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Human Hepatocyte Transplantation Corrects the Inherited Metabolic Liver Disorder Arginase Deficiency in Mice

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Abstract

The transplantation, engraftment, and expansion of primary hepatocytes have the potential to be an effective therapy for metabolic disorders of the liver including those of nitrogen metabolism. To date, such methods for the treatment of urea cycle disorders in murine models has only been minimally explored. Arginase deficiency, an inherited disorder of nitrogen metabolism that presents in the first two years of life, has the potential to be treated by such methods. To explore the potential of this approach, we mated the conditional arginase deficient mouse with a mouse model deficient in fumarylacetoacetate hydrolase (FAH) and with Rag2 and IL2-Rγ mutations to give a selective advantage to transplanted (normal) human hepatocytes. On day –1, a

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uroplasminogen-expressing adenoviral vector was administered intravenously followed the next day with the transplantation of 1×10^6 human hepatocytes (or vehicle alone) by intrasplenic injection. As the initial number of administered hepatocytes would be too low to prevent hepatotoxicity-induced mortality, NTBC cycling was performed to allow for hepatocyte expansion and repopulation. While all control mice died, all except one human hepatocyte transplanted mice survived. Four months after hepatocyte transplantation, 2×10^{11} genome copies of AAV-TBG-Cre recombinase was administered IV to disrupt endogenous hepatic arginase expression. While all control mice died within the first month, human hepatocyte transplanted mice did well. Ammonia and amino acids, analysed in both groups before and after disruption of endogenous arginase expression, while well-controlled in the transplanted group, were markedly abnormal in the controls. Ammonium challenging further demonstrated the durability and functionality of the human repopulated liver. In conclusion, these studies demonstrate that human hepatocyte repopulation in the murine liver can result in effective treatment of arginase deficiency.

Keywords

arginase deficiency; hyperargininemia; ammonia; urea cycle disorder; treatment; cellular transplant

2.1 Introduction

Arginase deficiency is a rare metabolic disorder resulting from a loss of arginase 1 (*ARG1*), the final enzyme in the urea cycle, which is the major pathway for the detoxification of ammonia in terrestrial mammals. *ARG1* is expressed most prevalently in hepatocytes and red blood cells. Through *ARG1* in the liver, in coordination with the other enzymes of the cycle, nitrogen is sequestered as urea [1]. Arginine undergoes hydrolysis by *ARG1* to produce ornithine which then re-enters the cycle as urea and is excreted as waste in the urine.

ARG1 deficiency usually presents later in life beginning in late infancy to the second year with microcephaly, spasticity, seizures, clonus, loss of ambulation (often manifesting as spastic diplegia that may be indistinguishable from cerebral palsy), and failure to thrive associated with hyperargininemia [2]. The neurologic manifestations seen in arginase deficiency may arise from the accumulation of arginine and its metabolites or may result from hyperargininemia, in which several guanidino compounds (putative neurotoxins) increase, though the exact cause is not known. Patients typically avoid the catastrophic hyperammonemic crises characteristic of the other urea cycle disorders and thus tend to survive much longer [1]. At present, long-term therapy rests on the provision of a restricted protein diet and administration of nitrogen scavengers. While these treatments together can partially alleviate *ARG1* deficiency, there is no completely effective therapy available today.

Because of the marginally effective therapies that are available for this disorder, new strategies, including preclinical application of gene therapy to treat this disorder, have been applied and proven successful, albeit with limitations [3–7]. The extensive episomal loss of adeno-associated viral vectors (AAV) in rapidly dividing tissues such as the neonatal liver, results in minimal residual hepatic AAV expression of arginase in adulthood and thus the

treated animals remain quite vulnerable to ammonia and at risk for hyperammonemia and death [7].

Because of these limitations, other approaches, including that of cellular therapy, deserve attention and an evaluation of their potential efficacy in treating arginase deficiency and other disorders of the liver that present early in life. In this report, we describe the therapeutic efficacy of hepatocyte transplantation to treat the biochemical defect of arginase 1 deficiency in an adult conditional murine knockout model and demonstrate the marked improvement in ammonia-mediated vulnerability with exogenous challenging by this method of treatment.

3.1 Materials and Methods

3.1.1 FRG-CKO Arginase Mouse

Animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). FRG (Fah$^{-/-}$/Rag2$^{-/-}$/Il2r$\gamma^{-/-}$) mice on the C57BL/6 background were obtained from Yecuris (Tualatin, OR) and after a breeding colony was established, mice were mated with a conditional arginase knockout mouse (Stock number 8817) on the C57BL/6 background obtained from The Jackson Laboratory (Bar Harbor, ME). Previous studies by our group [8] and others [9] have demonstrated the utility of this model to replicate human arginase deficiency (except for the frequency of hyperammonemia) and its mortality in the floxed model ~3–4 weeks after initiation of cre-lox recombination. Mating was performed until all 4 genes were homozygous and these mice were used for the studies conducted herein and were designated as FRG-CKO Arginase.

Genotype screening was performed by PCR. Genomic DNA was prepared from an ear tip by standard methods (5-PRIME ArchivePure™ DNA Purification Kit, 5 Prime Inc, Gaithersburg, MD) and subjected to PCR. Screening for the conditional arginase allele was performed per the Jackson Lab strain technical support documents (https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS_CODE:23468,008817).

FAH is screened for by three primers: FAH Common primer: 5′ TTG CCT CTG AAC ATA ATG CCA AC 3′; FAH Mutant reverse primer: 5′ GGA TTG GGA AGA CAA TAG CAG GC 3′; FAH WT reverse primer: 5′ TGA GAG GAG GGT ACT GGC AGC TAC 3′ with 35 cycles: 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min followed at completion by 72°C for 5 mins with a mutant band present at 150 bp and wild type at 250 bp.

RAG2 is screened for by three primers: Rag2 Mut forward 2: 5′ CGG CCG GAG AAC CTG CGT GCAA 3′; Rag2 WT forward 2: 5′ GGG AGG ACA CTC ACT TGC CAG TA 3′; Rag2 common reverse 2 5′ AGT CAG GAG TCT CCA TCT CAC TGA 3′ with 35 cycles: 95°C for 30 sec, 62°C for 45 sec, and 72°C for 1 min with 72°C for 5 mins at completion. The mutant band is 300 bp while the wild type is 225 bp.

IL2rγ is screened for by three primers: Il2rg common forward 5′ CTG CTC AGA ATG CCT CCA ATT CC 3′; Il2rγ mutant reverse 5′ GGT CGC TCG GTG TTC GAG GCC AC 3′; Il2rγ wt reverse 5′ ACC GTT CAC TGT AGT CTG GCT GC 3′ with 35 cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec with a 5 mins 72°C extension at completion. The mutant band is 309 bp while the wild type is 473 bp.

Mice were maintained on 5% dextrose water (Fisher Scientific) with 16 mg/L 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) (Yecuris) with alternating Bactrim (Yecuris) added for *Pneumocystis carinii pneumoniae* prophylaxis.

3.1.2 Cellular Transplantation

All animal procedures were performed according to institutional guidelines. Adult FRG-CKO arginase mice were administered 1.25×10^9 pfu/25g body weight of Ad:uPA liver transplant enhancer (Yecuris, #20-0029) via retro-orbital injection 24 hours prior to hepatocyte transplantation. Mice were given a Carprofen MediGel (Clear H_2O, Westbrook, ME) one day prior to surgery for pain control. Fresh human hepatocytes (Yecuris) were received in a transport buffer on ice and prepared according to the distributor's manual. The hepatocytes were washed by centrifugation at 140 g for 5 mins at 4°C followed by resuspension in HCM media (Lonza, CC-1398). Hepatocytes were counted using a cell counter (Nexcelom, Lawrence, MA) and resuspended in HCM media for a concentration of 1×10^6 cells/300μl. Hepatocytes were kept on ice until transplantation.

The FRG mouse to be transplanted was anesthetized in an isoflurane chamber during the am and were not fasted prior. The area of the incision in the left subcostal region was clipped of hair and prepped with 70% EtOH and Betadine. The mouse was placed on a sterile surgery pad on a heated mat for the cellular transplantation procedure. A 0.5cm transverse left subcostal incision was made through the skin, muscle and peritoneum. The spleen was identified and grasped gently with forceps to pull it out of the incision onto sterile gauze. A small part of the spleen was tied gently using a 4-0 silk tie taking care not to avulse the nub of spleen. 1×10^6 hepatocytes were slowly injected into the spleen using a 28.5G insulin syringe through the tied off splenic nub and loop of suture, so as to minimize bleeding from the puncture site. The suture was cut and the spleen was placed back into the abdomen. The peritoneum and muscle layers and subsequently the skin were closed using a 4-0 Vicryl suture. The mouse was recovered on a heated mat in a recovery cage. Controls, receiving saline, were sibling littermates and all mice were 3–4 months of age at transplantation with both male and female mice represented in these studies.

3.1.3 NTBC Cycling

Prior to transplantation, FRG-CKO Arginase mice were maintained on 16mg/L NTBC (Yecuris) water. 24 hours prior to transplantation, the drinking water was switched to water with no NTBC for seven days, after which mice were given water with 8mg/L NTBC for three days. This was followed by cessation of NTBC for 3 weeks. If mice lost >20% of body weight, they were administered water with 8mg/L NTBC for 1–3 days. This cycle was repeated for a total of about 120 days, after which mice were kept indefinitely on water with no NTBC.

3.1.4 Adeno-Associated Viral Vectors

AAV8-TBG-Cre, a serotype 8 adeno-associated viral vector expressing cre recombinase under the thyroxine binding globulin promoter (hepatocyte-specific), was purchased from the University of Pennsylvania Vector Core (Philadelphia, PA).

3.1.5 Biochemical Analysis

Ammonia—Ammonia determination was performed from plasma samples per manufacturer's instructions (Abcam, Cambridge, MA). Optical density was measured at 570 nm using a microplate reader (iMark 16704, Bio-Rad, Hercules, CA). From the standards, a line was generated and sample results in μM were calculated.

Plasma Amino Acids—The concentration of amino acids was determined by high-performance liquid chromatography utilizing the Agilent 1260 Infinity HPLC system and pre-column derivatization with o-phthalaldehyde as previously described [10].

3.1.6 Immunohistochemistry for FAH

Tissues were fixed in 10% buffered formalin and embedded in paraffin blocks from which 4μm liver sections were transferred to slides. Slides were baked at 40°C overnight in incubator, dewaxed in xylene and hydrated through an alcohol gradient (100%, 95%, 90%, 70%) and dH$_2$0. For antigen retrieval, slides were incubated at ~95°C in 10 mM sodium citrate buffer (pH 6.0) for 20 mins. Next, slides were soaked in PBS-T with 0.03% H$_2$O$_2$ for 10 mins and blocked with horse serum (Vector labs, Burlingame, CA) for 30 mins. Slides were then incubated for 1 h at 37°C with anti-FAH antibody (Yecuris) diluted 1:1000 in antibody diluent solution (Ventana, Roche, Germany). After washing in PBS, slides were incubated at RT with the ImmPRESS anti-Rabbit IgG (Vector) for 30 mins. After washing in PBS, tissue sections were incubated in DAB peroxidase substrate solution (Vector). Slides were counterstained in hematoxylin for 5 mins, washed in running tap water and then dried and mounted in Permount. Slides from wild type and FRG mice were used as positive and negative controls respectively.

3.1.7 Immunofluorescence for Arginase and FAH

Tissues were similarly fixed and embedded and antigen was retrieved as above. Next, tissues were blocked with horse serum (Vector) for 30 mins and then incubated overnight at 4°C with rabbit anti-FAH antibody (1:1000; Yecuris), goat anti-arginase (1:100; Abcam) in antibody diluent solution (Ventana). After washing in PBS, slides were incubated at room temperature with the horse Alexa Fluor anti-rabbit 594 and horse Alexa Fluor anti-goat 488 (ThermoFisher, Waltham MA) for 30 mins. After washing in PBS, tissue sections were mounted in vectashield mounting media with DAPI (Vector) and visualized.

3.1.8 Ammonium Challenge

Ammonia challenging was performed as previously described [5].

3.1.9 Arginase Assay

Hepatic arginase activity was measured in cell lysates of the liver as previously described [11].

3.1.10 Human Albumin ELISA

Human albumin levels in mouse plasma were determined by ELISA (Bethyl Laboratories, Montgomery, TX, Catalog number E80-129) according to the manufacturer's instructions. Human albumin concentration was determined by 4-parametric logistic regression with human albumin standards utilizing the analysis software at www.elisaanalysis.com.

3.1.11 Behavioral analyses

FRG-CKO arginase control and human hepatocyte transplanted FRG-CKO arginase mice were studied. 7–9 mice per genotype were used for each behavioral test. Mice were tagged with an ear tag number typically on the left ear. Experimenters were blind to the genotype during testing and analysis. All of the behavioral tests were performed in the UCLA Behavioral Testing Core and analyzed with the MedAssociates (Fairfax, VT) Fear Conditioning software or TopScan (Clever Sys Inc., Reston, VA, USA) automated system software.

3.1.12

SHIRPA (or SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; Imperial College School of Medicine at St Mary's Royal London Hospital, St Bartholomew and the Royal London School of Medicine; Phenotype Assessment) was performed as previously described [5]: As a primary screen of an individual mouse, we used the SHIRPA as a quantitative and standardized method to obtain phenotypic data about individual animal's general health and performance [12]. This primary observational screen based on a modified Irwin profile [13] was performed as described previously [14]. Behavioral and functional profile screen included quantitative scoring to detect defects in gait or posture, motor control and coordination, changes in excitability and aggression, autonomic function such as lacrimation, piloerection, defecation and muscle tone. To provide a quantitative assessment, the parameters in this test were given a quantitative score.

3.1.13 Accelerating Rotarod

To assess motor coordination and ability of the mice to handle a complex motor task that involves balance, coordination and motor skill learning, we used an accelerating Rotarod (Rotamex; Columbus Instruments, Ohio, USA). All subjects received a pre-training habituation on the apparatus for 2 minutes each at a constant speed of 1 RPM. This habituation pre-training took place two hours before testing. Each trial started at a constant forward rotational speed of 1 RPM and then increases by 0.5 RPM every 2 seconds until either the subject falls off or the speed reaches 60 RPS. Trials were completed after experimental subjects fell off the rod or 300 s at a maximum speed of 60 RPM had elapsed, whichever came first. They received three trials at this accelerating speed with

approximately 20 minutes in between trials. Primary measure of interest was latency to fall. Results are presented as mean ± standard deviation.

3.1.14 Light/Dark Box

This test is used to assess anxiety-like behaviors using mouse's natural preference for dark spaces. The apparatus consists of Plexiglass box with two separate compartments. The light compartment measures 10 inches wide, 12 inches long and 12 inches deep with white walls and floor with bright illumination and no ceiling. The dark compartment is 10 inches wide, 6 inches long and 12 inches deep with black walls, floor and ceiling so that light does not penetrate the compartment. There is a 2 x 2 inch doorway between the two compartments with color-matched door. The door can be controlled manually from outside the box via a pulley (a string attached to a nut). The subjects were placed in the dark compartment with the door closed and allowed to habituate for 5 minutes. The door was then opened allowing an opening for entrance into the light compartment and behavior was recorded for another 5 minutes. Latency to enter the light compartment with all four paws, percent time spent in the light compartment and number of bouts of entry into the light compartment were measured in this test to determine levels of anxiety-like behaviors.

3.1.15 Cued Fear Conditioning

This task measures learning by utilizing Pavlovian conditioning and allows a mouse to make an association between a neutral stimuli (tone, CS [conditioned stimulus]) when paired with an aversive stimuli (shock) in a particular context. When the mouse is placed in a novel context and provided with the same CS, it associates the CS with an aversive stimulus and displays freezing response. Freezing is defined as 'absence of movement except for respiration [15]. For the acquisition phase of fear conditioning, mice are placed in a conditioning chamber for 200 seconds before the onset of the discrete conditioned stimulus (CS; 20 s, 2800 Hz, 80 dB tone). At the end of the CS, animals receive a short, mild foot-shock unconditioned stimulus (US; 0.70 mA, 2 s). Animals are exposed to three pairings of the CS and US, with an inter-trial interval of 202 sec. After the CS-US pairings, the mice are left in the conditioning chamber for another 202 s and then placed back in their home cages. 24 h later, mice are placed in a novel context with new odors, textural audio, visual and spatial cues but the same CS is played for measuring cued fear. To measure contextual fear, mice are placed in same context where they received aversive stimulus and freezing is measured for 8 minutes. No shocks are given during either of the two test sessions (See Supplementary Figure 1).

3.1.16 Statistical analyses

The data are presented as the arithmetic mean ± standard deviation. Statistical analyses including Kaplan-Meier survival curve (with Log-rank test), one-way ANOVA, two-way ANOVA and unpaired Student's *t*-test were performed using GraphPad Prism Software 6.0 (La Jolla, CA). For one-way ANOVA, Tukey's multiple comparison test and for two-ANOVA Sidak's multiple comparison test were used. Statistical significance was assigned for a value of $p < 0.05$.

4.1 Results

4.1.1 Model and Experimental Design

These studies were designed to determine if transplantation and repopulation of the murine model of the conditional arginase knockout could lead to 1) survival, 2) control of plasma ammonia and arginine, and 3) reduced vulnerability to increases in plasma ammonia. Furthermore, we sought to examine if this therapy, at least in the short term, would prevent the development of any behavioral abnormalities after knockout of endogenous hepatic arginase expression and the potential development of hyperargininemia and hyperammonemia. In order to perform these studies and make these determinations, we needed to 1) develop the model for transplantation, 2) successfully transplant human hepatocytes to these mice and discontinue the small molecule inhibitor (NTBC) protection of the endogenous FAH$^{-/-}$ hepatocytes, and then 3) eliminate any remaining endogenous arginase expression from the liver such that animal survival and urea cycle function would be solely based on the transplanted human hepatocytes.

Developing a mouse model that would accept human hepatocytes gained success when deletion of the common γ-chain of the IL2 receptor was shown to lead to the inability of mice to reject human cells. While insufficient alone to produce chimeric animals, Grompe and colleagues took advantage of the fumarylacetoacetate hydrolase knockout as a defect in the tyrosine catabolic pathway was known to lead to the accumulation of fumarylacetoacetate, a toxic metabolite resulting in injury to native hepatocytes [16]. This injury provided a selective growth advantage to Fah-expressing transplanted hepatocytes [17]. However, with the rapid accumulation of fumarylacetoacetate death of affected animals can occur before adequate human hepatocyte repopulation. To prevent animal demise while providing time for exogenously administered hepatocytes to divide and repopulate the liver, continuous injury and death can be prevented by administration of the small molecule inhibitor of tyrosine catabolism, NTBC. Hepatocyte repopulation can then successfully occur with cycling of NTBC with eventual complete withdrawal after adequate repopulation with human hepatocytes. These FRG mice (which are T and B cell deficient) are triply mutant for Fah, Rag2 and the common γ-chain of the interleukin 2 receptor.

The FRG-CKO arginase mouse was generated by mating the FRG mouse [16] and the conditional arginase knockout (CKO) [18] and screening for homozygosity of the 4 genes; subsequent intercrossing was performed until the complete knockout was obtained (Fah$^{-/-}$/Rag2$^{-/-}$/Il2rγ$^{-/-}$/Arg$^{flox/flox}$). While maintained on NTBC, these mice appeared identical in behavior and outward appearance compared to the conditional arginase knockout mouse (data not shown). In order to successfully achieve human hepatocyte transplantation, combining this model with features of the previously described urokinase plasminogen activator (uPA) model is necessary (as described by Grompe [19]), in this case, by administering an adenovirus expressing uPA 1 day prior to hepatocyte transplantation [16] (Figure 1). This was followed by human hepatocyte transplantation the subsequent day with NTBC cycling to cause transplanted hepatocyte proliferation *in vivo* as endogenous hepatocytes suffer continuous injury and death from Fah-mediated metabolites. At day 120, AAV8-TBG-Cre recombinase was administered to ensure complete disruption of any

arginase activity that might remain in native hepatocytes, ensuring that survival and urea cycle function were based in the transplanted human hepatocytes.

4.1.2 Animal Survival

The studies described were designed to demonstrate that hepatocyte transplantation could be effective in treating arginase deficiency in a conditional murine knockout of the disorder. In the first set of studies, FRG-CKO arginase mice (n = 14) were transplanted with human hepatocytes by intrasplenic injection after the administration of Ad-uPA one day prior. While FRG-CKO arginase control animals also received Ad-uPA the day prior (n = 8), these mice received only saline (vehicle) by intrasplenic injection. Cycling of NTBC was performed subsequently to examine survival in both groups (Figure 2A) and allow for engraftment, expansion and repopulation with human hepatocytes in the transplanted group. With NTBC cycling, all of the control (saline-administered) FRG-CKO arginase mice expired while only two hepatocyte administered mice died, both occurring between days 21 and 40; histologic examination of the liver demonstrated minimal repopulation in these mice (data not shown) as liver failure was likely the cause of their death.

To add further rigor to these experiments, a second set of studies was performed. When designing these experiments, we did not want to solely rely on the replacement of native hepatocytes with human hepatocytes as we wanted to also include a method for complete disruption of endogenous arginase activity (which if untreated, leads to death [8]). By utilizing the conditional arginase knockout mouse bred with the FRG model, we could take advantage of the lethal nature of the conditional disruption of hepatic arginase activity to result in hyperammonemia and elevated plasma arginine and glutamine (amongst other changes) as we have previously determined [8]. Therefore, at day 120, FRG-CKO arginase human hepatocyte transplanted mice (n = 10) and a new cohort of control (non-transplanted) FRG-CKO arginase mice (n = 8) were administered AAV8 with cre recombinase under the control of the thyroxine binding globulin (TBG) promoter (hepatocyte-specific). While all the control mice (Figure 2B) died by ~4 weeks after AAV-Cre administration, only one human hepatocyte transplanted mouse had died; liver histology demonstrated that there was engraftment but the foci were relatively sparse and small in size in this animal (data not shown). Together, these mice were followed for a total of more than 5 months before subjecting to other studies, euthanizing and collecting tissues.

4.1.3 Hepatic Repopulation with Human Hepatocytes

A series of studies was performed to support our findings of successful transplantation of functional human hepatocytes and to determine whether cellular transplantation was effective in treating the murine model of hyperargininemia.

Plasma was isolated and livers were procured from FRG-CKO arginase mice that were transplanted with human hepatocytes and administered AAV-Cre to compare with untransplanted animals. Human hepatocyte repopulation occurred to different extents in animals with some being nearly fully repopulated (Figure 3A) while others having substantially less repopulation (Figure 3B. In fact, repopulation occurs in some animals only sparsely (Figure 3C1, C2 [<25%]) while in others there is much greater repopulation (>75%

in Figure D1, D2) or nearly complete replacement (Figure E1, E2). Human repopulated cells show positive FAH staining (DAB) in a field of FAH negative native murine hepatocytes (counterstained with hematoxylin). Arginase expression varies depending on the percent of repopulation of arginase positive human hepatocytes in the field of murine arginase negative hepatocytes (see Supplementary Table 1).

With human hepatocyte repopulation, hepatic zonation that is present in normal liver was not detected in the repopulated liver (Figure 4A vs. 4B). Repopulated animals demonstrate human albumin in the circulation (n = 6) that was not detected in untransplanted controls (n = 8) (Figure 4C). Taking advantage of the final step of the urea cycle that metabolizes arginine into ornithine and urea, functionality of the enzyme was measured by determining the concentration of urea produced per milligram of liver. While urea production is normal in untreated FRG-CKO arginase liver (n = 7) (Figure 4D, black bar), it is nearly undetectable in untransplanted mice administered AAV-Cre (n = 8) (blue bar). In human hepatocyte repopulated mice (n = 7), arginase activity is detected at substantial levels (red bar). (One-way ANOVA, Tukey's multiple comparison test, $p < 0.05$ comparing FRG-CKO arginase +AAV-Cre with or without hepatocyte transplantation; $p < 0.0001$ comparing FRG-CKO wild type arginase mice with the other two groups.)

Ammonium challenging was performed to assess the physiological and behavioral response to exogenous nitrogen loading (Figure 4E). Animals were scored on a scale of 1–7 by a blinded observer as previously performed after administering 0.4M ammonium chloride [5]. Hepatocyte transplanted mice (n=7; 4 male, 3 female) had minimal alteration in their behavior (average score 6.0 ± 0.8; range 5 to 7) with exogenous nitrogen administration compared to control untransplanted mice (FRG-CKO arginase that were not administered AAV-Cre) (n=8; 4 male, 4 female) also undergoing challenging (6.9 ± 0.4; range 6 to 7) ($p = 0.02$). Baseline ammonia was higher in the hepatocyte transplanted mice (484.9 ± 42.5 μM) compared to the controls (276.3 ± 21.6 μM), as was the increase in plasma ammonia at 20 minutes (1604.1 ± 78.2 vs. 418.0 ± 24.6). However, both groups showed a substantial decline by 60 minutes after administration (875.2 ± 25.4 vs. 243.8 ± 29.7), demonstrating the metabolic ability of the functional transplanted human hepatocyte mass; while less than that of the control animals, the transplanted hepatocytes were able to reduce peak ammonia levels by nearly 50% in 40 minutes.

Selective amino acids important in the urea cycle and nitrogen metabolism were examined in human hepatocyte transplanted FRG-CKO arginase mice and untransplanted controls, before and after receiving AAV-Cre recombinase. Control animals (saline-injected, untransplanted) (n = 6) demonstrated low glutamine and arginine (Figure 5A, black bar) prior to AAV-Cre. However with disruption of hepatic arginase activity, both amino acids (blue bars) markedly increased as did plasma ammonia (Figure 5B, blue bar) (n = 6) ($p < 0.0001$, by 2 way ANOVA with Sidak's multiple comparison tests). Human hepatocyte transplanted mice (n = 8) fared quiet differently. While plasma arginine had a small but not statistically significant increase, plasma glutamine slightly declined (Figure 5C). Though plasma ammonia did increase and was statistically significant, the amount was small compared to the marked increase of the untransplanted controls (from 276.5 ± 9.6 μM to 484.9 ± 35.6 μM in the

hepatocyte transplant group vs. 352.1 ± 32.6 μM to 3386.5 ± 17.8 μM in the untransplanted group) (p < 0.0001) (Figure 5D).

Immunofluorescent staining was performed with untransplanted and transplanted FRG-CKO arginase mouse livers (with or without AAV-Cre recombinase administration) to demonstrate repopulation and to show that the source of hepatic arginase is from transplanted human hepatocytes in those mice having received AAV-Cre recombinase (Figure 6). Liver sections from control FRG-CKO arginase mice (untransplanted) were immunostained; hepatocytes were negative for FAH and positive for arginase as expected (Figure 6A). Immunostaining was performed with FRG-CKO arginase mice transplanted with human hepatocytes. Mice that did not undergo endogenous arginase disruption by administration of AAV-Cre demonstrate that transplanted human hepatocytes are positive for FAH in a field of FAH negative murine hepatocytes (Figure 6B, top panel); the second panel demonstrates that the entire field (both transplanted human and native murine hepatocytes) is positive for arginase. After administration of AAV-Cre recombinase, native (endogenous) murine arginase is disrupted and only the foci of human hepatocytes is both FAH positive (Figure 6C, top panel) and arginase positive (Figure 6C, second panel).

4.1.4 Behavioral Analysis after Human Hepatocyte Repopulation and Discontinuation of NTBC

We performed four types of assessments to examine for the potential behavioral or cognitive deficits after human hepatocyte transplantation after AAV-Cre recombinase administration; the human hepatocytes are providing the complete urea cycle function. These tests assessed the animal's overall physical activity (SHIRPA), general motor coordination (rotarod), anxiety-like behaviors (light/dark box), and learning and memory (cued fear conditioning). Together, deficits in these tests provide insight into dysfunctions in brain areas such as prefrontal cortex, hippocampus, amygdala and cerebellum. FRG-CKO arginase age-matched animals not receiving transplanted human hepatocytes or AAV-Cre recombinase were included as normal controls for comparison.

The SHIRPA general assessment (Table 1) showed no differences between the two groups except for tail elevation that was scored higher in the transplanted group, and body length which was smaller in the transplanted group (p 0.05 for these two studies; all others p > 0.05). Both cohorts of mice showed normal behavior in this wide ranging assessment.

In the fear conditioning tests, ANOVA revealed no significant differences between the FRG-CKO arginase control mice (black line or bar) with those with transplanted hepatocytes and cre recombinase expression (red line or bar) in cued or contextual fear (Figure 7A, 7B and 7C). The overall freezing response to the tones was found to be high in both groups of mice, slightly greater in the transplanted mice (**p = 0.0009) (see Supplementary Figure 2). Taken together, these data indicate that both groups had no deficits in learning and that treatment with hepatocyte transplantation to control plasma arginine, ammonia, and other metabolites is effective. While the number of mice used in contextual and cued fear were low, these results suggest that regions such as the amygdala and hippocampus that modulate this type of fear may not be affected by hepatocyte transplantation. However, further studies looking at specific roles of these regions will need to be conducted to further validate these findings.

In the light/dark box to examine 1) the duration in the light compartment (Figure 7D) and 2) latency to enter the light compartment (Figure 7E) as a measure of exploration and lack of anxiety, the two cohorts were similarly compared. No statistically significant difference was found in either of these two measures between the groups. These results indicate that low anxiety-like behaviors in transplanted arginase knockout mice. Further tests of anxiety will help assess these results further.

The accelerating rotarod was performed to assess motor function and balance. Comparing the two groups with regards to average time to falling off the rotating rod, there was no significant difference (Figure 7F). This test indicates that hepatic arginase activity supplied by the human hepatocyte mass may not affect motor functions but other tests of locomotion and motor tests will be carried out in the future confirm these findings.

5.1 Discussion

Primary hepatocyte transplantation, engraftment, and expansion have the potential to be an effective therapy for metabolic disorders including those of nitrogen metabolism. Studies of such therapeutic methods for urea cycle disorders in murine models are sparse. The purpose of the present investigation was to examine if hepatocyte transplantation would be effective in treating arginase deficiency in a conditional murine knockout model of the disorder. We sought to examine 1) if hepatocyte transplantation could lead to normal behavioral and cognitive activity in treated animals where endogenous hepatic arginase activity was eliminated knowing that complete loss results in ataxia, listlessness, and death [3, 8]; 2) if control of plasma ammonia and amino acids (including arginine and glutamine) was possible in transplanted animals; and 3) to determine the durability of the treated animal's response to ammonium loading with an eye towards understanding if substantial nitrogen vulnerability remains; we have found this to be a marked limitation in our other studies of treating arginase deficiency with AAV due to the substantial hepatic episomal copy loss that occurs [6, 20]. While one other group had attempted transplantation of wild type murine hepatocytes to the conditional arginase knockout mouse utilizing the cell inhibitor retrorsine and partial hepatectomy, their results were limited by low enzyme activity, limited lifespan extension and repopulation estimated only to be up to 5% [21].

In our investigations, we sought a different approach. We generated the FRG-CKO arginase mouse by taking advantage of the FRG mouse with T and B cell deficiency, allowing for immune suppression, and deficiency in fumarylacetoacetate hydrolase to give a selective advantage to transplanted hepatocytes, as described by the Grompe group [16, 17]. Breeding with the conditional arginase knockout and screening for homozygosity, we generated the $Fah^{-/-}/Rag2^{-/-}/Il2r\gamma^{-/-}/Arg^{flox/flox}$ mouse, amenable to hepatic arginase disruption with AAV8-TBG-Cre recombinase, similar to what our group previously reported with tamoxifen induction with a widespread cre deleter [8].

We performed a wide-ranging array of behavioral studies that would assess for subtle dysfunction related to metabolic abnormalities in hepatocyte transplanted mice (with absent native hepatic arginase activity) to assess the therapy beyond only biochemical analysis. Utilizing a sophisticated battery of tests, different aspects of brain function including motor

activity, anxiety-like behaviors, fear, learning, and memory were examined to compare hepatocyte transplanted mice without endogenous hepatic arginase activity with age-matched wild type arginase positive controls (FRG-CKO arginase mice). From our behavioral analysis, we detected no differences in learning, pain detection, general activity, cerebellar function, exploratory activity, posture, gait, motor control and coordination, muscle tone or autonomic function. The only small differences between the two groups were in animal length and tail elevation. While these tests indicate that brain areas that modulate learning, memory and motor functions may have normal functions, further studies will need to be conducted to confirm our findings.

Human hepatocyte transplantation left some animals with relatively sparse repopulation while others had >90% of their liver containing human hepatocytes. The presence of circulating human albumin varied based on the amount of repopulation as the degree of humanization is reported to correlate with human albumin levels (1 mg/ml albumin corresponds to about 20% human hepatocyte repopulation [22]). Our previous studies with both the constitutive arginase knockout [4] and the conditional arginase knockout [7] have shown that minimal arginase expression and ureagenesis is necessary for survival with murine arginase deficiency.

Cellular transplantation with disruption of endogenous hepatocyte arginase activity did result on average in a mild increase in plasma ammonia and arginine in these repopulation studies, likely because of the incomplete repopulation of the liver with normal human hepatocytes. However, glutamine did not increase, suggesting that the ammonia metabolizing capabilities of the transplanted hepatocytes was sufficient to control normal metabolic needs. Administration of ammonium to animals was performed to provide insight on the functioning of the urea cycle to examine the capability of the transplanted hepatic mass. While we do see a marked rise in plasma ammonia at 20 minutes in the transplanted group compared to arginase wild type controls, behaviour is only reduced one point and animals regained normal activity quickly. Furthermore, at 1 hour, plasma ammonia had declined nearly 50%. Importantly, this substantially reduced the nitrogen vulnerability of these animals; in our previous studies with the constitutive arginase knockout treated with AAV, there was tremendous episomal vector loss, and with ammonium challenging, there was persistently high plasma ammonia at one hour, with associated marked reduced behavioural scores, hypothermia, seizures, and death [5].

These studies demonstrate that the metabolic abnormalities present in arginase deficiency can be mitigated or markedly improved in mice with transplantation of normal human hepatocytes. In addition, nitrogen metabolism occurs in the context of normal behavior and learning in the transplanted mice and is effective in lowering exogenous ammonium challenges, markedly reducing vulnerability. Importantly, glutamine buffering is not necessary to keep plasma ammonia controlled. Taken together, these studies suggest that hepatocyte transplantation may be an effective intervention in the treatment of arginase deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

List of Abbreviations

FAH   fumarylacetoacetate hydrolase

FRG $Fah^{-/-}/Rag2^{-/-}/Il2r\gamma^{-/-}$ mouse

CKO   conditional knockout

NTBC   2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione

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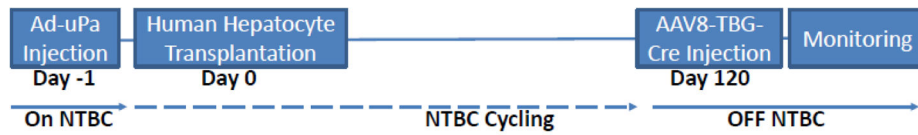
Figure 1. Design of Studies
Mice were administered a serotype 5 adenovirus intravenously on the day prior to the administration of 1×10^6 human hepatocytes. This was followed by cycling of NTBC to allow for human hepatocyte repopulation. AAV8-TBG-cre recombinase was administered at day 120 to disrupt expression of any remaining endogenous arginase in surviving murine endogenous hepatocytes. NTBC, (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione); Ad-uPA, adenovirus expressing urokinase plasminogen activator; AAV8-TBG-Cre, adeno-associated virus serotype 8 expressing cre recombinase under the control of the thyroxine binding globulin promoter.
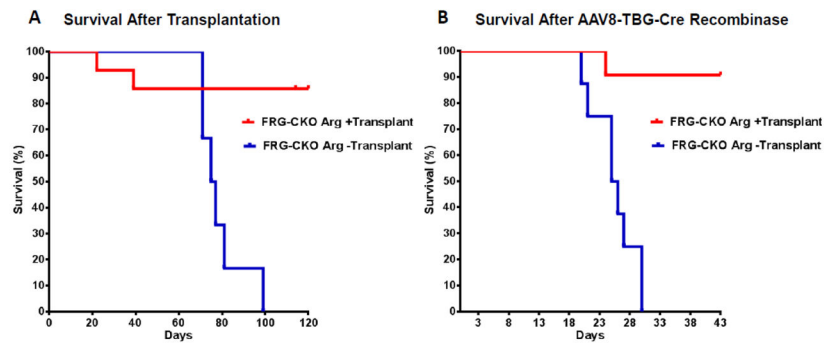
Figure 2. Survival of Human Hepatocyte Transplanted FRG-CKO Arginase Mice
A) Survival comparison of FRG-CKO arginase mice in days after transplantation of human hepatocytes (n = 14) or vehicle only (n = 8) (p = 0.0006 by log-rank). B) Survival comparison of human hepatocyte transplanted FRG-CKO arginase mice (n = 10) in days and non-transplanted control mice (n = 8) after receiving AAV8-TBG-cre recombinase to disrupt endogenous arginase expression in native hepatocytes (p = 0.0001 by log-rank).
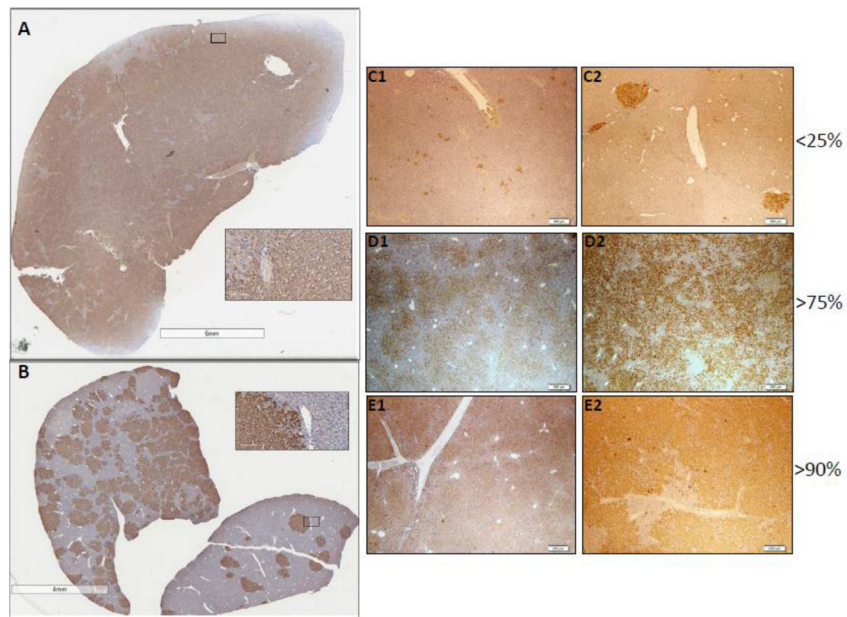
Figure 3. Human Hepatocyte Repopulation is possible in the FRG-CKO Arginase Liver
Repopulation of the murine FRG-CKO arginase liver is shown with A) nearly 100%
replacement, as indicated by FAH positive cells stained with DAB (brown) or B) at reduced
repopulation. (Inset: magnified detail of liver repopulation.) Various rates of repopulation
occur from low-level (C1, C2) to high levels of >75% (D1, D2) and in some with near
complete repopulation (E1, E2). (Each subgroup denotes representative images from
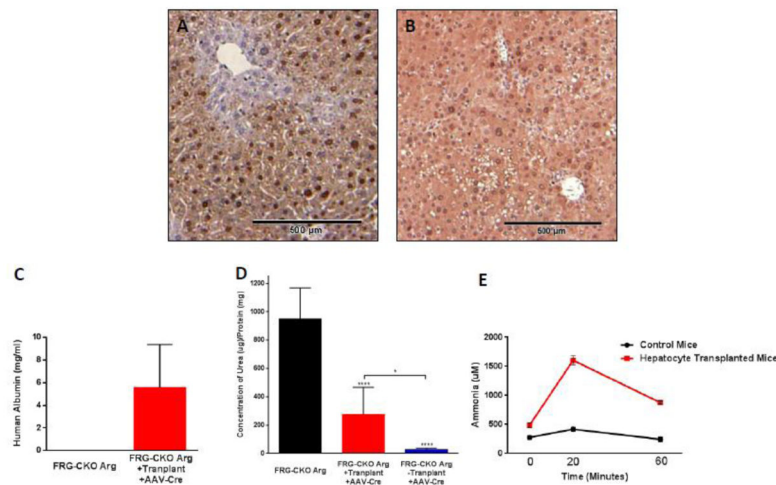different animals (i.e. C1 and C2 are different mice). (Bar = 200 μm)

Figure 4. Features of Hepatocytes and Liver Function with Human Hepatocyte Repopulation of the Conditional Arginase-Deficient Mouse

Hepatic Zonation: A) section of wild type murine liver demonstrating lack of arginase staining hepatocytes by immunohistochemistry at central vein while image of repopulated liver with human hepatocytes (B) shows homogenous arginase staining including hepatocytes surrounding the central vein. C) Detection of human albumin in transplanted mice (red bar) (n = 6) and lack of detection in untransplanted mice (n = 8). D) High level arginase activity (as detected by urea production) is present in FRG-CKO arginase liver (black bar) (n = 7) that is reduced to nearly undetectable levels with after administration of AAV-Cre recombinase (blue bar) (n = 8) (*p < 0.05 comparing FRG-CKO arginase +AAV-Cre with or without transplanted hepatocytes; ****p < 0.0001 comparing FRG-CKO arginase mice with the other two groups). After hepatocyte transplantation, functional arginase activity (red bar) from the human cells is present along with the ability to metabolize exogenously administered ammonia (E) (red line [n = 7]; black line represents arginase wild type controls [n = 8] [p < 0.0001 by 2 way ANOVA, with Sidak's multiple comparisons test]).
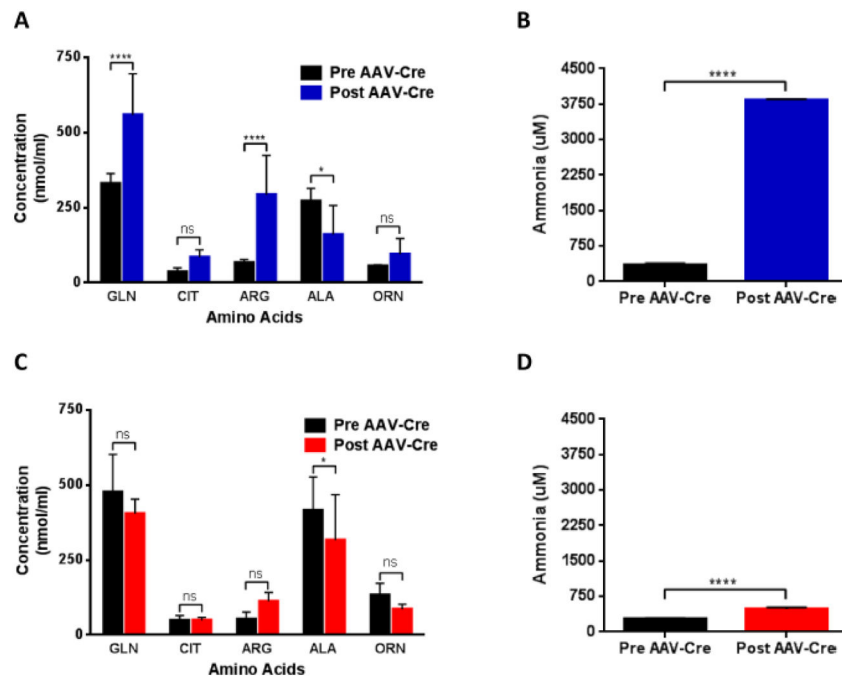
Figure 5. Marked Plasma Metabolite Alteration with Disruption of Endogenous Arginase with AAV-Cre Recombinase in Untransplanted Mice and with Minimal Alteration in Transplanted Mice

There is marked alteration of A) plasma urea cycle related amino acids (n = 6) and B) ammonia (n = 3) with disruption of hepatic arginase activity with AAV-Cre in untransplanted FRG-CKO arginase mice. Animals that were repopulated with human hepatocytes (n = 8) and received AAV-Cre recombinase had minimal alteration, and glutamine buffering capacity remained intact (C and D). (GLN = glutamine; CIT = citrulline; ARG = arginine; ALA = alanine; ORN = ornithine; AAV-Cre = AAV-TBC-Cre recombinase) (**** = p < 0.0001; * = p < 0.05)
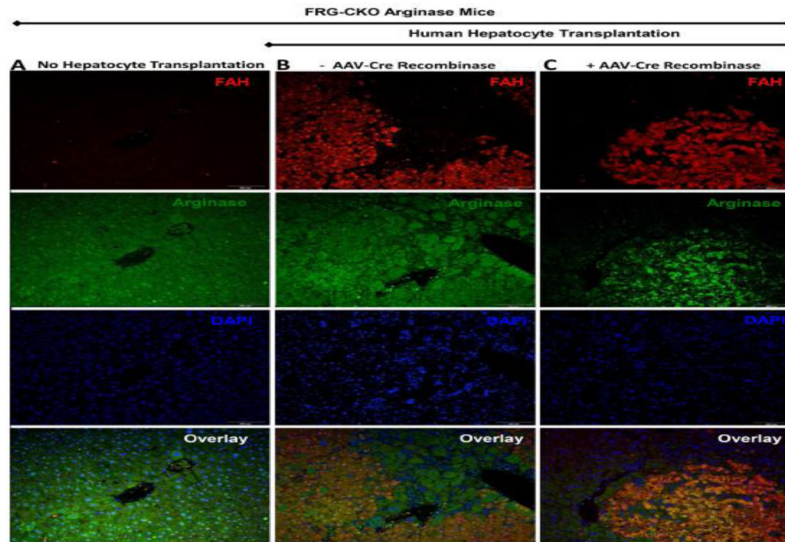
Figure 6. Immunohistochemistry Demonstrates Loss of Endogenous Hepatic Arginase Activity After Cre Recombinase With Repopulated Human Hepatocytes Expressing Both Arginase and FAH

A) Liver section from an FRG-CKO arginase mouse shows lack of hepatic FAH expression with intact arginase activity. B) Liver section from a human hepatocyte transplanted FRG-CKO arginase mouse shows restored hepatic FAH activity in transplanted cells and both native hepatic and transplanted hepatic arginase activity. C) After administration of AAV-Cre recombinase, liver sections show that endogenous native hepatic arginase activity is now disrupted with human hepatic nodules being positive for both arginase and FAH.
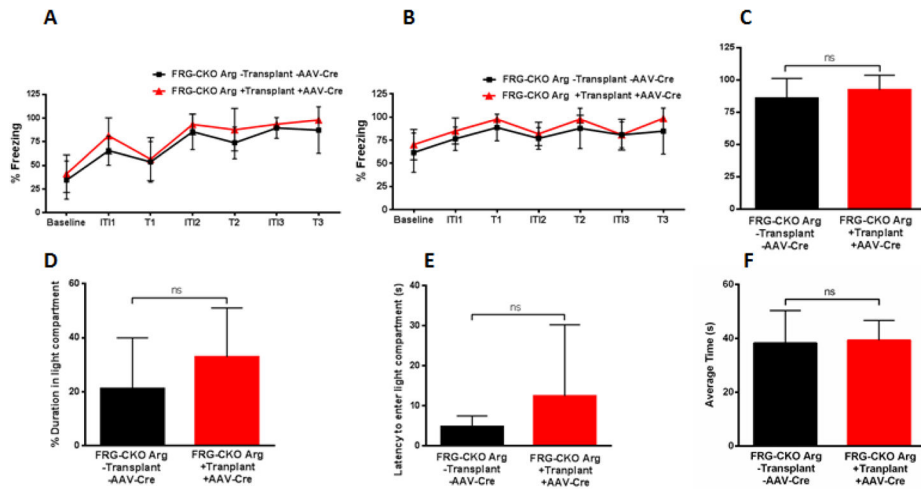
Figure 7. Behavioral Testing of FRG-CKO Arginase Human Hepatocyte Transplanted Mice Shows Normal Behavior of Repopulated Mice

Fear Conditioning (A–C; see also Supplementary Figure 1): Acquisition of fear (A) and cued fear tone test (B) showed no difference between human hepatocyte transplanted mice that were administered AAV-Cre recombinase and untransplanted FRG-CKO arginase mice (A: $p > 0.05$; B: $p > 0.05$). Contextual Fear (C), with measurement of freezing over 8 minutes in the original location of fear acquisition, was no different between the groups ($p = 0.36$). Similarly, light dark percent duration (D) and latency to enter the light compartment (E) were not different between the groups ($p = 0.19$ and $p = 0.22$, respectively) while accelerating rotarod (F), measuring average time to falling off, was also not different ($p = 0.84$).

Table 1

	Controls	Transplanted	p value
Viewing Jar			
Body position	3.22 ± 0.44	3.50 ± 0.30	0.26
Spontaneous activity	1.44 ± 0.73	0.88 ± 0.64	0.11
Respiratory rate	2.22 ± 0.44	2.25 ± 0.46	0.90
Tremor	0.33 ± 0.50	0.25 ± 0.46	0.73
Urination	0.11 ± 0.33	0.00 ± 0.00	0.36
Defecation	2.00 ± 1.66	1.88 ± 0.99	0.86
Arena			
Transfer arousal	3.89 ± 1.54	4.50 ± 0.53	0.30
Locomotor activity	14.56 ± 9.48	18.63 ± 4.03	0.28
Palpebral closure	0.00 ± 0.00	0.00 ± 0.00	
Piloerection	1.00 ± 0.00	0.88 ± 0.35	0.30
Startle response	0.56 ± 0.53	0.50 ± 0.53	0.83
Gait	0.33 ± 0.50	0.38 ± 0.52	0.87
Pelvis elevation	2.22 ± 0.44	2.25 ± 0.46	0.90
Tail elevation	1.00 ± 0.00	1.38 ± 0.52	**0.05**
Touch escape	1.44 ± 0.73	1.13 ± 1.13	0.49
Held by tail			
Positional passivity	0.00 ± 0.00	0.00 ± 0.00	
Trunk curl	0.11 ± 0.33	0.00 ± 0.00	0.36
Limb grasping	0.11 ± 0.33	0.00 ± 0.00	0.36
Abnormal behavior	0.56 ± 0.53	0.63 ± 0.52	0.79
Visual placing	2.78 ± 0.83	2.88 ± 0.64	0.79
Whisker brush	2.44 ± 0.73	2.25 ± 1.16	0.68
Whisker placement	2.33 ± 0.71	2.25 ± 1.04	0.85
Reflex			
Grip strength	2.22 ± 0.83	1.63 ± 0.74	0.14
Body tone	1.00 ± 0.00	1.00 ± 0.00	
Pinna reflex	0.44 ± 0.53	0.50 ± 0.53	0.83
Corneal reflex	0.89 ± 0.60	1.00 ± 0.00	0.61
Toe pinch	2.33 ± 1.00	1.63 ± 1.30	0.22
Wire maneuver	0.56 ± 1.01	0.50 ± 0.53	0.89
Supine Restraint			
Body length	9.41 ± 0.86	8.63 ± 0.44	**0.04**
Skin color	1.00 ± 0.00	1.00 ± 0.00	
Heart rate	1.11 ± 0.78	1.00 ± 0.00	0.69
Limb tone	1.67 ± 1.00	1.88 ± 0.83	0.65
Abdominal tone	1.00 ± 0.00	1.13 ± 0.35	0.30
Lacrimation	0.33 ± 0.50	0.25 ± 0.46	0.73
Salivation	0.00 ± 0.00	0.00 ± 0.00	

	Controls	Transplanted	p value
Provoked biting	0.67 ± 0.50	0.63 ± 0.52	0.87
Penlight vision	1.00 ± 0.00	1.00 ± 0.00	
Other			
Righting reflex	0.00 ± 0.00	0.00 ± 0.00	
Contact righting reflex	1.00 ± 0.00	1.00 ± 0.00	
Negative geotaxis	0.22 ± 0.44	0.63 ± 0.92	0.26
Fear	0.67 ± 0.50	0.63 ± 0.52	0.87
Irritability	0.67 ± 0.50	0.38 ± 0.52	0.26
Aggression	0.67 ± 0.50	0.63 ± 0.52	0.87
Vocalization	0.56 ± 0.53	0.88 ± 0.35	0.17