Generation of Genetically Modified Mice by Oocyte Injection of Androgenetic Haploid Embryonic Stem Cells

Hui Yang,1,6 Linyu Shi,1,6 Bang-An Wang,2,6 Dan Liang,1 Cuiqing Zhong,1 Wei Liu,2 Yongzhan Nie,4 Jie Liu,4 Jing Zhao,5 Xiang Gao,5 Dangsheng Li,3 Guo-Liang Xu,2,* and Jinsong Li1,*

1Group of Epigenetic Reprogramming, State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology
2Group of DNA Metabolism, State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology
3Shanghai Information Center for Life Sciences
Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
4State Key Laboratory of Cancer Biology and Institute of Digestive Diseases, Xijing Hospital, Fourth Military Medical University, Xi’an 710032, China
5MOE Key Laboratory of Model Animal for Disease Study, Model Animal Research Center, Nanjing University, Nanjing 210061, China
6These authors contributed equally to this work
*Correspondence: gbwu@sibs.ac.cn (G.-L.X.), jsli@sibs.ac.cn (J.L.)

SUMMARY

Haploid cells are amenable for genetic analysis. Recent success in the derivation of mouse haploid embryonic stem cells (haESCs) via parthenogenesis has enabled genetic screening in mammalian cells. However, successful generation of live animals from these haESCs, which is needed to extend the genetic analysis to the organism level, has not been achieved. Here, we report the derivation of haESCs from androgenetic blastocysts. These cells, designated as AG-haESCs, partially maintain paternal imprints, express classical ESC pluripotency markers, and contribute to various tissues, including the germline, upon injection into diploid blastocysts. Strikingly, live mice can be obtained upon injection of AG-haESCs into MII oocytes, and these mice bear haESC-carried genetic traits and develop into fertile adults. Furthermore, gene targeting via homologous recombination is feasible in the AG-haESCs. Our results demonstrate that AG-haESCs can be used as a genetically tractable fertilization agent for the production of live animals via injection into oocytes.

INTRODUCTION

In sexually reproductive organisms, haploid gametes—eggs and sperm—mediate genetic transmission to the next generation. However, both eggs and sperm are structurally specialized for fertilization and cannot divide in vitro, preventing genetic manipulation. An intriguing question is whether haploid cell lines from either sex can be generated, cultured in vitro, and used in place of gametes to create animal models (Kaufman et al., 1983; Latham et al., 2002; Modliriski, 1975; Tarkowski and Rorsman, 1976). embryonic stem cells (ESCs) established from these haploid embryos have turned out to display a diploid karyotype (Kaufman et al., 1983). In humans, near-haploid cell lines have been established from tumors (Carette et al., 2009; Kotecki et al., 1999; Sukov et al., 2010), potentially due to the existence of genetic mutations that stabilize the haploid genome. Recently, relatively stable haploid ESCs (haESCs) from mouse parthenogenetic embryos have been established and applied in forward and reverse genetic screens (Elling et al., 2011; Leeb and Wutz, 2011). These successes were made possible by the application of 2i culture conditions (Buehr et al., 2008; Nichols et al., 2009a, 2009b; Nichols and Smith, 2011; Ying et al., 2008), together with haploid cell enrichment using fluorescence-activated cell sorting (FACS) (Elling et al., 2011; Leeb and Wutz, 2011). However, it is still not clear whether haESCs with a specific genetic trait can be converted into a mouse model either through germline transmission after injection into blastocysts or through direct combination with gametes of the opposite sex. Furthermore, it is critical that these resultant mice can produce progeny themselves and deliver the genetic traits to the next generation. We reasoned that, if haESCs could be generated from androgenetic (AG) blastocysts and stably maintained in vitro, then they might be used to create mouse models simply by injecting these haESCs into oocytes, a procedure that is similar to intracytoplasmic sperm injection (ICSI).

In this study, we established protocols for the derivation of haESCs from androgenetic blastocysts. These cells display developmental potential in all examined aspects. More significantly, injection of these haESCs into oocytes led to the generation of fertile animals.

RESULTS

Derivation of haESC Lines from Androgenetic Blastocysts

To generate androgenetic haploid mouse embryos, we adopted two different approaches. In the first set of experiments, we...
Figure 1. Derivation of AG-haESCs

(A) Diagram for the generation of AG-haESCs from androgenetic blastocysts. The sperm injected into enucleated oocytes carries an Oct4-EGFP transgene. hpa, hours postactivation.

(B) An injected oocyte at 1 hr postactivation. Note that the sperm head has undergone decondensation based on Hoechst staining.

(C) Epigenetic modification of the male pronucleus (PN) formed from a sperm injected into an enucleated oocyte. (Left) 5 hmC (red) and 5 mC (green) appear preferentially in the male or the female PN, respectively, in normal fertilized oocytes. (Right) DNA oxidation (5 hmC, red) also occurs in the male pronucleus formed from a sperm injected into an enucleated oocyte.

(D) Fluorescence and phase-contrast image of androgenetic morulas and blastocysts developed from the injection of Oct4-EGFP sperm. Scale bar, 100 μm.

(E) Establishment of haES cell line (represented by AGH-OG-3) after three rounds of FACS enrichment for haploid cells. A DAPI filter was used to detect signal of Hoechst-stained DNA. (Right) FACS data of diploid control ESCs for comparison.

(F) Colony morphology of established AG-haESC line (AGH-OG-1). Scale bar, 50 μm.
performed nuclear transfer (NT), in which a haploid sperm head from Oct4-EGFP transgenic mice (C57BL/6 background), instead of a somatic nucleus, was injected into an enucleated oocyte (Figure 1A). The sperm head underwent decondensation and DNA oxidation based on Hoechst staining of the resulting pronucleus and the appearance of a 5-hydroxymethylcytosine (5 hmC) signal (Gu et al., 2011) (Figures 1B and 1C), reflecting a profound remodeling of the donor nucleus in the enucleated oocytes. Among the 909 oocytes that were reconstructed, 194 (21%) developed into blastocysts in vitro (Figure 1D), similar to previously reported efficiency (Latham et al., 2002). After removal of the zona pellucida, blastocysts were cultured in a standard embryonic stem cell (ESC) culture system supplemented with 2i (Leeb and Wutz, 2011). Among 34 ES cell lines that were generated, 4 lines (referred to as AGH-OG-1 to AGH-OG-4) were identified and maintained with a subpopulation of haploid cells through multiple rounds of FACS to enrich for haploid cells (Figures 1E and 1F). In the second set of experiments, androgenetic haploid blastocysts were generated by removal of the female pronucleus from oocytes fertilized by Actin-EGFP transgenic male mice (Figure S1A available online). From 490 manipulated fertilized oocytes, we obtained 82 blastocysts (Figure S1B) and generated 5 ES cell lines. After multiple rounds of FACS following passageing in vitro, one haploid ES cell line (referred to as AGH-EG-1) was obtained (Figures S1C and S1D). In summary, we derived 5 AG-haESC lines, which could be expanded in vitro for more than 30 passages. Notably, no AG-haESC line with the Y chromosome was observed in this study (Figure 1G), confirming the earlier observations that haploid or diploid Y chromosome-bearing androgenetic embryos could not develop to the blastocyst stage (Latham et al., 2000, 2002). Karyotyping of these ESCs revealed that all of them had a haploid set of 20 chromosomes (Figures 1H and S1E) at different passages (Table S1). Comparative genomic hybridization (CGH) of AG-haESC lines confirmed that the haploid cells sustained genome integrity (Figures 1I and S1F and Table S2).

Pluripotency of AG-haESCs
AG-haESCs showed colony morphology similar to normal diploid mouse ESCs. Immunostaining analysis revealed that ESC cell markers, including Nanog, Oct4, Sox2, and SSEA1, were expressed in haES clones (Figure 2A) and FACS-derived cells with haploid DNA content (Figure 2A). Next, we compared the gene expression profile of AG-haESCs with those of normal ESCs and mouse embryonic fibroblasts (MEFs) from male individuals. To avoid the influence of diploidized cells on the expression profile, we collected samples after FACS of cells in the G1/G0 phase. Clustering of these cells based on microarray expression data showed a high correlation between AG-haESCs and the diploid ESCs, but not MEFs (Figures 2B and S2B). To test the developmental potential of AG-haESCs, we injected the Oct4-EGFP (AGH-OG)- and Actin-EGFP (AGH-EG)-marked AG-haESCs into diploid blastocysts of ICR origin. AG-haESCs could contribute to the germline, as judged by Oct4-EGFP expression in gonads of day 13.5 embryos (Figure 2C). Live-born chimeric mice with a high degree of somatic contribution by ESCs were derived, and some of these chimeras survived to adulthood (Figure 2D). To determine whether haploidy can be maintained in chimeras during development, we injected FACS-purified Actin-EGFP-marked AG-haESCs into blastocysts and analyzed the DNA content of progeny of the injected cells at different developmental stages. We found that 2% of the enhanced green fluorescent protein (EGFP)-labeled cells isolated from day 6.5 embryos were still haploid, but no haploid cells could be detected in the embryos at later stages (Figures S2C-S2E and 2E). We also tested whether haploidy can be maintained upon the differentiation of AG-haESCs in vitro. In vitro differentiation of Oct-EGFP-marked AG-haESCs by retinoic acid treatment for 6 days showed that both the undifferentiated and differentiated populations contained haploid cells (data not shown). These data indicate that haploidy can exist, at least transiently, in differentiated cells. Nevertheless, repeated sorting is essential to maintaining haploidy in ESCs (Elling et al., 2011). At present, one cannot exclude the possibility that some essential differences may exist between ESCs and somatic cells (Kim et al., 2011), which could contribute to the maintenance of haploidy in ESCs. Taken together, our results demonstrate that haESCs derived from androgenetic blastocysts, despite showing a tendency of diploidization, are pluripotent as normal diploid ESCs.

Partial Maintenance of Paternal Genomic Imprints in AG-haESCs
Because paternal imprints established at the primordial germ cell stage are propagated through fertilization and persist during embryonic development, we next examined whether paternal imprints are maintained in these AG-haESCs. We first compared expression of imprinted genes in the haploid and control diploid ESCs. As expected from the androgenetic origin of AG-haESCs, all paternally imprinted genes, thus maternally expressed genes, were downregulated except for the H19 gene (Figures 3A and 3B). In contrast, maternally imprinted genes (expressed from the paternal allele) were upregulated (Figures 3A and 3B). These observations suggested that the AG-haESCs largely maintained a typical paternal imprinting status. To further assess epigenetic inheritance, we performed bisulfite sequencing to analyze the methylation profile of two paternally imprinted genes, Gtl2 and H19, and one maternally imprinted gene, Snrpn. Whereas the differentially methylated region (DMR) of H19 retained methylation at a reduced level, methylation at the Gtl2 DMR was largely...
Figure 2. Pluripotency of AG-haESCs
(A) Expression of ES cell markers in AG-haESCs. Representative immunostaining images of haES colonies are shown. Scale bar, 50 μm.
(B) Gene expression profiles of AG-haESCs. Gene expression profiles were clustered using all genes, and the Pearson correlation coefficient was calculated (indicated by red). Five different AG-haESC lines cluster together, showing highly similar expression profiles. AG-haESCs show gene expression similar to the control diploid E14 ESCs (male) but markedly different from mouse embryonic fibroblasts (MEFs, male). rep1, rep2, and rep3 indicate biological replicates.
(C) Gonads in day 13.5 embryos generated by injection of Oct4-EGFP-marked AG-haESCs into wild-type blastocysts. (Top) Bright-field image of gonads. (Bottom) The same gonads under fluorescent illumination, showing Oct4-EGFP-positive cells. Scale bar, 100 μm.
(D) Left A chimeric mouse produced by injection of Actin-EGFP-marked AGH-EG-1 ESCs into normal diploid blastocysts. The extra-embryonic tissues were host derived and thus not green fluorescent. (Right) A 7-week-old chimeric mouse obtained from the injection of AGH-OG-2 haESCs (C57BL/6, black) into ICR blastocysts (albino).
(E) Flow analysis of DNA content of EGFP-positive and EGFP-negative cells isolated from a day 13.5 chimeric embryo. For FACS, a DAPI (for the Hoechst staining signal) and a FITC filter (for EGFP) were used. See also Figure S2.
Figure 3. Paternally Imprinted State of AG-haESCs

(A) List of imprinted genes with more than 2-fold expression difference between AG-haESCs and normal diploid ESCs E14, based on microarray analysis. P, paternal allele; M, maternal allele.

(B) Expression of imprinted genes measured by quantitative real-time PCR (qPCR). The expression levels in AG-haESCs were relative to those in E14 cells, which were set to 1. *0.01 < p < 0.05; **0.001 < p < 0.01. Error bars represent SD.

(C) Methylation analysis of the DMRs of Gtl2, H19, and Snrpn in mouse tail, sperm, and AG-haESCs (AGH-OG-3, passage 15). Open and filled circles represent unmethylated and methylated CpG sites, respectively.

See also Figure S3.
Figure 4. AG-haESCs Support Full-Term Embryonic Development when Injected into Oocytes

(A) Diagram of intracytoplasmic AG-haESC injection (ICAHC) toward SC mice. After activation of the injected oocyte, the second polar body (PB) and pseudopolar body (PPB) are excluded, respectively, from the oocyte and metaphase haESC nucleus, which results in a diploid embryo. The AG-haESCs used for injection carry the EGFP transgene (green). PPN, pseudopronucleus derived from the injected haESC; hpa, hours postactivation.
intact (Figure 3C). In contrast, the DMR of the Snrpn gene was free of methylation (Figure 3C), reflecting the androgenetic origin of the haploid cells. Methylation at the H19 DMR appeared to be dynamic in cultured AG-haESCs, as the level fluctuated among different passages (Figure S3). It should be noted that loss of imprinting at the H19 locus was also observed in standard diploid mouse and human ESCs upon prolonged culturing (Dean et al., 1998; Humpherys et al., 2001; Rugg-Gunn et al., 2005, 2007). Taken together, parental genomic imprints can be generally maintained in AG-haESCs, albeit more dynamically and less reliably at certain loci.

**AG-haESCs Support Development following Injection into Oocytes**

We next tested whether AG-haESCs could be used in place of sperm to support full-term development of mouse embryos upon injection into mature oocytes. To this end, we performed intracytoplasmic AG-haESCs injection (ICAHCI) (Figure 4A). In this process, the genome of the resultant embryos combines genomic material from the oocyte and the sperm-originated AG-haESC. This is similar to artificial fertilization technology, such as ICSI and round spermatid injection (ROSI), in which sperm or round spermatid is injected into oocytes, respectively. However, AG-haESCs, unlike sperm whose harbors horey-activation factors (Kuretake et al., 1996), lack the ability to activate the oocytes. Thus, reconstituted oocytes from ICAHCI were activated in medium containing Sr²⁺ as in standard mouse NT (Lin et al., 2011; Wakayama et al., 1998, 1999; Yang et al., 2010). In a preliminary experiment, small cells, which were surmised to be in the G1 phase (Wakayama et al., 1998, 1999), or FACS-selected haploid cells at the G1 phase were used as donors for injection. We found that most injected oocytes could not develop to the blastocyst stage in vitro (Table S3). Because oocytes that are reconstructed using metaphase ESCs as a donor develop into blastocysts at significantly higher frequency from ICAHCI using the cells of later passages (from S3). Because oocytes that are reconstructed using metaphase ESCs as a donor develop into blastocysts at a rate of 51% (Figure 4C and Table S3), similar to that in control ICSI experiments (Table S3). To examine whether these blastocysts had a normal euploid karyotype, we analyzed 19 ESC lines established from 40 blastocysts by measuring their DNA content using FACS. Seventeen ESC lines were diploid (Figure S4A and Table S4), reflecting a successful ICAHCI procedure. The other two cell lines were triploid (Figure S4A and Table S4), suggesting that a few diploidized AG-haESCs had been erroneously selected for injection.

We next tested the developmental potential of these ICAHCI embryos by transferring two-cell embryos or blastocysts into oviducts or uteri of pseudopregnant females, respectively. Out of 553 transferred two-cell embryos and 424 blastocysts derived from all five AG-haESC lines (passage 7 to passage 22), a total of 46 live pups were recovered by caesarean section (C section) at 19.5 days of gestation (Figures 4D and S4B and Table 1). All of them were females, as expected from the injection of X-bearing AG-haESCs. Genotyping revealed that they carried the EGFP transgene that originated from the AG-haESCs (Figure S4C). We refer to these mice as semicloned (SC) (Yanagimachi, 2005; Yi et al., 2009) because they were derived by combining an AG-haESC donor with a normal oocyte. The rate of SC mice born was ~4.5% of the transferred blastocysts or ~4.9% of the transferred two-cell embryos (Table 1), similar to the rate of standard NT using conventional ESCs (Li and Mombaerts, 2008; Wakayama et al., 1999). However, differing from cloned mice generated from ESCs that display an overgrowth phenotype (Rideout et al., 2001), SC mice were either normal with a typical newborn body weight or developmentally retarded (Figures 4D, 4E, and S4B). All retarded pups died within 1 hr of birth, reminiscent of what has been observed in bimaterial mice generated by the construction of oocytes from fully grown oocytes and nongrowing oocytes that contain a single deletion of the H19 DMR (Kono et al., 2004) or double deletions of the H19 DMR and the Dlk1-Dio3 intergenic germ-line-derived DMR (Kawahara et al., 2007). We then examined the methylation status of imprinted genes at birth and observed that methylation was absent at the H19 DMR in growth-retarded mice, whereas it was normal in alive newborn mice (Figures 4F and S5A–S5C). Interestingly, growth-retarded pups were obtained at a higher frequency from ICAHCI using the cells of later passages (from AGH-OG-3, Table 1) that harbored a more severe loss of the H19 methylation imprint (Figure S3E). Consistently, gene expression analysis showed that normal SC mice had similar expression patterns to control mice in two pairs of interrelated imprinted
genes (Igf2 and H19, and Dlk1 and Gtl2), whereas growth-retarded pups exhibited a significantly lower Igf2 expression in major organs than control mice (Figure S5D).

Normal-weight SC pups were successfully nursed by foster mothers, and most of them (14/18) grew to adulthood (Figure 4G).

To test whether the SC mice could deliver the Oct4-EGFP transgene to the next generation by germline transmission, we dissected one newborn and one 4-week-old SC pup derived from AGH-OG-1 and observed that ovaries and germinal vesicle (GV) oocytes were EGFP positive (Figure 5A). Furthermore, one SC mouse, which was superovulated and mated with a normal B6D2F1 male, delivered a litter of 16 pups (Figure 5B). Oct4-EGFP-positive pups accounted for 50% (8/16), conforming to the expected Mendelian ratio (Figure 5C), as the SC mother was heterozygous for Oct4-EGFP. Importantly, transgene-positive pups were found among both female and male progeny (Figure 5D), indicating that female SC mice derived from AG-haESCs are capable of normal gametogenesis. These data demonstrate that AG-haESCs, when injected into oocytes, can introduce genetic traits into the resulting SC mice, who further transmit to offspring.

**Gene Targeting in AG-haESCs**

Having demonstrated the developmental potential of AG-haESCs by both blastocyst injection and ICHACI, we next examined whether specific genetic alterations can be introduced into these haESCs through homologous recombination. To avoid potential perturbation in cellular function, a conditional gene targeting strategy was used to modify the locus of Vwce, a gene encoding a “von Willebrand factor C and EGF domains”-containing protein presumably involved in the Wnt-signaling pathway (Du et al., 2010). The targeting construct contains a left and a right homologous arm of 4.9 and 5.6 kb, respectively, flanking a PGK-neo drug selection cassette and a genomic region of 3.5 kb encompassing exons 2–4 (Figure 6A). Upon electroporation with the linearized construct, cells were selected in G418 and ganciclovir for gene targeting. Control experiments, in which cells were selected only in G418, indicated an average of a 6-fold enrichment with the G418/ganciclovir double-selection regime. When 90 double-resistant colonies, derived from two separate experiments, were analyzed by PCR using primer pairs P1-P2 and P3-P4, 43 positive clones were found (Figure 6B). Among these, 12 clones (13%) were identified to contain only the targeted allele, whereas the rest had an additional wild-type allele, as judged on the PCR result from the primer pair P5-P6. From four randomly picked targeted clones, AG-haESC lines (referred to as AGH-Vwce) were established through consecutive passages, followed by FACS sorting for haploid cells (Figure 6C). The identification of stable clones that contain a significant haploid subpopulation carrying the targeted allele but

<table>
<thead>
<tr>
<th>ES Cell Line</th>
<th>Passage Number</th>
<th>Embryo Stage</th>
<th>Number of Embryos Transferred</th>
<th>Number of Implantations (% of Transferred Embryos)</th>
<th>Number of Growth-Retarded Pups (% of Transferred Embryos)</th>
<th>Number of Normal Pups (% of Transferred Embryos)</th>
<th>Number of Pups Surviving to Adulthood (% of Transferred Embryos)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGH-OG-1 p22</td>
<td>two-cell embryo</td>
<td>68</td>
<td>31</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AGH-OG-3 p14</td>
<td>two-cell embryo</td>
<td>102</td>
<td>41</td>
<td>1</td>
<td>3**</td>
<td>NDb</td>
<td></td>
</tr>
<tr>
<td>p15</td>
<td>two-cell embryo</td>
<td>45</td>
<td>25</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>p17</td>
<td>two-cell embryo</td>
<td>112</td>
<td>62</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>p22</td>
<td>two-cell embryo</td>
<td>102</td>
<td>43</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AGH-EG-1 p22</td>
<td>two-cell embryo</td>
<td>124</td>
<td>52</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

| Subtotal | 553 | 254 (45.9) | 15 (2.7) | 12 (2.2) | 6 (ND) |
| AGH-OG-1 p7 | blastocyst | 80 | 37 | 2 | 1 | 1 |
| p9 | blastocyst | 37 | 24 | 2 | 0 | 0 |
| AGH-OG-2 p7 | blastocyst | 86 | 62 | 1 | 5 | 4 |
| AGH-OG-4 p9 | blastocyst | 44 | 23 | 1 | 1 | 1 |
| p11 | blastocyst | 68 | 39 | 1 | 1 | 1 |
| AGH-EG-1 p10 | blastocyst | 85 | 53 | 1 | 1 | 1 |
| p16 | blastocyst | 24 | 16 | 2 | 0 | 0 |

| Subtotal | 424 | 254 (59.9) | 10 (2.4) | 9 (2.1) | 8 (1.9) |
| Sperm | blastocyst | 56 | 40 (71.4) | 0 (0) | 17 (30.4) | 14 (25) |

| aThese three normal mice were dissected for gene expression analysis on the day of recovery. See also Figures 4, S4, and S5. | bNot determined. |
lacking the wild-type allele indicates that homologous recombination has occurred in haploid cells following electroporation of the targeting construct and thus that AG-haESCs are amenable to standard gene targeting manipulation.

We next tested whether the gene-targeted AG-haESCs have the capacity to support full-term embryo development. We constructed 444 embryos via ICAHCI using Vwce-targeted AG-haESCs, and from these embryos, we obtained one live-birth SC pup (Figure S6A). Genotype analysis confirmed the existence of the targeted allele in the whole body of the pup (Figure S6B). Unfortunately, the pup was growth retarded and died shortly after birth, which might be expected considering the loss of H19 imprint in the pup and in the original Vwce-targeted AG-haESCs (Figure S6C). The overall low success rate of obtaining live-birth gene-targeted pups is mostly likely due to the loss of imprint (including the H19 locus) in the Vwce-targeted AG-haESCs, which represented a late-passage line (passage 28) and had been through an additional period of culturing due to selection of the targeted allele. Nevertheless, our results provide the proof of principle that genetically modified animals can be obtained via the ICAHCI technology using AG-haESCs that harbor a modified allele.

**DISCUSSION**

Recently, two independent groups (Elling et al., 2011; Leeb and Wutz, 2011) have established haESCs from mouse embryos, enabling functional screening for genes involved in diverse cellular and developmental processes (Schimenti, 2011). In both reports, haESCs were generated from parthenogenetic blastocysts, implying that these cells probably maintain a maternal imprinting status that is established during oogenesis. If such is the case, then these haESCs may not support full-term development of embryos after injection into MII oocytes because the resulting embryos are parthenogenetic and would fail to develop to term (McGrath and Solter, 1984; Surani and Barton, 1983; Surani et al., 1984, 1990). The possible strategies to generate mice from parthenogenetic haESCs are either to replace the chromosome-spindle complex (CSC) of MII oocytes with that from haESCs, followed by ICSI into reconstituted oocytes (Liu et al., 2001), or to inject haESC into diploid blastocysts to derive chimeric mice with germline transmission. However, these strategies are technically complicated or have not been successfully tested (Elling et al., 2011; Leeb and Wutz, 2011). This leaves an open question of whether haESCs can be converted into mouse models (Schimenti, 2011), which is a prerequisite for extending genetic analysis at the cellular level to the organism level. In our study, we generated multiple haESC lines from androgenetic blastocysts, entering an egg with maternal imprints, resulting in a new organism. However, both parthenogenetic and androgenetic embryos fail to develop to term (McGrath and Solter, 1984; Schimenti, 2011).
Surani and Barton, 1983; Surani et al., 1984, 1990), showing that completion of embryogenesis requires the contribution of both the male and the female gamete genomes of different parental imprints. AG-haES cell lines, derived from sperm, are capable of "fertilization" of oocytes and can be used in place of sperm to support full-term development of mouse embryos after injection into oocytes, presumably thanks to the maintenance of typical male imprinting status in these cells. The efficiency of SC mice derivation by ICAHCI is lower than that of ICSI, suggesting that differences in epigenetic features, including parental methylation imprints, exist between AG-haESCs and sperm. Further investigation into the underlying differences will not only aid our understanding of the early reprogramming events following natural fertilization, but may also yield clues to improve the sperm-like features of AG-haESCs for more efficient generation of SC animals via ICAHCI.

SC mice fall into two readily distinguishable groups based on the birth weight of pups and placentas: the growth-retarded mice that could not survive the early postnatal period and those with normal birth size that could grow to adulthood and establish fertility. A plausible interpretation is that AG-haESCs used for ICAHCI are a heterogeneous population containing both epigenetically normal cells functionally equivalent to a mature sperm and aberrant cells carrying errors in genomic imprinting that are less compatible with embryonic and/or postnatal development. This distinction of subpopulations is indeed reflected by the methylation variation at the $H19$-imprinted gene seen in AG-haESCs (Figures 3C and S3). Consistently, the smaller SC pups born from ICAHCI are characterized by hypomethylation at $H19$, whereas the mice with normal size are marked with the expected paternal methylation (Figures 4F and S5). It is known that the in vitro culture environment can cause alterations in the imprinting status of $H19$ in human (Rugg-Gunn et al., 2007) and mouse diploid ESCs (Humpherys et al., 2001), especially during prolonged passaging (Rugg-Gunn et al., 2005), and that abnormal imprinting would result in aberrant development of ESC-derived embryos (Dean et al., 1998). Therefore, a future task is to define appropriate derivation and culture conditions that would enable the relatively stable maintenance of imprinting marks in AG-haESCs, which would facilitate the potential

Figure 6. Genetic Manipulation in AG-haESCs
(A) Strategy for homologous recombination to target the Vwce gene. Coding exons are shown as black boxes, and the 5' noncoding part of exon 1 is shown as a blank box. The Flipase recognition target (Frt) sites flanking the neo selection marker are shown as blank triangles, and the loxP sites flanking the target region are shown as gray triangles. PCR primers used for genotyping AG-haESC clones are shown as horizontal arrows. Correctly targeted haESC clones would give rise to a 4.9 kb fragment using primers one and two (from the region spanning the left arm) and a 5.6 kb fragment using primers three and four (from the region spanning the right arm). Diploid targeting can be detected by using primers P5-P6 as an additional shorter product and would arise from the untargeted wild-type allele lacking the right loxP site. AG-haESCs from AGH-EG-1 (passage 28) were used for gene targeting.
(B) Confirmation of gene targeting in AGH-Vwce cells. The PCR genotyping result of genomic DNA isolated from drug-selected AG-haESC lines is shown. PCR primer pairs used are indicated at the left. A targeted normal diploid ES cell line was used as a control to reveal the existence of two alleles (lane one) and the untargeted AG-haESC line as negative control (lane two).
(C) Propagation of targeted AGH-Vwce lines with a stable haploid population. Targeted cell lines were established from G418-resistant clones through multiple passages and FACS enrichment for haploid cells. See also Figure S6.
applications of these cells. One intriguing application is the generation of gene-modified animals via ICAHCl in other species for which conventional ESCs can be obtained but fail to support the production of chimeras (and thus, gene-modified animals), such as the monkey (Tachibana et al., 2012).

In summary, we have demonstrated that the AG-haESCs can be used as a genetically tractable fertilization agent for yielding live-born mice via injection into oocytes (the ICAHCl technology). By being amenable to gene manipulations and supporting transmission of genetic information to offspring, these haploid cells open new avenues for the generation of genetically modified animals. The next challenge is to improve the sperm-like features of the AG-haESCs by optimizing their epigenetic makeup without compromising their genetic integrity and proliferative capacity.

**EXPERIMENTAL PROCEDURES**

**Animal Use and Care**

All animal procedures were performed under the ethical guidelines of the Institute of Biochemistry and Cell Biology.

**Preparation of AG-Haploid Embryos**

Spermatozoa of Oct4-EGFP transgenic male mice (C57BL/6 background) were collected and prepared for ICSI according to the methods performed previously (Kimura and Yanagimachi, 1995; Yang et al., 2011). B6D2F1 (C57BL/6 × DBA2) female mice were used as oocyte donors. To generate AG-haploid embryos, we used two strategies. In the first strategy, standard NT (Lin et al., 2011; Wakayama et al., 1998) was performed, in which somatic donors were replaced by sperm heads. Briefly, oocytes were obtained 14 hr after human chorionic gonadotropin injection and enucleated in a droplet of HEPES-CZB medium containing 5 μg/ml cytochalasin B (CB) using a blunt Piezo-driven pipette. After enucleation, a single sperm head was injected into oocyte cytoplasts. The reconstructed oocytes were cultured in CZB medium for 1 hr and then activated for 5-6 hr in activation medium containing 10 mM Sr2+. Following activation, all of the reconstructed embryos were cultured in potassium simplex optimization medium (KSOM) with amino acids at 37°C under 5% CO2 in air. In the second strategy, B6D2F1 female mice were mated with Actin-EGFP transgenic male mice (C57BL/6 background), and zygotes were harvested at PN3–4 stages by piercing the zona pellucida using Piezo drive (Prime Tech) and aspirating using a micromanipulator (Gu et al., 2011). The zygotes containing a male pronucleus were used for the ES cell injection. More than 100 ESCs were picked up at the end of the injection pipette, and about 10–15 ESCs were injected into blastocyst cavity. The blastocysts were kept in KSOM with amino acids until embryo transfer. Eight to ten injected blastocysts were transferred into each uterine horn of 2.5 dpc of pseudopregnant ICR females. Pregnant recipients were subjected to caesarean section on day 19.5 of gestation.

**Injection of AG-haESCs into Diploid Blastocysts**

Blastocyst injection was performed as described previously (Jiang et al., 2011). Briefly, diploid blastocysts were collected from the uterus of superovulated ICR females at 3.5 days postcoitum (dpc) and kept in KSOM medium with amino acids until hESCs injection. Prior to blastocyst injection, AG-haESCs were trypsinized, resuspended in Dulbecco’s modified Eagle’s medium without LIF, and kept on ice. A flat-tip microinjection pipette was used for the ES cell injection. More than 100 ESCs were picked up at the end of the injection pipette, and about 10–15 ESCs were injected into the blastocyst cavity. The blastocysts were kept in KSOM with amino acids until embryo transfer. Eight to ten injected blastocysts were transferred into each uterine horn of 2.5 dpc of pseudopregnant ICR females. Pregnant recipients were subjected to caesarean section on day 19.5 of gestation.

**DNA Content Analysis of Cells from Chimeras**

Chimeric fetuses generated by injection of AGH-EG-1 into diploid blastocysts were dissected at days 6.5, 8.5, 10.5, and 13.5 of gestation. Dissociation to single cells was performed by treatment with 0.05% trypsin/EDTA at 37°C for 30 min. Cells were fixed in 4% PFA in 4°C water for 30 min. The cells were treated with a fluorescein isothiocyanate (FITC) filter (for GFP) were used. For FACS, a DAPI filter (for the Hoechst staining signal) and a FITC filter (for GFP) were used.

**Quantitative Reverse Transcription PCR**

Total RNA was isolated from the cells using Trizol reagent (Invitrogen). One microgram of total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (TOYOBO). Real-time quantitative PCR reactions were set up in triplicate using the SYBR Green Realtime PCR Master Mix (TOYOBO) and run on a Bio-Rad CFX96. All of the gene expression levels were normalized to the internal standard gene, Gapdh. Primer information is presented in Table S5.

**Bisulfite Sequencing**

To obtain mouse sperm DNA and tail genomic DNA, samples were pretreated with dithiothreitol for 3 hr (for sperm only), following proteinase K lysis and phenol-chloroform extraction. For DNA methylation analysis in FACs-derived AG-haESCs, cumulus cells, and oocytes, bisulphite conversion was performed in agarose beads as described (Hajkova et al., 2002). The PCR products were cloned into pMD19-T vectors (Takara), and individual clones were sequenced by Invitrogen, Shanghai. Bisulphite primer information is presented in Table S5.

**Intracytoplasmic AG-haESCs Injection**

To generate SC embryos, AG-haESCs at G1 or M stage were used for intracytoplasmic injection. AG-haESCs were trypsinized, washed three times with...
HEPES-CZB medium, and suspended in HEPES-CZB medium containing 3% (w/v) polyvinylpyrrolidone. In the first set of experiments, small AG-haESCs, which were presumably at G1 stage or FACS-derived G1 phase AG-haESCs, were selected for injection. In the second set of experiments, AG-haESCs were arrested at M phase by culturing in medium containing 0.05 μg/ml demecolcine for 8 hr. Each nucleus from G1 phase donor cells or M phase chromosomes from M phase cells was injected into an MI- arrested oocyte using a Piezo-drill micromanipulator. The reconstructed oocytes were cultured in CZB medium for 1 hr and then activated for 4–6 hr in activation medium without GB. Following activation, all of the reconstructed embryos were cultured in KSOM medium with amino acids at 37°C under 5% CO2 in air.

**Embryo Transfer and Cesarean Section**

ICAHCI embryos were cultured in KSOM medium until the two-cell stage or blastocyst stage. Thereafter, 15–20 two-cell embryos or 8–10 blastocysts were transferred into each oviduct or uterus of pseudopregnant ICR females at 0.5 dpc or 2.5 dpc, respectively. Recipient mothers were euthanized at 19.5 days of gestation, and the pups were quickly removed from the uteri. After cleaning fluid from their air passages, the pups were kept in a warm box supplied with oxygen. Surviving pups were raised by lactating mothers.

**Microarray Analysis**

RNA from biological triplicates of diploid ESCs (E14), five independently derived haploid ESCs (AGH-OG-1 passage 14, AGH-OG-2 passage 15, AGH-OG-3 passage 12, AGH-OG-4 passage 14, and AGH-EG-1 passage 15), and duplicates of mouse embryonic fibroblasts (MEFs) from male individuals were extracted using the RNeasy kit (QIAGEN). Gene expression analysis on Affymetrix GeneChip 430 2.0 arrays was performed by Imagenes. The data were extracted using GeneSpring GX software (Agilent Technologies). The labeling and hybridization were performed at the Shanghai Biochip Company according to the protocols in the Affymetrix GeneChip 3′ IVT Express Kit User Manual. The relatedness of transcription profiles was determined by calculating the Pearson correlation coefficient (r).

**Gene Targeting**

To generate the Vwce targeting vector, DNA fragments for the 5′ and 3′ homology arms were cloned from a BAC clone from mouse (C57BL/6) genomic DNA by the standard recombining (recombination-mediated genetic engineering) technique. The left arm consisted of a 4.9 kb 5′ region (including exons 1), and the right arm was a 5.6 kb fragment covering the 3′ coding region of exons 5 and 6. The two arms were confirmed by sequencing. The AG-EG-1 haESC cell line (passage 25) was used for gene targeting. Approximately 3 hr after a medium change, the ESCs were trypsinized and resuspended as a single-cell suspension at a density of -1 × 10⁷ cell/ml in the Ca²⁺/Mg²⁺-free PBS and then electroporated with 25 μg of the pL253-Vwce targeting vector in a 0.4 cm wide sterile cuvette. The condition for a typical single pulse was 260 V, 500 μF. After storing at room temperature for 5 min, the cells were plated on a 10 cm dish with neomycin-resistant MEF feeder cells. After 24 hr, drug selection was started by replacing medium with ES selection medium containing about 200 μg/ml G418 and 2 μM ganciclovir, and the selection medium was changed every day. Colonies were picked 10–12 days later. The harvested colonies were trypsinized and transferred to a 24-well plate with ES selection medium containing 100 μg/ml of G418 and 2 μM of ganciclovir. After 3–5 days in culture, G418-resistant colonies were screened for homologous recombination by long-range PCR using primers (P1–4) spanning the left and right recombination arms, respectively. The primer sequences are listed in Table S5.

**Statistic Analysis**

Differences of gene expression levels between groups were analyzed by means of Student’s t test. All statistical analyses were done applying SPSS software 13.0.

**ACCESSION NUMBERS**

Gene expression and CGH data sets can be accessed as the GEO reference series GSE35787 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35787). This series includes the GSE35785 (mRNA expression data from AG-haESC, E14, and MEF) and the GSE35786 (CGH analysis of AG-haESCs) data sets.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and five tables and can be found with this article online at doi:10.1016/j.cell.2012.04.002.

**ACKNOWLEDGMENTS**

We thank M. Dym, X. Sun, and C. Walsh for critical reading of the manuscript. This study was supported by grants from the Ministry of Science and Technology of China (2009CB941101 to J.L. and G.-L.X., 2011CB11304), the National Natural Science Foundation of China (30730058 to G.-L.X.), and the “Strategic Priority Research Program” of the Chinese Academy of Sciences (XDA01010403 to J.L.; XDA01010301 to G.-L.X.).

Received: January 31, 2012
Revised: March 21, 2012
Accepted: April 4, 2012
Published: April 26, 2012

**REFERENCES**


Figure S1. AG-haESCs Derived from Androgenetic Blastocysts Generated by the Removal of Female Pronucleus from Fertilized Oocytes, Related to Figure 1 and Tables S1 and S2

(A) Diagram for the derivation of AG-haESCs from androgenetic blastocysts generated by the removal of female pronucleus from fertilized oocytes. The sperm carries an Actin-EGFP transgene.

(B) Images of a fertilized oocyte removed of the female pronucleus and a blastocyst expressing the Actin-EGFP transgene transmitted from the sperm. Left, a control zygote immunostained for 5 hmC (red) in the male pronucleus and for 5 mC (green) in the female pronucleus; middle, a fertilized oocyte upon the removal of the female pronucleus; and right, image of an androgenetic blastocyst showing EGFP expression. PB, polar body.

(C) HaESC line AGH-EG-1 established after several times of sorting.

(D) Colony morphology of AGH-EG-1. Scale bar, 50 μm.

(F) CGH analysis of AGH-EG-1 showing the normal haploid complement of 20 chromosomes (19 + X).
Figure S2. Pluripotency and Diploidization of Mouse AG-haESCs, Related to Figure 2

(A) Immunostaining for pluripotency markers (Nanog, Oct4, Sox2, and Ssea1) of AG-haESCs upon FACS enrichment for haploid cells.

(B) Scatter plots showing high correlation in gene expression between AG-haESCs and control diploid ESCs (E14, male). Diploid denotes diploid ESCs (E14, three replicates). Haploid, five AG-haESC lines. MEF, mouse embryonic fibroblasts (from male individuals, two replicates).

(C) Flow analysis of DNA content of EGFP-positive and EGFP-negative cells isolated from day 6.5 chimeric fetuses. For FACS, a DAPI (for the Hoechst staining signal) and a FITC filter (for EGFP) were used. Scale bar, 100 μm.

(D) Flow analysis of DNA content of EGFP-positive and EGFP-negative cells isolated from day 8.5 chimeric fetuses. Scale bar, 500 μm.

(E) Flow analysis of DNA content of EGFP-positive and EGFP-negative cells isolated from day 10.5 chimeric fetuses. Scale bar, 500 μm.
Figure S3. Methylation at the DMR of the H19 Gene Varied in AG-haESCs, Related to Figure 3

(A) Methylation state of imprinted genes (H19, Gtl2, and Snrpn) in MII oocytes.
(B) Methylation state of imprinted genes (H19, Gtl2, and Snrpn) in cumulus cells.
(C) Methylation profiles of the H19 gene in AGH-OG-1 ES cell line. Passage numbers are indicated in bracket.
(D) Methylation profile of the H19 gene in AGH-OG-2 ES cell line.
(E) Methylation profile of the H19 gene in AGH-OG-3 ES cell line.
(F) Methylation profile of the H19 gene in AGH-EG-1 ES cell line.

The confirmation of expected methylation status at the three imprinted genes in MII oocytes and cumulus (A and B) provided validation of bisulfate sequencing analysis.
Figure S4. SC Mice Generated from AG-haESC Lines, Related to Figure 4 and Tables 1, S3, and S4

(A) FACS analysis of two representative ES cell lines (a diploid and a triploid) derived from different ICAHCl blastocysts. Cells were stained with PI prior to FACS analysis. The derivation of a few triploid ES cell lines from blastocysts indicates that some diploid cells were erroneously picked for ICAHCl.

(B) SC mice recovered at day 19.5 of gestation. Asterisks indicate growth-retarded SC mice that died one hour after birth. The cell lines used in ICAHCl are indicated above.

(C) Genotyping of SC pups for the EGFP transgene. Note that all SC pups are positive for EGFP, indicating that they inherit the genetic trait from the original haESCs.
Figure S5. Methylation and Expression Analysis of Imprints in SC Pups, Related to Figures 4 and S4 and Table 1

(A) Methylation analysis of Snrpn in growth-retarded SC and normal SC mice. Open and filled circles represent unmethylated and methylated CpG sites, respectively.

(B) Methylation analysis of Gtl2 in growth-retarded SC and normal SC mice.

(C) Methylation analysis of H19 in three growth-retarded SC, and two normal SC mice.

(D) Transcription analysis of imprinted genes (Igf2, H19, Gtl2, and Dlk1) in growth-retarded (n = 3) and survived (n = 3) SC mice. The transcription level of Igf2 (upper left) was significantly lower in lung and heart of growth-retarded SC mice compared with normal SC mice and control newborn mice. The expression values (means ±SD) were normalized to that of Gapdh. *: 0.01 < p < 0.05; **: 0.001 < p < 0.01. Compared to control mice, the transcription level of H19 (upper right) was similar in normal SC mice, but slightly higher in retarded SC mice. The transcription levels of Gtl2 (lower left) and Dlk1 (lower right) were not significantly different among normal SC mice, retarded SC, and control mice.
**Figure S6. A Live-Birth SC Pup Generated after ICAHCl of AGH-Vwce Cells, Related to Figure 6**

(A) An SC pup obtained after injection AGH-Vwce cells into oocytes (top). Both placenta and fetus are EGFP-positive, indicating that the injected cell carries EGFP-transgene. Bottom, the ovary dissected from the SC pup (right, EGFP-positive) and the control pup (left, EGFP-negative).

(B) Genotyping analysis of the pup.

(C) Methylation state of the H19 DMR in the SC pup and cells used for injection.