Transplantation of Gene-Edited Hepatocyte-like Cells Modestly Improves Survival of Arginase-1-Deficient Mice

Yuan Yan Sin,1 Laurel L. Ballantyne,1 Christopher R. Richmond,1 and Colin D. Funk1

INTRODUCTION

Progress in gene editing research has been accelerated by utilizing engineered nucleases in combination with induced pluripotent stem cell (iPSC) technology. Here, we report transcription activator-like effector nuclease (TALEN)-mediated reincorporation of Arg1 exons 7 and 8 in iPSCs derived from arginase-1-deficient mice possessing Arg1 KO alleles lacking these terminal exons. The edited cells could be induced to differentiate into hepatocyte-like cells (iHLCs) in vitro and were subsequently used for transplantation into our previously described (Sin et al., PLoS ONE 2013) tamoxifen-inducible Arg1-Cre arginase-1-deficient mouse model. While successful gene-targeted repair was achieved in iPSCs containing Arg1 KO alleles, only minimal restoration of urea cycle function could be observed in the iHLC-transplanted mice compared to control mice, and survival in this lethal model was extended by up to a week in some mice. The partially rescued phenotype may be due to inadequate regenerative capacity of arginase-1-expressing cells in the correct metabolic zones. Technical hurdles exist and will need to be overcome for gene-edited iPSC to iHLC rescue of arginase-1 deficiency, a rare urea cycle disorder.

Arginase deficiency is a rare urea cycle disorder with hyperargininemia and profound neurological impairment as hallmark features.20 Arginase-1-deficient mouse models have been created, and they exhibit a profound lethal phenotype approximately 2 weeks after birth in the global knockout (KO) mice or after induction of tamoxifen-induced gene KO in adult mice (about 2 weeks after induced KO).21–23 Recently, we generated induced pluripotent stem cells (iPSCs) from our inducible arginase-1-deficient mouse model carrying a deletion of Arg1 exons 7 and 8 (Arg1 KO), which results in defective function of arginase-1.24 We showed that the deletion was repaired using CRISPR/Cas9, in combination with an excisable piggyBac transposon system to target corrective sequences to the endogenous Arg1 locus. In the current study, we report on...
TALEN-mediated correction of the Arg1<sup>d</sup> alleles in iPSCs and their successful differentiation to hepatocyte-like cells (HLCs) coupled with piggyBac transposon selection technology for seamless genetic manipulation, followed by secondary expansion to propagate the repaired iPSC-derived HLCs (named iHLCs hereafter). The genetically repaired iHLCs were transplanted to restore arginase-1 expression in Arg1<sup>d</sup> mice, and survival of the arginase-1-deicient mice was extended by up to a week in some mice. Although an elevation in arginase-1 expression was observed in the iHLC-transplanted mice, urea renewal capacity and their differentiation potential, we examined a TALEN-mediated correction of the Arg1<sup>d</sup> locus (Figure 1E).

**RESULTS**

**Design of TALEN-Targeting Strategy for Correcting Arg1-Deficient (Arg1<sup>d</sup>) iPSCs**

We designed a TALEN pair targeting intron 6 of the Arg1 locus at a region adjacent to the position targeted previously by CRISPR/Cas9. Each monomer contains an array of RVDs to bind the target DNA sequences (Figure 1A). The TALEN expression constructs showed good transfection efficiency when introduced into mouse iPSCs by electroporation (Figure 1B). Surveyor nuclease cleavage to detect NHEJ events of PCR products from the target region produced two bands in the 162- to 186-bp range, with insertion or deletion (indel) frequencies up to 18% in mouse iPSCs (Figure 1C), similar to that with CRISPR/Cas9. The TALEN pair target sites were selected to utilize a BspHI restriction site located within the spacer region to determine editing efficiency. The loss of the BspHI recognition sequence was demonstrated by the presence of uncleaved PCR products compared to the control (Figure 1D). Sequencing results confirmed that the TALENs induced DSBs at the predetermined position in the Arg1 locus (Figure 1E).

**TALEN-Mediated Reincorporation of Deleted Exons via HDR**

By taking advantage of iPSCs and their propensity for unlimited self-renewal capacity and their differentiation potential, we examined a TALEN-mediated targeted knockin approach for deleted exons 7 and 8 of Arg1 in Arg1<sup>d</sup> iPSCs. TALEN-expressing vector delivery, together with a linearized targeting vector, which consists of exons 7 and 8 cDNA fused to an RFP monomeric-encoding segment with piggyBac inverted terminal repeats (ITRs) flanking a PGK-Puro-TK cassette, was performed (Figure 2A). An HDR stimulatory compound, L-755,507, was added into the culture to enhance TALEN-mediated HDR efficiency. Upon cleavage by TALENs and subsequent HDR with the repair template, exons 7 and 8 should be reincorporated into the Arg1 locus. Eleven puromycin-resistant iPSC colonies were obtained and screened for proper targeting by PCR. We designed two primer pairs spanning the junction between the insert and endogenous sequence in both homologous arms as indicated in Figure 2A to examine correct integration. Of the 11 clones analyzed, two were correctly targeted (clones #T4 and #T10; efficiency of 18.2%) for both PCR primer sets (Figure 2B). These two clones were subjected to piggyBac-mediated excision of the integrated selection cassette. Upon ganciclovir selection, PCR screening results showed good transfection efficiency. Images were acquired 24 hr post-electroporation. Scale bars, 95 μm.

![Figure 1. TALEN-Mediated Gene Targeting in Arg1<sup>d</sup> Mouse iPSCs](image-url)

(A) Schematic diagram showing the site of Arg1 gene modification using TALEN set 7/8. The TALEN 7/8 pair was designed to target intron 6 of Arg1. Each TALEN arm consists of a DNA-binding domain with repeat variable di-residues (RVDs) corresponding to DNA binding sequence proceeded by a 5’ T nucleotide and a 17-bp spacer region containing a BspHI recognition site (highlighted in yellow) to assay activity. TALE repeat domains are colored to indicate the identity of the RVD. Site-specific double-stranded breaks (DSBs) are generated upon dimerization of fused FokI endonucleases. (B) Electrotransfection to deliver 7 μg of each TALEN and pMAX-GFP into cells to assess transfection efficiency. Images were acquired 24 hr post-electroporation. Scale bars, 95 μm. (C) Surveyor nuclease assay for detection of NHEJ-induced indels resulting from DSBs. The cleavage products were shown as extra bands (between 162 and 186 bp) indicated by the arrows. Mutation frequencies (indels %) were calculated by measuring the band intensities. (D) BspHI digestion results. The cut products were shown as extra bands (169 bp + 183 bp). (E) Sequencing data from PCR amplicons of TALEN-modified genomic DNA showing a few examples of NHEJ-mediated indel mutations at the desired location. The wild-type sequence is shown above in red. Deleted bases are indicated by colons, and inserted bases are shown by lowercase letters in blue. The net change in length caused by each indel mutation is to the right of each sequence.
of 17 ganciclovir-resistant colonies revealed three with biallelic repair (Figure 2C). All three completely repaired clones were from the #T4 line (Figure 2D). Our TALEN-mediated targeting experiments demonstrated HDR knockin efficiency comparable to that of the CRISPR/Cas9 system.24 Sequence analysis showed the presence of a single TTAA sequence26 at the site of transposon excision in the repaired cell lines (clones #T4–7, #T4–12, #T4–17), an evidence for scarless removal of selectable marker cassettes following successful HDR (Figure 2E).

Transplantation of Repaired iHLCs Extends Survival in Some Mice after Induced Arginase-1 Loss of Expression

To facilitate iPSC-mediated ex vivo gene therapy, efficient differentiation into the proper cell type after gene repair is essential. Considering the challenges in coaxing iPSCs to mature functional hepatocytes in culture in our recent work,24 we attempted to test whether iHLCs can mature after transplantation and if Arg1 gene editing could lead to the restoration of functional arginase in the liver. Repaired clones (#T4–7 and #T4–12) were subjected to hepatic differentiation using a stepwise protocol, which includes hepatic specification, hepatoblast formation, and iHLC expansion (Figure 3A). At the end of the 25-day differentiation protocol, 2 million iHLCs were transplanted into Arg1-Cre mice via splenic administration. More importantly, two cycles of retrorsine treatment followed by partial hepatectomy was performed prior to transplantation to stimulate engraftment and regenerative capacity.27 Mice were then allowed to recover for 15 weeks, at which time the 5-day sequential tamoxifen-induced global Arg1 gene disruption was carried out. Kaplan-Meier survival curves of Arg1<sup>D</sup> mice versus iHLC-transplanted mice began to diverge on day 14 after the last dose of tamoxifen. In fact, the
survival of the iHLC-transplanted mice was extended up to an extra week (day 22 post-tamoxifen) compared to the Arg1−/− mice, which died at the usual time point (around 14 days post-tamoxifen) from a wasting phenotype21–23 (Figure 3B). Successful excision of exons 7 and 8 of Arg1 induced by tamoxifen administration in experimental mice was verified by PCR genotyping of tail samples for the diagnostic band of 195 bp (Figure 3C). Using primers flanking the Arg1 and RFP domains, liver tissue samples obtained from iHLC-transplanted mice yield bands at 668 bp, indicating the presence of the repaired allele (Figure 3D).

**Recovery of Arginase Expression in Livers Repopulated with Transplanted Repaired iHLCs**

Real-time qPCR analysis was carried out to evaluate Arg1 mRNA in liver using primers in the region encoded by exons 7 and 8. Our data show significantly increased Arg1 transcripts in liver tissues of iHLC-transplanted mice compared to non-transplanted mice, confirming the restoration of full-length Arg1 mRNA expression after gene correction (Figure 4A). However, the level was still distinctly lower than in livers obtained from wild-type mice. After excision of the ITR-flanked PGK-Puro-TK cassettes by piggyBac transposase (PBx), the C-terminal Arg1-RFP should be expressed under the control of the endogenous Arg1 promoter. Hence, hepatocytes expressing fusion protein Arg1-RFP were analyzed by western blot analysis. While wild-type liver samples showed a strong immunoreactive 37 kDa Arg1 band, appreciable amounts of Arg1-RFP fusion product were detected in the iHLC-transplanted mice (Figure 4B). Next, we performed immunohistochemical staining in liver sections to detect restored arginase-1 protein and examine repopulation efficiency. As shown in Figure 4C, the results clearly demonstrate that transplantation of the edited iHLCs improves arginase-1 expression as compared to Arg1−/− mice. Moreover, the transplanted iHLCs did not develop into hyperplastic nodules. Interestingly, scattered distribution of arginase-1 expression was observed throughout the regenerated liver parenchyma in the iHLC-transplanted mice with no signs of colocalization with glutamine-synthetase surrounding the central veins. As in sections from wild-type and Arg1−/− mice, immunostaining of liver slices obtained from iHLC-transplanted mice showed only the hepatic marker albumin (Alb) expression and lack of immature hepatocyte-specific marker alpha-fetoprotein (Afp) expression, providing evidence of in vivo maturation of iHLCs. Together, our data indicate that genetically corrected mouse iPSCs can express the Arg1 protein once they differentiate into mature hepatocytes. Finally, we assessed the potential of restored Arg1 for improving functional enzyme activity. Although iHLC-transplanted mice showed significantly higher arginase-1 activity when compared to Arg1−/− mice, the level is still considerably low compared to normal activity (Figure 5A). No significant differences were observed in blood arginine levels in the iHLC-transplanted mice compared to Arg1−/− mice at both baseline (4 days post-tamoxifen administration) and humane endpoint, when arginine levels are substantially increased (Figure 5B).

**DISCUSSION**

In this study, we demonstrate TALEN-mediated gene editing to repair the dysfunctional Arg1−/− allele in iPSCs, in concert with transplantation-based studies. Our data reveal that TALEN-mediated site-specific genome modification in mouse iPSCs was similar in efficiency with the CRISPR/Cas9 system.24 Our application of TALEN-mediated gene repair highlights the feasibility and potential for gene-editing strategies using engineered cell therapies, albeit with modest improvement in survival after iHLC transplantation in the arginase-1 KO mouse model. qPCR and western blot analyses verified the presence of repaired cells expressing arginase-1. Nevertheless, transplantation of repaired iHLCs only resulted in about 5% repopulation of livers, and the lifespan of the transplanted mice could only be modestly extended by up to a week in some mice. The transplanted
cells failed to fully recapitulate the normal liver distribution of arginase-1 in the correct metabolic zones, hence leading to marginal urea cycle function and elevation of blood arginine.

The major challenges highlighted by this study are the low rates of engraftment and the lack of hepatocyte repopulation in the correct liver zones. There are two main metabolic zonations in the liver. Metabolic activities such as glycolysis, lipogenesis, and xenobiotic disposal preferentially localized in perivenous (PV) areas encode glutamine synthetase, GLT1 (a glutamate transporter), and RHBG (an ammonia transporter).28 In contrast, key enzymes of the urea cycle such as ARG1 and carbamoyl-phosphate synthase (CPS1) are preferentially expressed in the periportal (PP) regions.

Intrasplenic transplantation of hepatocytes, which we employed in this study, was reported to facilitate cell integration in PP locations.29 We also performed partial hepatectomy to create a growth advantage for transplanted cells.27 However, we could not achieve optimum engraftment and functional regeneration for long-term therapeutic effects. The success of the hepatocyte infusion protocol is likely to depend on engraftment of sufficient numbers of hepatocytes in the PP loci for optimum arginase-1 enzyme activity. There are several questions to be addressed: (1) What is the optimal differentiation stage of iHLCs to achieve the highest level of engraftment and urea cycle function? (2) As the remaining hepatocytes after partial hepatectomy still have extensive proliferative potential, will they constrain competitive advantage for the transplanted cells? (3) Is there senescence or any immunogenic response to the newly corrected gene product of transplanted iHLCs? One of the major concerns of iPSC development is the potential tumorigenicity of iPSCs and their progeny.32 However, recent findings using in vitro differentiated iPSC-derived cells have sparked optimism over their therapeutic potential. There was no evidence of immune rejection of iPSCs that have matured to an adult fate, including endothelial cells, hepatocytes, and neuronal cells upon transplantation into syngeneic mice.33,34 The discrepancies between those studies may be attributable to different iPSC lines used in their experiments. In this regard, it should be noted that in vitro differentiated gene-edited cells may have distinctive immunogenicity due to genetic manipulation and their long culture time. It remains to be determined whether iHLCs derived from gene-edited iPSCs used in our transplantation studies were immunogenic and if transient immunosuppression is required following transplant. (4) Can zonal regeneration of gene-edited hepatocytes be manipulated to improve therapeutic efficacy? Several signaling pathways have been identified to direct zonal organization including Wnt/β-catenin35,36 and its antagonistic pathway, Ras/MAPK/Erk.37 Further investigation is needed to refine our current understanding in shaping desired metabolic zonation.

In summary, this report demonstrates another successful application of gene editing in iPSCs. Despite the lack of efficient metabolic functional repair after transplantation, the application of gene correction not only
into HLCs might still be a realistic goal for ex vivo gene therapy of liver diseases with further experimental optimization. To our knowledge, these results represent the first description of transplantation using cells derived from HDR-mediated repair of iPSCs for arginase-1 deficiency. The development of efficient targeted gene editing using TALENs and CRISPR/Cas9 systems could open exciting new avenues for arginase-1 gene therapy.

MATERIALS AND METHODS

Mice and Cell Source

The inducible Arg1-deficient mouse strain (herein referred to as Arg1-Cre mice), derived from parental strains Arg1fl/Y (JAX strain 008817, C57BL/6-Arg1tm1Pmu/f) and CreER2 (JAX strain 008463, B6.129-Gt(ROSA)26Sortm1(Lev/)J) were injected intraperitoneally (i.p.) on 5 sequential days with tamoxifen to induce global Arg1 deficiency (herein referred to as Arg1Δ mice) as previously described. All procedures were reviewed and approved by the Queen’s University Animal Care Committee (approval #Funk-2011-048-R1-A4) and conformed to the Guidelines of the Canadian Council on Animal Care.

Design and Assembly of TALENs Targeting the Mouse Arg1 Gene

A pair of TALENs (left TALEN 5’-GCTTTGTGTGGCGAGT-3’, right TALEN 5’-TCTTGACATTCTCTGCA-3’) were designed and assembled to target a region close to exon 6 in the Arg1 gene using the GoldmanTALEN scaffold method. The TALEN target site comprises binding sites for two TALE-FokI fusion proteins that are each preceded by a T at the 5’ end and a unique restriction enzyme (BspHI) recognition site within the spacer region. Specificity of TALENs was examined using NCBI Primer-BLAST. Potential off-target sites were assessed in silico using the Paired Target Finder tool on the TAL Effectr Nucleotide Targeter 2.0 web interface to scan the mouse genome for sequences containing mismatches within the TALEN pair target site. The repeat variable di-peptide (RVD)-containing units for TAL-7 (NN HD NG NG NN NG NN NG NN HD NN NI NI NN NG) and TAL-8 (NG NN NI HD NI NN NN NI NI NG NN NG HD NI NN NN NI) were assembled using the Golden Gate approach. After assembly, the RVDs were cloned into the pC-Goldy TALEN destination vector for expression in mammalian cells.

Functional Evaluation of TALEN Cutting Efficiency

Arg1Δ iPSCs (3 × 10⁶) were electroporated with equal amounts of each TALEN plasmid DNA (7 μg each) to induce double-stranded breaks (Gene Pulser System, 250 V, 500 μF, 0.4 cm cuvettes) (Bio-Rad, Mississauga, ON, Canada). Genomic DNA from the electroporated cells was isolated 2 days later. The genomic region surrounding the TALEN target site (within intron 6 and 3’ UTR of the Arg1 gene) was amplified with Phusion high-fidelity DNA polymerase (NEB, Ipswich, MA). Primers used were as follows: forward 5’-CTAACCGTCATTACCTCACTCTG-3’, reverse 5’-GCACGTCTAAAGCCCAGAGATTC-3’. Purified PCR products were subjected to a re-annealing process using a step gradient (95°C–25°C over 30 min) to enable heteroduplex formation. For mismatch cleavage assays, the annealed products were treated with Surveyor nuclease (Transgenomics, Omaha, NE), and resultant cleavage products (two bands at 162 bp and 167–185 bp) were analyzed in 8% Tris-borate-EDTA polyacrylamide gels. The indel efficiency was quantified based on the relative band intensities measured using Quantity One Software (Bio-Rad, Mississauga, ON, Canada). The indel percentage was calculated using the following formula: 100 × (1 – (b + c)/(a + b + c))¹/², wherein a is the intact band and b and c represent the Surveyor nuclease digestion products. The PCR products were also assessed for gene modification by BspHI (NEB, Ipswich, MA) digestion, which gave rise to 183 and 169 bp fragments. To confirm TALEN-mediated gene modification, PCR amplicons were subcloned into pCR 2.1-TOPO TA vector (Invitrogen, Carlsbad, CA) and individual colonies were subjected to sequence analysis.

TALEN-Mediated Gene Targeting and Excision of Selection Markers Using PBx

A custom-designed repair targeting vector, with Arg1 homology arms, which consists of exons 7 and 8 cDNA fused to the coding sequence of monomeric RFP, a hybrid PGK-EM7 promoter, a positive-negative drug resistance cassette carrying a puromycin-thymidine kinase resistance gene, and a self-cleaving 2A peptide (T2A), all flanked by the piggyBac transposon ITRs (Transposagen,
Differentiation of Mouse iPSCs into HLCs In Vitro

TALEN-mediated gene-edited cells were differentiated into HLCs using a modified stepwise protocol as previously described. In brief, mouse iPSCs were trypsinized into single-cell suspensions and resuspended in advanced RPMI containing 10% fetal bovine serum (FBS) (Wistat, St-Bruno, Quebec, Canada), 100 ng/mL activin A (R&D Systems, Minneapolis, MN), and 50 ng/mL Wnt3a (R&D Systems, Minneapolis, MN). One million cells per well were seeded into 12-well plates coated with 2% Matrigel (BD Biosciences, Mississauga, ON, Canada) and incubated at 37°C/5% CO2 for direct definitive endoderm (DE) induction. FBS was reduced to 0.2% on the following day until day 5. On day 6–10, the medium was replaced with hepatic commitment medium (advanced RPMI, 2% FBS, 50 ng/mL BMP4 (PeproTech, Montreal, Quebec, Canada), 20 ng/mL FGF-2, (PeproTech, Montreal, Quebec, Canada). On day 11–15, the medium was replaced with advanced RPMI containing 2% FBS, and 20 ng/mL hepatocyte growth factor (HGF; PeproTech, Montreal, Quebec, Canada) to promote the expansion of early hepatic progenitor cells. Cells were maintained in advanced RPMI supplemented with 2% FBS, 20 ng/mL HGF, 20 ng/mL Oncostatin M (R&D System, Minneapolis, MN), 50 nM dexamethasone (Sigma-Aldrich, St. Louis, MO), and 1× insulin-transferrin-selenium (Corning, Corning, NY) for at least 10 days prior to transplantation.

iHLC Transplantation Studies

Twelve-week-old female Arg1-Cre mice were used as recipients. The mice were conditioned with retransorine (70 mg/kg i.p., twice at 2 week intervals) (Sigma-Aldrich, St. Louis, MO) prior to transplantation. Two-thirds partial hepatectomy was performed after the last retransorine dose to create a selective growth advantage for transplanted cells. In brief, mice were anesthetized with isoflurane/O2 and 20 mg/kg subcutaneously (s.c.) of Tramadol for pre-surgery analgesia. The left lobe and median lobes were ligated with a silk filament at the base before resection. Two million iHLCs (donor cells) were suspended in 100 μL of Hanks’ Balanced Salt Solution (HBSS) and injected slowly over 20–30 s into the lower pole of the spleen. All operations were performed on a heating pad with sterile surgical techniques. Immediately after surgery, mice received 1 mL of warmed saline and 2.5 mg/kg Meloxicam. Five hundred microliters of saline, Tramadol, and Meloxicam were administered for the next 3 days. The following experimental groups were defined: group 1-wild-type (n = 3), group 2-Arg1 knockout (n = 3), and group 3-transplantation of repaired iHLCs (n = 10). Fifteen weeks later, allowing sufficient time for donor cell engraftment, the 5-day sequential tamoxifen-induced Arg1 KO regimen was carried out. Changes in body weight of mice were carefully monitored daily during the experimental period. Humane endpoints were defined as body weight loss of >15% relative to the weight at the time of final tamoxifen administration, accompanied by hunched posture. The endpoint took place between days +11 to +14 with the mean at day +13. Hence, lifespan extension of mice that underwent iHLC transplantation was recorded from this time point (day +13).

PCR Genotyping

Genomic DNA from tail biopsies was extracted by standard protocols and subjected to PCR for genotyping using primer sets (Integrated DNA Technologies, Coralville, IA) as follows: F1 5’-TGCGAGTT CATGACTAAGTTT-3’, R1 5’-AAAGCTCAGGGTGAATCG-3’, and R2 5’-GCCAGTGTTAAGCGAGCT-3’. Cycle parameters were as follows: denaturation at 94°C for 30 s, annealing at 64.5°C for 1 min, and elongation at 72°C for 1 min for 35 cycles.

Liver Engraftment Analysis by Immunohistochemistry

Liver tissues were collected immediately at the time mice were sacrificed. Paraformaldehyde-fixed and paraffin-embedded mouse liver sections (4 μm thick) were stained following standard immunohistochemical protocols, performing heat-induced antigen retrieval (10 mM citrate buffer [pH 6.0], 0.02% TWEEN 20) before incubation with primary antibodies. IHC analysis (rather than direct fluorescence detection of red fluorescent protein [RFP]) was preferred as livers exhibit high autofluorescence. Primary antibodies used in this study were as follows: Arg-1 (Abcam, Cambridge, MA, ab91279, 1:200), glutamine synthetase (Abcam, Cambridge, MA, ab64613, 1:200), Alb (Abcam, Cambridge, MA, ab19196, 1:400), Afp (Novus Biomedical Technologies, Coralville, IA).
Biologicals, Littleton, CO, #NBP-762755, 1:200). Fluorescent secondary antibodies, Alexa Fluor 488 goat anti-rabbit immunoglobulin G (IgG) (Molecular Probes, Eugene, OR, #A-11068, 1:400), Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes, Eugene, OR, #A-11005, 1:400), and Texas Red goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, #111-075-144, 1:200) were used for primary antibody detection. Slides were then dehydrated and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA). Visualization was performed with a fluorescent microscope (Leica, DM IRB, Richmond Hill, ON).

Gene Expression Analysis
LIVER tissues were pulverized in liquid nitrogen prior to RNA extraction. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), followed by RNA cleanup using a GeneJET RNA Purification Kit (Fisher Scientific, Unionville, ON, Canada) and treated with DNase I (1 μg/μL, Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. RNA quality was assessed with the RNA 6000 Nano Kit (Agilent Technologies, Mississauga, ON, Canada). cDNA was synthesized from 1 μg of total RNA using an iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada). qPCR was performed using a thermal cycler (Applied Biosystems Model 7500) with SYBR Green PCR master mix (Bio-Rad, Mississauga, ON, Canada). Western Blot Analysis
Liver tissues were pulverized in liquid nitrogen and homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer (Millipore, Bedford, MA), including protease inhibitor cocktail (Roche, Mississauga, ON, Canada). Twenty-micrograms of centrifuged, clarified protein samples were subjected to western blot analysis and probed with rabbit polyclonal anti-Arg1 antibody (C-terminal) (Abcam, Cambridge, MA, #ab91279, 1:10,000), mouse monoclonal anti-RFP antibody (Rockland Immunochemicals, Limerick, PA, #200-301-3795, 1:2,000), and mouse monoclonal anti-z-tubulin antibody (Sigma-Aldrich, St. Louis, MO, #T5168, 1:5,000) used as loading control. Immunoreactive proteins were detected using horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (Sigma-Aldrich, St. Louis, MO, 1:5,000) and visualized by enhanced chemiluminescence detection (GE Healthcare, Mississauga, ON, Canada).

Arginase Activity Assay and Biochemical Analysis
Arginase activities of all samples were assayed as described previously.21,30 One unit of activity is defined as 10 nmol urea/μg protein. Whole-blood samples were obtained from the submandibular vein on day 4 and 14 following the final tamoxifen (or vehicle) injection and humane endpoint (for mice surviving beyond day 14 post-tamoxifen administration). Drops of blood were collected into microcapillary tubes prior to transfer onto the Whatman 903 filter paper cards (GE Healthcare, Mississauga, ON, Canada). Sample preparation was based on the method described previously30 prior to analysis by mass spectrometry.

Statistical Analysis
All experiments were performed at least in three biological replicates. Survival curves were computed in each group of mice using the Kaplan-Meier method and compared across groups using the log rank test. All results are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Means were compared using the two-tailed Student’s t test. p values of <0.05 were considered statistically significant.

ACKNOWLEDGMENTS
Y.Y.S. is supported by a fellowship from the Urea Cycle Disorders Consortium (UCDC; U54HD061221), which is a part of the NIH Rare Disease Clinical Research Network (RDCRN), the National Center for Advancing Translational Science (NCATS), and the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD). This work was supported by a microgrant from the Rare Disease Foundation (ORDR), the BC Children’s Hospital Foundation (BCCHF) (#18-19 to Y.Y.S.). C.D.F. is supported by the Canada Research Chairs program and the Canadian Institutes of Health Research (CIHR) (MOP-341036).

REFERENCES


