**Transplant Date: August 9, 2021**

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

**Study Number:** AMI-018-006-123

**Title of Study:** Transplantation with various species of primary hepatocytes to address if a xeno-incompatibility issue with the FRG rat and human hepatocytes.

**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. 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) allow for rat hepatocytes to enter senescence (0.4mg/L) prior to the very low NTBC concentration(0.05mg/L) where selective pressure is applied for the human hepatocytes to expand. Using this cycle, we have seen survival at ≥ 80% in the first 50-days post-transplant

To date, with the testing of many variations in the NTBC cycling regime, we have not been able to improve survival ≥ 70 days post-transplant. Due to the immune compromised state of the animals and immunohistochemistry data we have confirm the disease state is not graft versus host disease. One hypothesis for the low survival is a xeno-incompatibility issue between the FRG rat and expanded human hepatocytes. This may be due to species incompatibility within a critical signaling pathway or a toxic metabolite produced from the transplanted hepatocytes that induces the disease state. Cumulative data from a health evaluation study indicates cachexia and hypoglycemia caused by (1) impaired rat hepatocyte function and (2) rat proximal tubule injury may be the cause of morbidity. To address if these pathologies correlate with human hepatocyte expansion, we will use hepatocytes from various species, including human, mouse, rat and pig. We have confirmation from collaborators, Markus Grompe and Scott Naugler, that mouse hepatocytes transplanted into the FRG rat engraft and robustly expand while maintaining high survival. The mouse hepatocytes would act as a positive xenograft control to determine if hepatocytes from a different species can engraft, expand to ≥ 50% hepatocyte replacement index, and rescue the HT-1 phenotype of the FRG rat – this is a critical control for testing the xeno-incompatibility theory. The pig would be the third species, phylogenetically further removed from the rat, to determine if similar morbidity to human hepatocytes occurs as the pig hepatocytes expand in the FRG bioreactor. A fifth group, mock transplant, will be used for determining if the NTBC regime is contributing to the low survival seen at ≥ 70 days post-transplant. This group will receive Ad:uPA pre-conditioning and the same laporatomy and injection into the splenic pulp except transplant media, at an equivalent dose volume compared to hepatocytes will be used in place of hepatocytes. All animals will be placed on the same NTBC cycling regime, Cycle 11. The main endpoint for this study is survival and hepatocyte expansion at ~ 90 days post-transplant.

In addition, each individual animal in all 5 groups will be evaluated at the specified points in the NTBC cycling regime post-transplant. Since this is a pilot study and the groups sizes are small, we will use blood and urine as the matrix for evaluating metabolic function, nutritional state as well as liver and renal injury. Serum and urine will be collected at ~ 30-, 50-, 70- and 90-days post-transplant. Comprehensive liver clinical chemistries for metabolic function and liver/kidney injury, amino acid composition to correlate nutritional state, succinylacetone/tyrosine as contributors to liver/kidney injury, urine for total protein and amino acid analysis as well as specific gravity will be analyzed and reported. If body condition score is ≤ 3 and body weight loss ≥ 30% or a clinical score of ≤ 3.0, animals will be euthanized and blood, urine, kidney, liver and spleen collected as stated in Appendix A. At the end of the study, >100 days post-transplants, all remaining animals will undergo the necropsy process described in Appendix A.

All FRG rats will be on 5LJ5

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 pre-conditioned using 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. *The viral and pHH lot used in this study can be found in the FRG rat Compiled Information Google sheet in the tab marked “Study Tracker” on the Ambys G drive*.

|  |  |  |  |
| --- | --- | --- | --- |
| Group | # of FRG rats | Ad:uPA (pfu/grams of BW) | Hepatocyte source |
| A | 8 | 5E+7 | Commercial human hepatocytes |
| B | 8 | 5E+7 | Commercial Lewis rat hepatocytes |
| C | 8 | 5E+7 | Commercial C57Bl6 mouse hepatocytes |
| D | 8 | 5E+7 | Mayo clinic/Ambys produces pig hepatocytes |
| E | 8 | 5E+7 | Hepatocyte media |

**Day -1:** In the AM, ALL rats will be dosed with 5E+7 pfu/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 above chart.

**Day 0:** Each FRG rat will be dosed with 5E+6 viable cells/ gram of body weight and volume calculated from a 25E+6 viable cells per milliliter suspension. The volume of hepatocytes suspension determined by body weight will be dispensed into individual sterile 1.5ml Eppendorf tubes and transferred to 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 drinking water containing the specificed concentration of NTBC on the NTBC cycle indicated on 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.

**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 ~ 30, 50, 70 and 90**: On the days indicated in the chart below and 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. The animals will be induced to a surgical plane of anesthesia with 3-5% isoflurane and ~500µL of whole blood collected via the retro-orbital vein. Serum will be isolated and 210µL dispensed sterile 1.5mL microfuge tubes before storing at -20C.

|  |  |  |
| --- | --- | --- |
| Phase | Days post-transplant | Day of collection ± 1 days |
| 1 | ~30 | September 8, 2021 |
| 2 | ~50 | September 29, 2021 |
| 3 | ~70 | October 20, 2021 |
| 4 | ~90 | November 10, 2021 |

As stated above in the study design, if the body condition is ≤ 3 and body weight loss > 30% or a clinical score of ≥ 3.0, animals will be euthanized and blood, urine, kidney, liver and spleen collected as stated in Appendix A.

**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