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Male CD81 Knockout Genotype Disrupts Mendelian Distribution of Offspring

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CD81 is an integral membrane protein in the tetraspanin superfamily that serves as an adaptor protein. CD81 is also a maternally imprinted gene that is found in a regulated cluster of genes on mouse chromosome 7. Among offspring produced from heterozygous breeding pairs, CD81$^{null/null}$ mice grew at the same rate as CD81$^{+/+}$ and CD81$^{+/null}$ mice. Because of an inhibition in sperm–egg fusion, CD81$^{null/null}$ female mice are much less fertile than CD81$^{+/+}$ and CD81$^{+/null}$ mice. However, no published study has detailed the effect of the male CD81 genotype on the genotype and sex distribution of offspring. We set up breeding pairs of heterozygotic (C.129-Cd81^{tm1} N7) female mice and male mice with CD81$^{+/null}$, CD81$^{+/+}$, or CD81$^{null/null}$ genotypes. The survival and development of CD81$^{+/null}$, CD81$^{+/+}$, and CD81$^{null/null}$ offspring were monitored and compared. Compared with those of heterozygous male breeders, CD81$^{null/null}$ pups were born at a less-than-expected ratio from CD81$^{null/null}$ males. Sex distribution did not differ among pups sired by CD81$^{null/null}$ compared with CD81$^{+/null}$ mice. The data suggest that the effect of the CD81$^{null/null}$ paternal genotype on offspring is manifested early in development or in utero.

CD81 is found on mouse chromosome 7 among a cluster of maternally imprinted genes.[24] Imprinting of this gene occurs in the placenta, not in the embryo.[15,28] The protein is an integral membrane protein in the tetraspanin superfamily that serves as an adaptor protein.[14] The extracellular domain of CD81 interacts with cell-surface proteins, and the 3 intracellular domains interact with other molecules within the cell.[4] CD81 is a required receptor for hepatitis C virus infection[34] and originally was identified because of its association with CD19 and CD21 molecules in B cells.[22] CD81 also interacts with the T cell receptor and its coreceptors' components, CD8, CD4, and CD2.[17,27] Although much is known about how CD81 affects B cell activation and its role as an HCV receptor, other specific functions and interactions are not well understood.

CD81 has previously been shown to play a role in development. Homozygous null female mice develop fertilized embryos 40% less frequently than do CD81-expressing female mice.[26] CD81 apparently participates in oocyte–sperm fusion, but it has a different role than the related tetraspanin molecule, CD9.[13,18] CD9 and CD81 appear to have complementary roles.[26]

We established a breeder colony of CD81 knockout mice at our institution in 2004 and have maintained records of births and development of CD81 offspring over the last 5 y. As expected, female CD81$^{null/null}$ mice were infertile. However, examination of data for litters sired by CD81$^{null/null}$ male mice revealed unexpected skewing of genotypes. We report these data here.

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Materials and Methods

All mice were maintained ad libitum on a commercial diet (LabDiet 5015, Purina, St Louis, MO) and water. Breeder pairs and pups were monitored daily, and survival to weaning was greater than 90%. All animal experiments were approved and monitored by the Institutional Animal Care and Use Committee of Kansas State University.

C.129-Cd81^{tm1} N7 heterozygotic mice (CD81$^{+/null}$) were obtained from Shoshana Levy (Stanford University, Palo Alto, CA). These mice have a targeted disruption in the CD81 gene that was created by homologous recombination of a neomycin-resistance gene into a segment beginning in the intron between exons 1 and 2 and ending in the intron between exons 7 and 8 in strain 129 mice.[16] These mice were backcrossed to BALB/c mice by the Levy group, and this line has been maintained by our group since 2004. After the initial heterozygote crosses, we bred CD81$^{+/null}$ and CD81$^{null/null}$ male mice with their CD81$^{+/null}$ female siblings.

C.C3-Tlr4^{Lpsd}/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). These mice have a point mutation located within the BB loop of Tlr4 which inhibits the cellular response to LPS.[23,30] C.C3-Tlr4^{Lpsd}/J mice had been backcrossed more than 20 generations to the BALB/cJ background.

Mice were genotyped by using tail tissue. For genotyping, 200 µL lysis reagent (DirectPCR Lysis Reagent, Viagen Biotech, Los Angeles, CA) and 0.2 mg/mL proteinase K (Sigma, St Louis, MO) were added to each tube containing sample. The tubes were rotated in a hybridization oven (Hybrid National Labnet, Woodbridge, NJ) at 55 °C for 6 h (2×10^6 cells) and 18 h (1 mm tail tissue). Lysates then were incubated in an 85 °C water bath for 45 min and stored at 4 °C until DNA was analyzed by PCR. DNA sample concentrations were determined spectrophotometrically (ND1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE). All samples were diluted to an optimal concentration

Primer	Amplicon (bp)	Accession no.	Sense (5′→ 3′)	Antisense (5′→ 3′)
S14	402	M11241	CAA TCC GCC CAA TCT TCA TCC C	GAC GAC GTT CAG AAA TGG CAC C
Neo	254	U02434	CCA TGA TAT TCG GCA AGC AGG CAT	ATC CAT CAT GGC TGA TGC AAT GCG
CD81	542	NC000073	CTC AAC TGT TGT GGC TCC AAC	CCA ATG AGG TAC AGC TTC CC
HeJ	476	AF177767	GTA TAT GTG AAA CAT CAG AAA TTC CT	CAT GTT TGA GCA ATC TCA TAT TCA A
β-actin	600	00NM-007393.1	ATG GAT GAC GAT ATC GCT	ATG AGG TAG TCT GTC AGG T
SV40 T	209	NC001669	GGA AAG TCC TTG GGG TCT TC	CTG ACT TTG GAG GCT TCT GG

Figure 1. Primers used to identify gene expression by PCR or RT-PCR.

of 500 to 1000 ng/μL, and a total of 2.5 to 5.0 μg DNA was used per reaction. Mice were genotyped by using primers specific for sequences in exons 6 and 7 of CD81 (Figure 1). Primers specific for the *neo* gene (Figure 1) were used to confirm targeted disruption of the CD81 gene. Primers specific for the 14S ribosomal protein gene[25] (Figure 1) were used to assess the quality of the DNA sample when *neo*- or CD81-specific PCR reactions were negative. Because of their differences in length, amplicons were run in tandem on ethidium bromide-(Ethidium Bromide Tablets, 10 mg, MidSci, St Louis, MO) stained 1.5% agarose (Agarose, Low EEL 500 GM, Fisher Scientific, Pittsburgh, PA) gels in tandem. cDNA was amplified by using Go*Taq* Flexi DNA Polymerase (Promega, Madison, WI) and specific primers (Integrated DNA Technologies, Coralville, IA; Figure 1).

C.C3-Tlr4^{Lpsd}/J and C.129-Cd81^{tm1} mice were crossed to generate mice deficient in both CD81 and Tlr4. F_1 mice were brother–sister-mated to create mice potentially homozygous at both loci. Male and female mice for breeding were selected for homozygosity at the Tlr4 gene. Breeding males were either CD81$^{+/null}$ or CD81$^{null/null}$, and breeding females were heterozygous for CD81 for reasons described above. Tlr4 genotyping was done by using methodology that allowed us to distinguish all 3 Tlr4 alleles.[32] cDNA was amplified by using GoTaq Flexi DNA Polymerase (Promega) and specific primers (Integrated DNA Technologies; Figure 1). Briefly, cDNA underwent restriction enzyme digest to reveal restriction fragment length polymorphisms created by the C-to-A conversion at nucleotide 39,609 (accession no., AF177767) by using *Hsp*92II (Promega). Digested wild-type Tlr4 cDNA produced products of 422 and 54 bp. Digested heterozygous Tlr4 cDNA produced fragments of 422, 320, 102, and 54 base pairs. Digested point-mutated cDNA produced fragments of 320, 102, and 54 bp. The digested cDNA was resolved on ethidium bromide-stained, 1.5% agarose gels.

Weight differences were analyzed by paired *t* test and linear regression; genetic data were evaluated by χ^2 test (Statmost, Detaxiom Software, Los Angeles, CA).

Results

We initially used C.129-Cd81^{tm1} N7 heterozygotic mice to establish a CD81-knockout mouse colony and assessed the birth and development of CD81$^{+/+}$,CD81$^{+/null}$ and CD81$^{null/null}$ mice that were born to heterozygotic breeding pairs. Nonbreeding mice in our early cohorts were weighed approximately every 2 wk over the course of 1 y. For each mouse, weight was recorded and plotted against the number of weeks since birth. The data from the colony were compiled according to genotype (Figure 2). CD81$^{null/null}$ pups grew at the same rate as did CD81$^{+/+}$ and CD81$^{+/null}$ mice, although some female mice appeared to lag behind their CD81$^{+/null}$ and CD81$^{+/+}$ litter mates in weight (Figure 2).

In our efforts to increase the percentage and number of CD81$^{null/null}$ mice born, male CD81$^{null/null}$ mice were used as breeders. When CD81$^{+/null}$ female mice crossed with CD81$^{null/null}$ male mice, the genotypes of the offspring were not present at the expected 1:1 ratio of CD81$^{+/null}$:CD81$^{null/null}$ ($P < 0.05$; Table 1). Of the 160 mice examined, 102 were CD81$^{+/null}$ compared with 58 CD81$^{null/null}$ mice (that is, approximately 2:1). Although the number of female mice born exceeded the number of male mice (93 versus 67), the male:female distribution was not significantly ($P > 0.10$) different from the expected 1:1 ratio. In comparison, when CD81$^{+/null}$ mice were used as sires, the distribution of homozygotes and heterozygotes did not deviate from expected ratios (Table 1).

Our group is interested in innate immunity, and we have developed mice with multiple defects in genes thought to contribute to innate and acquired immunity.[3,11,32] Therefore, we began an effort to cross C.C3-Tlr4^{Lpsd}/J and C.129-Cd81^{tm1} mice. C.C3-Tlr4^{Lpsd}/J mice carry a mutation resulting in a proline-to-histidine substitution in the BB region of toll-like receptor 4 (Tlr4). This mutation occurred naturally at the Jackson Laboratories in the 1960s in C3H/HeJ mice.[29] Another group then backcrossed the defect into the BALB/c background.[31] Therefore, we had access to a second group of breeders that had a breeding history independent of those that had been backcrossed to the BALB/c background for

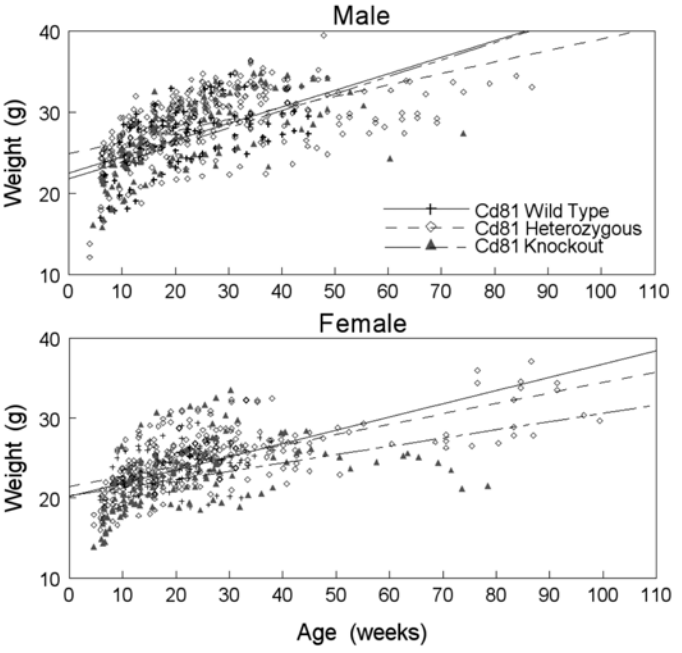

Figure 2. Growth of CD81$^{+/+}$, CD81$^{null/null}$, and CD81$^{+/null}$ female (top) and male (bottom) mice born to heterozygotic breeding pairs.

Table 1. Effects of parental expression of CD81 on observed offspring genotypes.

Parental cross	Offspring			
	Genotype	No. Expected	No. Observed	*P*
$CD81^{null/null}$:$Tlr4^{Lpsd/Lpsd}$ × $CD81^{+/null}$:$Tlr4^{Lpsd/Lpsd}$	$CD81^{+/null}$:$Tlr4^{Lpsd/Lpsd}$	30.5	42	
	$CD81^{null/null}$:$Tlr4^{Lpsd/Lpsd}$	30.5	19	<0.05
$CD81^{+/null}$:$Tlr4^{Lpsn/Lpsn}$ × $CD81^{+/null}$:$Tlr4^{Lpsn/Lpsn}$	$CD81^{+/+}$: $Tlr4^{Lpsn/Lpsn}$	23.5	26	
	$CD81^{+/null}$:$Tlr4^{Lpsd/Lpsd}$	47	50	
	$CD81^{null/null}$:$Tlr4^{Lpsd/Lpsd}$	23.5	18	not significant
$CD81^{null/null}$:$Tlr4^{Lpsn/Lpsn}$ × $CD81^{+/null}$:$Tlr4^{Lpsn/Lpsn}$	$CD81^{+/null}$:$Tlr4^{Lpsd/Lpsd}$	80	102	
	$CD81^{null/null}$:$Tlr4^{Lpsd/Lpsd}$	80	58	<0.05

only 7 generations (C.129-$CD81^{tm1}$ N7); these mice were selected for the *Tlr4*^{Lpsd} allele. When using C.C3-$Tlr4^{Lpsd}$/J sires (which had been backcrossed for more than 7 generations) that were homozygous $CD81^{null/null}$, 42 of the 61 offspring were heterozygous at the CD81 gene. This proportion represents, a significant ($P < 0.05$) skewing from the expected Mendelian distribution (Table 1). Together, our data strongly suggest that male expression of CD81 may affect genotype distribution among offspring.

Discussion

We discovered that the frequency of successful birth of $CD81^{null/null}$ mice was about 50% that expected when offspring were sired by $CD81^{null/null}$ fathers. This skewed birth distribution has been followed by our group for more than 5 y in 36 breeding pairs and by using $CD81^{null/null}$ sires bred onto 2 semiindependent BALB/c stocks. Although CD81 expression is important for fecundity in female mice, male CD81 knockout mice reportedly are fertile,[26] but it was unclear whether the investigators examined the genotype distribution from the crosses of those male mice. In addition, at the time of development of the CD81 knockout mouse, CD81-null mice reportedly were born in the expected Mendelian ratios and developed without problems.[16] However, the parental genotypes used in those crosses are unclear.

We found that $CD81^{null/null}$ mice develop similarly to their CD81-expressing littermates, regardless of the sire's genotype. Some investigators have suggested that $CD81^{null/null}$ mice with a C57BL background do not survive well,[12] and we see few neonatal fatalities of $CD81^{null/null}$ pups on the BALB/c background. Compared with other strains, BALB/c mice are less aggressive during pregnancy[21] and have lower aggressive responses during stress than do C57BL/6 mice.[20] These data suggest that providing that satisfactory husbandry including mouse background are present, once mice are born, the CD81 deficiency does not affect vital functions or can be complemented by other tetraspanins or members of other molecular families.[2,5,33] Because the present study is semiretrospective, we have yet to determine whether $CD81^{null/null}$ embryos are fertilized less successfully than are controls or whether embryos are spontaneously aborted during the 21-d gestation period of mice.

Because the mice used to establish our breeding colony (C.129-CD81tm1 N7) had been backcrossed to the BALB/c background for only 7 generations, genetic interactions from the mixed BALB/c and 129 backgrounds of the knockout[16] may have played some role in this process. Although we cannot rule out this possibility, it is unlikely because the genotypes of offspring from our C.C3- $Tlr4^{Lps\text{-}d}$/J × C.129-cd81 ^{tm1} crosses exhibited a similar 2:1 ratio of heterozygous:homozygous-null births in mice fathered by $CD81^{null/null}$ fathers. The C.C3-$Tlr4^{Lpsd}$/J mice were backcrossed into the BALB/c background for 20 generations. Therefore, once we made the $CD81^{null/null}$ mice in that cross, those offspring were the equivalent of an N8 generation with respect to BALB/c background.

CD81 is one of several genes in the IC2 region of chromosome 7 that are maternally imprinted in the placenta but not the embryo.[15,28] CD81 appears to act as a growth regulator in brain,[6,8,12] macrophages,[19] and other cells.[9] Therefore, epigenetic mechanisms may contribute to the skewed genotype distribution. One hypothesis to explain the recent evolutionary development of imprinting in mammals is that it is a mechanism to control embryonic growth by altering the placenta.[1] *Ipl* is another linked gene in the IC2 region that is maternally imprinted.[7] Disruption of the *Ipl* gene affects placental size but not the distribution of the genotypes born.[7] Disruption of the paternal regulatory region of IC2 with the KvDMR1 deletion led to the restoration of paternal CD81 allelelic expression in the placenta.[15] Therefore, the paternal allele plays some as-yet-unknown role in the regulatory process. Regardless, how the CD81 genotype of the sire affects the genotypic distribution of the progeny mice remains unknown. One hypothesis is that because CD81 affects cellular growth,[6,8,9,12,19] the absence of CD81 in half of the embryos would alter the fetal–maternal balance,[10] causing spontaneous abortion of the embryos demanding the most resources. This hypothesis is consistent with the idea that fetal–placental interactions control fetal growth.[1] Additional experiments are needed to confirm this hypothesis.

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