SUPPLEMENTARY INFORMATION

Allogeneic Bone Marrow Transplantation in the Absence of Cytoreductive Conditioning Rescues Mice with Beta Thalassemia Major

Running title: Noncytoreductive BMT rescues mouse model of CA

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SUPPLEMENTARY RESULTS

Establishment of the humanized CA mouse model

Mice do not produce a distinct fetal hemoglobin, rather mice initiate high-level expression of their adult β-globin genes early in fetal development. Therefore, homozygous deletion of the murine adult β-globin genes results in *in utero* death at approximately embryonic day 14.5.[1, 2] In an attempt to mimic the postnatal onset of anemia that is observed in Cooley's anemia (CA) patients, the mouse adult β-globin genes were replaced by a human $\gamma^{HPFH}\beta^0$ globin gene cassette in mouse embryonic stem (ES) cells (Figure S1). The human γ-globin gene promoter contained the Greek-type hereditary persistence of fetal hemoglobin (HPFH) mutation (guanine to adenine transition at promoter position -117bp) to postpone the fetal-to-adult hemoglobin (Hb) switch and the human β-globin gene contained a splice donor site mutation (guanine to adenine transition in the first base of intron 1) to generate a nonfunctional β^0 thalassemic allele.[3-5] Heterozygous human $\gamma^{HPFH}\beta^0$ knock-in (KI) mice were generated from the targeted ES cells and bred to CMV-*Cre* transgenic mice[6] to delete the hygromycin marker gene (Figure S1A). DNA from both heterozygous and homozygous $\gamma^{HPFH}\beta^0$ KI mice was analyzed by Southern blot to confirm the modification of the mouse β-globin locus (Figure S1B). These $\gamma^{HPFH}\beta^0$ KI mice were further humanized by breeding to human α2α1-globin gene KI mice[3-5] to produce animals that survive solely upon human Hb.

Transgenic mice that contain the entire human β-globin locus complete the human fetal-to-adult Hb switch early in fetal development.[7, 8] To investigate if the temporal control of human γ-to-β globin gene switching in the $\gamma^{HPFH}\beta^0$ KI mice more closely mimics that seen in humans, the Hb switching pattern of humanized compound heterozygous β-thalassemia trait mice ($\alpha_2\alpha_1/\alpha_2\alpha_1$, $\gamma^{HPFH}\beta^0/\gamma\beta^A$) was analyzed over time (Figure 1A). Human γ-globin levels were 64.1 ± 1.3% (Mean ± SEM, n = 13) of total β-like globin chains in the newborn β-thalassemia trait mice.

These high levels gradually decline to 10.5 ± 0.6% (Mean ± SEM, n = 16) in five-week-old mice and are expressed stably thereafter (Figure 1A). These data demonstrate that the human $\gamma^{HPFH}\beta^0$ globin gene cassette delays the human fetal-to-adult globin gene switch in KI mice and mimics the postnatal Hb switching pattern observed in humans.

Fully humanized homozygous CA mice ($\alpha_2\alpha_1/\alpha_2\alpha_1$, $\gamma^{HPFH}\beta^0/\gamma^{HPFH}\beta^0$) were generated by brother-to-sister breeding of humanized β-thalassemia trait mice. Homozygous CA mice survive solely upon human fetal Hb at birth and synthesize no β-globin chains or adult Hb upon switching to their nonfunctional human β^0 globin allele. The postnatal survival of homozygous CA mice was analyzed over time (Figure 1B). The majority of these mice (80%) die from 10 to 20 days after birth due to the severe anemia (Figure 1B and Table 2). The median life span of 17 days is comparable to a 1½ to 2 year life span for an untreated CA patient.

Growth velocity and fertility of humanized CA mice

Humanized CA mice have variable levels of stunted growth depending on the severity of the anemia during their limited life span. The body weight of CA mice is significantly lower than their littermates ($p<0.01$). The majority of CA mice (80%) are the smallest pups in their litter (Figure S2). We measured the growth velocity of transplanted CA mice and ten non-anemic control littermates that were fostered to three dams in equal numbers (Figure S3). They were weighed 1, 2, 4, and 8 weeks post-transplantation. In the first week the body weights of the transplanted CA mice were significantly lower than the littermates ($p<0.01$). From the second week post-transplantation through adulthood, there was no significant difference between the transplanted CA mice and the littermate control mice (Figure S3).

As survival of untreated CA mice is significantly shorter than the time to sexual maturity, reproduction in the absence of intervention is not possible. Furthermore, the toxic myeloablative conditioning used in allogeneic transplantation leaves recipients sterile (data not shown). To

determine whether transplanted humanized CA mice could sire offspring, a homozygous CA males were bred to heterozygous trait females. Females gave birth to litters containing both CA and heterozygous mice. The sizes and variability of these litters are consistent with those of heterozygous crosses (Table S3)

Summarized humanized CA mouse and control transplantations

Table 1 and Table S1 contain a summary of hematopoietic chimerism and survival information for various transplant experiments. Animals were analyzed for donor chimerism by assessing the levels of GFP+ hematopoietic cells by flow cytometry. Periodic tail tip bleeds were used to evaluate RBC chimerism. At sacrifice, whole blood and bone marrow were stained after lysis of RBCs. In order to determine if HSPC chimerism could be monitored without sacrifice of the recipient, we compared donor granulocyte chimerism in the peripheral blood to the donor HSPC chimerism in the bone marrow. Peripheral blood Gr-1 positive granulocytes and bone marrow LSK cells showed similar percentages of GFP fluorescence (Table 1 and Table S1). Noncytoreductive transplants were performed without irradiation of newborn CA recipient mice. Transplants performed without prior injection of recipients with anti-CD122 and/or Diprotin A treatment of donor BMCs yielded no long-term survival. Nonmyeloablative transplants were performed after irradiation of newborn recipient CA mice. Due to increased radiation sensitivity in newborn CA mice, supportive RBC transfusions were given over three weeks following the nonmyeloablative transplants.

Nonmyeloablative Bone Marrow Transplantation

Control nonmyeloablative transplants were performed by exposing recipient mice to 400 rads of X-ray irradiation with a XRAD 320 (Precision X-Ray Inc., N. Branford, CT) on the second day of life. Radiation was administered at a dose rate of 100 rad/min over 4 consecutive minutes. A single dose of 1.5×10^7 BMCs from a GFP transgenic mouse[9] was delivered via the facial vein

following irradiation. After irradiation newborn CA mice required supportive RBC transfusions during donor cell engraftment. Supportive RBCs were obtained from C57BL/6 mice, The Jackson Laboratory, Bar Harbor, ME). Donor RBCs for transfusions were obtained from 2,2,2-tribromoethanol (Sigma-Aldrich) anesthetized mice via cardiac puncture prior to euthanasia. Starting the day after transplantation, three supportive RBC transfusions (75% hematocrit) were given to sustain animals during engraftment of donor marrow. The three weekly transfusions were administered via intraperitoneal injection with increasing volume each week (50 μL, 200 μL, and 300 μL). In order to distinguish transfused RBCs from GFP positive transplant-derived RBCs, nonfluorescent C57Bl/6 RBCs were used for transfusions. While donor HSPC chimerism was quite variable (range of 15.1-97.1% GFP+ $Lin^{-}Sca1^{+}ckit^{+}$ cells), transplant-derived RBC chimerism rose to 99% by nine weeks post-transplant. (Table 1 and Table S1).

SUPPLEMENTARY DISCUSSION

In this report we demonstrate that a single postnatal injection of allogeneic bone marrow in the absence of cytoreductive conditioning can rescue from death a novel preclinical animal model of CA. Stable low-level donor hematopoietic chimerism is achieved that is sufficient to populate over 90% of the peripheral RBCs. Transplanted CA recipients have a marked improvement of their anemia and anemia-induced pathological changes in the spleen and liver, exhibit no growth retardation or graft versus host disease, and are fertile. This study describes a method in mice for the consistent engraftment of donor hematopoietic cells and amelioration of anemia for β-thalassemia major in the absence of toxic conditioning regimens.

The humanized $\gamma^{HPFH}\beta^{0}$ KI CA mouse model produced for these studies is similar to our earlier reported $\gamma\beta^{0}$ and $\gamma^{HPFH}\delta\beta^{0}$ KI CA models.[3, 4] All of these models survive solely on human Hb at birth and succumb to lethal anemia upon completion of their fetal-to-adult Hb switch. The

presence of the HPFH mutation in the γ-globin gene promoter extends the short perinatal lifespan of the $\gamma\beta^0$ CA mice for a couple weeks making the $\gamma^{HPFH}\delta\beta^0$ and $\gamma^{HPFH}\beta^0$ CA models more amenable for postnatal studies. Genotypically, the $\gamma^{HPFH}\delta\beta^0$ and $\gamma^{HPFH}\beta^0$ CA models differ by the presence or absence of the human minor adult δ-globin gene, respectively. Phenotypically these models are very similar, except that the $\gamma^{HPFH}\beta^0$ CA mice maintain higher fetal and no minor adult Hb A2 in their adult RBCs. While it has not been tested we expect the $\gamma^{HPFH}\delta\beta^0$ CA model could also be rescued by BMT in the absence of cytoreductive conditioning.

Supplementary References

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1

Knock-in of a delayed human fetal-to-adult globin gene switching cassette into the mouse β-globin locus. A. Gene targeting strategy to replace mouse β-globin genes with human globin genes in ES cells. The unmodified murine β-globin locus is shown at the top with the locus control region (LCR) upstream of the functional primitive embryonic (εY and βh1) and definitive adult (β^{maj} and β^{min}) globin genes and pseudogenes (βh0 and βh2). The human $\gamma^{HPFH}\beta^{0}$ targeting vector is designed to replace 16kb of mouse sequence containing the adult globin genes with 8.7kb of human sequence containing mutant human fetal (γ^{HPFH}) and adult (β^{0}) globin genes and a 2kb hygromycin (hyg) marker gene flanked by LoxP sequences (black triangles). The herpes simplex virus thymidine kinase (tk) gene is included upstream for negative selection. Homologous recombination, denoted by the crossed lines, occurs within the 5' and 3' mouse homology regions (grey lines) to generate a $\gamma^{HPFH}\beta^{0}$hyg KI allele in the mouse β-globin locus. After breeding to CMV-Cre transgenic mice the marker gene is deleted leaving a single LoxP sequence downstream of the $\gamma^{HPFH}\beta^{0}$ KI allele. **B.** Southern blot analysis of DNA from wild-type, heterozygous $\gamma^{HPFH}\beta^{0}$ KI, and homozygous $\gamma^{HPFH}\beta^{0}$ KI mice demonstrates correct gene targeting using both upstream (5') and downstream (3') probes. DNA was restricted with XbaI or EcoRI (R1) for the 5' and 3' probes, respectively. Note that the 3' probe hybridizes to sequences downstream of both the β^{min} and β^{maj} globin genes in the wild-type and heterozygous mice.

Figure S2

Newborn humanized CA mice ($\gamma^{HPFH}\beta^{0}/\gamma^{HPFH}\beta^{0}$) are normal size, but become runted compared to their non-anemic littermates after birth. A. Newborn (left panel) and 18-day-old (right panel) litters showing CA pups (labeled CA) and their non-anemic littermates. At birth CA

pups may be slightly pale, but there is no significant difference in size compared to their littermates. As the human fetal Hb levels decline with age the CA pups become severely anemic and are significantly smaller than their littermates at the time of euthanasia before death. **B.** Plot of body weight distributions of CA mice and their littermates at the time of euthanasia before death. To control for differences in offspring number and size between litters, the body weight of a CA pup is only compared to the other pups within the same litter. For each of the 7 litters in this figure the pup with the largest weight is set to 100% and the smallest pup is set to zero. The weights of the remaining pups are listed as a percentage of the difference between the largest and smallest pup. Eighty percent of the time the CA pups are the smallest in the litter.

Figure S3

Growth velocities of CA mice normalize after BMT in the absence of cytoreductive conditioning. The weights of transplanted CA mice were compared to un-transplanted control littermates over 8 weeks. Humanized CA mice were significantly smaller than their non-anemic control littermates 1 week post-transplant. After 2 weeks post-transplant CA mouse weights were no longer significantly different from their control littermates. Values represent mean ± s.d., $n \geq 5$. *P* values were calculated by Student's *t*-test. n.s., not significant

Figure S4

Histology of organs with CA disease-related pathology from additional transplanted CA mice. A. Peripheral blood smears were prepared and stained with Wright-Giemsa, spleens and livers were fixed and stained with hematoxylin and eosin (H&E) and Prussian Blue (livers only). Shown are the images from additional CA mice transplanted without cytoreductive conditioning. The peripheral blood smears of the additional animals are normocytic and normochromic similar

to non-anemic mice (shown in main text). The spleens of these transplanted mice have reduced erythroid expansion with normal red and white pulp architecture. There is little evidence of extramedullary erythropoiesis in the liver and iron deposition is not apparent. **B.** Histological sections of intestine, skin, and lung of wild-type control and transplanted CA mice were examined for evidence of GVHD. Five months post-transplantation skin, intestine, and lung were fixed and stained with hematoxylin and eosin. No GVHD was observed in CA mice after BMT in the absence of cytoreductive conditioning. Scale bar is 100μm for the intestine and 50μm for the lung and skin.

Figure S5

Histology of organs that can exhibit GVHD histopathology. Histological sections of intestine, skin, and lung of additional transplanted CA mice were examined for evidence of GVHD. Five months post-transplantation skin, intestine, and lung were fixed and stained with hematoxylin and eosin. No GVHD was observed in CA mice after BMT in the absence of cytoreductive conditioning.

SUPPLEMENTARY FIGURES

Figure S1

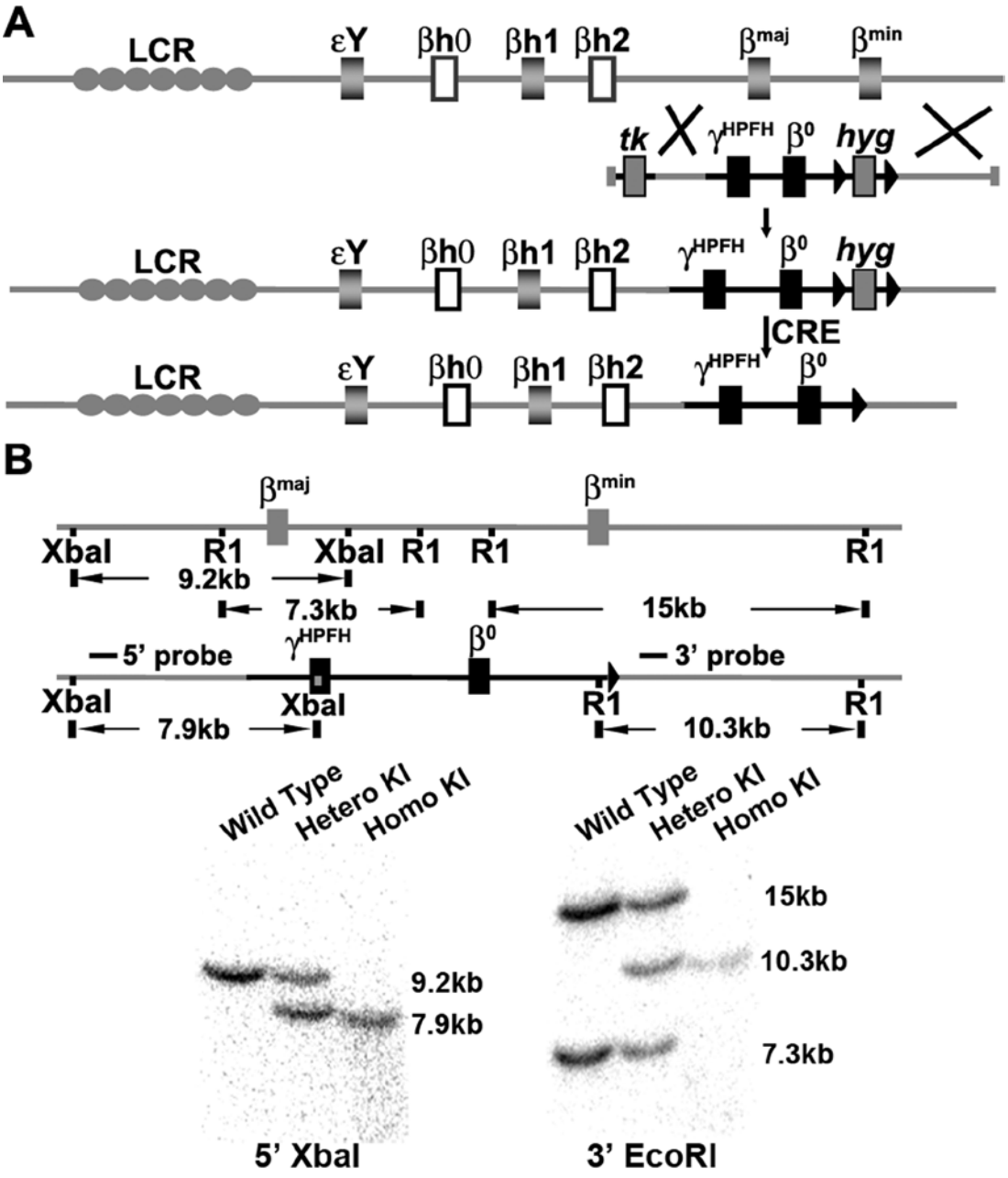


Figure S2

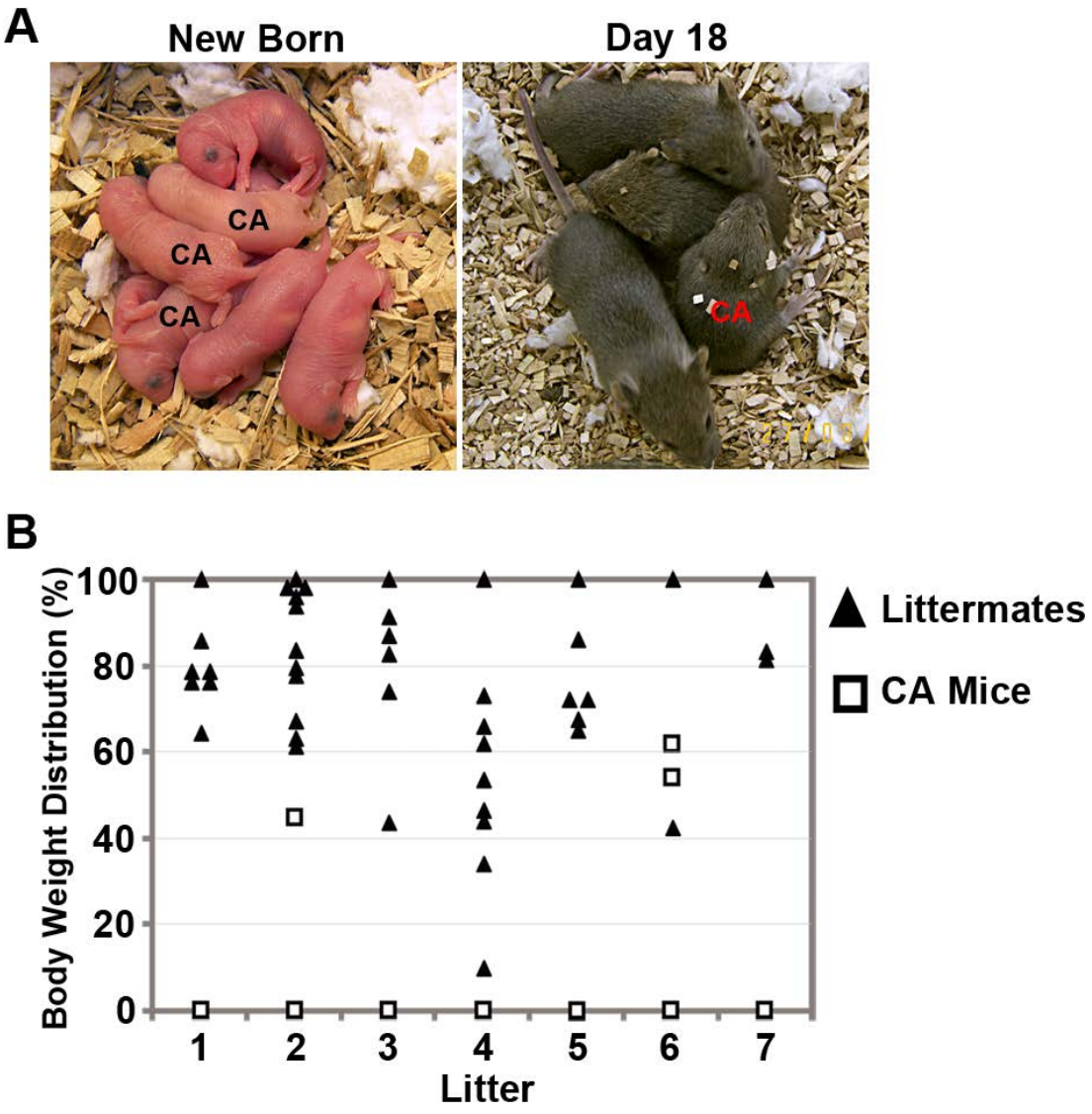

A
New Born
Day 18
CA
CA
CA
CA
B
Body Weight Distribution (%)
100
80
60
40
20
0
1
2
3
4
5
6
7
Litter
Littermates
CA Mice

Figure S3

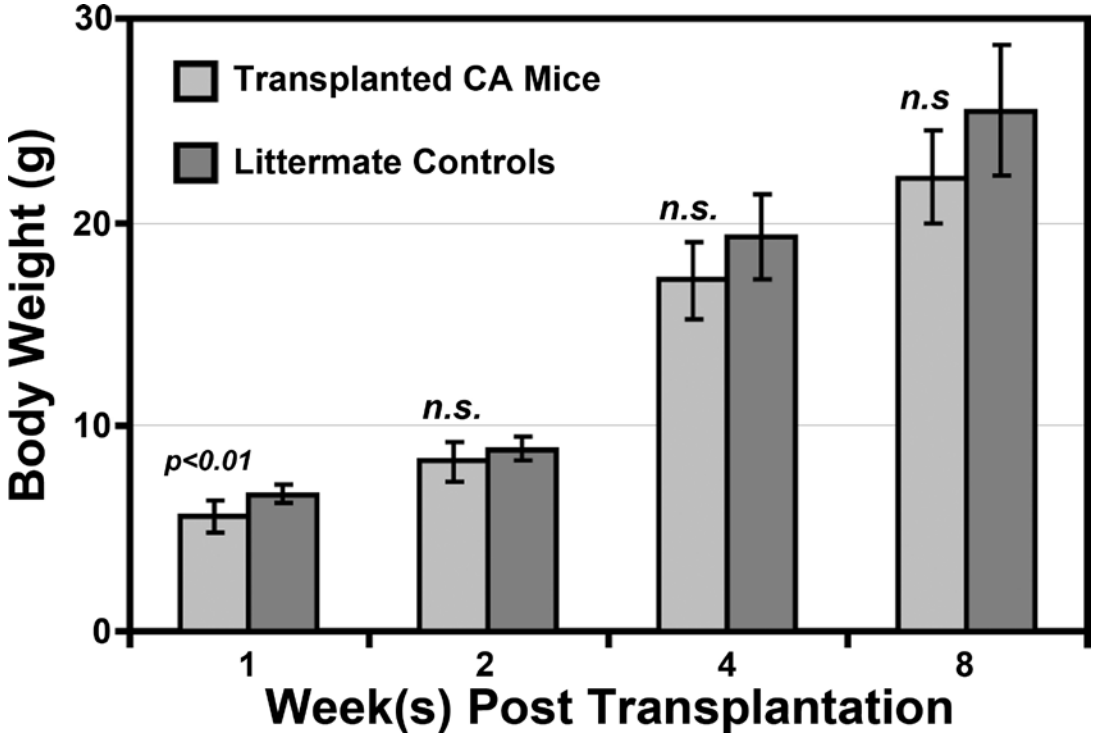

Transplanted CA Mice
Littermate Controls
Body Weight (g)
30
20
10
0
p<0.01
n.s.
n.s.
n.s
1
2
4
8
Week(s) Post Transplantation

Figure S4

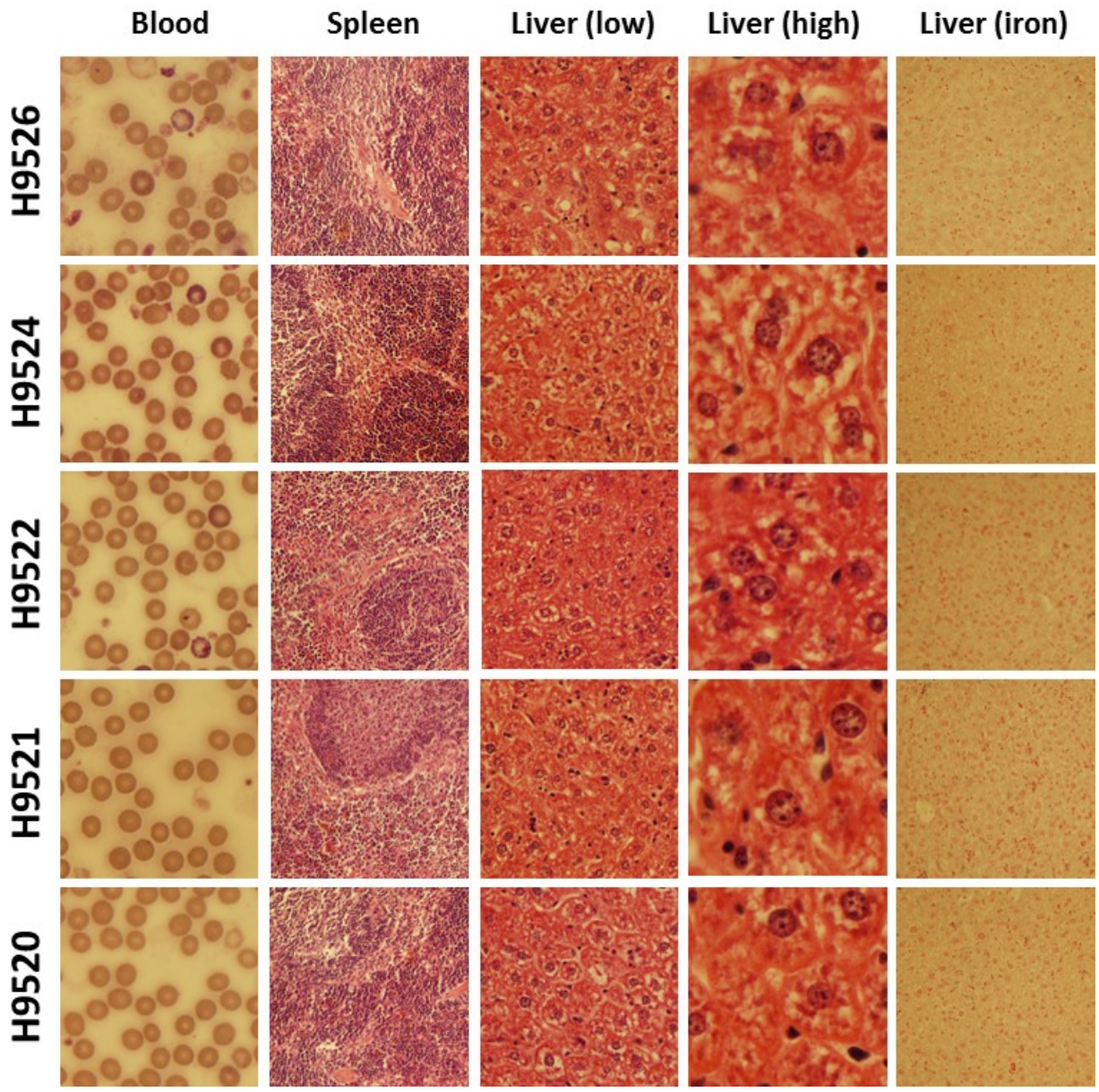
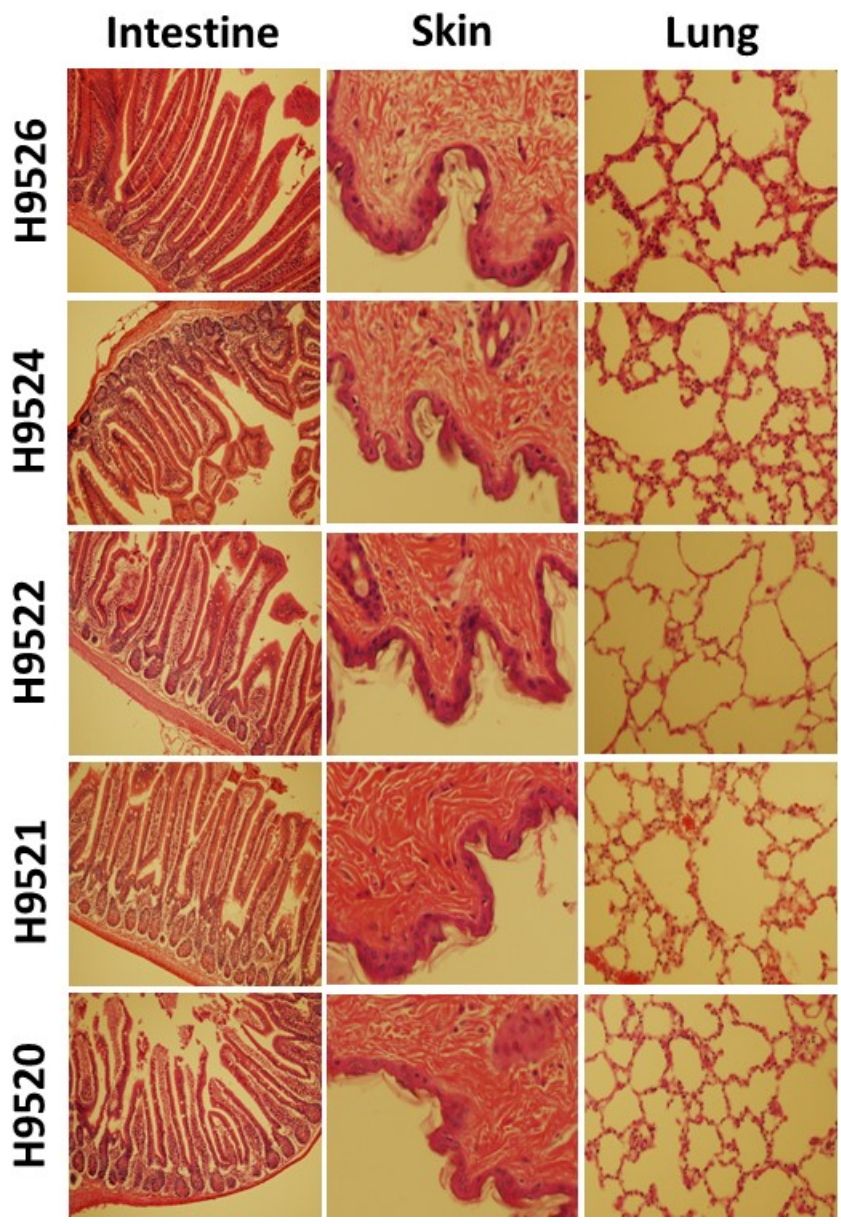

Blood
Spleen
Liver (low)
Liver (high)
Liver (iron)
H9526
H9524
H9522
H9521
H9520

Figure S5



SUPPLEMENTARY TABLES

Table S1. Summary of hematopoietic chimerism in noncytoreductive and nonmyeloablative transplants

Mouse ID	Genotype	Transplant Conditions	RBC GFP%						LSK GFP%	Gr-1 GFP%
			2 weeks	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks		
H9520	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		69.5	79.5	91.0	98.7	99.1	98.5	15.7	10.6
H9521	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		78.5	83.5	91.3	93.0	95.6	90.2	3.5	1.4
H9522	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		69.1	71.5	89.0	92.5	93.2	95.1	5.2	3.4
H9524	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		88.1	92.7	97.0	99.3	99.1	98.9	8.3	7.8
H9526	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		45.5	65.5	73.4	78.7	84.4	76.3	1.5	0.8
I5211	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		83.0	93.9	93.7	95.1	95.3	93.6	4.9	4.5
I5213	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		71.0	73.3	76.7	80.3	81.7	69.3	0.5	1.1
I7145	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		28.5	45.4	88.3	96.4	95.4	91.3	1.9	1.9
I7151	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		66.3	60.7	88.2	75.8	60.4	59.3	1.7	4.6
I7225	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		63.6	81.9	93.5	92.7	89.7	93.7	2.1	1.8
I7226	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		54.2	83.2	90.4	90.2	90.9	90.7	7.1	9.4
I7415	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		77.6	97.2	97.0	95.7	96.6	97.9	9.7	10.1
I5212	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/+$		0.3	0.1	0.0	0.0	0.0	0.0	ND	0.0
I5214	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/+$	anti-CD122, Diprotin A	9.9	7.5	5.5	5.6	4.7	4.2	ND	1.2
I5216	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/+$		0.2	0.2	0.0	0.0	0.0	0.0	ND	6.0
I5217	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/+$		5.0	3.5	3.1	3.0	2.7	2.7	ND	3.9
I5309	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/+$		0.0	0.0	0.0	0.0	0.0	0.0	ND	0.0
I5312	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/+$		1.4	1.0	0.3	0.2	0.2	0.0	ND	0.1
I5314	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/+$		3.6	0.5	1.0	1.7	10.8	1.6	ND	1.0
I5315	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/+$		0.6	0.6	0.0	0.0	0.0	0.0	ND	ND
I5316	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/+$		2.8	0.2	0.0	0.0	0.0	0.0	ND	ND
I5319	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/+$		7.2	2.9	3.8	0.3	4.1	2.2	ND	ND
I5210	$\alpha_2\alpha_1/\alpha_2\alpha_1, +/+$		13.0	9.1	7.9	7.5	6.7	5.7	ND	0.0
I5215	$\alpha_2\alpha_1/\alpha_2\alpha_1, +/+$		9.0	7.9	5.4	5.7	5.6	6.1	ND	6.4
I5313	$\alpha_2\alpha_1/\alpha_2\alpha_1, +/+$		3.0	0.3	0.9	0.0	0.4	1.4	ND	1.3
I5310	$\alpha_2\alpha_1/\alpha_2\alpha_1, +/+$		0.0	0.0	0.0	0.0	0.0	0.0	ND	0.0
I5311	$\alpha_2\alpha_1/\alpha_2\alpha_1, +/+$		0.2	0.0	0.0	0.0	0.0	0.0	ND	0.0
I5318	$\alpha_2\alpha_1/\alpha_2\alpha_1, +/+$		0.0	0.0	0.0	0.0	0.0	0.0	ND	ND
I5320	$\alpha_2\alpha_1/\alpha_2\alpha_1, +/+$		0.1	0.0	0.0	0.0	0.0	0.0	ND	ND
I7146	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		45.4	-	-	-	-	-	-	-
I7147	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$	Diprotin A	28.5	-	-	-	-	-	-	-
I7224	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		47.0	44.6	-	-	-	-	-	-
I7152	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$	anti-CD122	45.4	27.8	-	-	-	-	-	-
I7153	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$		28.5	-	-	-	-	-	-	-
				6 weeks	**9 weeks**	**12 weeks**	**15 weeks**	**25 weeks**		
I6092	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$			69.3	99.2	99.8	99.8	99.8	22.7	39.2
I6198	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$			93.2	99.8	99.9	99.9	99.9	63.7	79.8
I6827	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$	400 rad, supportive RBC transfusions		85.9	99.9	99.9	99.9	99.9	90.0	75.9
I6828	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$			86.1	99.8	97.2	97.0	98.8	15.1	18.8
I6829	$\alpha_2\alpha_1/\alpha_2\alpha_1, \gamma^{HPFH_0}\beta/\gamma^{HPFH_0}\beta$			77.9	99.9	99.9	99.9	99.9	97.1	83.7

ND = not determined

Table S2. Transplanted CA mouse fertility

Litter	Litter Size	Number of CA pups	Number of Heterozygous pups
1	5	3	2
2	8	3	5
3	3	1	2
4	3	0	3
5	4	1	3