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Summary

The Functional Annotation of Animal Genomes (FAANG) project aims to identify genomic regulatory elements in both sexes across multiple stages of development in domesticated animals. This study represents the first stage of the FAANG project for the horse, *Equus caballus*. A biobank of 80 tissue samples, two cell lines and six body fluids was created from two adult Thoroughbred mares. *Ante-mortem* assessments included full physical examinations, lameness, ophthalmologic and neurologic evaluations. Complete blood counts and serum biochemistries were also performed. At necropsy, in addition to tissue samples, aliquots of serum, ethylenediaminetetraacetic acid (EDTA) plasma, heparinized plasma, cerebrospinal fluid, synovial fluid, urine and microbiome samples from all regions of the gastrointestinal and urogenital tracts were collected. Epidermal keratinocytes and dermal fibroblasts were cultured from skin samples. All tissues were grossly and histologically evaluated by a board-certified veterinary pathologist. The results of the clinical and pathological evaluations identified subclinical eosinophilic and lymphocytic infiltration throughout the length of the gastrointestinal tract as well as a mild clinical lameness in both animals. Each sample was cryo-preserved in multiple ways, and nuclei were extracted from selected tissues. These samples represent the first published systemically healthy equine-specific biobank with extensive clinical phenotyping *ante-* and *post-mortem*. The tissues in the biobank are intended for community-wide use in the functional annotation of the equine genome. The use of the biobank will improve the quality of the reference annotation and allow all equine researchers to elucidate unknown genomic and epigenomic causes of disease.

Keywords biorepository, genome regulation, horse, necropsy, nuclei isolation, tissue collection

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Introduction

The Encyclopedia of DNA Elements (ENCODE) Consortium was established in 2003 to identify functional elements within the human genome (ENCODE Project Consortium 2004). At the conclusion of the pilot phase, in 2007, it was abundantly clear that the human genome was composed of more than just protein-coding genes (ENCODE Project Consortium *et al.* 2007). Information gained from the project allowed researchers to assign a biochemical function to 80% of the genome (ENCODE Project Consortium 2012). According to the ENCODE website, as of July 2017 ENCODE data have been utilized in over 700 publications linking functional data to disease in humans (ENCODE Project Consortium 2017). The number of publications will continue to expand as additional genomic regions associated with disease are identified. Realizing the need for better annotation to advance discovery in animal species, the animal genomic research community developed the Functional Annotation of Animal Genomes (FAANG) Consortium.

The FAANG Consortium, organized in 2014, is working to use the assays and analysis techniques developed during the ENCODE project to annotate the majority of functional elements in the genomes of domesticated animal species (Andersson *et al.* 2015). Biobanks have been created for human (Bao *et al.* 2013) and porcine datasets (Abbott 2015; Albl *et al.* 2016). These biobanks have aimed to standardize tissue collection and preservation to improve downstream molecular analyses. The FAANG consortium has worked to standardize tissue sets, methods of collection, assays and meta-data analyses (Tuggle *et al.* 2016). The necessity of these datasets in the horse cannot be overstated.

Putative causal variants in coding regions have been identified for simple, Mendelian diseases in the horse such as hyperkalemic periodic paralysis (Ptacek *et al.* 1994), hereditary equine regional dermal asthenia (Tryon *et al.* 2007) and dwarfism in Frisian horses (Leegwater *et al.* 2016). However, complex inherited diseases, such as fracture risk (Blott *et al.* 2014), osteochondrosis (van Grevenhof *et al.* 2009; Lykkjen *et al.* 2010; Teyssedre *et al.* 2012; McCoy *et al.* 2016) and recurrent laryngeal neuropathy (Dupuis *et al.* 2011) have failed to be localized to coding regions of the genome despite extensive research. Better annotation of genes and functional elements within the genome will help to identify variations causative of complex diseases such as these. The objective of the current study was to create a biobank of tissues from two systemically healthy Thoroughbred mares to be used in the functional annotation of the equine genome.

Materials and methods

Animals

Two Jockey Club registered Thoroughbred mares deemed systemically healthy by the University of California Davis (UCD) Veterinary Medical Teaching Hospital (VMTH) were donated to the project. The horses (ECA_UCD_AH1 and ECA_UCD_AH2) were five and four years of age respectively at the time of necropsy. Neither mare had a history of traumatic injuries nor had they been bred. The animals were more than one year out of athletic training programs prior to euthanasia. All protocols were approved by the UCD Institutional Animal Care and Use Committee (Protocol #19037). A pedigree for both animals was obtained prior to their donation to the project.

Clinical phenotyping

Both mares were evaluated at the UCD VMTH prior to euthanasia. Clinical evaluations were performed by a board-certified internist (CJF), surgeon (SAK) and ophthalmologist (ML) for signs of abnormalities in dermatologic, cardiopulmonary, gastrointestinal, neurologic, urogenital, lymphatic, orthopedic or ophthalmic systems. Videos were taken of the lameness and neurological examinations. A complete blood count and serum biochemistry were also performed on each horse.

Karyotyping

Metaphase chromosome spreads were prepared from Pokeweed-stimulated blood lymphocyte cultures according to standard protocols (Raudsepp & Chowdhary 2008). Chromosomes were stained with Giemsa for initial counting. Sex chromosomes were identified by CBG banding (Arrighi & Hsu 1971). Refined chromosome analysis and karyotyping were carried out by GTG banding (Seabright 1971). Twenty cells were captured and analyzed for each experiment using ISIS V5.2 (MetaSystems GmbH) software.

PCR analysis of sex chromosomes

Genomic DNA was isolated from blood lymphocyte cultures. PCR analysis was carried out using primers for the equine *sex determining region Y* (*SRY*) and *androgen receptor* (*AR*) gene, as described earlier (Raudsepp *et al.* 2004, 2010).

Peripheral blood mononuclear cell collection and preservation

Peripheral blood mononuclear cells were isolated from 10 ml of heparinized blood using Histopaque® and density gradient centrifugation (Hida *et al.* 2002).

Fluid collection

Blood was collected *ante-mortem* from an intravenous-catheter into EDTA (ethylenediaminetetraacetic acid), heparin and additive-free vacutainers. The blood was shielded from light, preserved on ice and returned to the lab for

centrifugation. Immediately *post-mortem*, cerebrospinal fluid (CSF), synovial fluid and urine were collected. CSF was collected at the atlanto-occipital site, as previously described (Finno *et al.* 2015). Synovial fluid was collected via syringe aspiration of the carpal and tarsal joints from ECA_UCD_AH1. For ECA_UCD_AH2 synovial fluid was collected via syringe aspiration of the carpal joint only. All blood, CSF and synovial fluid samples were centrifuged at 2000 ***g*** for 10 min at 4 °C, and the supernatant was flash frozen in 1-ml aliquots. Plasma, serum and buffy coat were transferred to storage tubes and flash frozen. Urine was freely caught postmortem. All fluid samples are stored at −80 °C.

Tissue collection

Tissue samples for cell culture were collected concurrently with CSF and synovial fluid. All tissues to be collected were assigned to one of seven collection stations based on organ system (Table 1). Organs or organ samples were labeled and delivered to the stations by veterinarians ensuring proper identification. All tissues were examined by a board-certified pathologist (VKA) for any evidence of gross pathology. Additionally, tissue samples for histopathology were collected. Two sets of samples were then collected for the biobank: the first set was directly proximal and the second set distal to the sampling site for histopathology (Fig. 1). For each biobank sample, 1-cm^3 aliquots of tissues were collected. At least two, and a maximum of 12, aliquots of each tissue were preserved.

Tissue preservation

After collection, the samples were preserved multiple ways. Histopathologic samples were labeled and preserved in 10% buffered formalin. All but two of the proximal site samples were flash frozen in liquid nitrogen and stored at −80 °C until further use. The two remaining proximal site samples were maintained on ice before cross-linking and preservation for ChIP-seq using a modification of the iDEAL chip-seq kit protocol from Diagenode®. The samples collected distal to the histopathologic sites were sectioned and flash frozen and are stored at −80 °C. Nuclei isolation was carried out, according to the standards developed in the mouse ENCODE project (Yue *et al.* 2014), on 16 of the tissues from a tertiary sample collected distal to the histopathologic site (Table 2).

Cell isolation and preservation

Immediately *post-mortem*, skin above the gluteal muscle and the medial aspect of the proximal hindlimb were cleaned with phosphate-buffered saline (PBS), shaved and sterilized. Full-thickness strips were excised from each region and stored in media appropriate for isolation of either epidermal keratinocytes or dermal fibroblasts (Caldelari & Muller 2010; Raimondi *et al.* 2011).

Table 1 Tissues from each organ system of the body were sorted into stations based on when they became available during a necropsy. This organization of tissues into stations allowed for an expedited collection process.

Station	Organ system collected
Station 1	Cell culture biopsies
Station 1B	Integumentary, abdominal and thoracic
Station 2	Musculoskeletal
Station 3	Neurological
Station 4	Respiratory and cardiovascular
Station 5	Gastrointestinal
Station 6	Urogenital

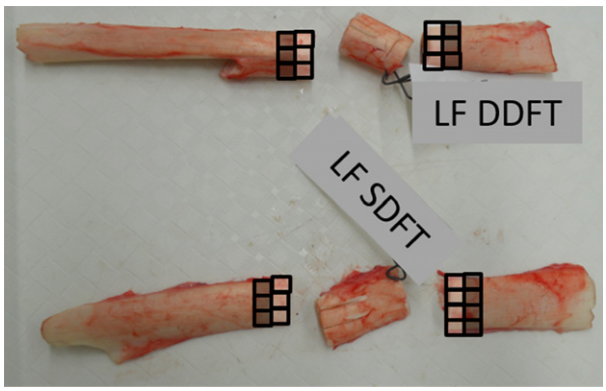

Figure 1 Depiction of collection sites using superficial and deep digital flexor tendon of the left forelimb. The middle sections of tissue were labeled and preserved in formalin for pathological evaluation. The light squares represent proximal samples and the dark squares represent the distal samples.

Keratinocyte cultures were established from proximal hind-limb biopsies after being sectioned into 0.5 cm × 1 cm pieces and treated with dispase (Roche) for 24 hours at 4 °C. The epidermis was then separated from the dermis and incubated in CnT Accutase100 (CELLnTEC Advanced Cell Systems AG) at room temperature. The keratinocytes were detached, collected in a cell suspension, passed through a cell strainer, centrifuged and resuspended to count. Keratinocytes were seeded at 5×10^4 cells/cm^2 in CnT-09 complete medium (CELLnTEC Advanced Cell Systems AG), which was changed every two days until confluence. To passage, keratinocytes were treated with Accutase100, centrifuged, counted and re-seeded at 5×10^4 cells/cm^2. Keratinocytes were cryopreserved after passages 2 and 3 (ECA_UCD_AH1) or passage 4 (ECA_UCD_AH2) using CnT-CRYO-50 (CELLnTEC Advanced Cell Systems AG), according to manufacturer instructions, and were stored in cryotubes in liquid nitrogen (Caldelari & Muller 2010).

To collect dermal fibroblasts, skin biopsies were washed three times in ice cold PBS containing 3× antibiotics (100× penicillin and streptomycin, Sigma-Aldrich). Dermis was then separated, and 2–3 mm^2 fragments were placed into a 24-well tissue-culture-treated plate. Complete medium

Table 2 A complete list of tissues collected from ECA_UCD_AH1 and ECA_UCD_AH2.

Integumentary system	**Cardiovascular system**	**Digestive system**
Neck skin	*Left lung**	Tongue
Dorsum (over back) skin	Heart left atrium	Epiglottis
*Loin adipose**	*Heart left ventricle**	Esophagus
Gluteal adipose	Heart right atrium	Stomach
	Heart right ventricle	Duodenum
Musculoskeletal system	Mitral valve	Jejunum*
Gluteal muscle	Tricuspid valve	Ileum
Sacrocaudalis muscle	Aortic valve	Cecum
*Longissimus muscle**	Pulmonic valve	Right ventral colon
Rib bone marrow	Trachea	Left ventral colon
Long bone marrow		Left dorsal colon
Coronary band	**Nervous system**	Right dorsal colon*
Hoof wall	Cornea	Small colon
*Lamina**	Retina	
Metacarpal bone diaphysis	Frontal cortex*	**Urogenital System**
Sesamoid bone	*Parietal cortex*	Urinary bladder
Cartilage	Occipital cortex	Uterus
Superficial digital flexor*	Temporal cortex	*Ovary**
Suspensory ligament	Pituitary	Oviduct
Deep digital flexor	Cerebellum vermis	Cervix
	Cerebellum Lateral Hemisphere*	Vagina
Abdominal/thoracic organs	Pons	Mammary gland
Sciatic nerve	Thalamus	
*Liver**	Hypothalamus	**Cell Culture**
Spleen*	Dura mater	Keratinocytes
Adrenal cortex	Corpus callosum	Fibroblasts
Adrenal medulla	C1 spinal cord	
Kidney cortex	C6 spinal cord*	**Other**
Kidney medulla*	T8 spinal cord	Peripheral blood mononuclear cells[1]
Larynx	L1 spinal cord	
Pancreas	L6 spinal cord	
Thyroid	Dorsal root ganglia	
Lymph node*		

Tissues with nuclei isolated and preserved are denoted with an asterisk (*). Tissues in **bold** have been prioritized based on the FAANG guidelines for RNA-seq and ChIP-seq of four histone modifications (H3K4me3, H3K4me1, H3K27ac and H3K27me3).
[1]Sample only collected from UCDAH1.

(Dulbecco's Minimum Essential Medium, 20% fetal bovine serum, 2× non-essential amino acids, 2 mM L-glutamine, 2× penicillin/streptomycin, 2 μg/ml amphotericin B and 1 μg/ml fluconazole) was added to cover each tissue fragment. For the first week, complete medium was added to keep the tissue covered. Afterwards, medium was changed weekly until confluence, at which point the cells were trypsinized, counted and seeded in a 12-well plate. With each passage, cells were seeded in a larger well size: 1.9 cm^2 (primary), 3.8 cm^2 (passage 1), 9.5 cm^2 (passage 2), 25 cm^2 (passage 3) and 75 cm^2 (passage 4). Dermal fibroblasts were cryopreserved after passages 3 and 4 for both horses using DMSO-based freezing medium (Raimondi *et al.* 2011) and stored in liquid nitrogen.

Pathological evaluation

The tissues preserved in formalin were embedded in paraffin, sectioned and evaluated by a board certified veterinary pathologist (VKA). The sections were stained using hematoxylin and eosin (HE) then visualized using light microscopy.

Results

Clinical phenotyping

The lameness examinations revealed a grade 2 out of 5 lameness in both horses (Table S1). ECA_UCD_AH1 was lame on the right hind leg. No tissues were collected from this leg. ECA_UCD_AH2 was bilaterally lame on both forelimbs (see supplementary video at https://youtu.be/OrfyVtYr1iQ), but it was noted that the horse's front shoes were removed the day prior to the exam. Results of the serum biochemistry, complete blood count, neurological exam (see supplementary Video at https://youtu.be/seWYe69ZhUs) and ophthalmic exam were all within normal limits (Table S1). A karyotype and PCR analysis of sex chromosomes was completed for each individual (TR) with no abnormalities detected. Interpretation of all clinical

examinations determined both mares were systemically healthy at the time of euthanasia.

Tissue and fluid collection and preservation

All tissues were collected within three hours of euthanasia. Eighty tissues and six body fluids were collected (Table 2). The samples were preserved via one of four methods and subsequently stored at −80 °C. Serum, plasma, urine, CSF, synovial fluid and buffy coat were aliquoted after centrifugation and stored at −80 °C. Dermal fibroblasts and epidermal keratinocytes established from skin biopsies were cryopreserved in liquid nitrogen (Table S2). Peripheral blood mononuclear cells's were isolated from UCDAH1 only and stored at −80 °C (Table 2).

Pathology report

The conclusions from the pathology report of each tissue from each horse are listed in Table S3. Most of the tissues collected showed no significant abnormalities. However, the gastrointestinal tract of both horses contained substantial, subclinical, eosinophilic and lymphocytic inflammatory cell infiltrate in the lamina propria and submucosa (Fig. S1). This inflammation extended from the duodenum through the small colon in both horses (Table S3).

Discussion

Biobanks have been created for samples from healthy humans (Triendl 2003; Ronningen *et al.* 2006; Jaddoe *et al.* 2007; Roden *et al.* 2008; Sudlow *et al.* 2015), diseased humans (Triendl 2003; Garcia-Merino *et al.* 2009), a diabetic pig model (Albl *et al.* 2016), cancer cell lines (Barretina *et al.* 2012; van de Wetering *et al.* 2015) and canine mammary tumors (Milley *et al.* 2015). In humans, the list continues to grow, but the availability of published biobanks for domesticated animals is much more limited (Groeneveld *et al.* 2016). The 80 tissue samples, two cell lines and six body fluids in the biobank described here are intended to be used by the equine research community in the functional annotation of the equine genome. This report also provides guidelines on the tissue sampling and collection to ensure congruency with future biobanks. Samples from the biobank may be used by any interested researchers to further the annotation of the equine genome.

The phenotyping and histopathologic results are intended to provide context for future sequencing and related molecular analyses. The lameness observed in both horses was evaluated for plausible causes to determine if samples should be excluded from the biobank. No plausible cause of the right hind lameness for UCDAH1 could be elucidated. Therefore, all musculoskeletal limb samples were collected from the left legs for UCDAH1. UCDAH2 had her shoes removed the day prior to her lameness exam, which could have contributed to the lameness. Hoof tester examination was positive bilaterally on UCDAH2's soles, and the lameness improved with the addition of shoes prior to euthanasia. For this reason, it is suspected the lameness was due to the shoe removal and the samples were included in the biobank.

Underlying pathology will have a significant impact on the annotation in abnormal tissues. For example, the eosinophilic and lymphocytic infiltration within the gastrointestinal tract of these two horses may result in a difference in observed gene expression and epigenetic modifications compared to healthy animals, as the presence of these inflammatory cells changes the cellular composition of the tissue. The presence of eosinophils and lymphocytes is indicative of underlying inflammation; therefore, transcriptional changes such as an increase in the expression of interleukins, cytokines and chemokines (Davanian *et al.* 2012; Brady *et al.* 2015) should be expected in future RNA-sequencing data. Along with an elevation of transcription of these genes, it is possible that histone modifications, methylation, transcription factor binding and open chromatin regions will also be altered because these modifications influence transcription (ENCODE Project Consortium *et al.* 2007). Observation of inflammatory cells *post-mortem* underscores the importance of histological evaluation in the establishment of a biobank.

This is the first published report of a systemically healthy equine specific biobank and the first non-human biobank to include extensive *ante-* and *post-mortem* phenotypic data. The use of this biobank in the functional annotation of the equine genome will lead to advances in both equine and human medicine. Similar to ENCODE pioneering the field of personalized human medicine, FAANG will advance individualized care of animals for production and companion purposes. Researchers can access sample availability and associated metadata on data.faang.org. It is recommended to then contact the corresponding author to coordinate shipping of samples. Accession information for each horse can be found in Table S1.

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Supporting information

Additional supporting information may be found online in the supporting information section at the end of the article.
Figure S1 A hematoxylin and eosin stained section of the duodenum of ECA_UCD_AH1 demonstrating the eosinophilic and lymphocytic infiltration observed in both horses.
Table S1 Results from clinical examinations and phenotyping of ECA_UCD_AH1 and ECA_UCD_AH2.
Table S2 Description of cryopreserved cells from cell culture.
Table S3 Detailed description of the histopathologic report for every tissue from each individual horse.