**Transplant Date: September 7, 2021**

**Protocol Number:** AMI-018-006

**Study Number:** AMI-018-006-127

**Title of Study:** Transplantation with PHH lot DJW and repeat NTBC cycle 38

**Primary Investigators:** Lisa Wilson

**Number of Animals:** 40. **Species:** Rat. **Strain:** SD FRG. **Gender:** Male & Female. **Age or weight range: 5-**6 weeks

**Purpose**:

In IACUC protocol AMI-018-006, the focus of the set of experiments is to utilize the immunodeficient rat model of hereditary tyrosinemia type 1 (HT1) - which are deficient in the enzyme fumarylacetoacetate hydrolase (FAH) – as the model for expanding human hepatocytes. Hepatocytes that are FAH+ have a selective growth advantage over FAH- hepatocyte; therefore, we hypothesize that FAH+ human hepatocytes will grow and repopulate the FAH-KO rat liver. Proof-of-concept has already been demonstrated in the mouse model of HT1 (see Azuma et al. 2007 PMID: 17664939).

Studies are under way to improve the health and survival of transplanted FRG rats out to 90-125 days post-transplant while maintaining robust expansion of the transplanted primary human hepatocytes. In previous NTBC cycles there was an abrupt transition from NTBC at ≥ 1mg/L NTBC to 0.05-0 mg/L within the first 28 days of the cycle leading to a high mortality rate. We believe this mortality is due to a hypersensitivity to NTBC withdrawal (≤ 0.05mg/L) which leads to rapid apoptosis of the rat hepatocytes causing acute liver failure. Data from the most recent NTBC cycles in Phase 1 and 2, at -28- and -50-days post-transplant respectively, using the 0.4mg/L > 0.05mg/L NTBC (Cycles 4 and 11) has improved survival in the first 50-days post-transplant but survival declines to ≤ 50% starting at ~60 days post-transplant.

Study plans for AMI-018-006-124 and -125, animals from study 110 and 112 cycled on 4mg/L(3d)>0.05mg/L(5d) - Cycle 38 – had high survival at ≥ 70 days post-transplant, in addition to exponential increase in hAlbumin levels from day 50 to 71 indicative of pHH expansion and upon perfusion the gross appearance and associated vasculature of the liver was closer to normal when compared to livers from naïve animals, e.g., with clinical scores = 3.5 out of 4. In addition, clinical chemistry data on animals at 70d post-transplant show the major hepatic markers that influence metabolic function were at normal levels indicating the human hepatocytes can rescue the animals.

The quality of the human hepatocytes used in transplantation is critical for successfully generating highly humanized FRG rats for the downstream CMC cell therapy product. Due to the limited availability of high-quality commercial lots of human hepatocytes, we pre-screen all pHH donors in the FRGN mice to validate the lots meet the standard of repopulation in that model. Since this resource is so precious, in study AMI-018-006-126 we compared the standard cell dose of 5E+6 cells/100 g of body weight versus half that dose, 2.5E+6 cells/100g of body weight, using NTBC cycle 38. In study AMI-018-006-127, will repeat the 0.5X (2.5E+6 cells/100g of body weight) for all 40 FRG rats. Increasing the number of animals receiving the 0.5X dose allows for a larger “N” for statistical analysis as well as animals for necropsy at the end of Phase 1 and 3 for health evaluation.

By collecting additional data on survival, pHH expansion and clinical markers for the health evaluation (hepatic markers in serum, SA/Tyrosine, renal biomarkers and histology), we will be able to determine the efficacy of NTBC cycle 38 to support health.

All FRG rats will be on 5LJ5 diet

All FRG rats will begin on 16 mg/L NTBC + 820µg/mL Equisul in the drinking water

**Experiment Outline:**

N=40 FRG rats

Animals will be held for at least one week to acclimate following arrival.

Animals will be socially housed. On the rare case where one animal remains in a cage without a partner, additional cage enrichment (non-food) will be provided.

Using body weights collected the same day as preconditioning the rederived FRG rats from Envigo will be dosed with Ad-uPA 24h ± 2h prior to transplant and placed on the drinking water as specified in the NTBC cycling protocol located on the Ambys G-drive. On the day of transplant, the cryopreserved validated commercial PHH Lot will be prepared following the standard protocol used for transplantation.

**Day -1:** In the AM, ALL rats will be dosed using Vector Biolabs titer of **2.4E+11pfu/mL**. The dose will be calculated to be ~2.5E7pfu/gram of body weight Ad-UPA by IV tail injection. Ad-uPA will be provided to the IVS team for tail vein dosing. The NTBC drinking water will be changed as indicated in the chart.

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| --- | --- | --- | --- | --- | --- |
| **# of FRG rats** | **Ad:uPA dose (pfu/g of BW) IV by tail vein** | **Ad:uPA lot** | **pHH Donor** | **Cell dose by laparotomy into the splenic pulp** | **NTBC Cycle ID** |
| 40 | 2.5E7 | WBAO0007 | DJW | 2.5E6 viable cell/100g of BW | 38 |

**Day 0:** The volume of hepatocytes suspension determined by body weight will be in 1.5ml Eppendorf tubes and transferred on ice. Hepatocytes will remain on ice until time of injection. At time of injection, hepatocytes will be gently pipetted up/down x3 with P1000 pipet and sterile P1000 tip to get the cells in suspension (hepatocytes are large and will quickly pellet to the bottom of the tube). Hepatocytes will be drawn into a sterile 1cc syringe with a 27G needle and injected into the spleen via laparotomy method.

**Day 1 onwards:** Animals will be put on the short NTBC cycle as indicated above in the chart and as indicated in the NTBC water cycle sheet located on the Ambys’ G drive.

All animals post-transplant will be offered the nutritionally balanced Supreme mini treats supplement at the time of body weight collection as positive reinforcement (~1-2 pellets) and for supplementation of additional calories and vitamins at a dose of 6-8 pellets per animal.

The IVS team will record the date and quantity of supplement administration, and clinical observational scores.

**Day 28 and 71: Urine, blood and tissue collection**

On the days indicated in the chart below, the PI or IVS Supervisor with randomly select **2F and 2M** animals for health evaluation procedures.

1. Using the standard urine collection method, as much urine as possible will be collected from each animal and dispensed into 50µL aliquots before storing at -20C.
2. Induce a surgical plane of anesthesia with 3-5% isoflurane and from the vena cava collect ~500µL of whole blood into a K2EDTA tube for hematology by shipping to IDEXX on the same day, ≥ 2000µL into a serum separator to isolate serum for blood chemistries and SA/Tyrosine quantitation. Serum will be dispensed into 2 X 210µL and 1 X 80µL into sterile 1.5mL microfuge tubes before storing at -20C.
3. The liver, spleen and kidney will be isolated and processed as described in Appendix A.

|  |  |  |
| --- | --- | --- |
| Phase | Day of collection ± 1 days | Group A and B |
| 1 | October 8, 2021 | 2F, 2M |
| 3 | November 18, 2021 | 2F, 2M |

**Day 30 – 90**: All animals, collect 5µL whole blood for human albumin measurements via tail vein, tail or saphenous vein nick at the specified dates on the chart above and the NTBC water cycle sheet located on the Ambys’ G drive. **PI will communicate:**

**Day 50 huAlb readout:** hAlbumin **≤ 250µg/mL** will be removed from study and euthanized.

**Day 70 huAlb readout:** hAlbumin **≤ 750µg/mL** will be removed from study and euthanized.

**Day 0-125:** Body weights and clinical scoring will be measured and documented up to three times each week.

**Day >125:** IVS to notify PI and Perfusion Team of any remaining animals.

**Animal care and welfare:**

Refer to **the document titled “VETERINARY CLINICAL CARE AND REPORTING FOR FRG RATS”** for the evaluation and documentation of clinical status and palliative treatments**.** In reference to the group A and B assignments above, animals will be supplemented based on their group assignments.

**Additional Veterinary Testing:**

For veterinary health and surveillance screening, the Attending Veterinarian may request aseptic sample collection of tissues in lieu of, prior to or following perfusion without interference to primary study objectives, under general anesthesia. Once all samples have been collected, the animal will be euthanized via exsanguination/vital organ removal.

**Humanized FRG rats for perfusion, hepatocyte isolation and human hepatocyte purification:**

At ≥ 71 days post-transplant with human albumin concentrations of ≥ 2,500µg/mL, animals may be offered to the Product Development team for optimization of processes involving liver perfusion (refer to the document titled “Perfusion Procedure Guideline: Portal Vein and Inferior Vena Cava Approaches), hepatocyte isolation, human hepatocyte purification and in vitro characterization.

Once the cannula has been inserted into the caudal vena cava or portal vein, secured and blood starts to fill the head, ≥ 500µL whole blood will be collected for sera isolation. Alternatively, the blood may be collected from the tail vein based on a 50µL aliquot of sera will be dispensed into a pre-labeled sterile 1.5 mL tube for Tyrosine and Succinylacetone quantitation. Remaining sera will be transferred to a second pre-labeled sterile 1.5mL tube. Both aliquots should be stored at -20C until shipped for analysis.

Principal Investigator: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Head of Pharmacology (or alternate): \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**Appendix A: Processing blood, urine and tissue samples**

Blood collection

* Using Isoflurane, anesthetize the animal to a surgical plane of anesthesia (Induction 4-5%, maintenance 2-3%). Confirm with a firm toe pinch on both hind feet.
* Using operating scissors or other appropriately sized scissors, cut through the skin and muscle to open the peritoneal cavity.
* Using gauze, sweep the intestines out of the body cavity to expose the inferior vena cava.
* Using 5 mL (rat) syringe and 25 G needle puncture the inferior vena cava and slowly withdraw 4mL of blood from rat. Be careful to not collapse the vein by creating too much vacuum pressure.

Serum:

* Dispense ≤ 2mL whole blood volume into 4mL serum separator tube and allow to clot for at least 1 hour at room temperature.
* Centrifuge at 10,000 rpm for 5 minutes.
* Dispense 220µL into 4 X 1.5 sterile mL tube, store at -80C

Plasma:

* Dispense ≥ 1mL of whole blood in a 4mLK2EDTA anticoagulation micro tube (do not overfill or blood will clot).
* Gently invert 3 times (do not shake) to facilitate complete mixing with anticoagulant.
* Centrifuge at 10,000 rpm for 5 minutes.
* Dispense 80µL into 4 X 1.5 sterile mL tube, store at -80C

Urine during Necropsy

* Following collection of blood, collect urine (if present) via cystocentesis. Urine to be placed in labeled freezer compatible tube and store at -20oC

Collection of Liver

* Using blunt tipped scissors (curved if possible) separate the liver from the diaphragm, blood vessels and connective tissue and remove the liver from the body cavity.
* Make 4-5, 1cm nicks across the liver to facilitate fixation.
* Place whole liver in bottle of 10% NBF.

Collection of the Kidney

* Remove the adrenal glands, located in the fat cranial to the kidneys.
* Dissect the kidneys away from the fat and cut at the juncture of the kidney with the renal artery and vein.
* Transect the left kidney (transverse section) with a scalpel blade or straight razor.
* Longitudinally bisect the right kidney at the midline.
* Place into a bottle with 10% NBF