Rapidly generating knockout mice from H19-Igf2 engineered androgenetic haploid embryonic stem cells

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Haploid mammalian embryonic stem cells (ESCs) hold great promise for functional genetic studies and assisted reproduction. Recently, rodent androgenetic haploid ESCs (AG-haESCs) were generated from androgenetic blastocysts and functioned like sperm to produce viable offspring via the intracytoplasmic AG-haESCs injection into oocytes. However, the efficiency of this reproduction was very low. Most pups were growth-retarded and died shortly after birth, which is not practical for producing knockout animals. Further investigation suggested a possible link between the low birthrate and aberrant expression of imprinted genes. Here, we report the high-frequency generation of healthy, fertile mice from H19-Igf2 imprinting-locus modified AG-haESCs, which maintained normal paternal imprinting and pluripotency. Moreover, it is feasible to perform further genetic manipulations in these AG-haESCs. Our study provides a reliable and efficient tool to rapidly produce gene-modified mouse models and will benefit reproductive medicine in the future.

Keywords: Haploid ES cells; AG-haESCs; imprinted gene; ICAHCl; gene-modified mice

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Introduction

Recently, mammalian haploid embryonic stem cell (ESC) lines were successfully established in mouse, rat and monkey [1–4]. They displayed unique potential for functional genetic studies and hold great promise for assisted reproduction [5, 6]. Moreover, androgenetic haploid ESCs (AG-haESCs) were isolated from androgenetic rodent blastocysts and functioned like sperm to produce viable, fertile progeny after intracytoplasmic injection into mature oocytes [3, 7, 8], providing a new approach for rapidly producing genetically modified mice. However, the efficiency was extremely low, and many of these semi-cloned (SC) pups were growth-retarded and died shortly after birth, most likely due to instable paternal imprinting in these AG-haESCs.

Genomic imprinting has an essential role in mammalian development [9–11]. Among the ~150 reported murine imprinted genes, the H19-Igf2 locus was first identified and shown to be essential for normal fetal growth [12, 13]. Furthermore, viable bi-maternal mice were produced from reconstructed eggs containing fully grown oocytes and non-growing oocytes that harbored a deletion in the H19-Igf2 locus [14]. Interestingly, abnormal H19 imprinting was observed in the prolonged cultured AG-haESCs and growth-retarded newborns mentioned above [7, 8]. We therefore asked whether genetic modification of the H19-Igf2 locus in AG-haESCs could yield fertile transgenic mice at a high frequency.
Results

Genetic modification of the H19-Igf2 locus in AG-haESCs

The mouse H19 gene produces a 2.3 kb long non-coding RNA exclusively expressed from the maternal allele and physically linked to the Igf2 gene on chromosome 7. They are reciprocally imprinted. The imprinting control region (ICR) within the H19-Igf2 locus is essential for transcriptional insulation of the maternal Igf2 allele [15]. To disrupt H19 gene expression in AG-haESCs, we used clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 enhanced homologous recombination [16, 17] to knock out the 13 kb region of H19 that includes the transcription unit and the ICR (Figure 1a). We designed four single guide RNAs (sgRNAs) targeting different sites up- or downstream of the region and investigated the specificity of these sgRNAs via the Surveyor assay [18]. Cas9/sgRNA-4 and Cas9/sgRNA-7 transfection efficiently cleaved at the target loci and were used for gene targeting (Figure 1b). We co-transfected the pCCI-H19, Cas9-sgRNA-4 and Cas9-sgRNA-7 plasmids into AGH-OG-3 AG-haESCs harboring the Oct4 promoter-driven eGFP (Oct4-eGFP) transgene. The positive clones were examined by PCR (Figure 1c) and were further validated by Southern blot analysis (Figure 1d). Among the 96 picked colonies, 29 positive clones harbored the desired H19-ICR deletion (H19\(^\Delta\)). We randomly chose two clones containing considerable haploid cells for further experiments. The two AG-haESC lines, referred to as H19\(^{\Delta1}\) and H19\(^{\Delta2}\), were established through consecutive passages and multiple rounds of fluorescence-activated cell sorting (FACS) for haploid cells (Figure 1e). Karyotyping analysis showed that these cells contained a haploid set of 20 chromosomes (Figure 1f). Comparative genomic hybridization (CGH) analysis confirmed the genome integrity of the two haploid cell lines (Supplementary Table S1). Analyses of potential off-target regions showed that none of the eight predicted sites were mutated by CRISPR/Cas9 in the two-cell clones (Figure 1g).

Characteristics of H19\(^{\Delta}\) AG-haESCs

Cultured H19\(^{\Delta}\) AG-haESCs exhibited dome-shaped colony morphology similar to diploid mouse ESCs and expressed the pluripotency genes Oct4, Sox2, Nanog and SSEA1 (Figure 2a). These cells formed embryoid bodies when cultured in suspension (Figure 2b). The pluripotency was further tested by injection of the H19\(^{\Delta}\)AG-haESCs into diploid ICR blastocysts derived from mice with white coats. One chimeric mouse was obtained and survived to adulthood without obvious defects (Figure 2c). These studies provided evidence for the pluripotency of H19\(^{\Delta}\) AG-haESCs.

We then analyzed whether the H19-ICR deletion affected the imprinting status of AG-haESCs. We first examined the expression of H19, Igf2, Snrpn (maternally imprinted), Grb10 (paternally imprinted) and Gtl2 (paternally imprinted) in AGH-OG-3 and the two H19\(^{\Delta}\)haploid cell lines. As expected, the expression of H19 was nearly undetectable in the two H19\(^{\Delta}\) cell lines, whereas the Igf2 level was significantly upregulated (Figure 2d). Snrpn and Grb10 expression in H19\(^{\Delta}\)AG-haESCs was comparable to expression in AGH-OG-3 AG-haESCs (Figure 2d). Interestingly, the expression level of Gtl2 in the two H19\(^{\Delta}\) cell lines was varied. Gtl2 was expressed at low levels in H19\(^{\Delta1}\)AG-haESCs, whereas its expression in H19\(^{\Delta2}\) AG-haESCs was similar to AGH-OG-3 cells (Figure 2d). This demonstrated the intraclonal heterogeneity of Gtl2 expression in AGH-OG-3 cells. To further assess the DNA methylation profile of the imprinted genes, we performed bisulfite sequencing to analyze the ICRs of the Snrpn and Gtl2 loci. H19\(^{\Delta1}\) AG-haESCs showed normal methylation patterns similar to sperm in the ICRs of Snrpn and Gtl2, whereas late-passage (p65) AGH-OG-3 cells displayed abnormal methylation in the Gtl2 ICR. H19\(^{\Delta2}\) AG-haESCs harbored an abnormal methylation of the Gtl2 ICR similar to AGH-OG-3 cells (Figure 2e). The normal Gtl2 ICR methylation in H19\(^{\Delta1}\)AG-haESCs indicated that some late-passage AGH-OG-3 cells maintained normal imprinting of the Gtl2 ICR. The methylation status of the Snrpn and Gtl2 ICRs in H19\(^{\Delta1}\) and H19\(^{\Delta2}\)AG-haESCs was consistent with the expression of Snrpn and Gtl2 in these cells. These results indicated that H19-ICR deletion in AG-haESCs had a negligible impact on the imprinting status of genes except for the H19-Igf2 locus.

H19\(^{\Delta}\) AG-haESCs efficiently support the generation of healthy SC mice

We were interested in testing whether H19-ICR deletion in AG-haESCs increased the efficiency of producing offspring. We injected FACS-sorted G0- or G1-phase H19\(^{\Delta}\)AG-haESCs into pre-activated oocytes via intracytoplasmic AG-haESCs injection (ICAHCI). H19\(^{\Delta}\) AG-haESCs contributed to embryos, as judged by Oct4-eGFP expression in developed blastocysts (Figure 3a). We transferred 80 two-cell embryos from the H19\(^{\Delta1}\)AG-haESC line into two pseudopregnant ICR mice and obtained three female full-term pups.
Figure 1 Genetic modification of the H19-Igf2 locus in AG-haESCs. (a) Schematic representation of CRISPR-Cas9 assisted homologous recombination to target the H19-Igf2 locus in AG-haESCs (H19ΔAG-haESCs). The PGK-neo cassette was deleted by Cre (H19Δ-neoΔAG-haESCs). (b) Surveyor assay for Cas9-mediated cleavage up- and downstream of the H19 locus in AG-haESCs. (c) Validation of gene targeting in H19ΔAG-haESCs by PCR. (d) Confirmation of gene targeting in H19ΔAG-haESCs by Southern blot. (e) Establishment of the H19Δ1AG-haESC line after FACS enrichment for haploid cells. (f) Karyotype of H19Δ1AG-haESCs showing normal haploidy. (g) Identification of the potential off-targets of CRISPR-Cas9 in H19Δ1AG-haESCs.
All pups had normal body size and did not exhibit obvious developmental retardation after birth (Figure 3b). Two pups grew to adulthood with black (SC-Black) and agouti (SC-Agouti) fur, respectively (Figure 3c and d). Because $H19^{\Delta 1}$AG-haESCs had a C57BL/6 background, the black or agouti coat colors of SC mice depended on the oocytes used for ICAHCI, which were derived from CD-1 or B6D2F1 (C57BL/6 × DBA/2) mice, respectively. The SC mice delivered healthy progeny with litter sizes of 8–10 when mated with C57BL/6 males (Figure 3e and g). We obtained three SC-Black litters and two SC-Agouti litters. Approximately half of these carried the $H19$-ICR deletion, consistent with the expected Mendelian ratio (Figure 3f and h). The rate of SC mice born was ~4% of $H19^{\Delta 1}$AG-haESCs ICAHCI (Table 1) compared with the ~2.2% efficiency of generating SC mice from early passage AGH-OG-3 (passage 17) AG-haESCs ICAHCI and 0% from late-passage AGH-OG-3 cells (passage 22) [7]. Many of the AGH-OG-3 AG-haESC derived pups were growth-retarded and died within 1 h of birth [7]. However, no growth-retarded pups were produced from $H19^{\Delta 1}$AG-haESCs ICAHCI, even though the cells were from a very late passage (more than passage 56). The growth retardation of SC mice might be due to the loss of methylation in the $H19$ ICR.
and the consequent lower expression of Igf2. In SC mice generated by H19ΔAG-haESCs ICAHCI, H19-ICR was deleted, leading to the normal expression of Igf2. The methylation status of two other imprinted genes, Snrpn and Gtl2, was normal in SC mice (Figure 3i). These results demonstrated that H19ΔAG-haESCs could function like sperm to produce normal mice and transmit their genetic material to progeny. Thus, modification of the H19-Igf2 locus was sufficient to generate healthy and fertile SC mice at a high efficiency.

**Generation of genetically modified mice using H19Δ AG-haESCs**

To further analyze the feasibility of producing genetically modified mouse models from H19Δ AG-haESCs, we deleted a loxP flanked PGK-neo cassette by transient expression of Cre in the H19Δ AG-haESC line (Figure 1a). Among the 96 picked clones, the PGK-neo cassette was correctly deleted in most clones, as analyzed by PCR (Figure 4a). The remaining loxP site in the H19-Igf2 locus was further confirmed by DNA sequencing (Figure 4b). We then randomly chose 24 correctly deleted clones (H19Δ-neo^Δ) and performed FACS to determine the haploid subpopulation in these clones. The haploid ESC lines were established through consecutive passages, followed by two rounds of FACS for haploid cells (Figure 4c). The H19Δ-neo^Δ AG-haESCs had normal haploid karyotypes (Figure 4d and e), expressed pluri-potency markers (Figure 4f), and imprinted genes comparable to H19Δ AG-haESCs (Figure 4g). After injecting the sorted G0- or G1-phase H19Δ-neoΔ AG-haESCs into the pre-activated oocytes, we successfully obtained 50 two-cell stage embryos and transplanted them into pseudopregnant females. One full-term live pup derived from the H19Δ-neoΔ AG-haESC line was obtained (Table 1). Genotype analysis confirmed PGK-neo cassette deletion in the SC animal (Figure 4h). Our results demonstrated that H19Δ AG-haESCs can be used to rapidly generate gene-modified mice.
Table 1 Developmental efficiencies of ICAHCl embryos

| Donor | Passage number | Embryo stage | Number of transferred embryos | No. of normal pups (% of transferred embryos) | No. of pups surviving to adulthood (% of transferred embryos) | No. of Growth-retarded pups (% of transferred embryos) |
|-------|----------------|--------------|-------------------------------|-----------------------------------------------|---------------------------------------------------------------|--------------------------------------------------|
| OG-3  | p22            | Two-cell embryo | 102                           | 0                                             | 0                                                             | 3 (2.9)                                          |
| H19ΔI | > p56          | Two-cell embryo | 80                            | 3 (3.8)                                       | 2 (2.5)                                                       | 0                                                |
| H19ΔI-neoΔI | —        | Two-cell embryo | 50                            | 1 (2)                                         | ND                                                            | 0                                                |
| Round spermatids | —          | Two-cell embryo | 61                            | 5 (8.2)                                       | 5 (8.2)                                                       | 0                                                |

Abbreviations: AG-haESCs, androgenetic haploid embryonic stem cells; ICAHCl, intracytoplasmic AG-haESCs injection; ND, not determined. *The data were from Yang et al.*

Discussion

Although we do not know why the paternally imprinted gene H19 easily lost methylation in AG-haESCs, successful restoration of Igf2 expression by H19-ICR deletion faithfully improved the efficiency to generate viable SC mice. Other imprinted genes, such as Grb10, Dlk1 and Gtl2, also affect embryonic growth [11]. Moreover, Dlk1-Dio3 ICR deletion significantly increased the efficiency of generating parthenogenetic mice [14, 19]. It is tempting to investigate whether additional modification of these imprinted genes in AG-haESCs would increase the efficiency of producing SC mice, although the expression level and methylation status of these genes in AG-haESCs were comparable to those in sperm [7, 8]. During the submission of this work, an independent study [20] reported the highly efficient generation of healthy and fertile SC pups from AG-haESCs carrying deletions in the H19-DMR alone or in addition with Gtl2-DMR, confirming our findings. Gtl2-DMR deletion ensured the full silencing of the paternally imprinted gene Gtl2 in AG-haESCs, consistent with the normal imprinting status of Gtl2 in H19ΔI AG-haESCs. Modification of the H19-Igf2 locus in AG-haESCs faithfully improved the efficiency of generating healthy SC mice via the ICAHCl technology. Our work provides another approach for quickly generating gene-modified mice in addition to the VelociMouse [21] and CRISPR-Cas9 [22] technologies. The H19ΔI AG-haESCs have ‘sperm-like’ activity and can replace sperm in reproduction to overcome their limitations. Sperm are not able to propagate and are thus difficult to genetically modify in vitro. In addition, many established methods and resources for genetic studies of diploid ES cells are immediately applicable to H19ΔI AG-haESCs. This work provides inspiration for possible human AG-haESC research and may benefit male infertility in the future, similar to the contribution of spermatogonial stem cells [23].

Materials and methods

Mice

All mouse experimental protocols were approved by the Institutional Animal Care and Use Committee at Peking Union Medical College & Chinese Academy of Medical Sciences. All animal care and experimental methods were carried out in accordance with the institutional ethical guidelines for animal experiments. Male mice of C57BL/6 were used for sperm collection. B6D2F1 (C57BL/6 × DBA/2) and CD-1 female mice were used to provide oocytes for micromanipulation. Female mice of ICR were used to provide blastocysts for microinjection and were also used as pseudopregnant mice. C57BL/6 mice were used for animal mating.

AG-haESCs

Androgenetic haploid ES cell line AGH-OG-3 was obtained from Dr Jinsong Li’s lab in Shanghai (China). The cells were cultured in ESC medium supplemented with 15% fetal bovine serum, 1 000 U ml⁻¹ leukemia inhibitory factor, 3 μm CHIR99021 and 1 μm PD0325901.

DNA content analysis

The dissociated cells were incubated with 10 μg ml⁻¹ Hoechst 33342 at 37 °C for 30 min. Then the haploid 1n peak was purified by flow-cytometry (BD FACSAria III, San Jose, CA, USA). Flow-cytometric data were analyzed using the BD FACSDiva software.

Vector construction

For pCCI-H19 construction, the left and right homologous arms were amplified from bacterial artificial chromosome (RP23-209022). Eag I and Pac I restriction sites were...
added to the amplified left homologous arm, and Sma I and Xho I sites were added to the right arm. Eag I and Pac I digested left arm was cloned into pCCI18 backbone (digested with Not I and Pac I). The products were then digested with Xho I and ligated with Sma I and Xho I digested right arm.

**Construction of CRISPR plasmids**

The pX330 plasmid was bought from Addgene, Cambridge, MA, USA. The sgRNA-1, -2, -3, -4, -5, -6, -7 and -8 oligos were synthesized, annealed and ligated to the pX330 plasmid that was digested with Bbs I (New England Biolabs, Ipswitch, MA, USA). The sequences

Figure 4 Generation of ICAHCl offspring by H19<sup>1<sup>1</sup>-neo<sup>3</sup>AG-haESCs. (a) Subclones of PGK-neo knockouts in H19<sup>1</sup>AG-haESCs by PCR. The primer pairs P5–P6 were used. (b) DNA sequence of PCR products amplified from the H19 gene of H19<sup>1</sup>-neo<sup>3</sup>AG-haESCs. (c) Establishment of the H19<sup>1</sup>-neo<sup>3</sup>AG-haESC line after FACS enrichment for haploid cells. (d) Chromosome counting in H19<sup>1</sup>-neo<sup>3</sup>AG-haESCs showing normal haploidy. (e) The distribution of absolute chromosome numbers of H19<sup>1</sup>-neo<sup>3</sup>AG-haESCs under cell culture conditions. (f) Immunofluorescence staining of H19<sup>1</sup>and H19<sup>1</sup>-neo<sup>3</sup>AG-haESCs. Scale bar, 20 μM. (g) Expression of imprinted genes measured by quantitative reverse transcription PCR (RT-qPCR). AGH-OG-3 AG-haESCs were used as control. Error bars, ± s.d. n = 3. **P < 0.01. (h) PCR analysis of PGK-neo knockout in H19<sup>1</sup>-neo<sup>3</sup>SC mice.
of designed sgRNAs were listed in Supplementary Table S2.

**Surveyor assay**

The AG-haESCs were transfected with CRISPR-sgRNA plus pPB-puro plasmids and selected by puromycin for 36 h. Genomic DNA from wild-type and transfected AG-haESCs was extracted. PCR products were denatured, annealed and treated with T7EN1 (New England Biolabs). The primers used for PCR were listed in Supplementary Table S2.

The fragmentations of sgRNAs as follows:

| sgRNA   | Primers | Fragment size (bp) |
|---------|----------|--------------------|
| sgRNA-1 | S3+S4    | 100+230            |
| sgRNA-2 | S3+S4    | 120+220            |
| sgRNA-3 | S5+S6    | 130+240            |
| sgRNA-4 | S1+S2    | 130+180            |
| sgRNA-5 | S9+S10   | 220+130            |
| sgRNA-6 | S7+S8    | 100+200            |
| sgRNA-7 | S11+S12  | 120+220            |
| sgRNA-8 | S11+S12  | 140+200            |

**Gene targeting in AGH-OG-3 AG-haESCs**

The pCCI-H19, Cas9-sgRNA-4 and Cas9-sgRNA-7 plasmids were co-transfected into AGH-OG-3 AG-haESCs. The cells were selected by G418 (150 μg/ml) for 7 days. Colonies were picked and analyzed by PCR and flow cytometer sorting. The CAGGS-Cre plasmid was electroporated into H19ΔH19 AG-haESCs to delete the loxP flanked PGK-neo cassette.

**Southern blotting**

Wild-type and H19ΔAG-haESC genomic DNA was digested using BgII restriction enzyme. The digested DNA was subsequently separated on a 1% agarose gel for 5 h, and then transferred to a nylon membrane and hybridized with α-32P random primer-labeled probes.

**Prediction of potential off-targets**

The potential off-target regions were predicted using the online tool, http://crispr.mit.edu/. The regions with score >0.5 and mismatch ≤4 were considered as the potential off-target sites. They were amplified using High-Fidelity DNA Polymerase (New England Biolabs) and sequenced.

**Intracytoplasmic injection**

ICAHCI was described as previously [8]. In brief, mature oocytes were collected from the oviduct of super-ovulated female B6D2F1 and CD-1 mice and were pre-activated by 10 mM SrCl2 in calcium-free CZB medium for 30 min before microinjection. G0- or G1-phase purified AG-haESCs were collected and injected into oocytes separately. When the constructed embryos developed to the 2-cell stage in KSOM-AA medium (Sigma, St Louis, MO, USA), they were transferred to the oviduct of pseudopregnant ICR mice at 0.5 d.p.c.

**Comparative genomic hybridization analysis**

The genomic DNA of wild-type and H19ΔAG-haESCs was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and sent to the CapitalBio Corporation (Changping District, Beijing, China) for CGH analysis. The SurePrint G3 Mouse CGH 4 × 180 K microarrays (Agilent, Santa Clara, CA, USA) were used.

**Karyotype analysis**

Exponentially growing ES cells were incubated with 0.2 μg/ml -1 colcemid (Sigma) for 2–3 h at 37 °C. After trypsinization, the collected cells were incubated in 0.075 m KCl hypotonic solution for 15 min at 37 °C. Hypotonic solution-treated ES cells were fixed in fresh ice cold 3:1 methanol/acetic acid at room temperature and dropped onto the precleaned slides. The chromosome spreads were stained with Giemsa solution (10% v/v Giemsa to 7.0 pH phosphate buffer) for 5 min and washed in ddH2O. More than 30 metaphase spreads were analyzed.

**Immunostaining**

ES cells on coverslips were fixed in 4% paraformaldehyde/phosphate buffer saline (PBS) for 15 min at room temperature. The fixed cells were then permeabilized with 0.5% Triton X-100 for 10 min and were blocked in 3% bovine serum albumin for 1 h at room temperature. The cells were incubated overnight at 4 °C with primary antibodies, anti-oct3/4 (sc-5279; Santa Cruz, Dallas, TX, USA), anti-sox2 (sc-17320; Santa Cruz), anti-nanog (AB5731; Millipore, Billerica, MA, USA), and anti-SSEA1 (sc-21702; Santa Cruz). The cells were treated with the secondary antibodies for 1 h at room temperature. The nuclei were dyed with 4',6-diamidino-2-phenylindole (DAPI). Images were acquired by the fluorescent microscope (Zeiss, Oberkochen, Germany).

**Quantitative reverse transcription PCR**

Total RNA was extracted from AG-haESCs using Trizol reagent (Invitrogen, Waltham, MA, USA). 1.0 μg of total RNA was reverse transcribed using the PrimeScript II 1st strand cDNA synthesis kit (TaKaRa, Dalian, China). The real-time PCR reaction was performed using SYBR Premix Ex Taq II (TaKaRa) and run on Roche 480 Light Cycler. The amount of GAPDH expression was used to normalize all values.

**Bisulphite sequencing**

Genomic DNA of ESCs, sperm and mouse tail was treated with the EpiTect Bisulphite (Qiagen, Hilden, Germany) for bisulphite conversion according to the manufacturer's instructions. Differentially methylated regions (DMRs) of Snrpn and Gtl2 were amplified. The PCR products were cloned into pMD18-T vectors (TakaRa) and sequenced for unmethylated C to T conversion. Bisulphite primers are presented in Supplementary Table S2.

**Embryoid-body formation**

Embryoid bodies were formed from wild-type and H19Δ AG-haESCs. Cultured ES cells were dissociated with trypsin and sedimentated for 30 min at 37 °C. 1.5 × 104 cells were transferred to low attachment 90-mm-diameter bacteriological grade Petri dishes in differentiating medium containing high-glucose Dulbecco's modified Eagle's medium (Gibco, Waltham, MA, USA), 15% fetal bovine serum, 2 mM GlutaMax, 1% non-essential amino acids, and 100 μM β-mercaptoethanol.
embryoid bodies were replaced with fresh differentiation medium every other day.

Statistical analysis
The Student’s t-test was used to analyze significant differences. $P < 0.05$ was considered significant. The data analyses were performed using Prism GraphPad software.

Accession codes
CGH data are deposited at the Gene Expression Omnibus under accession number GSE67563.

Conflict of Interest
The authors declare competing financial interests.

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