TITLE: Pilot sheep tissue collection for the Ovine FAANG Project

Purpose: Tissues collected from ewes and wethers to be used for generation of ChIP-seq, DNA methylation (WGBS), and ATAC-seq data

Collection: Spring of 2018 at Washington State University, Pullman, WA, USA

1 Purpose / Introduction

To collect tissues from multiple healthy sheep, of both sexes, for subsequent extraction of RNA/DNA. Routine post-mortem dissection and examination (gross animal autopsy) is completed during the collection process by a veterinary pathologist. These tissues will be used for epigenetic assays as part of the pilot project in preparation for completing the assays on the Rambouillet_v1.0 genome reference sheep Benz_2616. Tissues will be used to produce histone methylation and CTCF ChIP-seq data, whole genome methylation data (WGBS), and ATAC-seq data for the Ovine FAANG Project.

2 Equipment/Reagents/Materials

Cryotubes for snap freezing tissues

Liquid Nitrogen dewars (insulated Styrofoam) filled with liquid nitrogen

Scalpel blade handles and sterile blades

Clean Forceps, Scissors, and Knives

Sterile plastic dishes (non-coated, such as Petri dishes)

Sterile PBS in liter bottles and empty sterile liter bottles

Sharps Bins

Non-sterile nitrile (or similar) gloves

Cryostor medium (cryopreservation medium)

Concentrated 10X DPBS (sterile filtered or autoclaved)

50 mL conical bottom tubes

Supplies appropriate for euthanasia: Blood collection set (tubing), sterile intravenous needles and syringes, sodium pentobarbital

Centrifuge

Laminar flow hood (biosafety cabinet)

Isopropanol bath slow-freeze containers

3 Principle

Post mortem examination is carried out at Washington State University by a single veterinarian trained in anatomic pathology and the team of researchers. Larger tissue specimens are harvested from the selected organs at the necropsy area then transferred to a clean work station for rinsing and aliquoting into small samples appropriate for storage until assay completion. For preservation of RNA and DNA the small tissue aliquots are either snap frozen in liquid nitrogen immediately, processed to nuclei, or slowly frozen to -80 °C for cell membrane preservation.

4 Procedure

4.1 Healthy yearling animals are group housed and fed routinely until immediately prior to euthanasia. Euthanasia is accomplished according to current American Veterinary Medical Association recommended guidelines and protocols approved by the Washington State University Institutional Animal Care and Use Committee. Intravenous sodium pentobarbital is administered via the jugular vein until the heart has stopped and no corneal reflex is present. Thereafter the jugular veins and carotid arteries are severed for exsanguination.

4.2 Tissues are quickly collected from the carcass by the post mortem team and transferred to the clean workstation for subsampling. Using a clean scalpel blade for each tissue, one to several, approximately 0.5 cm diameter samples are cut out, briefly rinsed with ice cold sterile 1X PBS (Phosphate Buffered Saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2PO4, 1.8 mM KH2PO4), aliquoted into individual 2 mL cryotubes, and promptly snap frozen in liquid nitrogen. Samples were transferred from liquid nitrogen directly into a -80 °C freezer for storage. Tissues collected from each animal include liver, cerebellum, spleen, rumen atrium, abomasum, jejunum, ileum, spiral colon, left cardiac ventricle (free wall), lung (left cranial apex), thymus, white adipose (back fat from lumbar region), longissimus dorsi, cerebral hemispheres (telencephalon/cerebral cortex), hypothalamus, and bone marrow (from the ribs). Tissues collected from intact ewes also included uterus (caruncle region), ovary, and mammary gland (quiescent state/nulliparous). Tissue subsamples are selected from regions of parenchyma, avoiding capsule, fibrous tissue septa, and large vessels as much as possible.

4.3 For cell isolation of alveolar macrophages, lungs are removed by the post mortem team and transferred to a separate collection station then rinsed of external blood with sterile DPBS. One liter rinses of sterile DPBS are poured into the transected trachea (grasp trachea with long forceps) near the bifurcation of the primary bronchi and the lungs are gently massaged with gloved hands for approximately 10 seconds. The transtracheal lavage fluid is then decanted from the lungs into 1 L sterile glass bottles. This process of lavage and decanting is repeated with a liter of fresh DPBS three times total for each animal (for a total of approximately 3 L of DPBS). Avoid collecting blood into the lavage fluid as much as possible by trimming great vessels and rinsing the lungs externally with additional DPBS as needed. Decanting of the lavage fluid from the glass bottles into 50 mL conical bottom tubes for cell isolation is performed in a laminar flow hood (biosafety cabinet). Continuing to work in a biosafety cabinet, lavage fluid is strained through 100-micron sterile mesh filters into new conical bottom tubes, avoid transferring mucus and surfactant material to the new tubes. Cells are collected from the lavage fluid by centrifugation at 400 x *g* for 10 minutes at room temperature. Supernatant is discarded and cell pellets are rinsed with 5 mL sterile DPBS and briefly re-spun. Supernatant is discarded then cells are resuspended in a small volume of DPBS (2-3 mL) by gentle rocking of the tubes. Then 18 mL of sterile water are added to lyse erythrocytes and susceptible cells (non-macrophages) within the suspension

while rotating gently for 30 seconds. The lysis is quenched by addition of 2 mL of 10X concentrated DPBS to restore isotonicity. The cell pellet is collected at the bottom of the tubes by centrifugation at 400 x *g* for 5 minutes (cell pellet should now be white to very pale tan), supernatant containing heme pigment is discarded. The cell pellet is washed again with approximately 40 mL of 1 x DPBS, gently resuspended, centrifuged briefly and the supernatant discarded. Cells are resuspended in a small quantity of 1x DPBS to obtain approximately 5 x 10^7 cells per mL and counted on an automated cytometer. Approximately 5 x 10^7 live cells are aliquoted with the addition of 1 mL of CryoStor media (CS10 catalog # C2874, Sigma-Aldrich) into 2 mL cryopreservation tubes. Tubes containing cell suspensions are placed within a Mr. Frosty system (Thermo Fisher Scientific) isopropanol bath and slowly frozen to -80 °C to preserve nuclear integrity.

5 Results

The snap frozen tissue samples will be utilized for ChIP-seq, DNA methylation, and ATAC-seq assays as detailed in those protocols as part of the Ovine FAANG Project pilot assays. Isolated alveolar macrophages that are slowly frozen will be used for ChIP-seq.