Genomic imprinting affects a subset of genes in mammals, such that they are expressed in a monoallelic, parent-of-origin–specific manner. These genes are regulated by imprinting control regions (ICRs), cis-regulatory elements that exhibit allele-specific differential DNA methylation. Although genomic imprinting is conserved in mammals, ICRs are genetically divergent across species. This raises the fundamental question of whether the ICR plays a species-specific role in regulating imprinting at a given locus. We addressed this question at the H19/Insulin-like growth factor 2 (Igf2) imprinted locus, the misregulation of which is associated with the human imprinting disorders Beckwith–Wiedemann syndrome (BWS) and Silver–Russell syndrome (SRS). We generated a knock-in mouse in which the endogenous H19/igf2 ICR (mIC1) is replaced by the orthologous human ICR (hIC1) sequence, designated H19/hIC1. We show that hIC1 can functionally replace mIC1 on the maternal allele. In contrast, paternally transmitted hIC1 leads to growth restriction, abnormal hIC1 methylation, and loss of H19 and Igf2 imprinted expression. Imprint establishment at hIC1 is impaired in the male germ line, which is associated with an abnormal composition of histone posttranslational modifications compared with mIC1. Overall, this study reveals evolutionarily divergent paternal imprinting at hIC1 between mice and humans. The conserved maternal imprinting mechanism and function at IC1 demonstrates the possibility of modeling maternal transmission of hIC1 mutations associated with BWS in mice. In addition, we propose that further analyses in the paternal knock-in H19/hIC1 mice will elucidate the molecular mechanisms that may underlie SRS.

Genomic imprinting is essential for mammalian development. Curiously, elements that regulate genomic imprinting, the imprinting control regions (ICRs), often diverge across species. To understand whether the diverged ICR sequence plays a species-specific role at the H19/Insulin-like growth factor 2 (Igf2) imprinted locus, we generated a mouse in which the human ICR (hIC1) sequence replaced the endogenous mouse ICR. We show that the imprinting mechanism has partially diverged between mouse and human, depending on the parental origin of the hIC1 in mouse. We also suggest that our mouse model is optimal for studying the imprinting disorders Beckwith–Wiedemann syndrome when hIC1 is maternally transmitted, and Silver–Russell syndrome when hIC1 is paternally transmitted.

**Significance**

Genomic imprinting is essential for mammalian development. Curiously, elements that regulate genomic imprinting, the imprinting control regions (ICRs), often diverge across species. To understand whether the diverged ICR sequence plays a species-specific role at the H19/Insulin-like growth factor 2 (Igf2) imprinted locus, we generated a mouse in which the human ICR (hIC1) sequence replaced the endogenous mouse ICR. We show that the imprinting mechanism has partially diverged between mouse and human, depending on the parental origin of the hIC1 in mouse. We also suggest that our mouse model is optimal for studying the imprinting disorders Beckwith–Wiedemann syndrome when hIC1 is maternally transmitted, and Silver–Russell syndrome when hIC1 is paternally transmitted.
Although interspecies compatibility of human IC1 was previously investigated in a transgenic mouse model (8), the human transgene failed to exhibit the expected imprinting pattern. The transgene acquired DNA methylation in male germ cells in a copy number-dependent manner, but the imprint was not stably maintained in somatic cells. In addition, the human H19 transgene was abnormally expressed on paternal transmission. Interpretation of these observations is complicated by transgene copy number variation, however. Furthermore, because long-range chromatin looping plays an essential role in H19/igf2 imprinting (10, 11), the transgene insertion site may influence the phenotype. Thus, it is imperative to test the functionality of the human IC1 element at the orthologous locus.

Here we generated a knock-in mouse model in which the endogenous mouse IC1 (mIC1) was replaced by human IC1 (hIC1). Our goal was to investigate the extent to which hIC1 can functionally replace mIC1. We found that hIC1 properly recapitulates mIC1 function on the maternal allele, whereas hIC1 fails to properly regulate the H19/igf2 locus on the paternal allele. hIC1 is incompletely methylated in the male germ cells of knock-in mice, which is associated with increased enrichment of dimethylation of histone H3 at lysine 4 (H3K4me2) on hIC1. Overall, this study reveals interspecies incompatibility of hIC1 in the mouse male germ line. Importantly, we show that aberrant histone modification composition at hIC1 may affect the proper establishment of DNA methylation at hIC1 during mouse germ cell development.

**Results**

**Generation of the H19<sup>hIC1</sup> Allele.** To determine whether hIC1 could functionally substitute for the orthologous mouse sequence, we replaced the endogenous mIC1 with hIC1 by gene targeting in embryonic stem (ES) cells (Fig. L4). Even though we obtained highly chimeric mice after blastocyst injection of the targeted ES cells, germ-line transmission of the targeted allele was inefficient. Only one female pup with the H19<sup>hIC1</sup> allele was live-born out of >250 agouti pups; all other agouti pups were wild-type, suggesting that the pups inheriting the H19<sup>hIC1</sup> allele might be dying prenatally. The single live-born female knock-in pup was of noticeably smaller size compared with its wild-type siblings and remained small.

The neonycin resistance cassette (NeoR) was excised by crossing the female to E1A-Cre male on a C57BL/6d (B6) background (Jackson Laboratories). Germ-line transmission of the targeted allele and excision of NeoR were confirmed by Southern blot analysis (Fig. 1B). When bred to a B6 male, the female knock-in mouse was fertile, and wild-type and knock-in progeny were born in the expected Mendelian ratios with no sex bias. Embryonic lethality on paternal transmission was again observed after NeoR excision. The use of knock-in males in a B6/CF1 mixed strain for paternal transmission did not resolve the embryonic lethality. These results rule out NeoR and pure B6 background as being solely responsible for the failure to obtain mutant pups. The H19<sup>hIC1</sup> allele was maintained through maternal transmission in a B6 background.

The Maternally Transmitted H19<sup>hIC1</sup> Allele Can Functionally Substitute for mIC1. To investigate H19 and igf2 imprinting when the targeted allele was maternally transmitted, we bred female H19<sup>hIC1/mIC1</sup> mice to B6 (CAST7) mice, which have a Mus musculus castaneus chromosome 7 on a B6 background (12). This cross allows the parental origin of H19 and igf2 expression to be distinguished in F1 progeny. Heterozygous H19<sup>hIC1/mIC1</sup> mice were compared with their wild-type littermates (H19<sup>+/+</sup>). The H19<sup>hIC1/mIC1</sup> and H19<sup>+/+</sup> mice were born in Mendelian ratios with no sex bias and no difference in neonatal weight (Fig. 2A). We assayed expression and IC1 methylation in neonatal livers, where H19 and igf2 are highly expressed, and detected monoallelic expression in all cases (Fig. 2B). Consistently, total expression levels of H19 and igf2 were statistically equivalent in H19<sup>hIC1/mIC1</sup> and H19<sup>+/+</sup> livers, as measured by quantitative real-time PCR (qRT-PCR) (Fig. 2C).

DNA methylation at hIC1 on the maternal allele and endogenous mIC1 on the paternal allele was measured by bisulfite mutagenesis of genomic DNA, followed by pyrosequencing. The maternal hIC1 was hypomethylated, as expected (Fig. 2D). Methylation at several other ICRs in H19<sup>hIC1+/+</sup> livers was normal, suggesting that the general imprinting machinery is functioning normally (Fig. S1A). Finally, hIC1 was properly hypomethylated in the oocytes of H19<sup>hIC1+/+</sup> females (Fig. 2E). We repeated these analyses in two sequential generations of the H19<sup>hIC1</sup> allele maternal transmission offspring and obtained the same results. Overall, these data illustrate that hIC1 can functionally replace mIC1 on the maternal allele.

The Paternally Transmitted H19<sup>hIC1</sup> Allele Leads to Abnormal Insulation at the H19/igf2 Locus. To investigate H19 and igf2 imprinting on the paternal allele, we bred male H19<sup>hIC1+/+</sup> mice to B6 (CAST7) mice.

**Fig. 1.** Targeting strategy to generate the H19<sup>hIC1</sup> allele. (A) Schematics of the endogenous locus, targeting vector (phIC1-neo), correctly targeted allele (H19<sup>hIC1+/+</sup>), and targeted allele after excision of the neo<sub>R</sub> cassette (H19<sup>hIC1−/−</sup>). Depicted are the IC1 (white rectangle) with CTCF-binding sites (black blocks) associated with methylation in male germ cells. The Maternally Transmitted H19<sup>hIC1</sup> allele can functionally replace mIC1. We found that hIC1 properly recapitulates mIC1 function on the maternal allele, whereas hIC1 fails to properly regulate the H19/igf2 locus on the paternal allele. hIC1 is incompletely methylated in the male germ cells of knock-in mice, which is associated with increased enrichment of dimethylation of histone H3 at lysine 4 (H3K4me2) on hIC1. Overall, this study reveals interspecies incompatibility of hIC1 in the mouse male germ line. Importantly, we show that aberrant histone modification composition at hIC1 may affect the proper establishment of DNA methylation at hIC1 during mouse germ cell development.
All live-born neonates were wild-type, similar to what was observed when breeding for germ-line transmission in chimeric mice, suggesting that paternal transmission of the H19\textsuperscript{mIC1} allele is embryonic lethal. To investigate this possibility, we isolated embryonic day (E)15.5 conceptuses. Although the H19\textsuperscript{mIC1} conceptuses were viable, the H19\textsuperscript{mIC1} embryos and placenta were smaller and weighed significantly less compared with those of H19\textsuperscript{+/+} (Fig. 3A).

H19\textsuperscript{mIC1} paternal transmission was barely detectable, indicating complete repression of H19\textsuperscript{+/+} in two-cell embryos compared with mature sperm (Fig. 4A). These results demonstrate that the DNA methylation is partially established at hIC1 during spermatogenesis, but is not maintained in preimplantation development.

**Increased Enrichment of H3K4me2 at hIC1 in Spermatogenic Cells.** To investigate factors that may inhibit complete establishment of DNA methylation at hIC1 during spermatogenesis, we examined histone posttranslational modifications at hIC1. Parental allele-specific histone modifications have been described at mIC1 in both somatic and germ cells (14–18). Several studies have suggested an antagonistic relationship between “activating marks,” such as dimethylation and trimethylation of histone H3 at lysine 4 (H3K4me2 and H3K4me3, respectively), and DNA methylation (17–20). Other studies have shown a strong relationship between “repressive marks,” such as trimethylation of histone H3 at lysine 9 (H3K9me3), and DNA methylation (21). In fact, H3K4 methylation is found preferentially on the hypomethylated maternal IC1, and H3K9me3 is found on the hypermethylated paternal IC1 in mouse and human somatic cells (11, 14). We hypothesized that depletion of H3K9me3, increased enrichment of H3K4me2, or both contribute to the inability to fully establish DNA methylation at hIC1.

Spermatogenic cells were fractionated by the STA-PUT method, and chromatin was isolated from a round spermatid-enriched fraction (22). ChIP–qRT-PCR analyses in round spermatids revealed that hIC1 had fivefold greater enrichment of H3K4me2 compared with H19\textsuperscript{+/+} embryos in both tissues (Fig. 3C and S2F). Similar results were observed in E9.5 whole embryos (Fig. S2B and C).

We next examined the extent to which methylation at hIC1 correlated with abnormal expression in heterozygous livers and placentas. hIC1 was completely unmethylated on the paternal allele, resembling the endogenous mIC1 on the maternal allele (Fig. 3D and S2F). This unusual methylation pattern was not due to a gross defect in the methylation machinery, because methylation at other ICRs was normal in H19\textsuperscript{+/+IC1} embryos (Fig. S1B).

Finally, to determine whether the hypomethylated state of hIC1 is associated with ectopic binding of CTCF on the paternal allele, we performed allele-specific chromatin immunoprecipitation (ChiP) followed by quantitative real-time PCR (ChIP–qRT-PCR) for CTCF in E12.5 mouse embryonic fibroblasts (MEFs). As expected, CTCF bound only to the unmethylated hIC1 on the maternal allele and did not bind to the methylated mIC1 on the paternal allele in H19\textsuperscript{IC1+/+} MEFs. In contrast, CTCF bound to both the unmethylated mIC1 on the maternal allele and the unmethylated hIC1 on the paternal allele in H19\textsuperscript{IC1+/+} MEFs (Fig. 3E). The results demonstrate that paternal hIC1 is unable to acquire or maintain the hypermethylated state of endogenous mIC1, fails to repress H19, and instead gains a CTCF-dependent insulator function.

**Incomplete Establishment of Genomic Imprinting at hIC1 During Spermatogenesis.** Because the paternal allele in E15.5 H19\textsuperscript{IC1+/+} embryos was hypomethylated, we assayed DNA methylation at earlier stages (Fig. S3). We did not detect any methylation at hIC1 as early as the blastocyst stage, suggesting that either methylation was not established during spermatogenesis or methylation was established but was lost during preimplantation development (Fig. S3). To distinguish between these possibilities, we examined DNA methylation at hIC1 in sperm of H19\textsuperscript{IC1+/+} males, and observed partial methylation (Fig. 4A and B). As positive controls, we analyzed methylation at endogenous hIC1 in human sperm samples from two fertile men as well as at endogenous mIC1 in H19\textsuperscript{IC1+/+} sperm and found that all were hypermethylated, as expected (Fig. 4A and B).

To explore whether the methylation at hIC1 in H19\textsuperscript{IC1+/+} sperm could be maintained after the first cleavage division, we assayed H19\textsuperscript{IC1+/+} two-cell embryos in which the zygote had undergone one round of mitosis. We found reduced methylation levels (close to wild-type levels) at hIC1 in two-cell embryos compared with mature sperm (Fig. 4A). These results demonstrate that the DNA methylation is partially established at hIC1 during spermatogenesis, but is not maintained in preimplantation development.
and placentas displayed an increased knockout models exhibited and is not due solely to the loss of displays biallelic and increased expression, 

Because null neonates are born smaller but viable (29, 30), suggesting Igf2 expression. Notably, these IC1 mutations (i.e., placentas and Igf2 play essential expression and growth defects (23, 24), we hypothesized Igf2 (31, 32).

Because null neonates are born smaller but viable (29, 30), suggesting Igf2 expression. Notably, these IC1 mutations (i.e., placentas and Igf2 play essential expression and growth defects (23, 24), we hypothesized Igf2 (31, 32).

Determined the extent to which these mutations contribute to the molecular and clinical phenotypes of BWS is challenging, because IC1 hypermethylation is mosaic in the patients, suggesting that not all cells are aberrantly DNA-methylated. Moreover, clinical phenotypes of the patients are highly variable, possibly as a consequence of either the mosaicism or the genetic background of the individuals (27). In this study, we have shown that maternal transmission of hIC1 can functionally replace mIC1 by properly regulating imprinted expression and hIC1 methylation. Thus, our findings raise the exciting possibility of modeling IC1 mutations associated with BWS in mice via maternal transmission.

In contrast, paternal transmission of hIC1 leads to loss of H19 and Igf2 imprinting; H19 displays biallelic and increased expression, and Igf2 is silenced. Offspring inheriting hIC1 paternaly also exhibit severe growth restriction. Of note, previous studies have shown that Igf2 null neonates are born smaller but viable (29, 30), suggesting that prenatal lethality of $H19^{+/hIC1}$ is not due solely to the loss of Igf2. Mice from two independent H19 knockout models exhibited overgrowth, suggesting a growth-suppressing role of H19 (31, 32).

In addition, ectopic expression of H19 caused late-gestation lethality (33). Thus, changes in both H19 and Igf2 may synergistically contribute to the severe growth restriction and prenatal lethality of $H19^{+/hIC1}$.

Discussion

Using a mouse model replacing endogenous mIC1 with hIC1, we have shown that the ability of hIC1 to functionally replace mIC1 depends on the parental origin of the hIC1 allele. Although the main aim of this study was to investigate interspecies compatibility of hIC1 in the mouse system, we anticipate that findings from this study also will provide insight into modeling and further examining imprinting disorders such as BWS and SRS.

Several groups have reported that a subset of patients with BWS carry mutations at IC1, and that these mutations are largely associated with IC1 hypermethylation, reduced H19 expression, and biallelic Igf2 expression. Notably, these IC1 mutations (i.e., microdeletions and point mutations) manifest BWS clinical phenotypes when the mutant allele is maternal in origin (9, 27, 28).

Abnormal Placental Morphology in $H19^{+/hIC1}$. Because $H19^{+/hIC1}$ embryos display similar phenotypes to those of many patients with SRS who present with IC1 hypomethylation, including altered H19 and Igf2 expression and growth defects (23, 24), we hypothesized that these embryos can serve as a model for SRS. Placental growth defects are prevalent among individuals with SRS (24); thus, we further characterized $H19^{+/hIC1}$ placentas to study potential mechanisms underlying SRS associated with IC1 hypomethylation. We performed histological analyses on E15.5 $H19^{+/hIC1}$ placentas to explore whether abnormal placentaion could contribute to embryonic growth restriction, given that H19 and Igf2 play essential roles in placental development (25, 26).

In addition to being smaller (∼74% of $H19^{+/+}$), $H19^{+/hIC1}$ placentas displayed an increased junctional/labyrinthine zone ratio, indicative of abnormal placenta morphology (Fig. S2 G and H).

Using a mouse model replacing endogenous mIC1 with hIC1, we have shown that the ability of hIC1 to functionally replace mIC1 depends on the parental origin of the hIC1 allele. Although the main aim of this study was to investigate interspecies compatibility of hIC1 in the mouse system, we anticipate that findings from this study also will provide insight into modeling and further examining imprinting disorders such as BWS and SRS.

Several groups have reported that a subset of patients with BWS carry mutations at IC1, and that these mutations are largely associated with IC1 hypermethylation, reduced H19 expression, and biallelic Igf2 expression. Notably, these IC1 mutations (i.e., microdeletions and point mutations) manifest BWS clinical phenotypes when the mutant allele is maternal in origin (9, 27, 28).

Determining the extent to which these mutations contribute to the molecular and clinical phenotypes of BWS is challenging, because IC1 hypermethylation is mosaic in the patients, suggesting that not all cells are aberrantly DNA-methylated. Moreover, clinical phenotypes of the patients are highly variable, possibly as a consequence of either the mosaicism or the genetic background of the individuals (27). In this study, we have shown that maternal transmission of hIC1 can functionally replace mIC1 by properly regulating imprinted expression and hIC1 methylation. Thus, our findings raise the exciting possibility of modeling IC1 mutations associated with BWS in mice via maternal transmission.

In contrast, paternal transmission of hIC1 leads to loss of H19 and Igf2 imprinting; H19 displays biallelic and increased expression, and Igf2 is silenced. Offspring inheriting hIC1 paternaly also exhibit severe growth restriction. Of note, previous studies have shown that Igf2 null neonates are born smaller but viable (29, 30), suggesting that prenatal lethality of $H19^{+/hIC1}$ is not due solely to the loss of Igf2. Mice from two independent H19 knockout models exhibited overgrowth, suggesting a growth-suppressing role of H19 (31, 32).

In addition, ectopic expression of H19 caused late-gestation lethality (33). Thus, changes in both H19 and Igf2 may synergistically contribute to the severe growth restriction and prenatal lethality of $H19^{+/hIC1}$.
expression was in-...expressed in the labyrinthine zone.

Igf2 was deleted and silenced in males with null females, which will help elucidate the transcript was found to result in growth restriction of embryos in late gestation. In a mouse model in which H19 was deleted and Igf2 expression was increased, both the placenta and the fetus were overgrown at E19 (35, 36). In humans, placental growth defects are common in individuals with BWS and SRS (24, 37). It was observed that H19+/+ males with IC1 hypomethylation are not completely erased in the male germ cells. (Note that H19 was necessarily transmitted maternally to generate offspring.) Consequently, the establishment of DNA methylation at hIC1 is inhibited. This finding adds to the growing consensus that H3K4 methylation marks are inhibitory to de novo DNA methylation in the germ line, whereas repressive histone marks do not play major role in the establishment of methylation at ICRs (14, 16–18, 20). However, it is equally possible that the hypomethylated state of DNA attracts H3K4me2 to hIC1 by an unknown mechanism. More detailed time course analyses of DNA methylation and H3K4me2 enrichment in the primordial germ cells and early-stage male germ cells will provide insight into this hypothesis. Alternatively, there might be an inherent difference between mIC1 and hIC1 in terms of acquisition of methylation. A noncoding transcript has been detected at mIC1 during methylation acquisition in male germ cells (41), suggesting a potential role of transcription in the establishment of methylation at mIC1. Whether the same holds true at hIC1 remains to be determined, however.

Finally, we have shown that the partially established methylation at hIC1 in the male germ line is not properly maintained during preimplantation development. We also have shown that CTCF ectopically binds to hIC1 on the paternal allele in somatic cells. Similar results have been reported for an earlier mouse model in which CpGs within the CTCF-binding sites at the mIC1 were mutated to abrogate methylation, while keeping the CTCF-binding motif intact (5). There, although methylation was properly established in the male germ cells at the mIC1, it was not maintained during pre-implantation development (5). These data suggest that CTCF binding inhibits the maintenance of DNA methylation in somatic cells, although the mechanism remains unknown. Alternatively, hIC1 might lack properties that allow the mouse imprint maintenance machinery to properly recognize the sequence. It is also possible that hIC1 contains inhibitory signals that block accessibility and/or activity of the mouse imprint maintenance machinery.

In conclusion, we have elucidated hitherto unreported principles regarding the conservation of ICR function at the H19/Igf2 locus and molecular mechanisms associated with SRS. First, evidence of incomplete histone reprogramming at hIC1 suggests that the mechanism regulating histone reprogramming at ICR is different between mouse and human. In this regard, it would be interesting to explore whether an IC1 ortholog of a species more closely related to mouse could recapitulate the wild-type epigenetic pattern on paternal transmission. Second, despite the fact that IC1 hypomethylation is the most common epimutation found in individuals with SRS (24), the molecular mechanism underlying the phenotype remains elusive. Obstacles to addressing this question include mosaicism of the epimutation in patients and the lack of a suitable genetic model system. We suggest that the paternal transmission of hIC1 in mice can be used to study the molecular mechanisms underlying SRS associated with IC1 hypomethylation. Future experiments, such as breeding H19IC1 mice with IC1 null females, will help elucidate the extent to which H19 contributes to the SRS-like phenotype. In addition, identifying pathways altered by IC1 hypomethylation may shed light on the physiology of SRS.

Materials and Methods

Targeting Vector. Detailed information on the hIC1 target vector is provided in SI Materials and Methods.
ES Cells and Mouse Generation, Breeding, and Genotyping. Details regarding ES cell targeting, Southern blot analyses, and mouse generation, breeding, and genotyping are provided in SI Materials and Methods.

Gene Expression Analysis. RNA isolation and cDNA synthesis was performed as described previously (42). For qRT-PCR, total expression levels of H19 and Igf2 were measured relative to the geometric mean of expression levels of Arbp (ribosomal phosphoprotein P0), Nano (non-POU domain-containing, octamer-binding protein), and Rpl13a (ribosomal protein L13A). For the y-axis on the graph, the mean value of wild-type is set arbitrarily as 1. Details are provided in SI Materials and Methods.

DNA Methylation Analysis. gDNA isolation from neonatal, embryonic, and germ cell samples; bisulfite treatment; and methylation analyses are described in detail in SI Materials and Methods.

Histology. Histological analysis was performed as described previously (43). ChIP

Mouse Spermatogenic Cell Fractionation. Round spermatid fractions of mouse spermatogenic cells were collected using STA-PUT in two independent replicates, and the purity of each fraction was verified as described previously (22, 44). Each collection used both testes of 12 heterozygous (H19<sup>wt</sup>/<sup>mut</sup>) male mice. The purity was measured as 87% for pool 1 and 86% for pool 2.

Isolation of MEFs. MEFs were isolated from individual day 12.5 embryos in a B6 background as described previously (15).

ChIP–qRT-PCR Analysis. ChIP–qRT-PCR was carried out as described previously (44); details are provided in SI Materials and Methods. Each ChIP signal was calculated as the percent input of each immunoprecipitated (IP) normalized to nonspecific IgG (percent input of IP – percent input of IgG). In Fig. 4C, the y-axis denotes the ChIP signal of each histone mark normalized to that of total H3 (e.g., percent input of H3K4me2/percent input of total H3).

ACKNOWLEDGMENTS. This work was supported by US Public Health Service Grants GM51279 to (M.S.B.) and HD068157 to (M.S.B. and S.L.B.); Advanced Imaging Research Center Grant 8700 (to F.C.); Telethon-Italy Grant GGP15131 (to A.R.); European Union Seventh Framework Programme, INGENUM 290123 (to F.C.); and National Institutes of Health Training Grant T32 GM07229 (to S.K.H.).