digested with 0.25% trypsin after being cultured for 5–7 days, ES-cell colonies usually appeared after 2–3 days. ahES cells were cultured in 2i medium and passaged with a split ratio of 1:3 every 2–3 days.

Purification of haploid ES cells. After being cultured for 4–5 passages, ahES cells were purified by flow-cytometry sorting as described previously[5]. ahES cells were collected and single-cell suspensions were obtained by repetitive pipetting and transfer through a 40-μM cell strainer. Haploid and diploid ES cells were incubated with Hoechst 33342 (Invitrogen) and 50 μM Verapamil (Sigma) for 30 min at 37 °C and sorted or analysed on a BD FACSAria II (BD Biosciences). Diploid (2*n*) ES cells were used as a control. Flow-cytometric data were analysed using the ModFit software (Verity Software House) following the manufacture's instructions. Standard G-banding chromosome analysis was carried out to detect whether the ahES cells had a normal karyotype. Approximately 30 separate metaphase spreads were examined for each cell line. The chromosome images were arranged according to a previously published G-banded karyotype ideogram[28].

Immunofluorescence microscopy, alkaline-phosphatase staining and western blot analysis. Immunostaining was carried out as reported previously[29]. In brief, samples were fixed with 4% paraformaldehyde (PFA) for 30 min at approximately 20–25 °C, washed 3 times with 1× PBS, and penetrated with 0.5% Triton X-100 for 30 min at approximately 20–25 °C. Non-specific sites were blocked with 500 μl 2% BSA for 1 h at approximately 20–25 °C. The samples were then incubated with the primary antibody overnight at 4 °C. The used primary antibodies included anti-Oct4 (Santa Cruz), anti-Sox2 (Santa Cruz), anti-SSEA1 (Millipore), anti-Nanog (Abcam), anti-Nestin (Millipore), anti-Doublecortin (Santa Cruz), anti-5mC (Abcam), anti-Tuj1 (Millipore) and anti-MAP2 (Millipore). Samples were washed with 1× PBS on the second day, followed by incubating with AlexaFlur 488-conjugated secondary antibody (diluted in 2% BSA) at approximately 20–25 °C for 1 h, and observed under confocal microscope (ZEISS, LSM 780 META). An alkaline-phosphatase-staining kit (Sigma) was used according to the manufacturer's instructions. Observations were made using an inverted microscope (Leica DMI3000B, Leica Co.).

For western blot analysis, protein samples were extracted from ahES cells, MEF cells and R1 ES cells, epiblast stem cells and epiblast-stem-cell-like cells. Equal amount of each protein sample were electrophoresed on SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (10%) gels, then transferred onto a polyvinylidene fluoride (PVDF) membrane. Blocked membranes were incubated with anti-Oct4 antibody (Santa Cruz), anti-Nanog (Abcam) or anti-Gata4 (Santa Cruz) at 4 °C overnight. After washing, horseradish peroxidise (HRP)-conjugated second antibody (1:1000 dilution; WAKO) was added to the membranes and incubated at approximately 20–25 °C for 1 h, and further washing was carried out. The immunoactivity was detected using an ECL detection kit (Millipore). The quantification of the results was accomplished using the computerized imaging program Quantity One (Bio-Rad).

Derivation of haploid epiblast-stem-cell-like cells. Derivation of haploid epiblast-stem-cell-like cells from haploid ahES cells was carried out as described previously[15], but with some modifications. ahES cells were dissociated using 0.25% trypsin and plated on the fibronectin-coated 4-well plates (5.6×10^4 cells per cm^2), cultured with N2B27 medium supplemented with 12 ng ml^{-1} bFGF, 20 ng ml^{-1} activin A, p53 inhibitor (2 μM) and y-27632 (5 μM). Haploid cells were purified by FACS sorting after 2–3 days post differentiation, then replated and cultured with N2B27 medium until confluent. Stable haploid epiblast-stem-cell-like cell lines (>85%) were established after 4 or more rounds of FACS sorting.

Embryoid-body formation and neural differentiation. Embryoid bodies were generated from ahES cells and diploid ES cells following protocols reported previously[29]. ES cells were digested with 0.25% trypsin, and suspended in gelatin-coated dishes for half an hour to remove feeder cells. The suspended ES cells were then collected and cultured as floating aggregations in the differentiation medium. The differentiation medium consisted of DMEM/F12 medium (GIBCO) supplemented with 10% knockout serum replacement (GIBCO), 0.1 mM non-essential amino acid (GIBCO), 2 mM Glutamax, 0.1 mM β-mercaptoethanol and 1 × penicillin–streptomycin solution. After 2 or 3 days, small aggregated embryoid bodies could be seen as spheres under the microscope. Neural differentiation was modified from a protocol reported previously[30]. Embryoid bodies were collected after culture on the differentiation medium for 6 days and attached on dishes coated with polyornithine (100 μg ml^{-1}) and fibronectin (10 ng ml^{-1}). Meanwhile, the culture medium was changed into N2 medium, which consisted of DMEM/F12 supplemented with 1 × N2, 2 mM glutamine and 1 × penicillin–streptomycin solution. After another 6 days, attached embryoid bodies expanded well and abundant neural precursors were formed. The expanded embryoid bodies were digested with 0.05% trypsin and transferred to the neural-stem-cell medium. The NSC medium consisted of DMEM/F12 medium supplemented with neurobasal medium (GIBCO) at a 1:1 ratio, 0.0025% bovine serum albumin fraction V, 0.5 × N2, 0.5 × B27, 2 mM glutamax, 0.1 mM β-mercaptoethanol, 10 μg ml^{-1} insulin, 1 × penicillin–streptomycin solution, 20 ng ml^{-1} FGF2 (R&D systems) and 20 ng ml^{-1} mouse EGF (Peprotech). Digested embryoid bodies were cultured as suspensions to generate purified neurospheres. The purified neural stem cells were then attached on dishes coated with polylysine (50 μg ml^{-1}) and laminine (5 μg ml^{-1}) for expansion[31]. All neural stem cells were characterized by morphological examination and immunostaining of specific markers. Differentiation was carried out in neuronal differentiation medium (N2B27 medium supplemented with 10 ng ml^{-1} human NT3 and 10 ng ml^{-1} human brain-derived neurotrophic factor (BDNF)) for 15 days and then cells were fixed with 4% paraformaldehyde for immunostaining.

Teratoma formation. Teratoma analysis was carried out to evaluate the pluripotency of ahES cells. Approximately 1×10^7 ahES cells were injected subcutaneously into the hind limbs of 6-week-old male severe-combined-immune-deficiency beige mice. After approximately 4 weeks, fully formed teratomas were dissected and fixed with 1× PBS containing 4% paraformaldehyde, embedded in paraffin, sectioned and stained with haematoxylin and eosin for histological analysis.

Diploid blastocyst injection. Chimaera generation was also carried out to evaluate the pluripotency of ahES cells *in vivo*. Super-ovulated female CD-1 mice (1.5 days post coitum (1.5 d.p.c.)) were killed to collect recipient embryos. Approximately 12–15 FACS-selected G0- or G1-phase ahES cells were microinjected into each blastocyst to produce a chimaeric embryo. After 1–4 h, these manipulated embryos were transferred into the oviduct of pseudopregnant CD-1 mice at 0.5 d.p.c. or 2.5 d.p.c. Chimaeras were identified by their coat colours or eGFP expression.

DNA-content analysis of *in vivo* differentiated ahES cells. Chimaeras derived by microinjection of ahES cells (AHGFP-4) into diploid CD-1 blastocysts were killed and dissected to perform DNA content analysis. Chimaeric fetuses (E6.5 and E12.5) and 4 organs (heart, liver, spleen and kidney) of adult chimaeras were dissected and digested into single cells with 0.05% trypsin with EDTA at 37 °C for 20 min. Cells were fixed in 4% PFA at 4 °C overnight. Fixed cells were incubated with 10 μg ml^{-1} Hoechst 33342 and 10 μg ml^{-1} RNase at 37 °C for 20 min. Flow-cytometric data were recorded on BD FACSAria II (BD Biosciences). The DNA content of differentiated ahES cells *in vivo* was analysed by ModFit software (Verity Software House) and graphed by Flowjo 7.6 software (Tree Star Institute).

Intracytoplasmic ahES-cell injection. The reproductive function of ahES cells was shown using the ICAI procedure. The ICAI procedure was modified from a previously reported round-spermatid-injection (ROSI)[18] procedure (Supplementary Fig. 10). In brief, matured MII oocytes were collected from the oviduct of super-ovulated 8-week-old female CD-1 mice. G0- or G1-phase and metaphase ahES cells were chosen as donors. To purify G0- or G1-phase cells, Hoechst 33342 staining method was applied to analyse the cell cycle of ahES cells, from which the

G0- or G1-phase cells were collected to perform the ICAI procedure. For ahES-cell injection, oocytes were pre-activated by 10 mM $SrCl_2$ in calcium-free CZB medium for 30 min before microinjection. The donor cells were then injected into oocytes separately to construct ICAI embryos by microinjection. The constructed embryos were activated by 10 mM $SrCl_2$ in calcium-free CZB medium at 37 °C with 5% CO_2 for 3 h. Completely activated embryos were transferred to KSOM-AA medium at 37 °C with 5% CO_2. The 2-cell stage or blastocyst stage ICAI embryos were transferred to the oviduct of psedudopregnant CD-1 mice at 0.5 d.p.c. At 13.5 days after embryo transfer, psedudopregnant CD-1 mice were killed to collect the E13.5 ICAI fetus. Images of the E13.5 ICAI embryos were captured using a Discovery V20 (ZEISS). Full-term pups derived from ICAI embryos were obtained through natural labour or caesarean section. To evaluate the development of ICAI embryos, the ROSI experiment was carried out as a control.

Comparative genomic hybridization analysis. CGH analysis was performed to detect any DNA copy number variants (CNVs) of ahES cells. Genomic DNA of 3 ahES-cell lines (AH129-5, AH129-NC1, AH129-N1) was extracted and equal amounts of DNA were used to hybridize to NimbleGen 3× 720K whole-genome tiling arrays (NimbleGen) with the male 129S2/SvPasCrl kidney DNA as a reference following protocols described previously[5]. The data were analysed using the company-provided (NimbleGen) software.

Global gene-expression analysis. Total RNA was extracted by Trizol (Invitrogen) as described previously[29]. Ten micrograms biotin-labelled cRNA (antisense RNA) was hybridized to GeneChip Mouse Genome 430 2.0 Array (Affymetrix). After hybridization and washing, the intensity of the fluorescence of the array chips was measured using the Affymetrix GeneChip Scanner GCS3000 according to the manufacturer's instructions. The expression-analysis file created from each chip scanning was imported into GeneSpring 11.5.1 (Agilent) for normalization and analysis. Normalization was carried out using MAS5. The Student's *t*-test was used to identify differently expressed genes with threshold of *P* value of <0.05 and a fold change of >1.5.

Simple sequence length polymorphism analysis. Simple sequence length polymorphism (SSLP) analysis was carried out as reported previously[29]. The sequences of primer pairs were adopted from the Mouse Genome Informatics website (http://www.informatics.jax.org/). DNA was extracted from tail tips of the ICAI fetuses and ahES cells. PCR products were separated by 3.5% agarose gels and visualized by gel imaging (Bio-Red) with ethidium bromide staining.

Bisulphite genomic sequencing. Genomic DNA was treated with the EpiTect Bisulphite Kit (Qiagen) according to the manufacturer's instructions. DMRs of *H19*, *Gtl2*, *Snrpn*, *Airn* and *Gnas* were amplified with nested primers (Supplementary Table 6). The first round of PCR was performed using 95 °C for 5 min; then 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. One microlitre of the first-round PCR product was used as a template for the second round of PCR, which was performed with 95 °C for 5 min; then 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The PCR products were cloned into pMD18-T vectors (Takara) and sequenced further for unmethylated C to T conversion. At least 10 randomly selected clones were sequenced and analysed for each gene.

Transgenic manipulation of ahES cells. To carry out transgenic manipulation in ahES cells, G0- or G1-phase haploid cells were purified by FACS sorting and cultured further to reach 5×10^6 cells (usually 1 to 2 passages). A total of 5×10^6 cells were electroporated with 25 μg plasmid carrying a PGK–*neo*^{r} cassette by Multiporator (Eppendorf) at 300 V and 300 μs, according to the manufacturer's instructions. Then the cells were plated onto 7 100-mm dishes coated with G418-resistant feeders and selected by 250 μg ml^{-1} G418 (Invitrogen) for 7 days. G418-resistant colonies were picked out for transgenic sub-cell-line derivation. Haploid transgenic cell lines carrying *neo*^{r} gene were used for transgenic ICAI mice production. Except for G418 resistance, the presence of transgenes was further confirmed by PCR amplification of the *neo*^{r} gene with specific primers: forward, 5′-CAGGTTCTCCGGCCGCTTG-3′; reverse, 5′-TCGCCGCCAAGCTCTTCAGC-3′. The PCR condition was 95 °C for 5 min, then 35 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C for 40 s.

Statistical analysis. Statistical analysis was carried out using SPSS 17.0 statistical software. The Student's *t*-test was used for statistical analysis. For all statistical analyses, a value of $P < 0.05$ was considered to be statistically significant.

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