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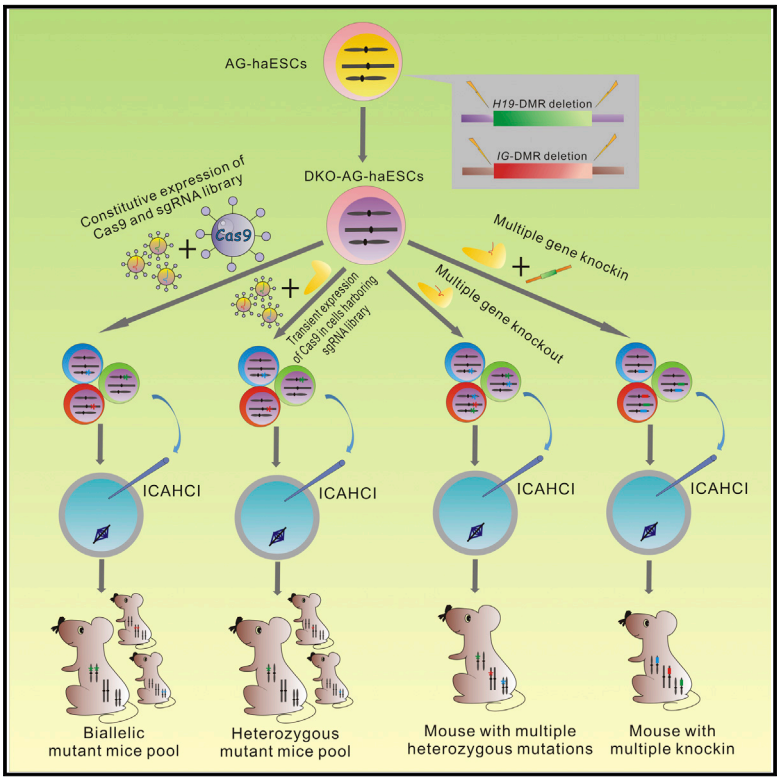
Cell Stem Cell

CRISPR-Cas9-Mediated Genetic Screening in Mice with Haploid Embryonic Stem Cells Carrying a Guide RNA Library

Graphical Abstract



Highlights

- Misexpression of imprinted genes hinders application of haploid mouse ESCs
- The mutation of two paternally imprinted genes improves semi-cloning efficiency
- Genetic manipulation using CRISPR-Cas9 efficiently leads to mutant mice
- Introduction of a CRISPR-Cas9-based library enables mutagenic screening

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In Brief

Li and colleagues show that the combined application of altered expression of two imprinted genes and CRISPR-Cas9-based genome editing allows the efficient and stable generation of gene-modified semi-cloned mice from androgenetic haploid embryonic stem cells. This approach has potential for mutagenesis and screening.

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CRISPR-Cas9-Mediated Genetic Screening in Mice with Haploid Embryonic Stem Cells Carrying a Guide RNA Library

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SUMMARY

Mouse androgenetic haploid embryonic stem cells (AG-haESCs) can support full-term development of semi-cloned (SC) embryos upon injection into MII oocytes and thus have potential applications in genetic modifications. However, the very low birth rate of SC pups limits practical use of this approach. Here, we show that AG-haESCs carrying deletions in the DMRs (differentially DNA methylated regions) controlling two paternally repressed imprinted genes, *H19* and *Gtl2*, can efficiently support the generation of SC pups. Genetic manipulation of these DKO-AG-haESCs in vitro using CRISPR-Cas9 can produce SC mice carrying multiple modifications with high efficiency. Moreover, transfection of DKO-AG-haESCs with a constitutively expressed sgRNA library and Cas9 allows functional mutagenic screening. DKO-AG-haESCs are therefore an effective tool for the introduction of organism-wide mutations in mice in a single generation.

INTRODUCTION

Mammalian haploid embryonic stem cells (haESCs) (Elling et al., 2011; Leeb and Wutz, 2011) hold considerable potential as a tool for genetic analyses (Horii et al., 2013; Kokubu and Takeda, 2014; Leeb et al., 2014). HaESCs fall into two readily distinguishable types based on their origin (Shi et al., 2012): parthenogenetic haESCs (PG-haESCs), which bear genomic material from the oocyte (Elling et al., 2011; Leeb and Wutz, 2011; Yang et al., 2013), and androgenetic haESCs, (AG-haESCs) whose genome is from the sperm (Li et al., 2012, 2014; Yang et al., 2012). While both PG-haESCs and AG-haESCs could in principle be used for forward or reverse genetic screens, mouse and rat AG-haESCs can also be used in place of spermatids to support full-term development of embryos upon injection into mature oocytes (intracytoplasmic AG-haESCs injection, ICAHCI), resulting in live animals referred to as semi-cloned (SC) animals. This capacity enables the extension of genetic analysis at a cellular level to the organism level in one step and may open up new avenues for efficient generation of gene-modified mice.

Recently, the CRISPR-Cas9 system from bacteria has been widely applied to rapid genome editing in several different species (Cong et al., 2013; Friedland et al., 2013; Mali et al., 2013; Niu et al., 2014; Wang et al., 2013). This system, consisting of the Cas9 nuclease and a single-guide RNA (sgRNA) targeting a specific gene, is relatively easy to implement compared to other gene-editing techniques (Doudna and Charpentier, 2014). Most recently, the CRISPR-Cas9 system has been applied for efficient loss-of-function screening in mouse and human cells (Koike-Yusa et al., 2014; Shalem et al., 2014; Wang et al., 2014).

It seemed to us that combined application of AG-haESCs and CRISPR-Cas9 technology could in principle enhance genetic analyses in mammals. However, previous studies have shown that

the efficiency of live-born SC animals was extremely low (around 4.5% of the transferred embryos in mouse and 1% in rat), and approximately 50% of SC mice displayed a growth-retarded phenotype and died shortly after birth (Li et al., 2012, 2014; Yang et al., 2012). Moreover, the overall success rate of obtaining live-born SC pups was reduced after long-term culturing of AG-haESCs, especially after the type of additional culturing period that is usually required for genetic manipulation. One possible cause of this low efficiency is aberrant expression of imprinted genes. These genes, which are expressed in a parental-of-origin specific manner, are postulated to be the main block to uniparental development (Barlow and Bartolomei, 2014) and underlie the requirement for both maternal and paternal genomes for full-term normal growth and development. Currently, approximately 150 imprinted genes are known in mice, and most are located in large clusters and regulated by differentially DNA methylated regions (DMRs) (Bartolomei, 2009). Consistent with this hypothesis, we previously observed loss of imprinting at the differentially methylated region (DMR) of one paternally repressed imprinted gene, *H19*, in both AG-haESCs and growth-retarded pups (Yang et al., 2012), implying that the aberrant paternal imprinting state may underlie the developmental defects of SC embryos. The maternally expressed *H19* gene is located adjacent to the paternally expressed *Igf2* gene and is regulated by a DMR that is methylated on the paternal allele and serves as a CTCF-dependent insulator that allows expression of maternal *H19*. The methylation status of this DMR determines whether *H19* (DMR unmethylated and active insulator) or *Igf2* (DMR methylated and inactive insulator) is expressed. Deletion of the *H19* gene or DMR leads to minimal if any adverse phenotypes in the mouse (Leighton et al., 1995; Thorvaldsen et al., 2002). Previous studies have also shown that deletion of the *H19* DMR (Kono et al., 2004) or the *H19* DMR and *Dlk1-Dio3* DMR (Kawahara et al., 2007) is the derivation of bimaternal mice. Taking all of this information into account, we reasoned that deletion of the *H19* DMR or perhaps both *H19* and *Dlk1-Dio3* DMRs from AG-haESCs might allow for more efficient generation of live SC animals using AG-haESCs.

In this study, we tested our hypothesis by removing the DMRs from *H19*, *Dlk1-Dio3*, or both and found that the double-knockout (DKO) AG-haESCs exhibited comparable ''fertilization'' capacity to round spermatids, reaching a success rate of 20% of transferred ICAHCI embryos. We further demonstrated that DKO-AG-haESCs can be used in combination with CRIPSR-Cas9 technology to generate mice with multiple genetic modifications and with an sgRNA library for genetic screening.

RESULTS

$H19^{\Delta DMR}/IG^{\Delta DMR}$ AG-haESCs Efficiently Support the Generation of Live SC Pups

To generate AG-haESCs harboring a DMR deletion in *H19*, we performed nuclear transfer (NT) as previously described (Yang et al., 2012), by injecting a haploid sperm head from Δ3.8kb-5′ *H19* mice (Thorvaldsen et al., 2002) into an enucleated oocyte instead of a somatic nucleus (Figure 1A). We derived three haploid cell lines (referred to as $H19^{\Delta DMR}$-AGH-1 to $H19^{\Delta DMR}$-AGH-3) from 250 reconstituted blastocysts (Figures 1B–1D and S1A) and then performed ICAHCI (Yang et al., 2012) using the $H19^{\Delta DMR}$-AGH cells as donors. From 1,443 transferred two-cell embryos from all three $H19^{\Delta DMR}$-AGH lines, we recovered a total of 86 apparently normal live pups and 39 growth-retarded pups by Caesarean section (C-section) at 19.5 days of gestation (Figures 1E and S1B–S1D; Tables 1 and S1). The rate of normal SC mice born was approximately 5.9% of the transferred two-cell embryos, significantly higher than the rate of control AG-haESCs, but 2.7% of the transferred SC embryos from $H19^{\Delta DMR}$-AGH cells were developmentally retarded.

The imprinted gene *Gtl2*, which resides in a large imprinted cluster on mouse chromosome 12, is normally expressed from the maternal allele and regulated by a paternally methylated DMR designated intergenic germline-derived DMR (*IG*-DMR). We previously observed that this gene was also expressed at higher levels in the organs of growth-retarded SC pups produced from wild-type (WT) AG-haESCs than in control mice (Yang et al., 2012). We then examined whether the growth-retarded pups from $H19^{\Delta DMR}$-AGH cells exhibited higher expression of *Gtl2* in major organs by performing qPCR. As expected, most examined organs in growth-retarded pups expressed *Gtl2* at higher levels than was expressed in normal pups (Figure S1E). Consistently, bisulfite sequencing analysis showed that growth-retarded SC pups exhibited hypomethylation at *IG*-DMR in the growth-retarded pups (Figures S1F and S1G). Interestingly, $H19^{\Delta DMR}$-AGH cells of later passage ($H19^{\Delta DMR}$-AGH-3, p16), which produced growth-retarded pups at a higher frequency, harbored a more severe loss of the *IG*-DMR methylation than the early passage cells ($H19^{\Delta DMR}$-AGH-3, p7) (Figure S1H; Table S1), implying that abnormal expression of *Gtl2* may be an underlying factor in AG-haESCs that contributes to the developmental failure of SC embryos generated from $H19^{\Delta DMR}$-AGH cells.

Because deletion of the *IG*-DMR from *Dlk1-Gtl2*-imprinted cluster in sperm did not appear to affect the development of the resultant progeny (Lin et al., 2003) and greatly promoted the development of bimaternal mice (Kawahara et al., 2007), we reasoned that the deletion of *IG*-DMR of *Dlk1-Gtl2* in $H19^{\Delta DMR}$-AGH cells could improve the capacity of $H19^{\Delta DMR}$-AGH cells to produce SC mice. To test this, we used CRISPR-Cas9 technology (Wu et al., 2013). We designed two sgRNAs targeting the 4.15-kb *IG*-DMR (termed *IG*-DMR-sgRNA1 and *IG*-DMR-sgRNA2) between *Dlk1* and *Gtl2* of *Dlk1-Gtl2* (Lin et al., 2003) (Figure 1F). pX330-*mCherry* plasmids expressing mammalian-codon-optimized Cas9 and *IG*-DMR-sgRNAs were transfected into haploid $H19^{\Delta DMR}$-AGH cells, and we then isolated 71 stable AG-haESC lines. DNA sequencing of PCR products obtained from the amplified targeted sites showed that *IG*-DMR had been successfully deleted in 58 of these lines (termed $H19^{\Delta DMR}$-$IG^{\Delta DMR}$-AGH-1 to $H19^{\Delta DMR}$-$IG^{\Delta DMR}$-AGH-58) (Figures 1G, S2A, and S2B). Off-target analysis of one line ($H19^{\Delta DMR}$-$IG^{\Delta DMR}$-AGH-2) showed no mutations at a total of 22 potential ''off-target'' sites (Table S2), predicted by searching the mouse genome according to a reported software tool (Hsu et al., 2013). ICAHCI analysis of $H19^{\Delta DMR}$-$IG^{\Delta DMR}$-AGH cells showed that 22.3% of SC embryos developed to term (Figures 1H, S2C, and S2D; Tables 1 and S1), similar to round spermatid injection (ROSI) performed by us (Table 1) and others (Kishigami

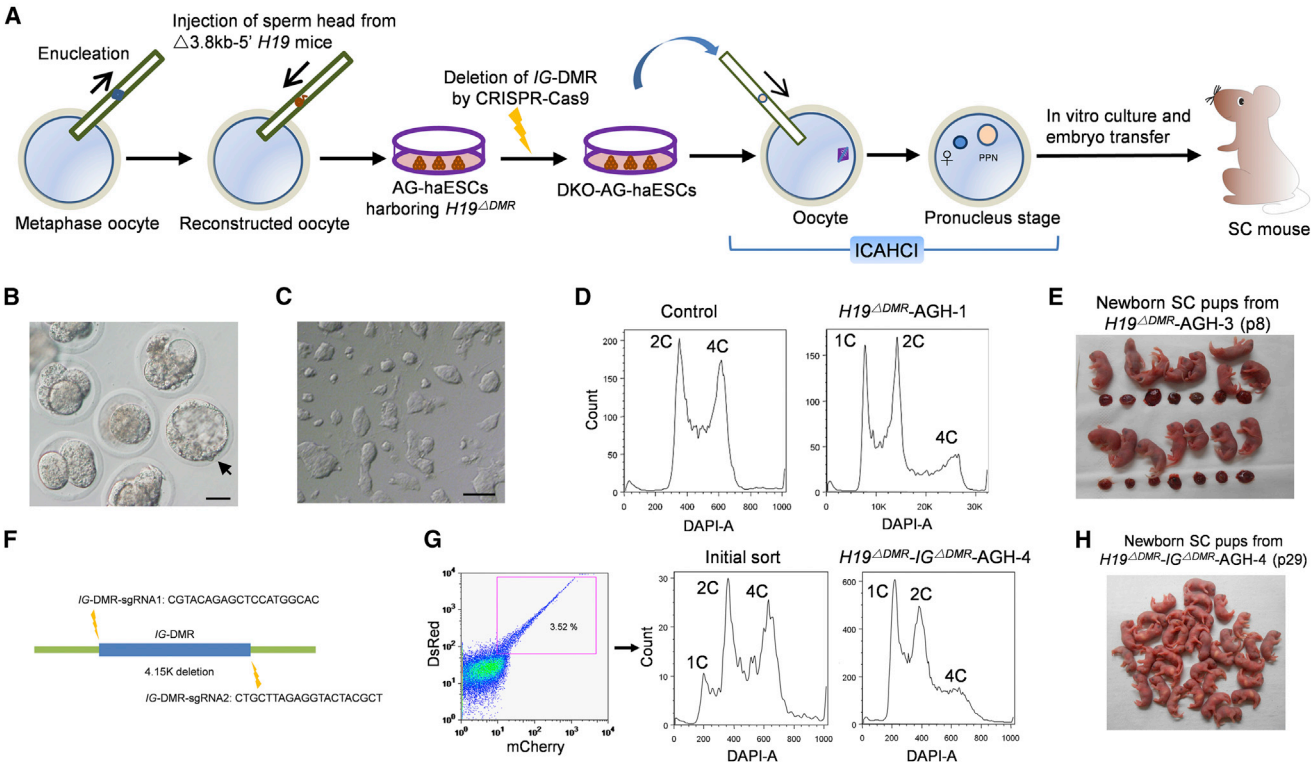

Figure 1. AG-haESCs Harboring Both *H19*$^{\Delta DMR}$ and *IG*$^{\Delta DMR}$ Efficiently Support the Generation of Live SC Pups

(A) Diagram of SC mice generated by ICAHCI using AG-haESCs carrying *H19*- and *IG*-DMR deletions. The sperm injected into enucleated oocytes carried a deletion of *H19*-DMR. PPN, pseudopronucleus derived from injected haESCs.

(B) Image of androgenetic embryos developed from an injection of *H19*$^{\Delta DMR}$ sperm into enucleated oocytes. A black arrow indicates a blastocyst, which had been used for ESCs derivation. Scale bar, 50 μm.

(C) Phase-contrast image of ESCs derived from one androgenetic blastocyst. Scale bar, 100 μm.

(D) Establishment of *H19*$^{\Delta DMR}$ -AGH cell lines (represented by *H19*$^{\Delta DMR}$ -AGH-1) after multiple rounds of FACS enrichment for haploid cells. A DAPI filter was used to detect the signal of Hoechst-stained DNA. The left panel shows FACS data of diploid control ESCs for comparison.

(E) SC pups from ICAHCI using *H19*$^{\Delta DMR}$ -AGH-3 cells (passage 8). Pups and placentas obtained by C-section from a pseudopregnant mouse at E19.5 are shown.

(F) Schematic of sgRNAs targeting for removal of *IG*-DMR. Dark green bar represents the deleted region (4.15 kb). The sequences of *IG*-DMR-sgRNA1 and *IG*-DMR-sgRNA2 are indicated.

(G) Generation of *H19*$^{\Delta DMR}$- *IG*$^{\Delta DMR}$-AGH cells. Left, mCherry-positive cells (3.52%), which were CRISPR-Cas9-transfected cells, were enriched and plated for derivation of AG-haESCs. Right: one established AG-haESC line (represented by *H19*$^{\Delta DMR}$- *IG*$^{\Delta DMR}$-AGH-4).

(H) SC pups from ICAHCI using *H19*$^{\Delta DMR}$- *IG*$^{\Delta DMR}$-AGH-4 cells (passage 29). Note that SC pups from *H19*$^{\Delta DMR}$- *IG*$^{\Delta DMR}$-AGH-4 cells were naturally delivered by mothers.

See also Figures S1 and S2 and Table S1.

et al., 2004). Strikingly, in contrast to the requirement for C-section to obtain SC mice when WT AG-haESCs or *H19*$^{\Delta DMR}$ -AGHs were used for ICAHCI, the pregnant females could deliver pups by themselves and almost all SC pups born from this set of experiments were alive and of normal size. These data demonstrate that DKO-AG-haESCs, derived after deletion of *H19* and *IG* DMRs in AG-haESCs, can support high-efficiency production of SC mice via ICAHCI.

The DMRs of *H19* and *Dlk1-Gtl2* Are Barriers to the High-Efficiency Generation of SC Mice in AG-haESCs

Having achieved the high-efficiency production of SC mice from AG-haESCs after deletion of the *IG*-DMR in *H19*$^{\Delta DMR}$-AGH cells, we next examined whether deletion of the DMR of *Dlk1-Gtl2* alone could enable high-efficiency production of SC mice in AG-haESCs. To this end, we generated AG-haESCs from *IG*-DMR KO mice (Lin et al., 2003) by performing NT. A total of eight haploid ESC lines were derived, in which two lines harbored a mutant *IG*-DMR (referred to as *IG*$^{\Delta DMR}$-AGH-1 and *IG*$^{\Delta DMR}$-AGH-2). ICAHCI analysis showed that *IG*$^{\Delta DMR}$-AGH cells were not efficient donors for producing SC pups (Tables 1 and S1); SC pups with normal size were rarely obtained (i.e., only 4 of 499 transferred SC embryos) (Figures 2A and S2E). Instead, most pups were growth retarded. Interestingly, methylation of the *H19* DMR was reduced in *IG*$^{\Delta DMR}$-AGH cells and absent in growth-retarded pups (Figures 2B, S2F, and S2G). We then removed the 3.8-kb DMR of *H19* (Thorvaldsen et al., 2002) in *IG*$^{\Delta DMR}$-AGH cells using the CRISPR-Cas9 system and generated 13 DKO-AG-haESC lines (termed *IG*$^{\Delta DMR}$-*H19*$^{\Delta DMR}$-AGH-1 to *IG*$^{\Delta DMR}$-*H19*$^{\Delta DMR}$-AGH-13) (Figures 2C

Table 1. Summary of In Vivo Development of ICAHCI Embryos Derived from Haploid Cells Carrying Different Gene Modifications

Donor Cell Type	Haploid ESC Line	Passage Number	No. of Embryos Transferred	No. of Growth-Retarded Pups (% of Transferred Embryos)	No. of Normal Pups (% of Transferred Embryos)
Single DMR KO AG-haESCs	*H19*$^{\Delta DMR}$-AGH cells	p8-p17	1443	39 (2.7)	86 (5.9)[c]
	IG$^{\Delta DMR}$-AGH cells	p8-p23	499	12 (2.4)	4 (0.8)
DKO-AG-haESCs	*H19*$^{\Delta DMR}$-*IG*$^{\Delta DMR}$-AGH cells	p19-p33	939	4 (0.4)	210 (22.4)
	IG$^{\Delta DMR}$-*H19*$^{\Delta DMR}$-AGH cells	p24-p28	544	5 (0.9)	105 (19.3)
	H19$^{\Delta DMR}$-*IG*$^{\Delta DMR}$-AGH-OG3 cells	p26-p37	510	1 (0.2)	87 (17.1)
	subtotal	p19-p37	1993	10 (0.5)	402 (20.2)[d]
DKO-AG-haESCs carrying *Tet 1*, *2*, and *3* triple mutations	Tet-TKO-DAH cells	p35-p37	407	4 (1.0)	59 (14.5)
DKO-AG-haESCs carrying *p53*, *63*, and *73* triple mutations	p53-TKO-DAH cells	p41-p46	660	2 (0.3)	111 (16.7)
DKO-AG-haESCs carrying *Tet1* and *3* Knockin	Tet1&3-KI-DAH-1	p40-p47	138	1 (0.7)	21 (15.2)
DKO-AG-haESCs carrying *Tet1*, *2*, and *3* Knockin	Tet-TKI-DAH-1	p47-p51	874	6 (0.7)	151 (17.3)
WT AG-haESCs	AGH cells[a]	p9-p20	294	3 (1.0)	2 (0.7)
	AGH-OG-3 cells[b]	p12-p26	379	6 (1.6)	7 (1.8)
Control	round spermatids		125	0	28 (22.4)

See also Figures S1–S5 and Tables S1 and S3.
[a]AG-haESCs generated in this study.
[b]AG-haESCs generated in our previous study.
[c]*H19*$^{\Delta DMR}$-AGH cells versus WT AG-haESCs ($p < 0.05$).
[d]DKO-AG-haESCs versus WT AG-haESCs ($p < 0.001$).

and S2H; Table S2). ICAHCI analysis of two lines showed comparable potential to that of *H19*$^{\Delta DMR}$-*IG*$^{\Delta DMR}$-AGH cells for generating normal SC pups (Figures S2I and S2J; Tables 1 and S1).

Next, we asked whether the capacity for high-efficiency production of SC mice could be re-established in late-passage WT AG-haESCs by removing the DMRs of *H19* and *IG* simultaneously. To this end, we chose a WT AG-haESC line, AGH-OG-3, which almost completely lost its ability to produce normal SC mice by ICAHCI by passage 22 (Yang et al., 2012) (Table S1). After transfection of plasmids expressing Cas9 and sgRNAs designed for targeting DMRs of *H19* and *IG* (Figures 1F and 2C) into passage 21 AGH-OG-3 cells, we generated 12 AG-haESC lines carrying deletions of both DMRs (termed *H19*$^{\Delta DMR}$-*IG*$^{\Delta DMR}$-AGH-OG3-1 to *H19*$^{\Delta DMR}$-*IG*$^{\Delta DMR}$-AGH-OG3-12) (Figures 2D and S3A; Table S2). Strikingly, after injection of haploid cells from two of these lines into oocytes, around 17% of the resulting embryos could develop normally to term (Figures S3B and S3C; Tables 1 and S1), indicating that WT AG-haESCs that had previously lost the ability to produce live SC mice regained it after removal of the *H19* and *IG* DMRs.

A total of 402 SC pups were born from three sets of DKO-AG-haESCs, at an average efficiency of 20.2% of transferred embryos. SC pups generated from DKO-AG-haESCs grew to adulthood and reproduced normally. Genotype analyses of 33 live-born pups from seven litters of progeny delivered by SC mice showed that 13 carried the mutant *H19*-DMR and 11 were WT (Figure 2E). Another nine pups, in which six carried mutant *IG*-DMR and three carried mutations in both *H19* and *IG* DMRs (Figure 2E), died shortly after birth, conforming to the expected phenotype of postnatal or neonatal lethality induced by maternal transmission of the *IG*-DMR deletion (Lin et al., 2003). These data demonstrate that removing the *H19* and *IG* DMRs does not impede normal development and reproduction in the resulting SC pups and elimination of the DMR deletions in the progeny of SC mice can be naturally obtained by further crossing with WT animals.

Next, we tested whether deletion of the DMRs changed gene expression in AG-haESCs. qPCR analysis showed that *H19* and *Gtl2* were downregulated, while *Igf2* and *Dlk1* were upregulated in DKO-AG-haESCs, as expected based on the DMR deletions (Figure S3D). Next, we compared the gene expression profiles of DKO-AG-haESCs with those of normal AG-haESCs and mouse round spermatids (RSs). Clustering of these cells based on RNA sequencing (RNA-seq) data showed a high correlation between DKO-AG-haESCs and WT AG-haESCs, but not RSs (Figures 2F and S3E). We further compared the expression patterns of other imprinted genes in DKO-AG-haESCs and control AG-haESCs and found that DKO-AG-haESCs and WT AG-haESCs exhibited highly similar expression profiles across all imprinted genes (Figure 2G). To further assess epigenetic inheritance, we performed reduced representation bisulfite sequencing (RRBS) to determine the methylomes at base resolution on a genome-wide scale. As shown in Figures

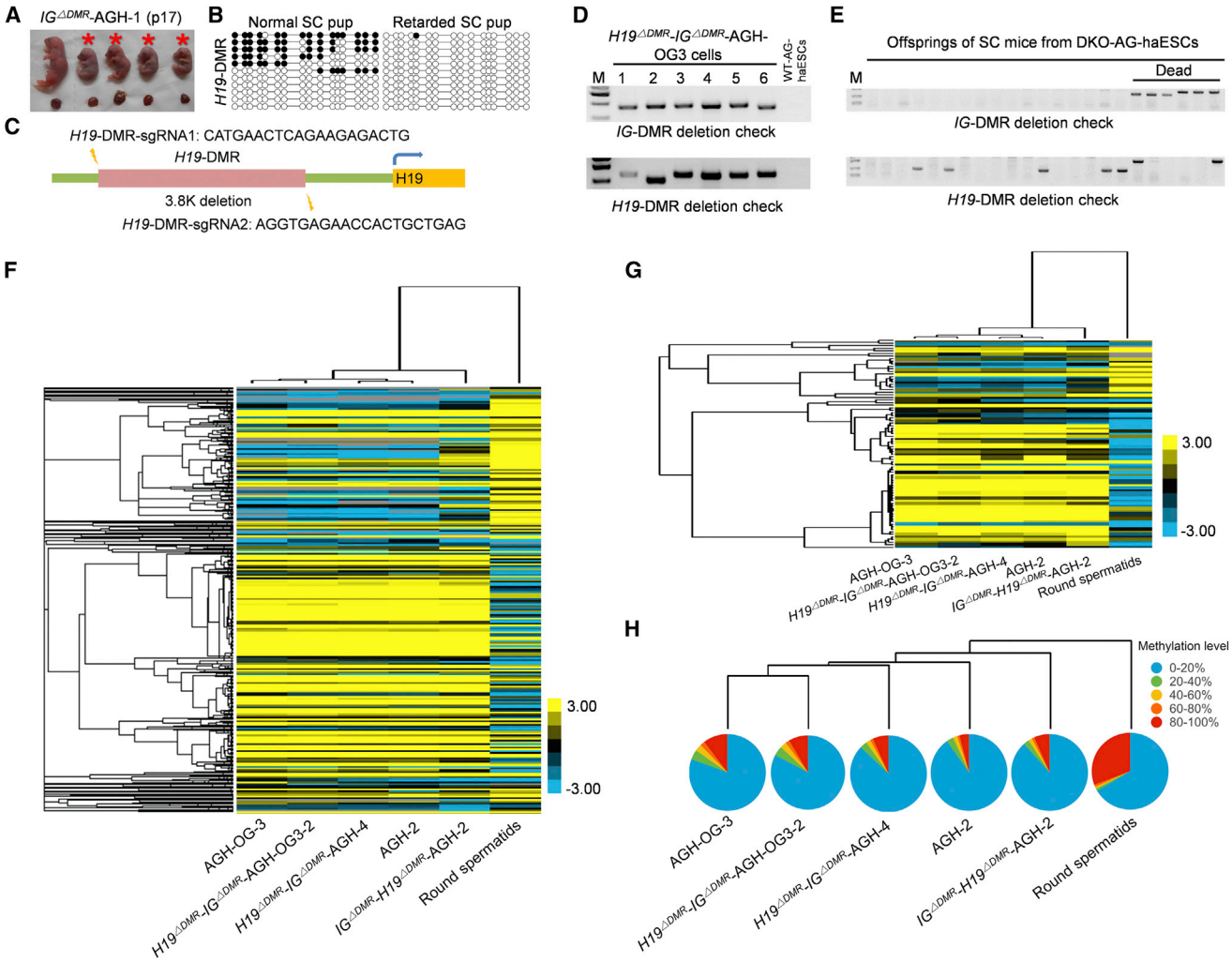

Figure 2. *H19* and *IG* DMRs Are Two Barriers to the High-Efficiency Generation of SC Pups in AG-haESCs

(A) SC pups from *IG*$^{\Delta DMR}$-AGH-1 cells (passage 17). Pups and placentas obtained by C-section from a pseudopregnant mouse at E19.5 are shown. Asterisks indicate growth-retarded SC pups that died shortly after birth.

(B) Methylation state of the *H19*-DMR in a normal SC pup (left) and a retarded pup (right) derived from *IG*$^{\Delta DMR}$-AGH-1 cells.

(C) Schematic of sgRNAs targeting for the removal of *H19*-DMR. A pink bar represents the deleted region (3.8 kb). The sequences of *H19*-DMR-sgRNA1 and *H19*-DMR-sgRNA2 are indicated.

(D) Genotyping analysis of *H19*$^{\Delta DMR}$-*IG*$^{\Delta DMR}$-AGH-OG3 cells. Note that these cells were generated by deletion of both *IG*-DMR and *H19*-DMR in WT AGH-OG-3 (passage 21) that have lost the ability to produce SC pups after injection into oocytes (Yang et al., 2012).

(E) Genotyping analysis of the progeny of SC mice derived from DKO-AG-haESCs. Note that pups carrying mutant *IG*-DMR or mutations in both *H19* and *IG* died shortly after birth.

(F) Gene expression profiles of DKO-AG-haESCs using RNA-seq analysis. Gene expression profiles were clustered using all expressed genes. Three DKO-AG-haESC lines generated by different strategies show highly similar expression profiles to the control AG-haESCs, although they are markedly different from mouse round spermatids (RSs). AGH-2 is a WT haploid cell line established in this study. AGH-OG-3 is a WT haploid cell line that was established in our previous study (Yang et al., 2012). To avoid the influence of diploidized cells on the expression profile, we collected samples after FACS of cells in the G1/G0 phase.

(G) Gene expression profiles of DKO-AG-haESCs based on imprinting genes. Three DKO-AG-haESC lines show highly similar expression profiles to the control AG-haESCs, although they are markedly different from mouse RSs.

(H) Methylation profiles of DKO-AG-haESCs based on RRBS analysis. The pies show different methylation levels of CpG sites in different colors.

See also Figures S2 and S3 and Table S1.

2H, S3F, and S3G, deletion of *H19* and *IG* DMRs did not change the methylation patterns in AG-haESCs based on the analyses of all detected CpGs and the promoter regions of all imprinted genes. Taken together, our results demonstrate that the DMRs of *H19* and *IG* are major barriers to high-efficiency production of SC mice in AG-haESCs and their deletion does not have notable negative effects on the gene expression profile of functionality.

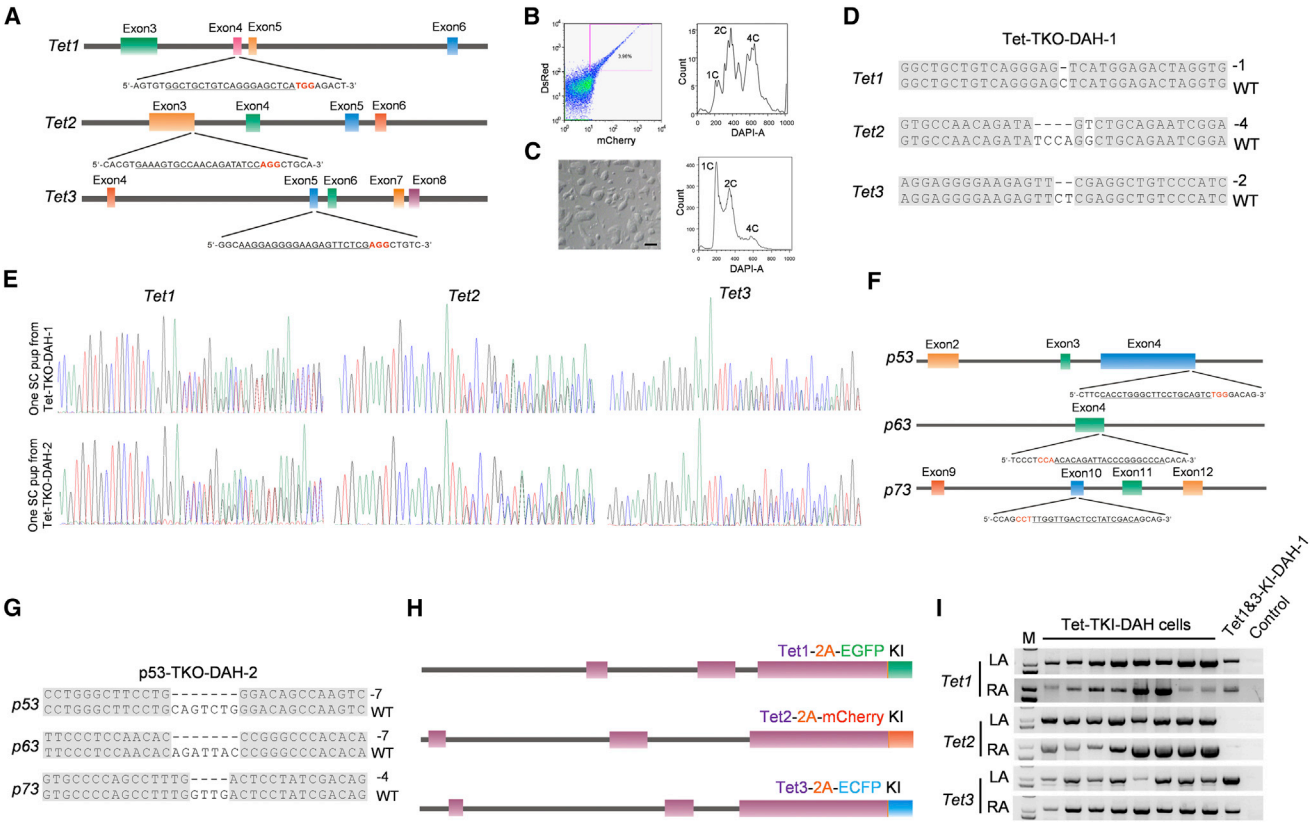

Figure 3. DKO-AG-haESCs Carrying Multiple Gene Modifications Support Efficient Generation of SC Mice

(A) Schematic of Cas9-sgRNA-targeting sites in *Tet1*, *2*, and *3*. The sgRNA-targeting sequences are underlined, and the PAM sequences are labeled in red.

(B) MCherry-positive cells (3.96%), which were CRISPR-Cas9-transfected cells, were enriched and plated for derivation of DKO-AG-haESCs carrying mutant *Tet* genes.

(C) Generation of Tet-TKO-DAH cells. Left: phase-contrast image of ESCs derived by expansion of single cells. Right: flow analysis of an established haploid cell line. Scale bar, 100 μm.

(D) The sequences of *Tet1*, *2*, and *3* in Tet-TKO-DAH-1 cells. Deletions are indicated with (−).

(E) DNA sequences of PCR products amplified from *Tet1*, *2*, and *3* genes of two SC pups generated from Tet-TKO-DAH-1 and Tet-TKO-DAH-2, respectively. Two peaks can be observed in the sequences of the SC pups carrying heterogeneous mutations in *Tet1*, *2*, and *3*.

(F) Schematic of Cas9-sgRNA-targeting sites in *p53*, *63*, and *73*. The sgRNA-targeting sequences are underlined, and the PAM sequences are labeled in red.

(G) The sequence of *p53*, *63*, and *73* in p53-TKO-DAH-2. Deletions are indicated with (−).

(H) Schematic of double-stranded vectors, including *Tet1-EGFP*, *Tet2-mCherry*, and *Tet3-ECFP*. *EGFP*, *mCherry*, and *ECFP* reporters fused with the last codon of the *Tet1*, *Tet2*, and *Tet3* genes, respectively.

(I) Genotyping analysis of Tet-TKI-DAH-1 cells.

See also Figures S4 and S5 and Table S3.

Generation of Mutant Mice Carrying Multiple Genetic Modifications Using DKO-AG-haESCs

Having demonstrated the stable developmental potential of DKO-AG-haESCs by ICAHCI, we next examined whether multiple genetic alterations can be introduced into these DKO-AG-haESCs, followed by ICAHCI to efficiently produce SC mice carrying multiple mutations. For this experiment, we first attempted disruption of *Tet1*, *Tet2*, and *Tet3* in DKO-AG-haESCs using the CRISPR-Cas9. We transfected constructs expressing Cas9 and three sgRNAs targeting *Tet1*, *2*, and *3* (Figure 3A) (Wang et al., 2013) into DKO-AG-haESCs, leading to 56 stable DKO-AG-haESC lines. DNA sequencing of PCR products corresponding to amplified targeted sites showed that *Tet1*, *2*, and *3* were successfully mutated in 18 cell lines (termed Tet-TKO-DAH-1 to Tet-TKO-DAH-18) (Figures 3B–3D and S4A). ICAHCI analysis of four of the cell lines showed that SC mice carrying mutations in *Tet1*, *2*, and *3* could be generated at an appropriate efficiency (Figures 3E and S4B; Tables 1 and S3). We further confirmed this by generation of DKO-AG-haESCs carrying mutations in the *p53* family (Figures 3F, 3G, and S4C) and efficient production of corresponding SC pups via ICAHCI (Figures S4D and S4E; Tables 1 and S3). These data demonstrate that DKO-AG-haESCs, unlike WT AG-haESCs that fail to produce live SC mice after being genetically manipulated in vitro (Yang et al., 2012), can efficiently and stably support the generation of SC mice following genome engineering.

Next, we tested whether precise gene-modification experiment by insertion of DNA constructs into multiple endogenous genes was feasible in DKO-AG-haESCs and could further lead to SC mice carrying corresponding genetic traits. For this, we

attempted to derive DKO-AG-haESCs carrying a fluorescent reporter constructs knocked into the endogenous *Tet1*, *2*, and *3* loci. We first transfected DKO-AG-haESCs with constructs expressing both Cas9 and two sgRNAs targeting *Tet1* and *3* (Figure 3A) and double-stranded donor vectors that were designed to fuse an *EGFP* reporter with the last codon of the *Tet1* gene and an *ECFP* reporter with the last codon of the *Tet3* gene (Figures 3H and S5A). A total of 150 DKO-AG-haESC lines were generated, and 10 and 7 cell lines carried the *EGFP* reporter gene in the *Tet1* and *ECFP* reporter gene in the *Tet3* loci, respectively (Figures S5B and S5C). One cell line, termed Tet1&3-KI-DAH-1, carried both *Tet1-EGFP* and *Tet3-ECFP* genes (Figures S5D and S5E). ICAHCI analysis with this line produced SC mice carrying the *Tet1-EGFP* and *Tet3-ECFP* knockin alleles with similar efficiency to ICAHCI with WT DKO-AG-haESCs (Figures S5F and S5G; Tables 1 and S3). Next, we transfected Tet1&3-KI-DAH-1 cells with constructs expressing both Cas9 and an sgRNA-targeting *Tet2* and a double-stranded donor vector of a *mCherry* reporter fused with the last codon of the *Tet2* gene (Figures 3A and 3H). From 130 established cell lines, we identified eight haploid cell lines with a *Tet2-mCherry* insertion (Tet-TKI-DAH-1 to Tet-TKI-DAH-8) (Figures 3I and S5H). We investigated the developmental potential of these Tet-TKI-DAH cells by ICAHCI and found that they reproducibly produced live SC pups after injection into oocytes (Figures S5I and S5J; Tables 1 and S3). Taken together, these results indicate that DKO-AG-haESCs are a feasible tool for multiple-site genetic modification using CRIPSR-Cas9 and ICAHCI to give pups carrying the corresponding genetic traits.

DKO-AG-haESCs Carrying an sgRNA Library Generate Heterozygous Mutant Mice

Recent studies have shown an application of genome-wide sgRNA libraries for loss-of-function screening in human and mouse cells (Koike-Yusa et al., 2014; Shalem et al., 2014; Wang et al., 2014). Based on that, we reasoned that DKO-AG-haESCs carrying an sgRNA library might be used to efficiently generate mutant mouse models by ICAHCI (Figure 4A; termed the "lenti-sgRNA+pX330" strategy). To test this, we employed a well-characterized, recently established mouse lentiviral sgRNA library (Koike-Yusa et al., 2014) that contains 87,897 sgRNAs targeting 19,150 mouse protein-coding genes. 1.0×10^7 FACS-enriched haploid cells from the $IG^{\Delta DMR}$-$H19^{\Delta DMR}$-AGH-2 cell line were infected with the genome-wide sgRNA lentiviral library. Two days later, transfected cells were selected by puromycin treatment, followed by transfection of pX330-*mCherry* plasmids expressing Cas9. Haploid cells expressing mCherry, in which Cas9 was successfully transfected, were enriched (Figure 4B) and used for subsequent ICAHCI analysis. To examine whether genes were mutated by CRISPR-Cas9 in haploid cells, we randomly analyzed seven haploid cell clones. All tested clones carried one sgRNA, and DNA sequencing analysis indicated that the targeted genes were mutated, reflecting a successful induction of gene mutation by CRISPR-Cas9 in haploid cells (Figures 4C and 4D).

Next, we tested whether these haploid cells that carried mutant genes support efficient generation of heterozygous mice by ICAHCI. A total of 114 SC pups were produced from three independent ICAHCI experiments (Figure 4E; Table 2), of which 82 SC pups carried only one sgRNA (Figure 4F). DNA sequencing of PCR products amplified from sgRNA-targeted sites indicated that 43 SC pups carried mutant genes at one targeted allele, of which 39 were frameshift insertion/deletion (indel) mutations that result in a loss-of-function allele (Figure 4G; Data S1). Interestingly, all mutant alleles exhibited one genotype, reflecting the fact that the gene was mutated in the individual haploid cells after transient expression of Cas9, which were then injected into WT oocytes to produce SC embryos. The remaining 39 SC pups carried an sgRNA but failed to show target site mutations, a frequency similar to previous observations in human cells (Zhou et al., 2014) or in mouse embryos (Wu et al., 2013). These data therefore demonstrate that DKO-AG-haESCs carrying an sgRNA library, when transiently infected with Cas9, can introduce genetic mutations into the resulting SC mice through ICAHCI, leading to the one-step generation of heterozygous mutant mice.

Generation of Biallelic Mutant Mice Using sgRNA Library DKO-AG-haESCs

Next, we tested whether DKO-AG-haESCs carrying an sgRNA library can be used to produce biallelic mutant mice by ICAHCI. For this approach, we performed ICAHCI first by injecting haploid cells carrying an sgRNA library into mature oocytes and then injected Cas9 mRNA into reconstructed oocytes (termed the "lenti-sgRNA+Cas9 injection" strategy). A total of 51 SC pups carrying one sgRNA were generated, and 22 of them carried mutant genes (Table 2; Data S1). DNA sequencing of PCR products with specific primers from tail genomic DNA showed that ten mice carried monoallelic modifications and 12 carried biallelic modifications (23.5% of total born SC pups). We then performed TA cloning and sequencing analysis of seven pups with biallelic mutations and found that around 63% of tested clones carried frameshift indel mutations (Table S4).

To increase the biallelic mutation rate of SC pups, we then conducted double Cas9 treatment by injecting haploid cells carrying sgRNA that been transiently transfected with pX330-*mCherry* plasmid (expressing Cas9) into mature oocytes followed by injection of Cas9 mRNA into reconstructed oocytes (Figures S6A and S6B) (termed the "lenti-sgRNA+pX330+Cas9 injection" strategy). We derived a total of 31 SC pups carrying one sgRNA, and 22 of them carried mutations (Figures S6C and S6D; Table 2; Data S1), of which 13 were biallelic (Figure S6E) (41.9% of total born SC pups) and 9 were monoallelic. TA cloning and sequencing analysis of five pups with biallelic mutations showed that around 79% of tested clones carried frameshift indel mutations (Figures S6F and S6G; Table S4).

As recent studies have shown that constitutive expression of Cas9 does not affect the viability of cells and mice (Koike-Yusa et al., 2014; Platt et al., 2014), we also tested a third strategy in which DKO-AG-haESCs with constitutive expression of Cas9 and an sgRNA library were generated by two rounds of drug selection and then used for production of SC mice by ICAHCI (Figures 5A–5D) (termed strategy of "lenti-Cas9+lenti-sgRNA"). We reasoned that this strategy would greatly enhance the likelihood of obtaining biallelic mutant mice from DKO-AG-haESCs. Among the 1,453 SC embryos that we reconstructed via ICAHCI, 272 (18.7%) developed to term in vivo (Figure 5E; Table 2), similar

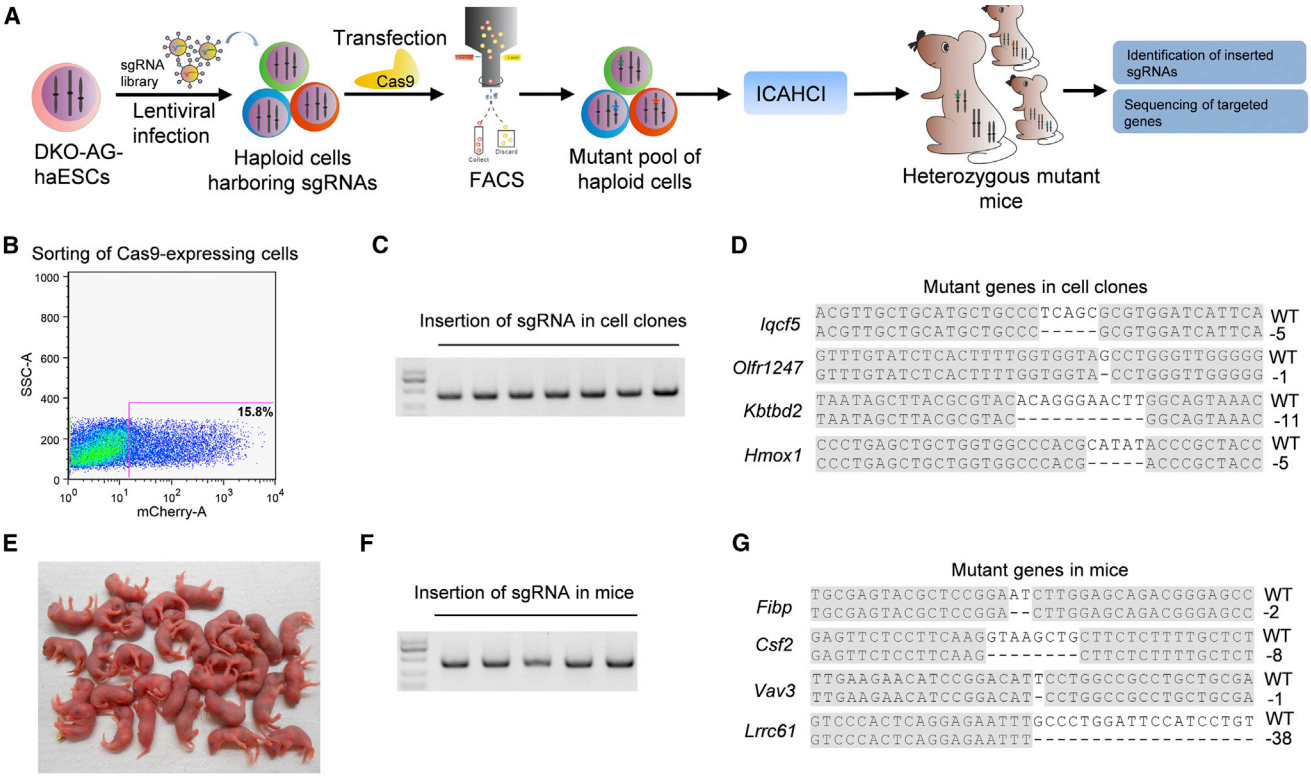

Figure 4. DKO-AG-haESCs Carrying an sgRNA Library Support the Efficient Generation of Heterozygous Mutant Mice in One Step

(A) Schematic of the efficient generation of heterozygous mutant SC mice via ICHACI using DKO-AG-haESCs carrying an sgRNA library in one step.

(B) Haploid cells expressing mCherry (15.8%), in which Cas9 were successfully transfected, enriched, and used for subsequent ICAHCI analysis.

(C) PCR analysis of sgRNA in haploid cell clones expanded from single cells. All tested clones carried sgRNA.

(D) The sequences of different targeted genes in cell clones. Gene modifications existed in the targeted genes in all tested clones.

(E) SC pups from DKO-AG-haESCs carrying an sgRNA library. Note that SC pups were naturally delivered by mothers.

(F) PCR analysis of existence of sgRNA in SC pups.

(G) The sequences of different targeted genes in SC pups. Gene modifications existed in the targeted genes in tested SC pups.

See also Data S1.

to the ICAHCI efficiency for WT DKO-AG-haESCs or DKO-AG-haESCs carrying different genetic modifications (Table 1), indicating that multiple rounds of genetic manipulations in DKO-AG-haESCs do not affect the developmental potential of resulting SC embryos. A total of 224 SC pups carrying one sgRNA were subjected to genotyping analysis (Figure 5F; Table 2; Data S1). The results showed that 143 of them carried mutations, of which 83 were biallelic (Figure 5G). We also produced 60 monoallelic mutant mice even though Cas9 is supposed to be constitutively expressed in this system, probably because of silencing of the lentiviral vectors (Ellis, 2005; Koike-Yusa et al., 2014). We performed TA cloning and sequencing of targeted genes in tails of 26 mutant mice and found around 66.3% of tested clones carried frameshift indels (Figures 5H and 5I; Table S4). To examine the whole-body mutation rate in the SC pups, we conducted genotyping in different organs, including brain, heart, kidney, liver, and lung dissected from four SC pups and found that all organs carried biallelic mutations. Finally, we performed TA cloning and sequencing in all of the organs of one SC pup that carried *Scube1* gene mutations. The results showed that more than 80% of tested clones carried frameshift indel mutations (Figure 5J). Interestingly, this mouse died 1 hr after birth, consistent with a previous report on *Scube1* mutant mice (Tu et al., 2008). Taken together, our results provide a proof of principle that mutant mice can be obtained via ICAHCI using DKO-AG-haESCs that harbor an sgRNA library and that this approach could be used for KO-based screens in mouse.

DISCUSSION

Our previous studies (Yang et al., 2012) showed that AG-haESCs can be derived from haploid AG-blastocysts and support the generation of SC mice via ICAHCI. However, AG-haESCs failed to produce live-birth SC pups upon long periods of culturing, especially through the course of in vitro genetic manipulation, probably due to the loss of paternal imprints. In this study, we show the high-efficiency generation of SC pups upon removal of DMRs of *H19* and *IG* in AG-haESCs, leading to stable production of live SC mice via ICAHCI at a rate of 20%, ~10 times higher than with the WT AG-haESCs even from early passages (Table 1) (Yang et al., 2012). Importantly, we also show that DKO-AG-haESCs can be used for genetic manipulation to produce, via ICAHCI, SC mice carrying multiple gene modifications at a comparable efficiency to WT

Table 2. Summary of SC Mice Derived from DKO-AG-haESCs Carrying an sgRNA Library

Strategies	No. of Embryos Transferred	No. of SC Pups (% of Transferred Embryos)	No. of SC Pups without sgRNA	No. of SC Pups Carrying sgRNA ($n \geq 2$)	No. of SC Pups Carrying One sgRNA	No. of SC Pups with Biallelic Mutation	No. of SC Pups with Monoallelic Mutation
lenti-sgRNA+pX330	580	114 (19.7)	11	21	82	0	43
lenti-sgRNA+Cas9 injection	306	51 (16.7)	0	4	47	12	10
lenti-sgRNA+pX330+ Cas9 injection	238	31 (13.5)	3	1	27	13	9
lenti-Cas9+lenti-sgRNA	1,453	272 (18.7)	22	13	237	83	60

See also Figure S6, Table S4, and Data S1.

DKO-AG-haESCs. Moreover, when combined with an sgRNA library, DKO-AG-haESCs can be used to generate monoallelic and biallelic mutant mice.

DKO-AG-haESCs have a number of advantages over existing technologies for production of gene-modified mice. First, DKO-AG-haESCs-mediated gene editing provides a unique system in which gene-modified DKO-AG-haESCs can be analyzed and pre-selected, ensuring the generation of SC pups with uniform modifications and avoiding somatic mosaicism induced by direct injection of CRISPR-Cas9 into zygotes (Niu et al., 2014; Wang et al., 2013). Second, thanks to the efficiency and reliability of SC mouse generation by ICAHCI, our system can produce sufficient numbers of offspring for analysis in one generation. Third, application of DKO-AG-haESCs carrying an sgRNA library and expressing Cas9 can be used for genetic screening. SC pups carrying different mutations can be easily identified by PCR of the integrated sgRNA (just like a "barcode" of the mutant mouse) using one pair of common primers for sgRNA. Application of this approach for large-scale screens based on a genome-wide production of mutant mice is not yet feasible at present, due to three significant hurdles: (1) the overall efficiency of generating biallelic mutants through DKO-AG-haESCs carrying sgRNA needs to be further improved; (2) it would be extremely time- and labor-consuming to generate a genome-wide mutant mouse library using this approach; and (3) the subsequent phenotype analysis of the mutant mice would be a complex task. In addition, culture of AG-haESCs and fertilization by ICAHCI are technologies that are not in use by many groups and successful application of this approach requires proficiency in them both. Nevertheless, we would like to propose that it is feasible to use our approach for medium-scale targeted screening at organism level, especially for developmental phenotypes, using the appropriate sgRNA libraries targeting pre-selected candidate genes.

In summary, the generation of SC mice using DKO-AG-haESCs is an efficient and simple method for producing mouse models carrying complex genetic modifications, such as mutations or knockin reporters in multiple members of a gene family. Moreover, together with an sgRNA library, DKO-AG-haESCs enable efficient generation of mutant mice in a single step. Future analyses will be needed to understand the detailed mechanism underlying the high-efficient generation of SC mice by DKO-AG-haESCs. In the meantime, we hope that the method we have developed will facilitate genetic analysis of development and modeling of diseases in mice.

EXPERIMENTAL PROCEDURES

Animal Use and Care

All animal procedures were performed under the ethical guidelines of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Derivation of AG-haESCs

For generation of AG-haploid embryos, spermatozoa of *H19*-DMR KO mice (C57/B6 background, homozygous) (Thorvaldsen et al., 2002) or *IG*-DMR KO mice (C57/B6 background, heterozygous) (Lin et al., 2003) were collected for NT according to the previously reported methods (Yang et al., 2012). Briefly, mature oocytes were obtained from superovulated B6D2F1 (C57BL/6 × DBA2) female mice 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 KSOM medium 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 into ESC medium for derivation of ESC lines as previously 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 LIF, 3 μM CHIR99021, and 1 μM PD0325901. 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 in 6-well plates for routine culture. To sort haploid cells, ESCs were trypsinized, washed by DPBS (GIBCO), and then incubated with 15 μg/ml Hoechst 33342 in a 37°C water bath. Subsequently, the haploid 1C peak was purified using BD FACS AriaII for further culturing.

CRISPR-Cas9-Mediated Gene Manipulation in Haploid Cells

To generate CRISPR-Cas9 plasmid for gene mutation, sgRNAs of target genes were synthesized, annealed, and ligated to the pX330-*mCherry* plasmid that was digested with Bbs I (New England Biolabs). AG-haESCs were transfected with corresponding pX330-*mCherry* plasmids, including sgRNA using Lipofectamine 2000 (Life Technologies) in accordance with the manufacturer's instruction manual. 48 hr after transfection, the haploid cells expressing red fluorescence protein were enriched with flow cytometry (FACS AriaII, BD Biosciences) and plated at low density. 4–5 days after plating, single colonies were picked for derivation of AG-haESCs. Haploid cell lines carrying expected genotypes were selected for further analysis by DNA sequencing of PCR products amplified from targeted sites (Table S5).

For the construction of double-stranded DNA donors, the sequences encoding *EGFP*, *mCherry*, or *ECFP* were amplified and ligated to the pMD19T vector, respectively. Subsequently, the sequences of left arms and right arms of targeted genes were inserted into the multiple cloning sites (MCS) of pMD19T-EGFP/mCherry/ECFP vectors, respectively.

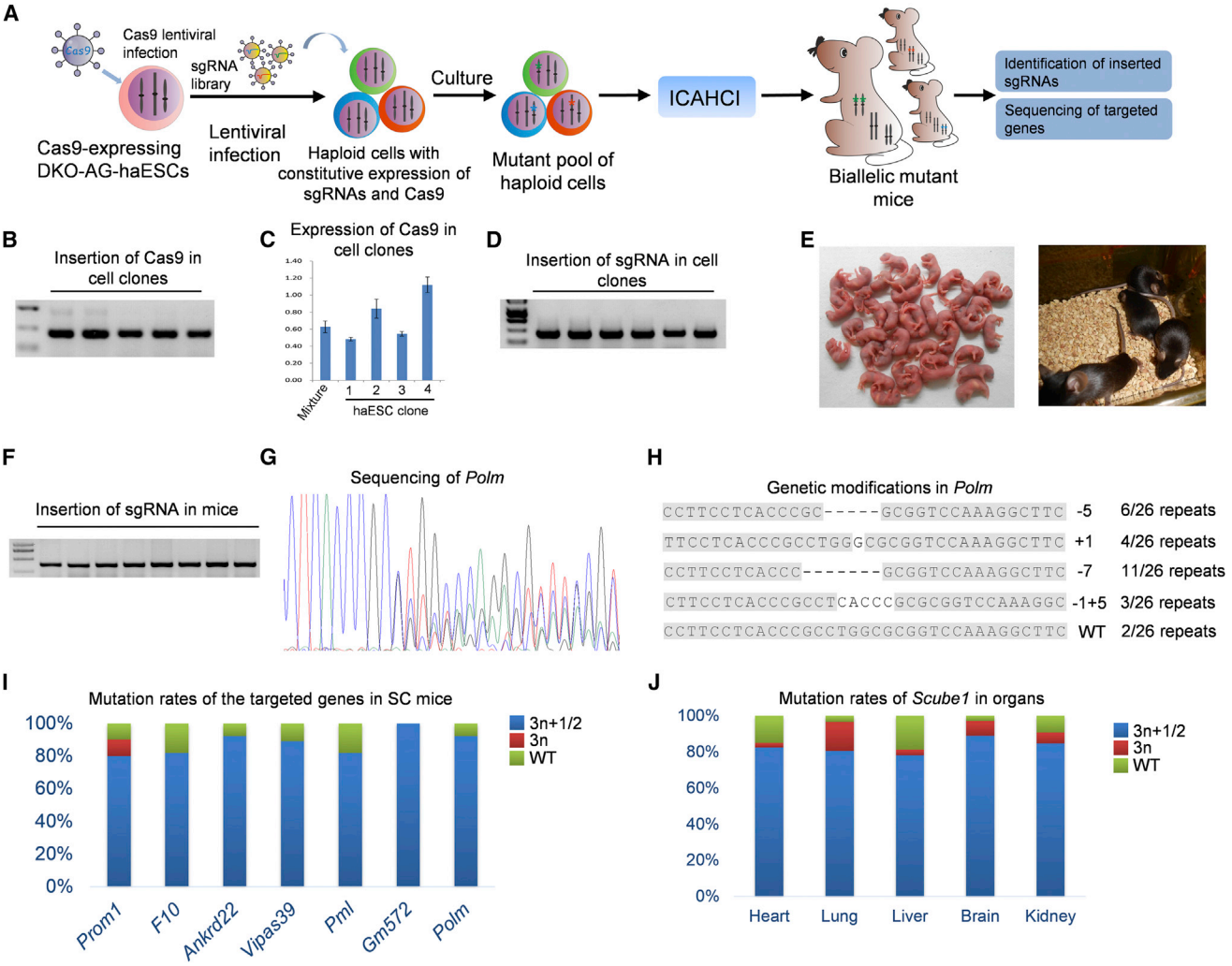

Figure 5. DKO-AG-haESCs Carrying Constitutively Expressed Cas9 and an sgRNA Library Support the Efficient Generation of Biallelic Mutant SC Mice in One Step

(A) Schematic of efficient generation of biallelic mutant SC mice via ICHACI using DKO-AG-haESCs carrying constitutively expressed Cas9 and an sgRNA library.

(B) PCR analysis of Cas9 in haploid cell clones expanded from single cells. All tested clones carried Cas9 transgene.

(C) qPCR analysis of Cas9 expression in cell clones expanded from single cells. Cas9 were expressed in all tested cell clones.

(D) PCR analysis of sgRNA in haploid cell clones expanded from single cells. All tested clones carried sgRNA.

(E) SC pups from DKO-AG-haESCs carrying constitutively expressed Cas9 and an sgRNA library. Note that SC pups were naturally delivered by mothers. Left: newborn SC pups. Right: adult SC mice.

(F) Identification of sgRNA in SC mice by PCR analysis.

(G) Generation of biallelic mutant mice via ICAHCI using DKO-AG-haESCs carrying constitutively expressed Cas9 and an sgRNA library. One represented SC mouse carries biallelic mutant *Polm* gene, indicated by multiple peaks in the sequence of PCR products.

(H) Sequence of the targeted *Polm* gene in the mouse tail by TA cloning and sequencing analysis. 24 of 26 tested clones carried frameshift insertion/deletion (indel) mutations.

(I) Summary of TA cloning and sequencing analysis of seven biallelic mutant mice. Over 80% of tested clones carried frameshift indels.

(J) TA cloning and sequencing analysis of different organs in one mouse carrying biallelic mutant *Scube1* gene. Over 80% of tested clones carried frameshift indel mutations. More than 30 clones were tested for each organ.

See also Figure S6, Table S4, and Data S1.

Lentiviral Infection of Haploid Cells

The lenti-sgRNA library and lenti-Cas9 plasmids were reported previously (Cong et al., 2013; Koike-Yusa et al., 2014) and provided by Addgene. For preparation of the lentivirus, the HEK293T cells in a 10-cm dish were transfected with 3 μg of a lentiviral vector (lenti-sgRNA library or lenti-Cas9) and 9 μg of ViraPower Lentiviral Packaging Mix (Invitrogen) by Lipofectamine 2000 Reagent (Invitrogen) in accordance with the manual. The supernatant was harvested 72 hr after transfection, concentrated with Lenti-Concentin virus precipitation solution (SBI), and then stored at −80°C. In order to determine the virus volumes for achieving an MOI of 0.3 to ensure that most cells receive single copy of the lentiviral vector, DKO-AG-haESCs in 24-well plates were incubated with different volume of lentivirus. Then the 10^8 suspended cells were infected in ESC medium supplemented with 8 μg /ml polybrene (Sigma) and optimal volume of lentivirus for 48 hr. After selection in ESC medium containing puromycin (Invitrogen) or blasticidin (Sigma), the cells were collected for genomic DNA extraction and sorted for injection. To mutate the DKO-AG-haESCs without

Cas9 integration, the cells were further transfected with pX330-*mCherry* plasmid and sorted with FACS to enrich the mCherry-positive cells.

ICAHCI, ROSI, and Embryo Transfer

To generate semi-cloned (SC) embryos, AG-haESCs arrested at M phase by culturing in medium containing 0.05 μg/ml demecolcine for 8 hr 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. Each nucleus from M-phase haploid cells was injected into an MII-arrested oocyte using a Piezo-drill micromanipulator (Movie S1). 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. ICAHCI embryos were cultured in KSOM medium for 24 hr to reach the two-cell stage. For ROSI, we adopted a reported protocol by Kishigami et al. (2004). 15–20 two-cell embryos derived from ICAHCI or ROSI were transferred into each oviduct of pseudo-pregnant ICR females at 0.5 days postcoitum (dpc). Recipient mothers were euthanized at 19.5 days of gestation, and the pups were quickly removed from the uteri (for embryos derived from WT AG-haESCs or AG-haESCs carrying single DMR deletion) or naturally delivered (for embryos derived from ROSI or DKO-AG-haECs). 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.

Statistical Analysis

Differences of generation of SC pups between groups were analyzed by means of Student's t test.

ACCESSION NUMBERS

All RNA-seq and RRBS data sets are available through GEO under the accession numbers GEO: GSE60072, GEO: GSE60075, and GEO: GSE60076.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, five tables, one movie, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2015.06.005.

AUTHOR CONTRIBUTIONS

J.L. and Y.W. conceived of the projects. C.Z. derived the haploid cell lines and prepared cells for injection. Q.Y. and Z.X. performed the ICAHCI experiments. M.B. and Y.W. performed the sgRNA-library-related experiments. R.D. analyzed the high-throughput data. W.T., Y.W., and Y.-H.X. performed embryo transfer and the molecular biological experiments. J.L., Y.W., L.Y., D.L., A.F.-S., M.B., L.-L.C., and R.D. analyzed and interpreted data. J.L., Y.W., D.L., M.S.B., and L.Y. wrote the manuscript.

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