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Liver-specific knockout of arginase-1 leads to a profound phenotype similar to inducible whole body arginase-1 deficiency

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ABSTRACT

Arginase-1 (Arg1) converts arginine to urea and ornithine in the distal step of the urea cycle in liver. We previously generated a tamoxifen-inducible Arg1 deficient mouse model (Arg1-Cre) that disrupts Arg1 expression throughout the whole body and leads to lethality ≈2 weeks after gene disruption. Here, we evaluate if liver-selective Arg1 loss is sufficient to recapitulate the phenotype observed in global Arg1 knockout mice, as well as to gauge the effectiveness of gene delivery or hepatocyte transplantation to rescue the phenotype. Liver-selective Arg1 deletion was induced by using an adeno-associated viral (AAV)-thyroxine binding globulin (TBG) promoter-Cre recombinase vector administered to Arg1 "floxed" mice; *Arg1*$^{fl/fl}$). An AAV vector expressing an Arg1-enhanced green fluorescent protein (Arg1-eGFP) transgene was used for gene delivery, while intrasplenic injection of wild-type (WT) C57BL/6 hepatocytes after partial hepatectomy was used for cell delivery to "rescue" tamoxifen-treated Arg1-Cre mice. The results indicate that liver-selective loss of Arg1 (>90% deficient) leads to a phenotype resembling the whole body knockout of Arg1 with lethality ≈3 weeks after Cre-induced gene disruption. Delivery of Arg1-eGFP AAV rescues more than half of Arg1 global knockout male mice (survival >4 months) but a significant proportion still succumb to the enzyme deficiency even though liver expression and enzyme activity of the fusion protein reach levels observed in WT animals. Significant Arg1 enzyme activity from engrafted WT hepatocytes into knockout livers can be achieved but not sufficient for rescuing the lethal phenotype. This raises a conundrum relating to liver-specific expression of Arg1. On the one hand, loss of expression in this organ appears to be both necessary and sufficient to explain the lethal phenotype of the genetic disorder in mice. On the other hand, gene and cell-directed therapies suggest that rescue of extra-hepatic Arg1 expression may also be necessary for disease correction. Further studies are needed in order to illuminate the detailed mechanisms for pathogenesis of Arg1-deficiency.

1. Introduction

In the urea cycle, arginase-1 (Arg1) catalyzes the distal step in the conversion of ammonia to urea. Patients with the genetic disorder of Arg1 deficiency display a unique phenotype relative to other urea cycle disorders, consisting of hyperargininemia and neurological deficits including some or all of the features of spastic diplegia, seizures, intellectual disability and failure to thrive [1,2]. Mouse models to study the human disorder have been created and all recapitulate the main biochemical feature of hyperargininemia [3–6]. However, the global Arg1 knockout (KO) exhibits a much more profound phenotype compared to the human disorder with all mice dying approximately 2 weeks after birth, presumably due to hyperammonemia [3]. To circumvent the neonatal lethality, three groups have generated similar, but not identical, inducible Arg1 KO mouse models with several differences that have been described recently [1,4–6]. These mice also die invariably 2–3 weeks after gene disruption. There are some differences in interpretation of the mechanisms for lethality with contributing factors including a wasting phenotype and also hyperammonemia [1].

We [7] and others [8–11] have focused on strategies to rescue the lethal phenotype of inducible and global Arg1 KO mouse models. Gene therapeutic approaches work best but none have rescued every feature of the genetic deficiency [7,9–11]. Understanding the mechanism(s) for the pathological features of genetic Arg1 deficiency in order to develop effective therapeutic strategies is paramount. An important question that remains unresolved is if it is the loss of Arg1 expression in liver, where Arg1 expression is by far the most abundant, that drives the

Abbreviations: Arg1, arginase-1; Arg1-eGFP, arginase-1 enhanced green fluorescent protein; AAV, adeno-associated virus; TBG, thyroxine-binding globulin; gc, genome copies; WT, wild-type; ip, intraperitoneal.

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neurological phenotype in humans and lethal phenotype in mice, respectively, or if it is due to the loss of extra-hepatic Arg1 expression that is also contributing to the pathological sequelae.

Herein, we interrogate this question by generating liver-specific Arg1 KO mice, in combination with various gene therapeutic efforts and hepatocyte cell transplant rescue experiments. While we have not yet determined specific mechanisms, we demonstrate that tissue-specific Arg1 deficiency can be just as severe as global Arg1 absence and have raised novel questions to be addressed in the field of rare urea cycle disorder research.

2. Materials and methods

2.1. Liver-specific Arg1 knockout mouse model

Arg1 floxed mice (*Arg1*$^{fl/fl}$; >8 week old male) with loxP sites flanking exons 7 and 8 of the *Arg1* gene [12] were injected with high (1.5×10^{11} genome copies (gc)), medium (5×10^{10} gc) or low (1.5×10^{10} gc) doses of an adeno-associated viral (AAV)-thyroxine binding globulin (TBG) promoter-Cre recombinase vector (obtained from University of Pennsylvania Gene Therapy Core Services; AAV8.TBG.PI.Cre.rBG) via the intraperitoneal (ip) route to induce liver-specific Arg1 deficiency.

2.2. Global inducible Arg1 knockout mouse model

Arg1-Cre mice, originally obtained from the Jackson Laboratory and bred in-house, derived from parental strains *Arg1*^{flox} (JAX 008817, C57BL/6-*Arg1*^{tm1Pmu}/J) and CreER^{T2} (JAX 008463, B6.129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT2)Tyj}$/J) (male, 12–16 weeks old) were injected ip on 5 sequential days with tamoxifen to induce global Arg1 deficiency as previously described [4,7]. All procedures were reviewed and approved by the Queen's University Animal Care Committee (Funk 2011-048) and conformed to the Guidelines of the Canadian Council on Animal Care. Unless otherwise specified, water and standard rodent chow (containing 21.8% protein, 9% fat, 2.2% fiber, 5% minerals by weight; PicoLab Mouse Diet 20 (5058)) were provided ad libitum. Specific signs of health deterioration due to arginase deficiency were present in mice, regardless of gender, approximately 24–48 h before exhibiting severe distress, which allowed for humane euthanization by CO_2 inhalation [4,7]. Humane endpoints were defined as body weight loss of >15% relative to the weight at the time of the final tamoxifen administration, accompanied by a slightly hunched posture. Some mice could lose substantial weight in a single day when approaching the endpoint resulting in some exceeding the 15% threshold.

2.3. Gene therapeutic delivery of Arg1-eGFP AAV vector

The AAV vector for transgene delivery has been previously described [7]. Briefly, the rh10 serotype vector expressing an Arg1-enhanced green fluorescent protein (Arg1-eGFP) transgene from a strong promoter (hybrid cytomegalovirus enhancer/chicken β-actin) was used in male 8 weeks old Arg1-Cre mice at 1.5×10^{11} gc injected two weeks prior to tamoxifen-induced Arg1 knockout to allow for adequate time for transgene expression.

2.4. Two-thirds hepatectomy and intrasplenic injection of C57BL/6 wild-type hepatocytes

Mice (female, >8 weeks old) were conditioned with retrorsine (Sigma-Aldrich; 70 mg/kg ip, twice at 2 week intervals), a cell cycle inhibitor, to block proliferation of native hepatocytes [13]. Two-thirds partial hepatectomy [14] was carried out 2 weeks after the last retrorsine dose to create a selective growth advantage for transplanted WT hepatocytes. Two million donor cells obtained from immunocompatible female C57BL/6 mice were suspended in 0.1 ml of media (high glucose DMEM supplemented with 15 mM HEPES (pH 7.4), 10% FBS and 100 nM dexamethasone) and injected slowly over 20–30 s into the lower pole of the spleen as described [13,14]. 15 weeks later, allowing sufficient time for donor cell engraftment, the usual tamoxifen-induced Arg1 knockout procedure was carried out.

2.5. Biochemical measurements and Arg1 enzyme activity assay

Blood was collected from the submandibular vein. A 3.2 mm circle from a dried blood spot sample collected on Whatman 903™ filter paper card was punched into the designated well of a 96-well plate. Sample preparation was based on the method described by Turgeon et al. [15] with minor modification. Briefly, amino acids were extracted using a methanolic solution containing the following isotope-labeled amino acid internal standards: ^{15}N,2-^{13}C-glycine, d4-alanine, d8-valine, d3-leucine, d3-methionine, ^{13}C6-phenylalanine, ^{13}C6-tyrosine, d2-ornithine, d2-citrulline and 5-^{13}C-d4-arginine-HCl (Cambridge Isotope Laboratories). After evaporation under nitrogen, the residue was derivatized using 3.0 N HCl in n-butanol at 60 °C for 20 min. Excess reagent was evaporated to dryness using nitrogen followed by reconstitution with 80% acetonitrile. A 10 μl portion of sample was subjected to analysis by mass spectrometry.

The analytical system used in this study consisted of a Waters TQ Detector equipped with electrospray ionization (ESI) source, Waters 1525 μ Binary HPLC Pump and a Waters 2777C Sample Manager (Waters). Tandem mass spectrometric (MS/MS) analysis of amino acids was achieved using a combination of selected reaction monitoring and neutral loss of mass to charge (m/z) of 102 scans, with the ESI source being operated in the positive ion mode.

Arg1 enzyme activity in liver homogenates was assayed as described previously [4,7]. One unit of activity is defined as 10 nmol urea/μg protein.

2.6. Western blot analysis

Liver tissues were homogenized in a solution containing T-PER solution and 1× HALT protease inhibitor cocktail (40 μl/mg tissue). Homogenates were further diluted in T-PER to a concentration of 1 mg/ml protein with 2× Laemmli buffer. Protein samples (20–30 μg) were subjected to Western Blot analysis. Proteins separated by electrophoresis in 10% TGX FastCast acrylamide gels (Bio-Rad) were transferred to PVDF membrane (Bio-Rad, TurboBlot system) and probed with rabbit polyclonal *anti*-Arg1 (C-terminal peptide; 1:10,000; Abcam #ab91279) and mouse monoclonal *anti*-α-tubulin (1:5000; Sigma #T5168) antibodies. Immunoreactive proteins were detected using HRP-conjugated goat anti-rabbit or anti-mouse secondary antibody (1:7500; Sigma) enhanced chemiluminescence signal. Digitized images were recorded with a FluorChem 8900 instrument (Alpha Innotech, San Leandro, CA). Some images underwent semi-quantitation with publicly available Image J software (Version 1.47, NIH).

2.7. Immunofluorescence and pathological analysis of liver sections

Liver sections (6 μm) were deparaffinized and rehydrated with toluene and ethanol by routine procedures. Antigen retrieval was performed by boiling slides in 10 mM citrate buffer, pH 6.0, 0.02% Tween-20 (Arg1 staining) or Tris/EDTA buffer, pH 9.0, 0.05% Tween-20 (CD4 staining). After PBS rinsing, sections were permeabilized with 1× PBS + 0.2% Triton X-100 for 10 min at room temperature then blocked with 2% normal goat serum (Cedarlane) in PBS for 30–45 min. Sections were incubated with rabbit polyclonal *anti*-Arg1 (1:100; Abcam #ab91279), rabbit monoclonal (EPR19514) anti-CD4 (1:250; Abcam #ab183685) in 1–2% goat serum, and mouse monoclonal *anti*-glutamine synthetase (1:200; Abcam #ab64613) in serum, for 1 h at room temperature or overnight at 4 °C. Secondary antibodies, goat *anti*-rabbit IgG FITC (Arg1; Cedarlane), goat anti-rabbit IgG (H + L)

Alexa Fluor 488 (CD4; 1:1000; Life Technologies #A11008) and goat anti-mouse IgG AlexaFluor 568 (GS; Life Technologies) were used. Slides were then dehydrated, mounted with ProLong Gold antifade reagent with DAPI (Invitrogen), and stored at room temperature until analysis. Visualization was performed with a fluorescent microscope (Leica, DM IRB, Richmond Hill, ON). Separate H&E sections, from both the gene therapy and hepatocyte transplantation studies, were examined by a pathologist blinded as to the identity of the samples.

2.8. Statistical analysis

All results are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, California, USA). Means were compared using the two-tailed Student's *t*-test. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Liver-specific knockout of Arg1

Injection of AAV-TBG-Cre into *Arg1* "floxed" ($Arg1^{fl/fl}$) mice induced a selective disruption of the *Arg1* gene in liver as evidenced by PCR genotyping of 11 separate tissues for the diagnostic band of 195 bp ($Arg1^{\Delta}$ allele indicative of loss of exons 7 and 8; Fig. 1A, lane 4). Western blot analysis of liver protein extracts revealed a loss of 80–95% Arg1 in the high- and mid-dose Cre-injected mice with almost no loss of Arg1 protein in 2 (of 3) low-dose Cre-injected mice (Fig. 1B and data not shown). Arg1 enzymatic activity in the extracts correlated with Arg1 protein levels (Fig. 1C). Some residual Arg1 immunostaining could be detected in discrete regions of liver sections in AAV-TBG-Cre low-dose injected mice that retained some enzyme activity (Fig. 1D, left). This staining was not co-localized with glutamine synthetase situated around central veins. In contrast, Arg1 staining in liver sections from WT mice was more widespread throughout the liver parenchyma (Fig. 1D, right).

We compared the phenotype of the liver-specific Arg1 KO mice with that of whole-body Arg1 KO mice that we had previously generated [4,7]. We observed a steady loss of body weight in the liver-specific Arg1 KO mice (high- and mid-dose Cre-injected) over the course of one week, which was virtually identical to what we had observed previously with global Arg1 KO mice that showed progressive loss of food intake/appetite [4,7] (Fig. 2A). The designated humane endpoint in the mice in these two groups (n = 13) was attained at day 21–22 post-Cre injection (Fig. 2B). All mice in the low-dose group were without symptoms at this time point. The only apparent difference between liver-specific KO of Arg1 and global Arg1 loss was that the former mice survived approximately 7–8 days longer (21 vs 13 days from the day of Cre-injection and last tamoxifen dose to induce Cre-mediated deletion, respectively).

Concomitant with weight loss from 2 weeks to 3 weeks post high- and mid-dosing with AAV-TBG-Cre, these liver-specific Arg1 KO mice showed an elevation in blood arginine levels, which was not directly correlated with the dose of AAV-TBG-Cre (Fig. 3).

3.2. Gene therapy to treat Arg1-deficient mice

Since the liver-specific KO mice appeared to show the same phenotype as global Arg1 KO mice, we decided to extend our studies with the latter group of mice to improve outcomes using our gene therapy protocol delivery of an rh10 serotype AAV-Arg1-eGFP vector previously described [7]. Here, we dosed male Arg1-Cre mice with this vector (n = 10) or an empty GFP-expressing vector (n = 3) two weeks prior to inducing the knockout with tamoxifen to allow for adequate expression of the transgene. All GFP vector-treated mice died at the usual time point (13 days from the last dose of tamoxifen) with virtually no Arg1 liver enzymatic activity (Fig. 4A, B). In contrast, only 2 of 10 mice, with low Arg1 enzyme activity, died at this early time point in the AAV Arg1-eGFP gene therapy group (Fig. 4A, B). The remaining 8 mice all survived long-term ≥3 months) and were euthanized at different time points for a variety of reasons (Table 1). Six of the long-term survivors had liver Arg1 enzymatic activity levels in the normal WT physiological range (8–11 units in standard enzyme assay; Fig. 4A), while two were in the supra-physiological range when euthanized. High expression of Arg1-

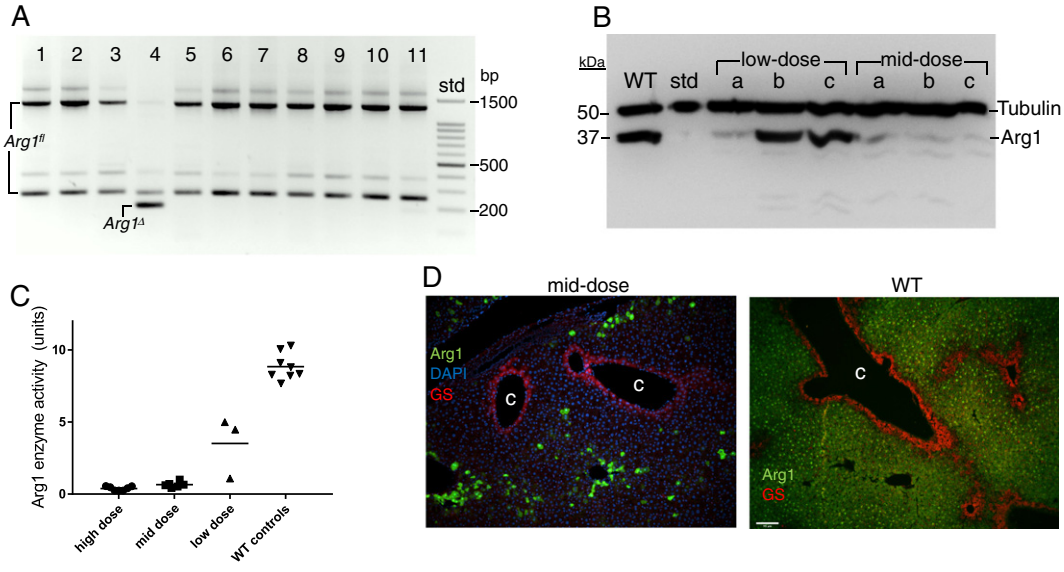

Fig. 1. AAV-TBG-Cre delivery results in loss of liver Arg1 expression. A. PCR genotyping of 11 tissues from a representative mouse injected with high-dose AAV-TBG-Cre. Lanes: 1, brain; 2, heart; 3, lung; 4, liver; 5, kidney; 6, spleen; 7, small intestine; 8, esophagus; 9, tongue; 10, skeletal muscle; 11, tail. The 195 bp band is diagnostic for the $Arg1^{\Delta}$ allele, while the 252 bp and 1.2 kb bands represent the $Arg1^{fl}$ allele. B. Western blot analysis of Arg1. Liver extracts from three low-dose Cre-injected mice express variable amounts of Arg1, whereas extracts from three mid-dose Cre-injected mice express almost no Arg1 protein. Tubulin is used as a loading control. C. Arg1 enzyme activity assay in the three groups of AAV-TBG-Cre-injected mice euthanized at day 21–22 post-injection (n = 7 high-, 6 mid-, 3 low-dose, respectively). None of the low-dose Cre-injected mice (n = 6) displayed symptoms requiring euthanization at this time point and three in this group were not sacrificed nor assayed for enzymatic activity. Liver Arg1 enzymatic activity from a cohort of untreated animals is shown for comparison on the right. D. Immunostaining for Arg1 (FITC, green) and glutamine synthetase (Alexa Fluor 568, red) in liver sections from a low-dose Cre-injected mouse (left panel) and a WT mouse (right panel).

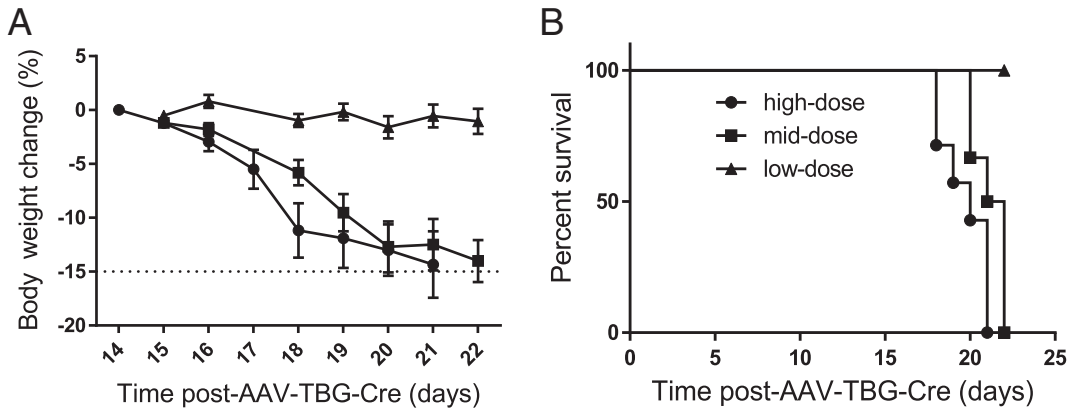

Fig. 2. Liver-specific Arg1 KO leads to weight loss and humane euthanization endpoint. A. Weight loss traces. B. Kaplan-Meier survival curves. Data for high- (n = 7), mid- (n = 6) and low-dose (n = 6) AAV-TBG-Cre-injected mice are shown.

eGFP could be detected in specific regions throughout the liver parenchyma in these successfully-treated mice including adjacent to glutamine synthetase-expressing cells (i.e. cell layer(s) surrounding central veins) with two examples shown (Fig. 4C; mouse R10 after 3 months of transgene expression and mouse R4 after 7 months).

Blood arginine levels at endpoint in the high-level Arg1-eGFP transgene-expressing mice (137 ± 26 μM; range 64–242 μM; n = 8) were not significantly different from untreated WT C57BL/6 mice (107 and 117 μM; n = 2) or untreated $Arg1^{fl/fl}$ mice (see Fig. 3; n = 15) and were significantly less than in the eGFP transgene-treated mice (807 ± 117 μM; n = 3, $p < 0.001$; see Table 1).

3.3. Hepatocyte transplantation to treat Arg1-deficient mice

We sought to determine whether transplantation of histocompatible Arg1-expressing WT C57BL/6 hepatocytes could rescue the phenotype of Arg1 deficiency. In pilot experiments, we tested various protocols that would enhance the efficacy of re-population of donor cells as other groups had carried out with retrorsine, a cell cycle inhibitor, combined with partial hepatectomy [14]. We selected two cycles of retrorsine treatment followed by two-thirds liver removal and infusion of 2×10^6 donor cells into the spleen. Mice were then allowed to recover for 15 weeks at which time tamoxifen-induced *Arg1* gene disruption was carried out. Non-retrorsine-treated mice with partial hepatectomy lost body weight and had to be euthanized 15 days post-tamoxifen. Mice in this group assayed for liver Arg1 activity displayed low enzyme activity (Fig. 5A, B). Of the 9 retrorsine-treated mice, 5 displayed significantly more enzyme activity (>2 units) than typical Arg1 deficient mice (<1 unit) and lifespan was extended up to an extra week (day 20 post-tamoxifen). These mice showed up to 5% re-population of liver Arg1 expression compared to WT controls as determined by immunostaining (Fig. 5C). Large numbers of "islands" positive for staining were detected throughout the regenerated liver parenchyma. After examination of liver sections, it was determined that all hepatocyte-transplanted mice showed normal lobular architecture with portal tracts and central veins with no detected inflammatory cell infiltrates. Blood arginine levels were significantly elevated 14–20 days post-tamoxifen treatment in the retrorsine-treated, repopulated recipient animals compared to "baseline" levels shortly after tamoxifen treatment or to vehicle-treated $Arg1^{fl/fl}$ mice (Fig. 5D).

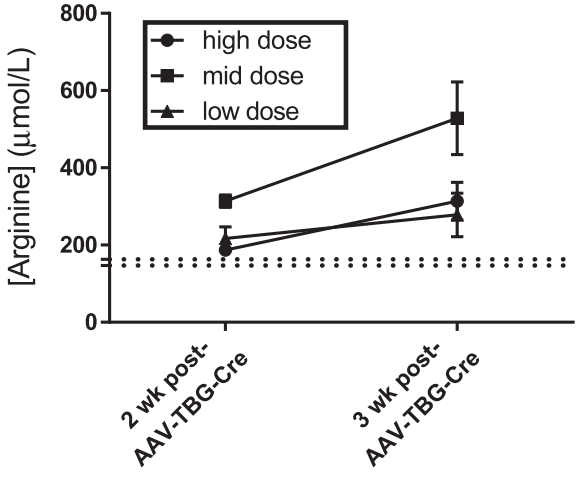

Fig. 3. Blood arginine levels at two time points post-AAV-TBG-Cre injection (n = 7 high-, 6 mid-, 3 low-dose, respectively). Samples were analyzed from dried blood spots by mass spectrometry as described in the Methods section. The two dotted lines represent arginine levels in two cohorts of untreated $Arg1^{fl}$ mice (n = 15 total) measured on two separate occasions.

4. Discussion

The key findings of the present studies are: (i) liver-specific KO of Arg1 in mice is lethal and leads to a phenotype that appears to be very reminiscent to that of the induced global Arg1 KO mouse phenotype; (ii) gene therapy with AAV vectors carrying an Arg1-eGFP expression construct can correct the phenotype in most male mice but this is not lifelong in duration; and (iii) syngeneic hepatocyte transplantation to restore Arg1 expression in the induced Arg1 deficient mouse model, despite radical steps to induce cell engraftment, remains a significant challenge.

The high- (1.5×10^{11} gc) and mid- (5×10^{10} gc) AAV-TBG-Cre dosing regimens were very efficient at producing liver-specific KO of Arg1 (>90% Arg1 protein loss; indistinguishable from background hepatocyte Arg1 enzyme activity level in global induced Arg1 KO mouse model). Liver-specific Arg1 KO mice appear to phenocopy the induced global Arg1 KO mice in most respects, to the best of our knowledge. A major biochemical factor in the tissue-specific KO is elevated blood arginine, which translates into a wasting phenotype. We had previously hypothesized how this might occur [4,7] but we are still lacking mechanistic insight. Arginine is a central amino acid in the regulation of mammalian cell physiology and can act as a sensor through various proteins to signal via the mammalian target of rapamycin complex 1 (mTORC1) to regulate cell growth/autophagy/nutrient sensing/protein synthesis [16,17]. It is possible that the wasting phenotype in the tissue-specific and global induced KO models is due to dysregulation of this pathway in the presence of elevated arginine levels. Blood ammonia levels rise after the wasting phenotype has been irreversibly initiated and may contribute to the very late-stages of demise of these mice [4,5,7].

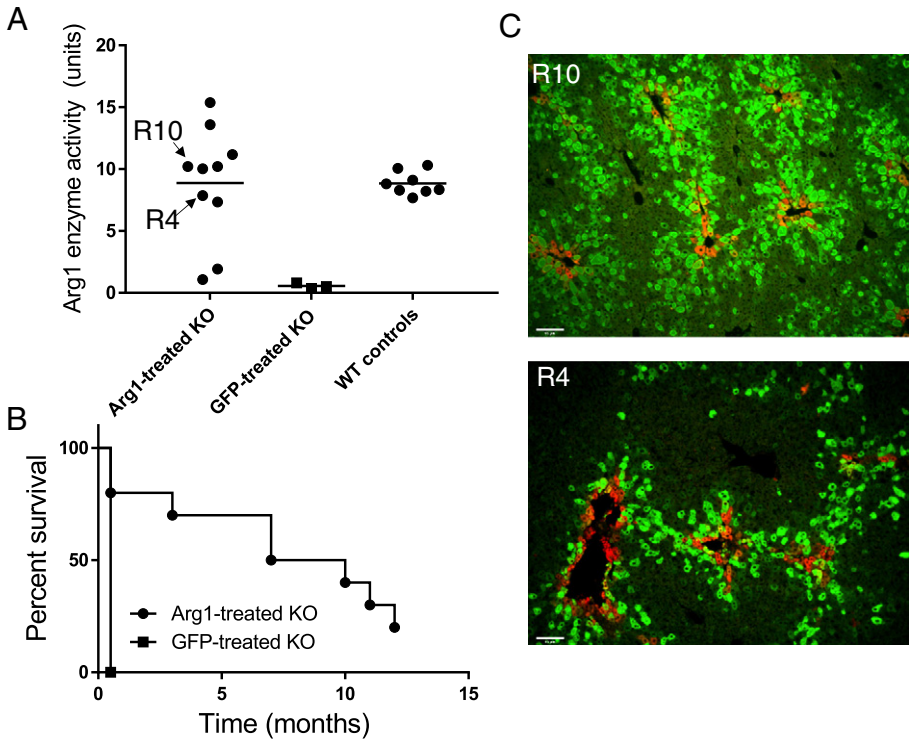

Fig. 4. AAV transgene delivery of Arg1-eGFP restores Arg1 hepatic enzyme activity and prolongs lifespan relative to control eGFP-treated mice. A. Arg1 hepatic enzymatic activity measured at endpoints in two groups (Arg1-eGFP treated, n = 10 and eGFP-treated, n = 3), as well as from a cohort of untreated animals (same as in Fig.1C) for comparison. B. Corresponding Kaplan-Meier survival curves for two groups of mice. C. Immunostaining for Arg1 (FITC, green) and glutamine synthetase (Alexa Fluor 568, red) in liver sections from representative mice; mouse R10 (euthanized after 3 months of transgene expression from unrelated skin lesions; top panel) and mouse R4 (euthanized after 7 months of transgene expression; bottom panel). See Table 1 for details on individual mice.

L-Arginine is also recognized as a very potent insulin secretagogue from pancreatic islet cells [18,19]. Moreover, injection of L-arginine at high doses (2 × 4 g/kg 1 h apart), but not the D-enantiomer, into C57BL/6 mice evokes acute pancreatitis and subsequent pancreatic injury, which is followed by lung damage [20]. It is possible that hyperargininemia in the liver-specific Arg1 KO mice is evoking pancreatic problems, which then affect feeding and metabolism.

Due to the complex and severe nature of the phenotype of Arg1 deficiency in mice, it has been very challenging to rescue completely all the features of the disorder. We have tried at least five different non-gene therapy approaches, which were largely unsuccessful [7]. Others [9,10] and our group [7] have had successes with AAV vector delivery in the "juvenile" model and in the global induced Arg1 KO model, respectively. Previously, in a study of 44 mice we showed partial rescue of ≈25% of male mice with no rescue of female mice using a rh10 serotype vector using a strong hybrid promoter [7]. Here, with 10 additional male mice, we demonstrated an 80% "correction" that was sustained over several months to a 1 year designated endpoint. We are not certain as to why there was greater success here than in our previous study since the same vector preparation was used for transgene delivery

Table 1
Treatment of Arg1-cre mice with an AAV-delivered Arg1-eGFP transgene extends lifespan of mice with tamoxifen-induced Cre-mediated Arg1 deficiency.

#	AAV Vector	Time of death (days)[a]	Clinical characteristics[a]	Blood [arg] (μM)[b]
R1	Arg1-eGFP	330	Loss of BW; a few lobular inflammatory foci with $CD4^+$ lymphocytes and Kupffer cells pericentral	121
R2	Arg1-eGFP	13	Loss of BW; mild portal and pericentral foci of mononuclear inflammation; some $CD4^+$ cells seen pericentrally	162
R3	Arg1-eGFP	210	Loss of BW; no inflammatory cells; no $CD4^+$ staining	64
R4	Arg1-eGFP	210	Loss of BW; mild portal predominant foci of mononuclear chronic inflammation including $CD4^+$ cells	64
R5	eGFP	13	Loss of BW	653
R6	eGFP	13	Loss of BW; one single focus of mononuclear cells including lymphocytes, plasma cells and Kupffer cells	1036
R7	eGFP	13	Loss of BW; no $CD4^+$ staining	733
R8	Arg1-eGFP	300	Bloody urine; undefined tumor; no $CD4^+$ staining	220
R9	Arg1-eGFP	13	Loss of BW; mild-moderate portal and periportal inflammation; minimal $CD4^+$ staining	489
R10	Arg1-eGFP	90	Skin lesions; no inflammation; no $CD4^+$ staining	242
R11	Arg1-eGFP	365	Hepatocellular adenoma; focal mild-moderate macrovesicular steatosis; no inflammation; no $CD4^+$ cells	172
R12	Arg1-eGFP	365	Moderate-severe macrovesicular steatosis; mild portal predominant foci of mononuclear chronic inflammation including $CD4^+$ cells; urinary tract blockage	88
R13	Arg1-eGFP	365	No overt problems; no inflammation; no $CD4^+$ cells	126

[a] Time of death/euthanization is relative to the last tamoxifen dose. Mice were euthanized when humane endpoints relating to loss of body weight (BW) were reached or when mice were on study for 1 year from the last tamoxifen dose. The clinical characteristics reported do not strictly identify exact cause of death. AAV integration can occur in the AAV-HCC locus of mice and initiate hepatocellular carcinoma in a small number of hepatocytes, which leads to clonal expansion [22]. The two tumors found in this study are likely consistent with vector integration in this locus and not due to the expressed arginase transgene. Liver sections of mouse R5 were not investigated.

[b] Blood arginine [arg] levels were measured at endpoint. Two non-treated C57BL/6 control mice at a comparable age (10 months) had levels of 107 and 117 μM.

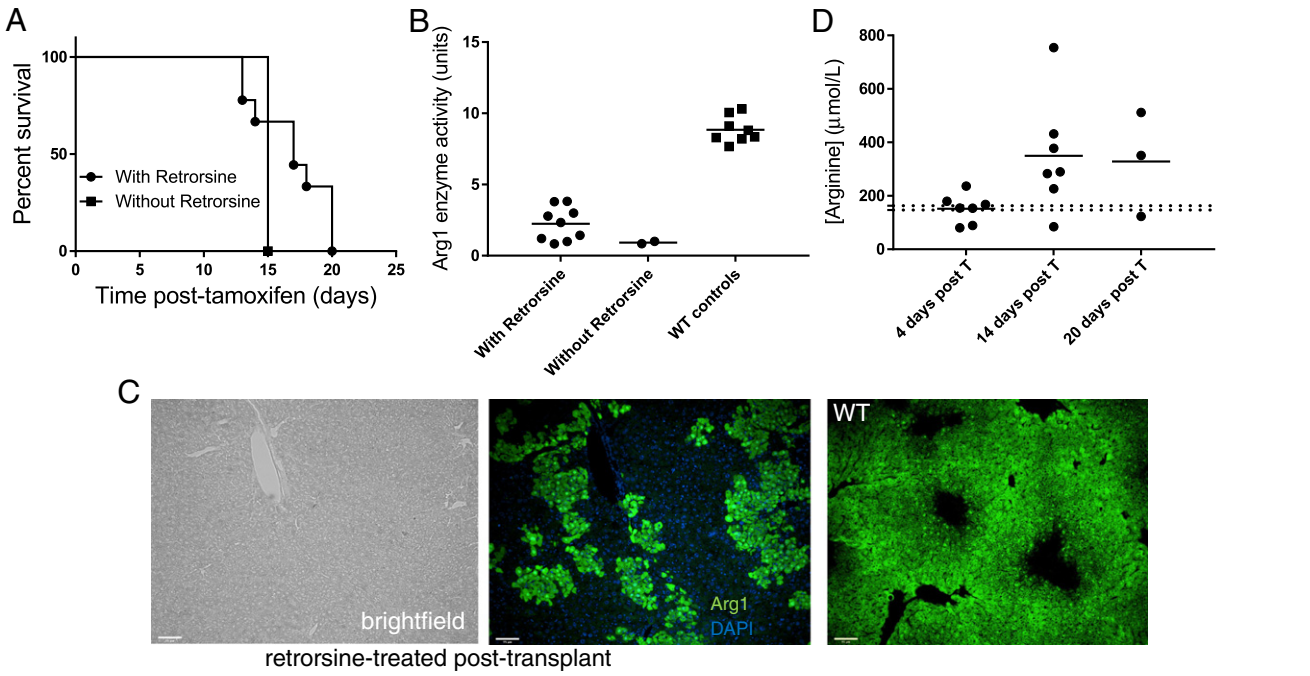

Fig. 5. WT Arg1-expressing hepatocyte delivery only modestly extends lifespan of induced Arg1 deficiency despite significant re-population of donor livers. A. Kaplan-Meier survival curves of mice (n = 9, retrorsine-treated; n = 3, non-retrorsine-treated). B. Arg1 enzyme activity in livers of hepatocyte-repopulated mice at endpoint, as well as from a cohort of untreated animals (same as in Fig.1C) for comparison. C. Arg1 immunostaining (FITC, green) in a liver section from a representative hepatocyte-transplanted Arg1-deficient recipient mouse 15 weeks post-transplantation (middle panel) and 20 days post-tamoxifen-induced Arg1 knockout, along with corresponding low-magnification brightfield image (left panel) and a section from a non-treated C57BL/6 WT control mouse (right panel). D. Blood arginine levels at three time points post-tamoxifen (T) in retrorsine-treated mice. The two dotted lines represent arginine levels in two cohorts of untreated $Arg1^{fl}$ mice (n = 15 total) measured on two separate occasions, as in Fig.3.

with only slightly varied "optimized" conditions. Arginase expression seems to be widespread throughout the liver parenchyma early after vector delivery in key cells carrying out urea cycle function and apparently in most hepatic zones (both portal and central vein regions) but appears to be lost in key portal circulation zones over time, presumably due to hepatocyte regeneration in these zones, with consequent loss of Arg1-transgene expression in these cells, which leads to loss of urea cycle function and elevation of blood arginine. There was no clear correlative indication of an immune response to either the AAV vector itself or to the expressed transgenes (either Arg1-eGFP or eGFP alone) with about half the mice showing inflammatory cell infiltrates early (day 13) and late (>3 months) and half showing no signs of $CD4^+$ lymphocyte infiltration (see Table 1). eGFP is only minimally immunogenic in C57BL/6 background mice [21].

In the hepatocyte transfer studies, we were unable to achieve sufficient, widespread expression of WT Arg1-expressing cells throughout the liver when we induced *Arg1* gene disruption with tamoxifen to rescue the knockout mice. Even though we created a selective growth advantage for the transplanted cells by partial hepatectomy and inhibition of proliferation of native cells expressing Arg1 floxed alleles with retrorsine, lifespan of the KO mice was extended by only several days. Five (of 9) hepatocyte-transplanted mice displayed reasonable total hepatic Arg1 expression (>15% of normal liver Arg1 enzyme activity), which we surmise should be sufficient to maintain essential urea cycle function. Thus, we can think of two scenarios as to why the mice are still not "rescued": (i) the transplanted cells that migrated to the liver after intrasplenic injection did not fully recapitulate the normal liver distribution of arginase and thus there is not sufficient Arg1 enzyme in the correct metabolic zones; (ii) that extra-hepatic Arg1 activity is also necessary for full "rescue" in this particular mouse model. This certainly raises more questions than answers. On the one hand, loss of liver arginase expression appears to be both necessary and sufficient to explain the lethal phenotype of the genetic disorder in mice. On the other hand, gene and cell-directed therapies are pointing towards the notion that rescue of extra-hepatic Arg1 expression may also be necessary for disease correction.

Significant challenges lie ahead to rescue the lethal phenotype of global induced Arg1 or liver-selective Arg1 deficiency in mice.

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