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

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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; Yan et al., 2000). Although mouse haploid embryos have been generated by different strategies in the last three decades (Kaufman,

1978; Latham et al., 2002; Modliński, 1975; Tarkowski and Rossant, 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
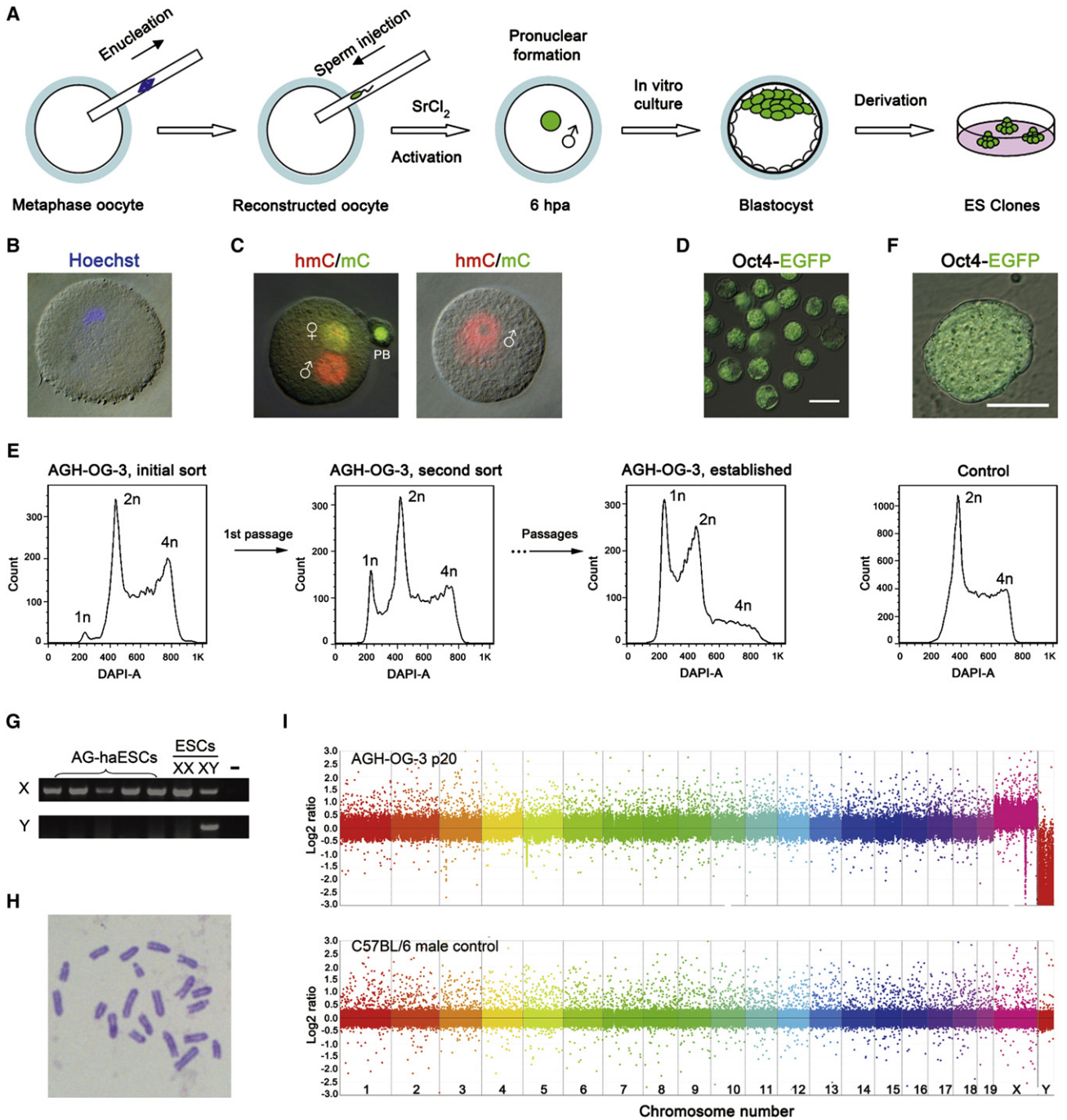
Figure 1. Derivation of AG-haESCs

(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 passaging 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 ES cell markers, including *Nanog*, *Oct4*, *Sox2*, and *SSEA1*, were expressed in haES clones (Figure 2A) and FACS-derived cells with haploid DNA content (Figure S2A). 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 develop-

mental 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-birth 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

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(G) Presence of X chromosome and absence of Y chromosome in the AG-haESC lines. The result of genomic PCR using primers specific for sex chromosomes is shown. Normal diploid ESCs (female XX and male XY) were used as a control.

(H) Karyotype of AGH-OG-3 showing the normal haploid complement of 20 chromosomes (19 + X).

(I) CGH analysis of haESCs (AGH-OG-3) and male mouse kidney cells (C57BL/6). (Top) haESCs versus kidney cells. (Bottom) Kidney versus kidney cells. No major genomic alterations (amplifications or losses) were detected in haESCs.
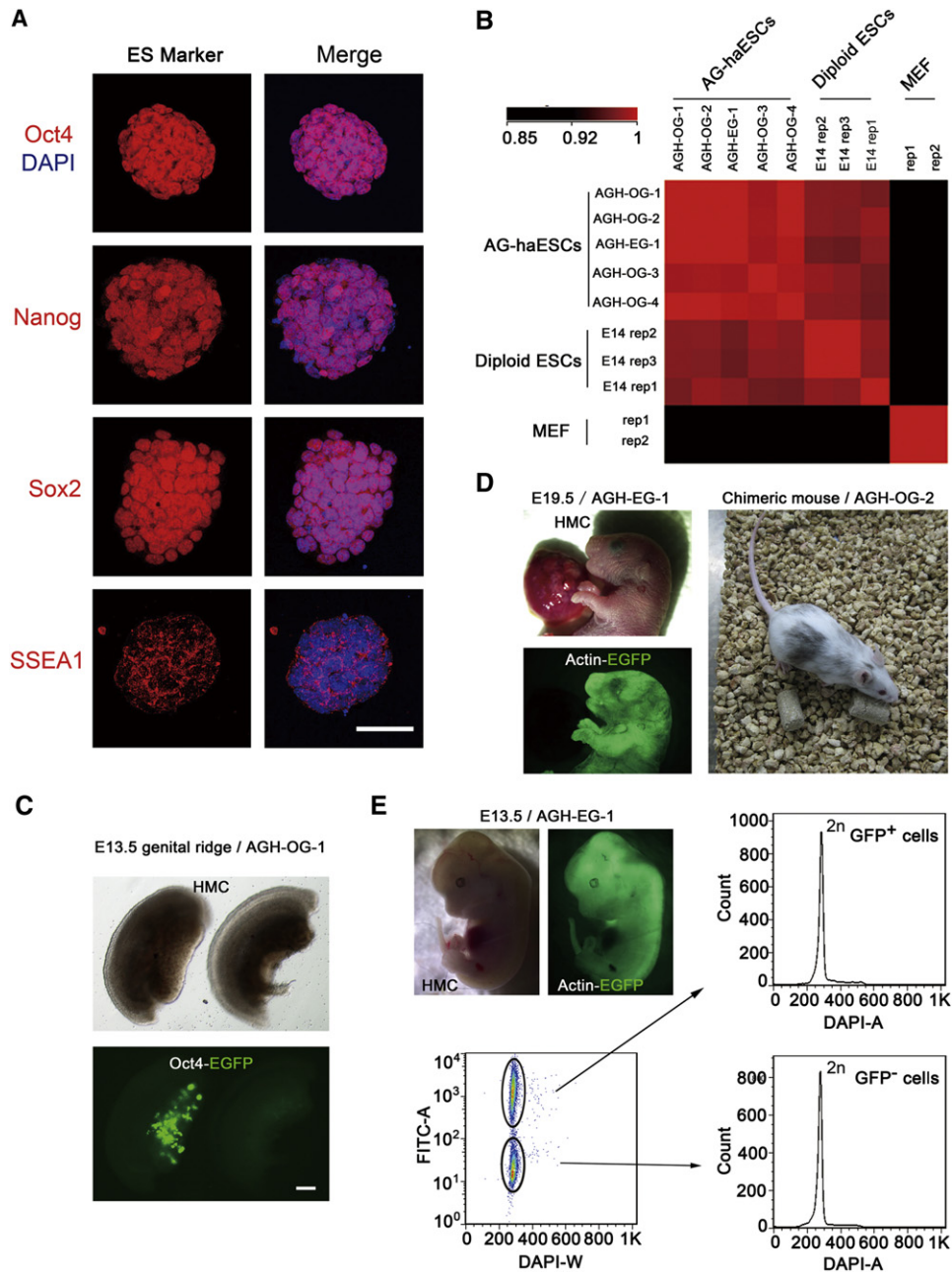
See also Figure S1 and Tables S1 and S2.

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.
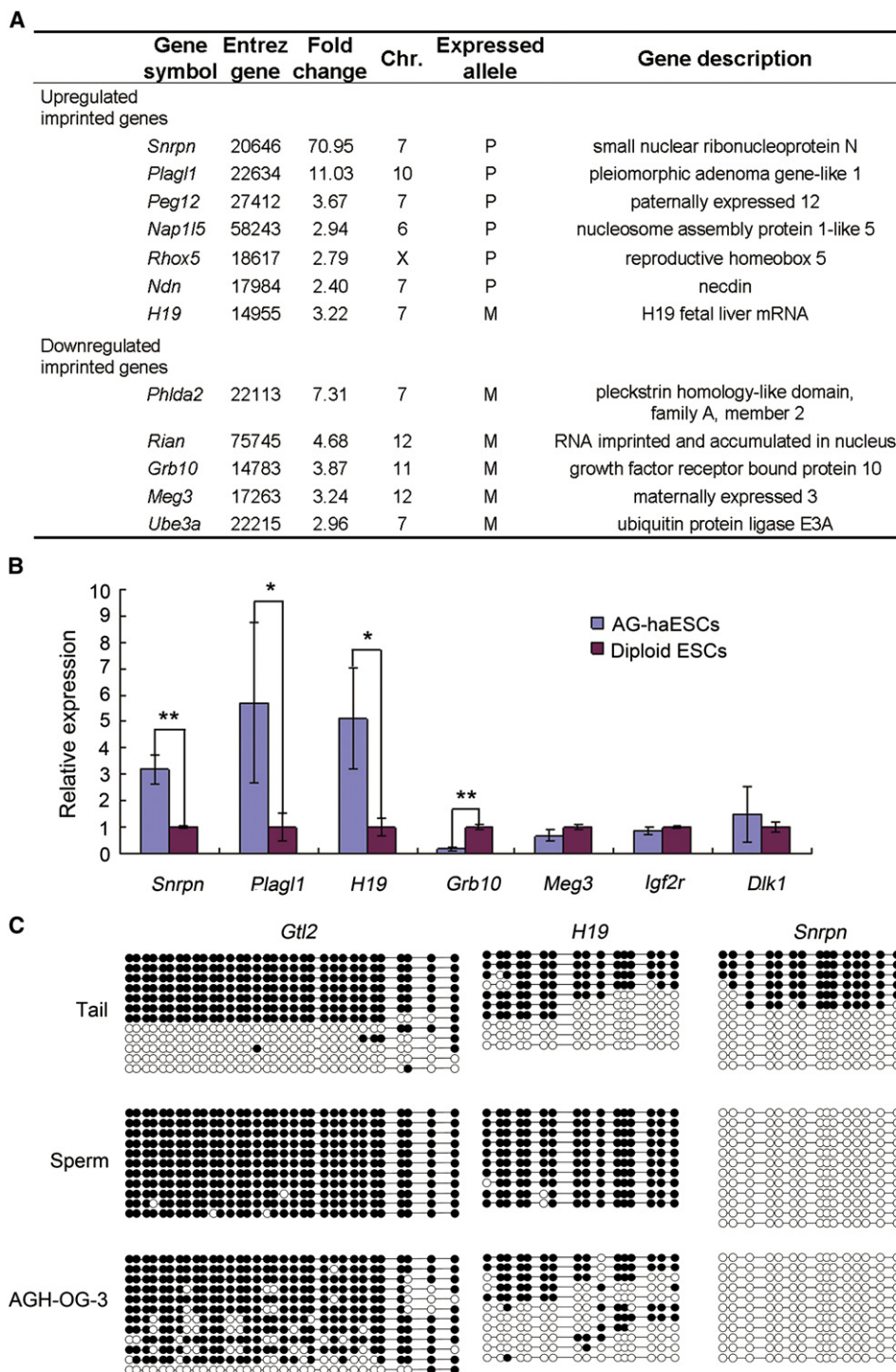
See also Figure S2.

A

Gene symbol	Entrez gene	Fold change	Chr.	Expressed allele	Gene description
Upregulated imprinted genes					
Snrpn	20646	70.95	7	P	small nuclear ribonucleoprotein N
Plagl1	22634	11.03	10	P	pleiomorphic adenoma gene-like 1
Peg12	27412	3.67	7	P	paternally expressed 12
Nap1l5	58243	2.94	6	P	nucleosome assembly protein 1-like 5
Rhox5	18617	2.79	X	P	reproductive homeobox 5
Ndn	17984	2.40	7	P	necdin
H19	14955	3.22	7	M	H19 fetal liver mRNA
Downregulated imprinted genes					
Phlda2	22113	7.31	7	M	pleckstrin homology-like domain, family A, member 2
Rian	75745	4.68	12	M	RNA imprinted and accumulated in nucleus
Grb10	14783	3.87	11	M	growth factor receptor bound protein 10
Meg3	17263	3.24	12	M	maternally expressed 3
Ube3a	22215	2.96	7	M	ubiquitin protein ligase E3A

B

C

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.
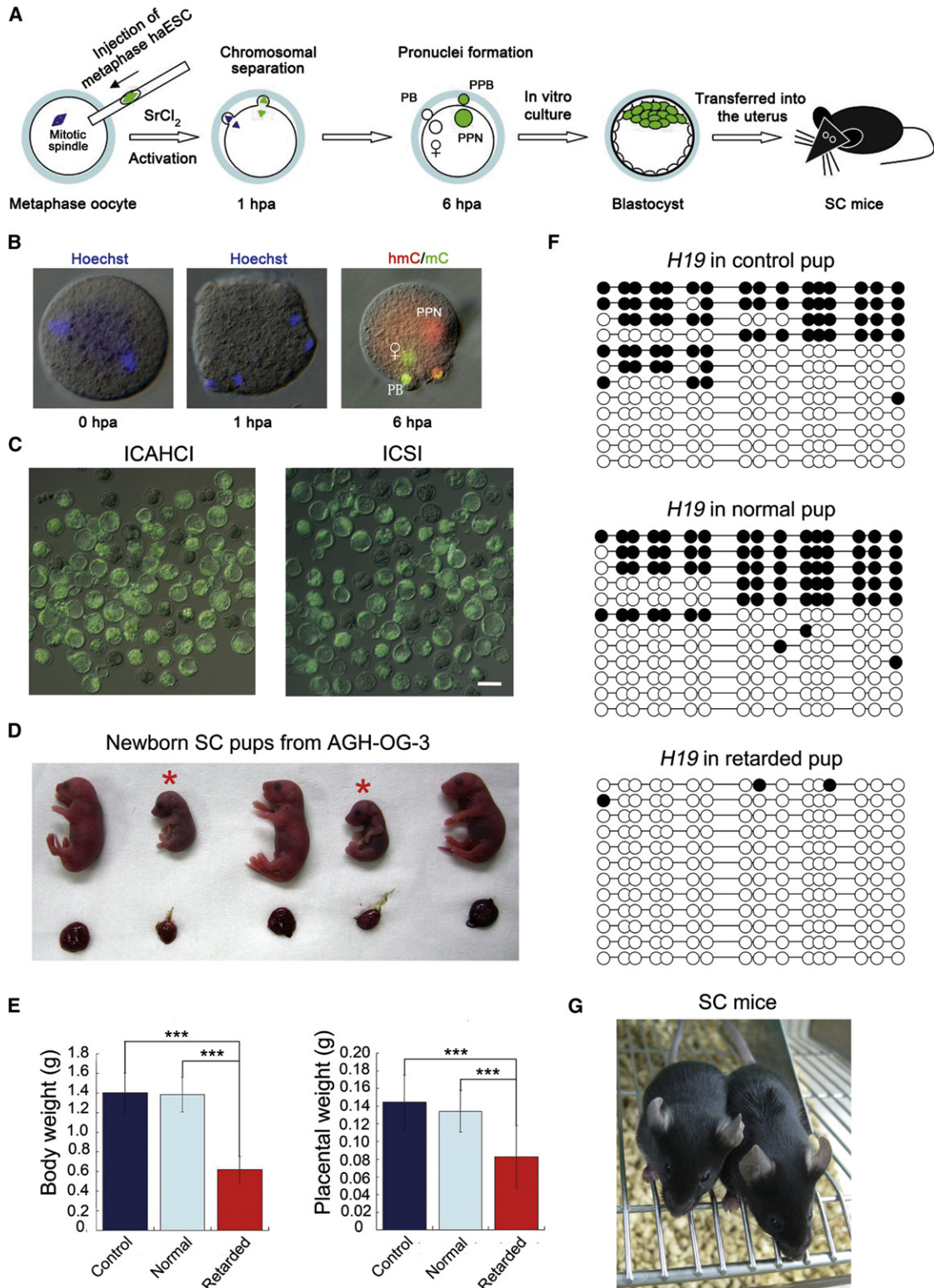
See also Figure S3.

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

(A) Diagram of intracytoplasmic AG-haESC injection (ICAHCI) 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 heads harbor oocyte-activation factors (Kuretake et al., 1996), lack the ability to activate the oocytes. Thus, reconstituted oocytes from ICAHCI were activated in medium containing Sr^{2+} 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 rates than interphase ESCs in nuclear transfer experiments (Ono et al., 2001a, 2001b), we synchronized AG-haESCs at metaphase and selected small cells, most of which were haploid cells in M phase, for ICAHCI. We observed that the injected haES nucleus formed a pseudopronucleus that underwent reprogramming like a male pronucleus (Figure 4B) (Gu et al., 2011). Moreover, the second polar body (PB) and the pseudopolar body (PPB) were excluded from the spindle-chromosome complex and the donor metaphase nucleus, respectively, resulting in a reconstructed embryo containing diploid DNA. Injected oocytes

developed 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 bimaternal 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 germline-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

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(B) Reprogramming of injected AG-haESC. (Left and middle) Hoechst staining images of reconstructed oocytes at 0 and 1 hr after activation. (Right) Immunostaining of a reconstructed oocyte 6 hr after activation. Preferential staining of 5 hmC (red) and 5 mC (green) was observed in PPN and female pronucleus (right), respectively.

(C) Blastocysts generated by injection of AGH-OG ESCs (ICAHCI) or sperm (ICSI) into oocytes. The donor haESCs and sperm carried the *Oct4-EGFP* transgene. Scale bar, 100 μm.

(D) SC pups from ICAHCI using AGH-OG-3 haESCs. Pups and placentas obtained by C section from a pseudopregnant mouse at E19.5 are shown. (Asterisks) Growth-retarded SC pups that died within 1 hr after birth.

(E) Body and placenta weights of SC pups at birth. Control pups were from ICSI. "Normal" SC pups had a similar birth weight (1.4 ± 0.18 g, n = 21) as the control ICSI pups (1.4 ± 0.2 g, n = 17) and could survive to adulthood. "Retarded" SC pups had a smaller size at birth (body weight: 0.6 ± 0.13 g, n = 22) and died within 1 hr after birth. Values are means ±SD. ***p < 0.001.

(F) Methylation state of the *H19* DMR in a control ICSI pup (top), a normal SC pup (middle), and a retarded SC pup (bottom).

(G) Two 8-week-old SC mice derived from ICAHCI of AGH-OG-2 cells.

See also Figures S4 and S5 and Tables 1, S3, and S4.

Table 1. In Vivo Development of ICAHCI Embryos

ES Cell Line	Passage Number	Embryo Stage	Number of Embryos Transferred	Number of Implantations (% of Transferred Embryos)	Number of Growth-Retarded Pups (% of Transferred Embryos)	Number of Normal Pups (% of Transferred Embryos)	Number of Pups Surviving to Adulthood (% of Transferred Embryos)
AGH-OG-1	p22	two-cell embryo	68	31	3	0	0
AGH-OG-3	p14	two-cell embryo	102	41	1	3[a]	ND[b]
	p15	two-cell embryo	45	25	2	3	2
	p17	two-cell embryo	112	62	2	2	1
	p22	two-cell embryo	102	43	3	0	0
AGH-EG-1	p22	two-cell embryo	124	52	4	4	3
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)

[a]These three normal mice were dissected for gene expression analysis on the day of recovery. See also Figures 4, S4, and S5.
[b]Not determined.

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 exam- ined 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 "*v*on *W*illebrand factor *C* and *E*GF 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 signifi- cant haploid subpopulation carrying the targeted allele but
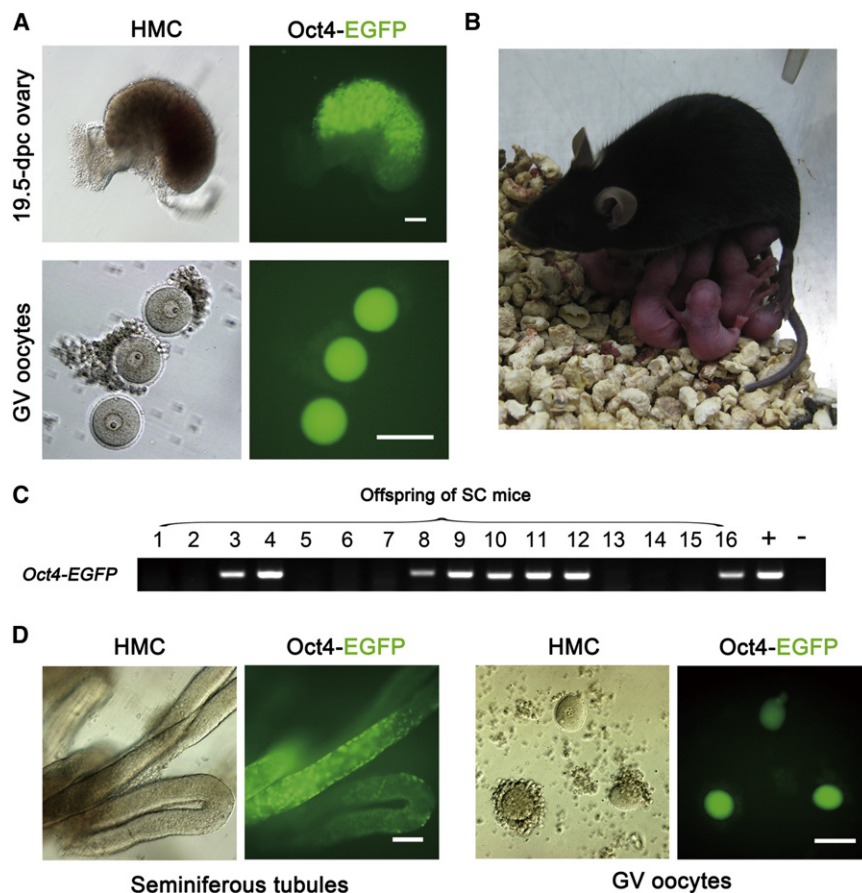
Figure 5. Genetic Transmission of AG-haESCs to Offspring of SC Mice

(A) Ovary (top) dissected from a newborn SC pup generated from AGH-OG-1 carrying an *Oct4-EGFP* transgene and GV oocytes (bottom) isolated from the ovary of a 4-week-old SC mouse. Green fluorescence indicates *Oct4-EGFP*-expressing germ cells. Scale bars, 200 μm (top) and 100 μm (bottom), respectively.

(B) Progeny with an SC mother derived from AGH-OG-1 cells.

(C) Genotyping analysis of the progeny. Note that 50% (8/16) of the progeny of an SC mother are *Oct4-EGFP* positive.

(D) Germline contribution of AG-haESC in both sexes of offspring of the SC mother. Two left panels show the *Oct4-EGFP* transgene expression in seminiferous tubules of a 1-week-old male F2 pup. *Oct4* expression is detected in the entire population of gonocytes in this newborn male, as expected (Pesce et al., 1998). The two right panels show *Oct4-EGFP* reporter expression in GV oocytes isolated from the ovary of a 2-week-old female F2 pup. The EGFP signal recapitulates the *Oct4* expression in developing oocytes. Scale bars, 100 μm.

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 and demonstrated that AG-haESCs could be used to support full-term embryonic development following injection into oocytes, which results in successful trait transmission into live animals.

During normal fertilization, a sperm carrying paternal imprints enters 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;
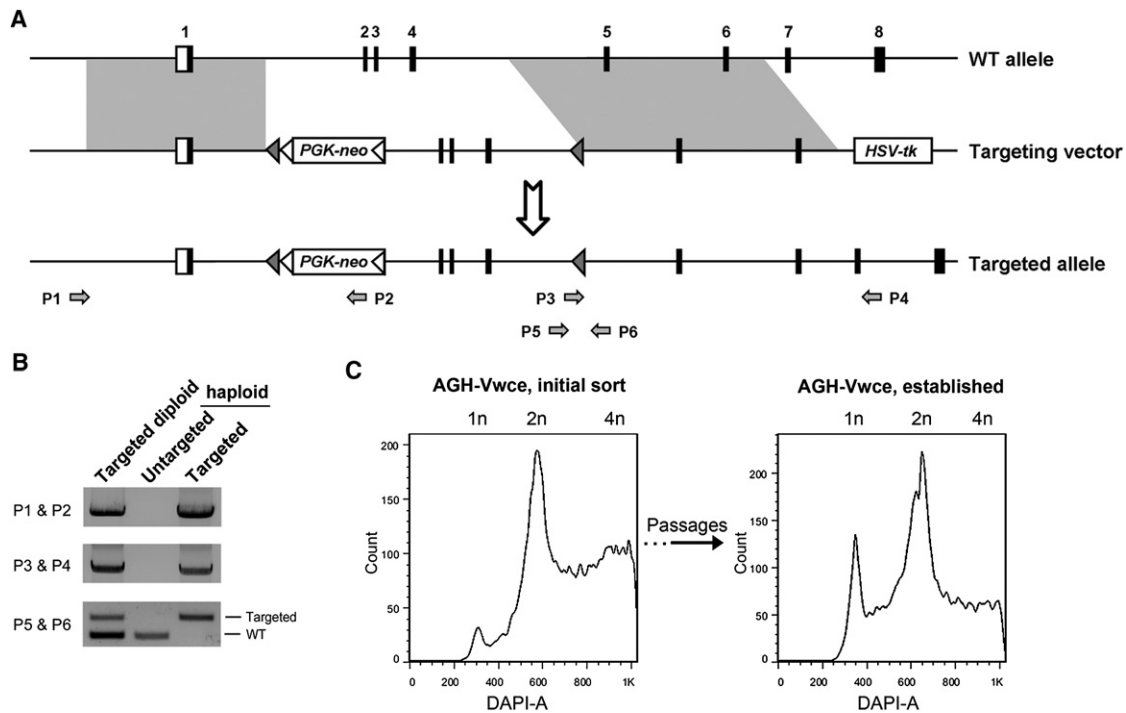
lacking the wild-type allele indicates that homologous recombination has occurred in haploid cells following electroporation of the targeting construct and thus that AG-haESCs are amenable to standard gene targeting manipulation.

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,

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 Flippase 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.

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

applications of these cells. One intriguing application is the generation of gene-modified animals via ICAHCI 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 ICAHCI 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 X 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 Sr^{2+}. Following activation, all of the reconstructed embryos were cultured in potassium simplex optimization medium (KSOM) with amino acids at 37°C under 5% CO_2 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 stage. Female pronuclei, which were distinguished from male pronuclei on the basis of their size and distance from the polar body, were removed from zygotes of 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 cultured in KSOM with amino acids at 37°C under 5% CO_2 in air. The reconstructed embryos that reached the morula or blastocyst stage by 3.5 days in culture were transferred to ES cell medium.

Derivation of AG-haESCs
Morulas or blastocysts were selected to generate ES cell lines as described (Yang et al., 2010). The zona pellucida was removed using acid Tyrode solution. Each embryo was transferred into one well of a 96-well plate seeded with ICR embryonic fibroblast feeders in ESC medium supplemented with 20% knockout serum replacement, 1,500 U/ml leukemia inhibitory factor (LIF), 3 M CHIR99021, and 1 M PD0325901 (Leeb and Wutz, 2011; Ying et al., 2008). After 4–5 days in culture, the colonies were trypsinized and transferred to a 96-well plate with a fresh feeder layer in fresh medium. Clonal expansion of the ESCs proceeded from 48-well plates to 6-well plates with feeder cells and then to 6-well plates for routine culture. To sort haploid cells, ESCs were trypsinized, washed by Dulbecco's Phosphate-Buffered Saline (GIBCO), and then incubated with 15 μg/ml Hoechst 33342 in a 37°C water bath. Subsequently, the haploid 1n peak was purified using BD FACS AriaII for further culturing. For analysis, after fixation in 70% ethanol, cells were

digested by 20 μg/ml RNase A and stained with 50 μg/ml propidium iodide (PI). Analytic flow profiles were recorded by BD LSRII SORP.

Karyotype Analysis
ESCs were incubated with 0.4 μg/ml demecolcine (Sigma) for 1 hr. After trypsinizaiton, the ESCs were resuspended in 0.075 M KCl at 37°C for 30 min. Hypotonic solution-treated cells were fixed in methanol:acetic acid (3:1 in volume) for 30 min and dropped onto precleaned slides. After being incubated in 5 M HCl, cells were stained with Giemsa for 15 min. More than ten metaphase spreads were analyzed.

Immunostaining
Cells on glass coverslips were fixed in PBS supplemented with 4% paraformaldehyde for 15 min at room temperature (RT). The cells were then permeabilized using 0.2% Triton X-100 in PBS for 15 min at RT. The cells were blocked for 30 min in 1% BSA in PBS. All primary antibodies against *Oct4* (sc-5279, Santa Cruz), *Nanog* (RCAB002P-F, Reprocell), *SSEA-1* (mab4301, Millipore), and *Sox2* (ab5603, Millipore) were diluted in the same blocking buffer and incubated with the samples overnight at 4°C. The cells were treated with a fluorescently coupled secondary antibody and then incubated for 1 hr at RT. The nuclei were stained with Hoechst 33342 (Sigma) for 5 min at RT.

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 uteri of superovulated ICR females at 3.5 days postcoitum (dpc) and kept in KSOM medium with amino acids until haESCs 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, following Hochest 33342 staining. EGFP-positive and EGFP-negative cells were isolated by FACS, and DNA content was determined by flow analysis. 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.

Bisulphite 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 MII-arrested oocyte using a Piezo-drill micromanipulator. The reconstructed oocytes were cultured in CZB medium for 1 hr and then activated for 5–6 hr in activation medium without CB. 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 analyzed 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*).

DNA samples (from AGH-OG-2, AGH-OG-3, and AGH-EG-1) for CGH experiments were extracted and sent to the CapitalBio Corporation (Changping District, Beijing) for CGH analysis using NimbleGen 3x720K mouse whole-genome tiling arrays with an average probe spacing of 3.5 kb. Adult male C57BL/6 kidney DNA was used as a reference.

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 recombineering (recombination-mediated genetic engineering) technique. The left arm consisted of a 4.9 kb 5′ region (including exon 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 haES 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.

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