



Published in final edited form as:

Anim Genet. 2018 December ; 49(6): 564–570. doi:10.1111/age.12717.

Generation of an equine biobank to be used for Functional Annotation of Animal Genomes Project

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Summary

The Functional Annotation of Animal Genomes (FAANG) project aims to identify genomic regulatory elements in both sexes and 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 plasma, heparinized plasma, cerebrospinal fluid, synovial fluid, urine and microbiome samples from all regions of the gastrointestinal and urogenital tracts were

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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

horse; genome regulation; tissue collection; nuclei isolation; necropsy; biorepository

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; Teyssevre *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 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 UC Davis Veterinary Hospital 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 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. 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-cm³ 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, 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 upper 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). Keratinocyte cultures were established from upper 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/cm² 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/cm². 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 & Müller 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 2–3mm² fragments were placed into a 24-well tissue-culture-treated plate. Complete medium (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 cm² (primary), 3.8 cm² (passage 1), 9.5 cm² (passage 2), 25 cm² (passage 3) and 75 cm² (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. The samples were preserved via one of four methods (Table 2) 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 for each tissue of 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 in the equine community to further the annotation of the 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 as 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to acknowledge members of the Zhou and Ross laboratories at UC Davis for their technical assistance

Funding Sources

Funding for sample collection was provided by the Grayson Jockey Club Foundation, USDA NRSP-8 and the UC Davis Center for Equine Health. Support for M.J.M. was provided by UC Davis Agriculture Experiment Station. Support for C.J.F. was provided by the National Institutes of Health (NIH) (1K01OD015134 and L40 TR001136). E.N.B was supported by USDA NIFA National Need Fellowship Award #20143842021796. All listed funding agencies provided support for sample collection or salary support for the investigators listed above. None of the funding agencies had any role in the design of the study, analysis, interpretation of the data or writing of the manuscript.

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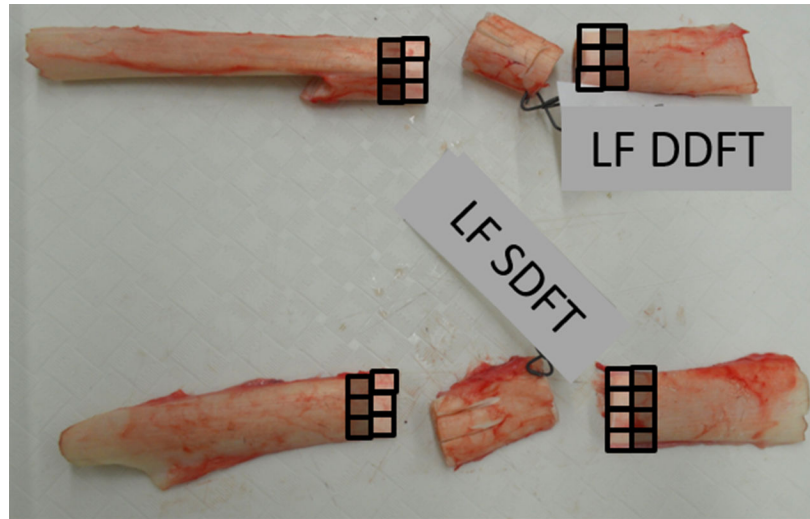


Figure 1. Depiction of collection sites using superficial and deep digital flexor tendon of the left forelimb. The middle sections of tissue were labeled and preserved in formalin for pathological evaluation. The white squares represent proximal samples and the black squares represent the distal samples.

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
Station 2	Musculoskeletal
Station 3	Neurological
Station 4	Respiratory and cardiovascular
Station 5	Gastrointestinal
Station 6	Urogenital

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Table 2.

A complete list of tissues collected from ECA_UCD_AH1 and ECA_UCD_AH2. 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).

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

^fSample only collected from UCDAH1.