AWARD NUMBER: W81XWH-20-1-0294

TITLE: Chromatin Accessibility and the Convergent Oncogenic Pathways of Angiosarcomas

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CONTRACTING ORGANIZATION: University of Minnesota, Twin Cities, Minneapolis, MN

REPORT DATE: July 2021

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2021	AnnualReport	01Jul2020-30Jun2021

4. TITLE AND SUBTITLE

Chromatin Accessibility and the Convergent Oncogenic Pathways of Angiosarcomas

5a. CONTRACT NUMBER
W81XWH-20-1-0294

5b. GRANT NUMBER
CA191225

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER
00081733

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Minneapolis, MN 55455-2070

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Angiosarcoma is a rare type of soft tissue sarcoma, with a prevalence of fewer than 300 cases in the US annually. Our understanding of the oncogenic mechanisms of aggressive angiosarcomas is rudimentary, and our ultimate goal is to develop appropriate and effective treatment options and protocols for patients with this disease. Angiosarcoma are genomically complex; however, they share a histological morphology that consists of disorganized, malignant vessel-forming cells. Our hypothesis is that chromatin accessibility is necessary to establish the mutational landscape, which consequently activates convergent signaling pathways that contribute to angiosarcoma development. We will establish chromatin accessibility and the transcriptomic landscape in angiosarcomas, develop *in vitro* tumor models to define molecular mechanisms that regulate convergent oncogenic pathways in angiosarcomas, and to determine if p53 deficiency in hemangioblasts contributes to angiosarcoma development. This project will impact our understanding of aggressive angiosarcomas and specifically enhance our basic knowledge of how morphologic convergence with genetic chaos arises and contributes to angiosarcoma development. This career development award also supports the PI, Dr. Kim's career goal to develop an independent, extramurally funded research program to advance our understanding of aggressive sarcomas.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	23	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

Angiosarcoma is a rare type of soft tissue sarcoma, with a prevalence of fewer than 300 cases in the US each year. These tumors are highly aggressive and metastatic, and more than half of the patients with this disease die within the first year of diagnosis. Angiosarcomas are genomically complex; however, they share a histological morphology that consists of disorganized, malignant vessel-forming cells. Our objective is to establish chromatin accessibility and the mutational landscape, which activate convergent signaling pathways contributing to angiosarcoma development. Specifically, we will develop tumor models to define molecular mechanisms that regulate convergent oncogenic pathways in angiosarcomas using induced pluripotent stem cells and genome engineering. From this approach, this project tests a new concept that could change the paradigms for addressing the fundamental oncogenic mechanisms of angiosarcomas.

2. KEYWORDS:

Angiosarcoma; rare cancer; induced pluripotent stem cell; CRISPR/Cas9; ATAC-seq; RNA-seq; chromatin accessibility; transcriptomics; hemangioblast; sarcoma modeling; single cell genomics

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The project has three specific aims. In this report for Year-1, we list specific aim 1 and 2 with pertinent major tasks and subtasks below. Details regarding the completion status are provided on section 5, Changes/Problems.

Specific Aim 1: To establish chromatin accessibility and the transcriptomic landscape in angiosarcomas.

Major Task 1: Generation of next generation sequencing data from human angiosarcomas project

Subtask 1: Submit HRPO and ACURO documentation to DoD
- Completed on Jun 3rd and Jun 25th, 2020

Subtask 2: Sample preparation of primary tissue samples for ATAC-Seq and RNA-Seq generation
- Partially completed (50% of completion) and ongoing

Subtask 3: Quality control analysis of samples and sequencing run
- Partially completed (25% of completion) and ongoing

Subtask 4: Initial bioinformatic analysis of sequenced data
- Partially completed (25% of completion) and ongoing

Subtask 5: Advanced bioinformatic analysis of ATAC-Seq and RNA-Seq
- Partially completed (25% of completion) and ongoing

Subtask 6: Project meetings
- Completed (100% of completion) for this period

Subtask 7: Career development for Dr. Kim (learning ATAC-Seq analysis and developing the application)
- Partially completed (50% of completion) and ongoing

Major Task 2: Establishment of angiosarcoma xenografts and generation of single cell-ATAC-Seq and -RNA-Seq

Subtask 1: Ordering and housing mice in animal facilities
- Deferred

Subtask 2: Culture and expand angiosarcoma (AS5 and ISO-HAS) cells
- Partially completed (25% of completion) and ongoing

Subtask 3: Mice xenograft experiment by transplantation of angiosarcoma cells
- Deferred

Subtask 4: Sacrifice mice, generation of histological samples, and harvest xenograft tumors to prepare single cells of tumors
- Deferred

Subtask 5: Generation of single cell-sequencing (scATAC-Seq and scRNA-Seq) data libraries
- Deferred

Subtask 6: Quality control analysis of samples and sequencing run
- Partially completed (10% of completion) and ongoing

Subtask 7: Initial bioinformatic analysis of sequenced data
- Partially completed (10% of completion) and ongoing

Subtask 8: Project meetings
- Completed (100% of completion) for this period

Subtask 9: Career development for Dr. Kim (learning scATAC-Seq and scRNA-Seq)
- Partially completed (25% of completion) and ongoing

Specific Aim 2: To develop in vitro tumor models to define molecular mechanisms that regulate convergent oncogenic pathways in angiosarcomas
Major Task 1: Gene engineering and differentiation in iPSCs

Subtask 1: Generation of iPSCs and preparation of reagents; commercially available iPSC cell lines (iPS12-10 and BYS-0110)
- Partially completed (75% of completion) and ongoing

Subtask 2: Engineering gene mutation (*TP53*, *PIK3CA*, *TP53*/*PIK3CA*) in iPSCs
- Partially completed (25% of completion) and ongoing

Subtask 3: Differentiation of hemangioblasts from engineered iPSCs
- Partially completed (50% of completion) and ongoing

Subtask 4: Functional validation of engineered cells
- Partially completed (50% of completion) and ongoing

Subtask 5: Cell line authentication and Mycoplasma screening (iPSCs-derived cells, HUVEC, fibroblasts, AS5, ISO-HAS)
- Partially completed (20% of completion) and ongoing

Subtask 6: Project meetings
- Completed (100% of completion) for this period

Subtask 7: Career development for Dr. Kim (acquisition of new experimental skills for iPSCs generation and genome engineering)
- Partially completed (25% of completion) and ongoing

Subtask 8: Career development for Dr. Kim (starting development of strategies to secure funding)
- Partially completed (25% of completion) and ongoing

What was accomplished under these goals?
We provide details regarding the impact of the COVID-19 pandemic on the milestones and accomplishment of the project on section 5, Changes/Problems.

Specific Aim 1: To establish chromatin accessibility and the transcriptomic landscape in angiosarcomas.
Major Task 1: Generation of next generation sequencing data from human angiosarcomas project
Subtask 1: Submit HRPO and ACURO documentation to DoD
- University of Minnesota IACUC protocol 2002-37901A was approved on 04/15/2020
- the University of Minnesota IRB determined that the proposed activity is not research involving human subjects as defined by DHHS and FDA regulations on 03/30/2020
- DoD ACURO protocol CA191225.e001 was approved as of 06/25/2020
- HRPO Log Number E01609.1a was approved for HRPO Concurrence With the Determination of Research Not Involving Human Subjects for the Protocol, as of 06/03/2020

Subtask 2: Sample preparation of primary tissue samples for ATAC-Seq and RNA-Seq generation
- Partially completed (50% of completion) and ongoing
We have frozen samples of human angiosarcoma and normal tissues stored at -80C as listed in **Table 1**. After rigorous protocol optimization for preparation, the previous tissue samples those will be processed for ATAC-seq and RNA-seq libraries generation within the next 6 months.

Table 1. Frozen samples of human angiosarcoma and normal tissues					
Specimen#	**Tumor/Normal Status**	**Anatomical Site**	**Age/Sex**	**Biobank Source**	**Note**
T001646_9	Angiosarcoma	Omentum	69F	Bionet	Metastasized tumor
T-02-289	Angiosarcoma	Adrenal gland	Unknown	CHTN	
ED76052T_003	Angiosarcoma	Ovary	40F	CHTN	
M4090402 A2	Angiosarcoma	Breast	65F	CHTN	From one patient
M4090402 B1	Angiosarcoma	Breast	65F	CHTN	
M4130787 A3	Angiosarcoma	Liver	85M	CHTN	
M4150020 A2	Angiosarcoma	Parotid	69F	CHTN	
T001595_6	Normal	Breast	80F	Bionet	
T010554_3	Normal	Skeletal Muscle	74M	Bionet	
T081659_4	Normal	Spleen	33M	Bionet	
CHTN = Cooperative Human Tissue Network					

Subtask 3: Quality control analysis of samples and sequencing run

- Partially completed (25% of completion) and ongoing

We generated ATAC-seq and RNA-seq data libraries from commercially available normal endothelial cells and osteoblast (HUVEC, HUAEC, HOB) to determine sequencing quality and to test bioinformatic algorithms as controls prior to using precious human angiosarcoma tissue samples.

ATAC-seq data: Cells were grown in normal culture condition with respective media for each cell line. Cells were lysed and treated with Tn5 transpose, and 10ul of purified DNA samples were submitted to the UMGC core for creation of ATAC-seq data libraries. Each sample was sequenced to a targeted depth of 50 million reads (2 × 50 pair-end) on NovaSeq. The mean quality score was above the cutoff of 30 for all sequencing libraries. The total number of sequencing reads and sequencing quality control metrics per sample is provided in **Table 2**.

Table 2. ATAC-seq sequencing reads and mean quality score		
Sample	**Total Sequences**	**Mean Quality Score**
HUAEC	56,578,449	34.3
HUVEC	56,655,860	35.0
HOB	52,167,490	35.0

Subtask 4: Initial bioinformatic analysis of sequenced data

- Partially completed (25% of completion) and ongoing

The sequenced reads data as FASTQ format were deposited to the MSI server storage. Using the original FASTQ files, we first checked the quality of the reads and the presence of the Nextera adapters (from reagent used for ATAC-seq library construction) using FastQC tool on Galaxy platform. Then, Cutadapt tool was used to trim sequences of the adapters. We mapped the trimmed reads (FASTQ format) to the human reference genome (GRCh38/hg38) with gene annotation files using Bowtie2. **Table 3** shows results of the trimming and mapping.

Table 3. Pre-processing metrics of ATAC-seq data			
	HUAEC	**HUVEC**	**HOB**
Total read pairs processed	56,578,449	56,655,860	52,167,490
Read 1 with adapter	4,242,734 (7.5%)	5,231,461 (9.2%)	3,464,030 (6.6%)
Read 2 with adapter	4,133,980 (7.3%)	5,269,274 (9.3%)	3,454,829 (6.6%)
Pairs that were too short	373,268 (0.7%)	106,728 (0.2%)	397,220 (0.8%)
Pairs written (passing filters)	56,205,181 (99.3%)	56,549,132 (99.8%)	51,770,270 (99.2%)
Percentage of read pairs mapped concordantly	94.1%	95.6%	94.0%

We then filtered sequence reads that map to the mitchondrial genome and that are not properly paired. We also removed read duplicates resulting from the PCR amplification using Picard MarkDuplicates tool.

To determine the quality of the ATAC-seq data, histograms of fragment size distribution per each sample were generated (**Figure 1**). X-axis indicates insert size (bp), which is the distance between read pair 1 (R1) and 2 (R2), and Y-axis represents insert size frequency (count). All HUAEC, HUVEC, and HOB data showed five major distinct peaks (1st peak around 50bp; 2nd peak around 200bp; 3rd peak around 400bp; 4th peak around 600bp; 5th smallest peak around 800bp). The clear DNA fragment length distributions indicate a good quality of the ATAC-seq data.

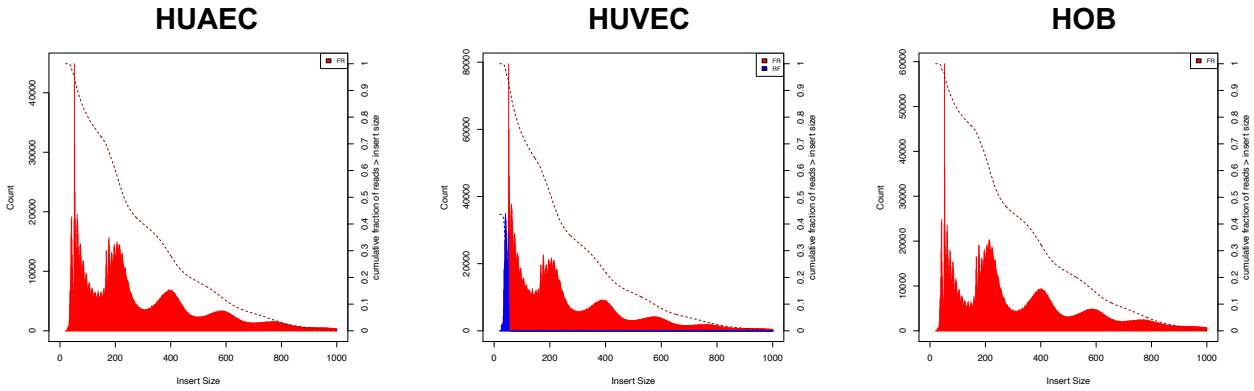

Figure 1. Histograms of DNA fragment size distribution in ATAC-seq data generated from HUAEC, HUVEC, and HOB.

Subsequently, we performed a peak calling, process to identify potential open chromatin regions using MACS2 software. The filtered BAM file was converted to BED format, and the single-end BED files were set to get coverage centered on the 5' extended 100bp each side. Then, bedgraph format data, which is output of call peaks with MACS2 was converted to bigwig format for better performance of visualization. Heatmap of coverage at transcription start sites (TSS) was created with deepTools plotHeatmap, and Figure 2 depicts the heatmaps centered on the TSS generated from HUAEC, HUVEC, and HOB. The heatmaps show genome-wide transcripts from all regions

on Y-axis, and the coverage is represented with a color code scale from red (no coverage) to blue (maximum coverage). TSSs are aligned in the middle of the heatmap with 2 kb gene distance (-1 to 1kb). The mean values at the TSS are also displayed on top of the heatmap. All the three samples show higher values on the left, representing accessible promoter regions of genes. From this approach, we found that accessible chromatin regions are detectable in ATAC-seq data generated from HUAEC, HUVEC, and HOB, suggesting that our protocols and pipeline for ATAC-seq is useful.

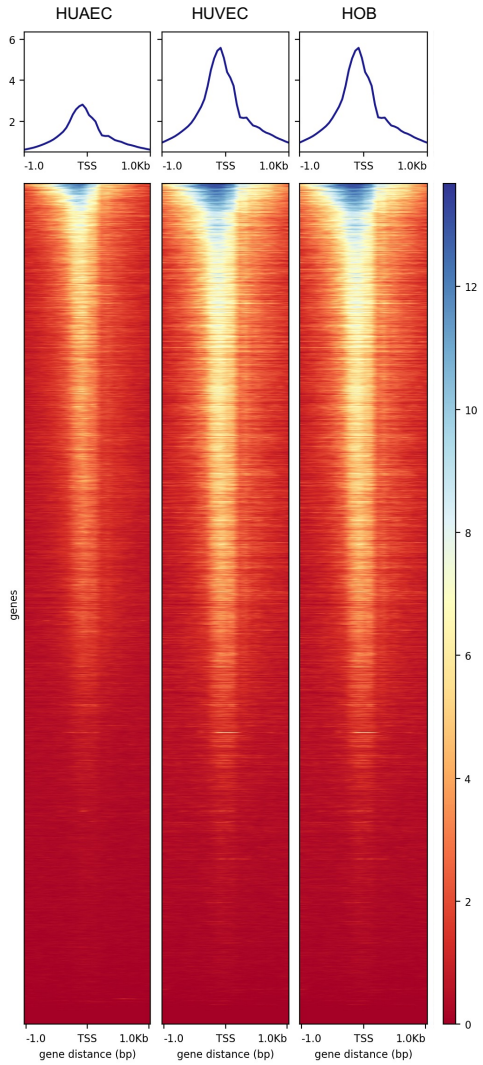

Figure 2. Heatmap of coverage at TSS in ATAC-seq from HUAEC, HUVEC, and HOB.

RNA-seq data: We extracted RNA from HUAEC, HUVEC, and HOB cells grown in the same culture condition with that for ATAC-seq. 1 ug of each RNA sample was submitted to the UMGC. and TruSeq stranded mRNA libraries were created and sequenced to a targeted depth of 20 million reads (2 × 150 pair-end) on NovaSeq. To determine reproducibility, we sequenced second batch of samples to a targeted depth of 50 million reads (2 × 50 pair-end). The mean quality score was above the cutoff of 30 for all sequencing libraries. The total number of sequencing reads and sequencing quality control metrics per sample from both batches is provided in **Table 3**.

Table 3. RNA-seq sequencing reads and quality

	Sample	Total Sequences	Mean Quality Score
Batch1	HUAEC	31,999,528	35.8
	HUVEC	27,894,783	35.9
	HOB	24,884,486	35.8
Batch2	HUAEC	70,774,722	37.3
	HUVEC	81,311,775	37.2
	HOB	66,881,952	37.2

Original FASTQ files were ran by the standard CHURP pipeline for RNA-seq data developed and maintained by the Research Informatics Solutions group at MSI. Raw count reads, output of the CHURP pipeline were filtered and normalized using DESeq2 R package. Normalized count values of 20,322 protein coding genes were log2-transformed to profile genome-wide global gene expression pattern by unsupervised hierarchical clustering analysis (average linkage). Each sample in duplicate shows a positive correlation between two batches by linear regression analysis (R^2 = 0.63 for HUAEC; R^2 = 0.72 for HUVEC; R^2 = 0.98) (**Figure 3A**; on next page). Heatmap also displays that the replicates are clustered together with comparable gene expression signature (**Figure 3B**; on next page): each value was scaled to standardize them by subtracting row means and dividing standard deviation (red bar = upregulated genes; green bar = downregulated genes).

Subtask 5: Advanced bioinformatic analysis of ATAC-Seq and RNA-Seq

- Partially completed (25% of completion) and ongoing

In addition to quality control analysis described above, we are currently comparing different ATAC-seq analysis tools. Since there is no gold standard bioinformatic pipeline for ATAC-seq analysis and it may depend on cell type and experiment, we will apply multiple methods listed below for ATAC-seq analysis generated from the upcoming datasets of human angiosarcoma and xenograft tumor tissue samples. ATAC-seq and RNA-seq data will be integrated to identify open chromatin regions associated with transcriptional regulation specific to angiosarcoma.

- ENCODE-DCC/atac-seq-pipeline (https://www.encodeproject.org/atac-seq/)
- esATAC R package (https://bioconductor.org/packages/release/bioc/html/esATAC.html)
- ATACseqQC R package (https://bioconductor.org/packages/release/bioc/html/ATACseqQC.html)
- ChrAccR R package (https://github.com/GreenleafLab/ChrAccR)

Subtask 6: Project meetings

- Completed (100% of completion) for this period

Subtask 7: Career development for Dr. Kim (learning ATAC-Seq analysis and developing the application)

- Partially completed (50% of completion) and ongoing

Dr. Kim, PI for this project acquired knowledge of bioinformatic algorithms for ATAC-seq analysis, and he tested performance of the analytic pipelines using Galaxy platform and multiple R packages. Data presented above were generated by Dr. Kim as part of learning practice. He will continue to enhance his skills to analyze ATAC-seq data by applying multiple resources in GitHub computational development community and Biostars bioinformatics forum.

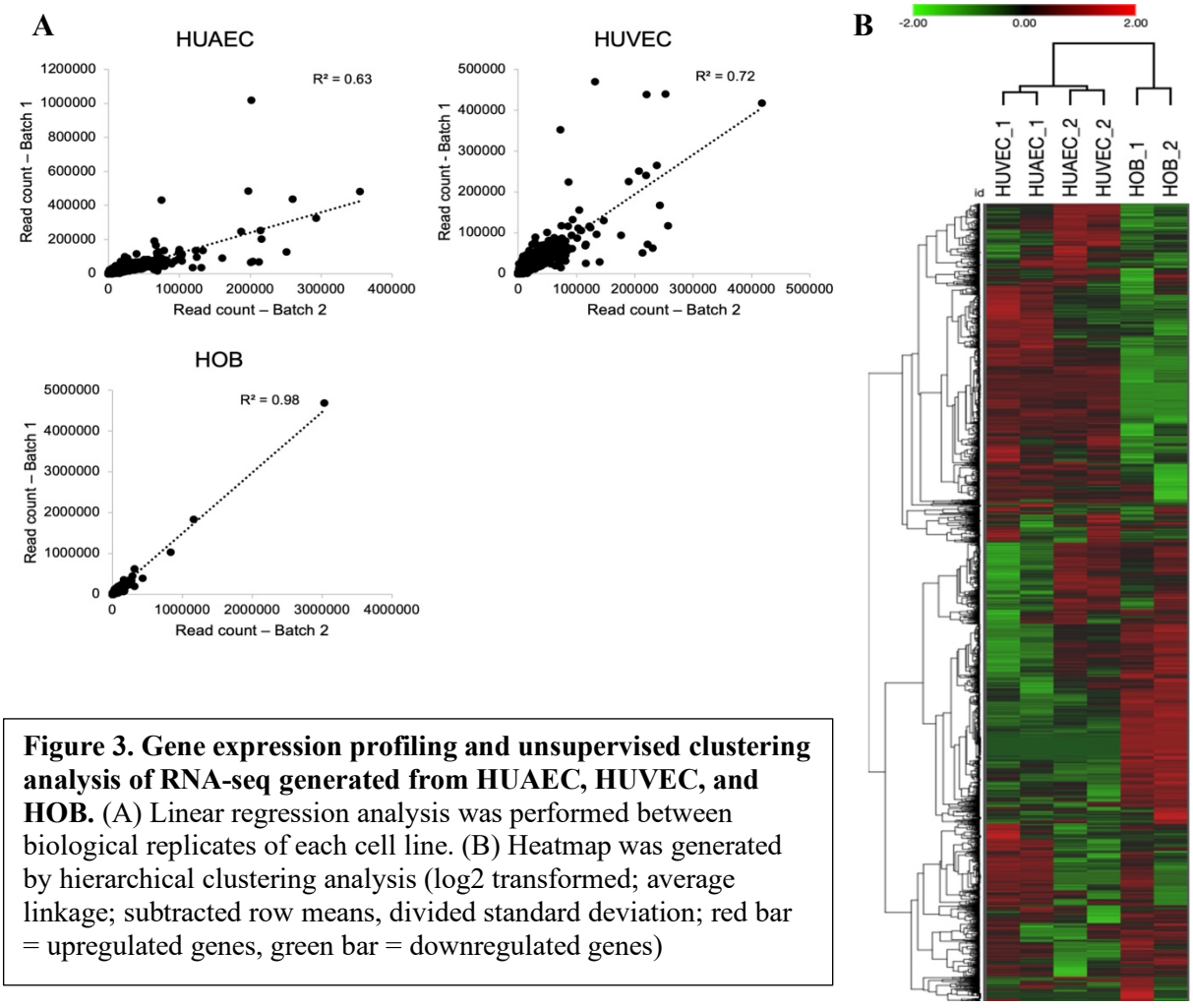

Figure 3. Gene expression profiling and unsupervised clustering analysis of RNA-seq generated from HUAEC, HUVEC, and HOB. (A) Linear regression analysis was performed between biological replicates of each cell line. (B) Heatmap was generated by hierarchical clustering analysis (log2 transformed; average linkage; subtracted row means, divided standard deviation; red bar = upregulated genes, green bar = downregulated genes)

Major Task 2: Establishment of angiosarcoma xenografts and generation of single cell-ATAC-Seq and -RNA-Seq

Subtask 1: Ordering and housing mice in animal facilities

- Deferred

Subtask 2: Culture and expand angiosarcoma (AS5 and ISO-HAS) cells

- Partially completed (25% of completion)

AS5 angiosarcoma cell line was authenticated with STR profiling by IDEXX Laboratories, Inc. on 01/01/20 as shown in **Figure 4**. After the authentication, AS5 cells were expanded to 10 million cells and 1.5 or 2 million cells per vial were inventoried at liquid nitrogen in the University of Minnesota Comparative Oncology group. ISO-HAS cells will be authenticated within next 3 months. After additional cell expansion, both AS5 and ISO-HAS cells will be re-authenticated before mouse xenograft experiments.

Marker Name	11	
	Sample Results	AS5
AMEL	X, Y	NA
CSF1PO	11, 13	NA
D13S317	10	NA
D16S539	9	NA
D18S51	14	NA
D21S11	28, 29	NA
D3S1358	15, 18	NA
D5S818	11, 12	NA
D7S820	9, 11	NA
D8S1179	12, 13	NA
FGA	22	NA
Penta_D	9, 11	NA
Penta_E	7	NA

Figure 4. Cell line authentication of AS5 angiosarcoma cells.

Subtask 3: Mice xenograft experiment by transplantation of angiosarcoma cells
- Deferred

Subtask 4: Sacrifice mice, generation of histological samples, and harvest xenograft tumors to prepare single cells of tumors
- Deferred

Subtask 5: Generation of single cell-sequencing (scATAC-Seq and scRNA-Seq) data libraries
- Deferred

Subtask 6: Quality control analysis of samples and sequencing run
- Partially completed (10% of completion) and ongoing

Subtask 7: Initial bioinformatic analysis of sequenced data
- Partially completed (10% of completion) and ongoing
Dr. Kim performed initial analysis using scRNA-seq datasets generated in Dr. Modiano (Career Guide for this project)'s lab to acquire bioinformatic skills for single cell analysis. More details for training purpose are described below in Subtask 8.

Subtask 8: Project meetings
- Completed (100% of completion) for this period

Subtask 9: Career development for Dr. Kim (learning scATAC-Seq and scRNA-Seq)
- Partially completed (25% of completion) and ongoing
Dr. Kim obtained knowledge of 10x single cell sequencing platform and output data type (barcodes.tsv.gz, features.tsv.gz, matrix.mtx.gz). He used scRNA-seq datasets generated from three peripheral blood samples from dogs with hemangiosarcoma, shared by Dr. Modiano. He applied BingleSeq R package, which is a comprehensive analytic tool for scRNA-seq data based

on Seurat's pipeline and monocle and SC3 R packages, to learn how to perform quality control and t-SNE clustering. He will use and enhance the skills to analyze data planned for this project.

Specific Aim 2: To develop in vitro tumor models to define molecular mechanisms that regulate convergent oncogenic pathways in angiosarcomas
Major Task 1: Gene engineering and differentiation in iPSCs

Subtask 1: Generation of iPSCs and preparation of reagents; commercially available iPSC cell lines (iPS12-10 and BYS-0110)
- Partially completed (75% of completion) and ongoing

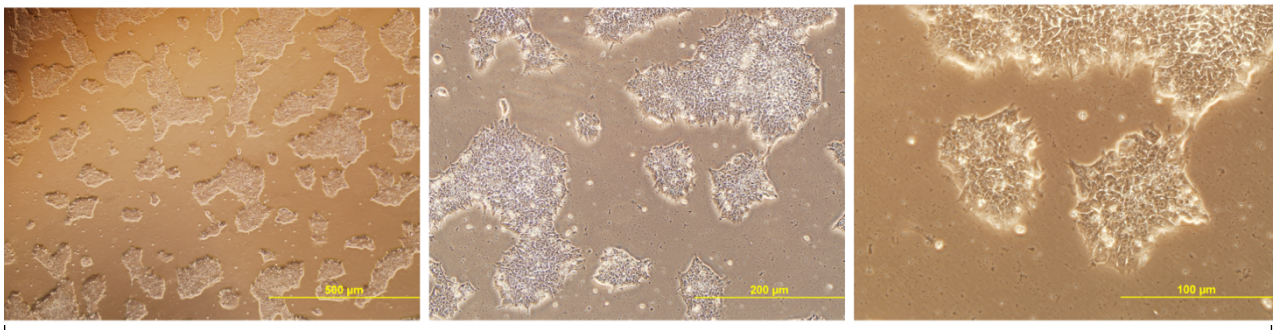

Figure 5. Maintenance of iPS12-10 cells. iPSCs were grown in mTeSR PLUS complete media on a Geltrex coated 6-well plate. Cells were incubated in normal culture condition (5% CO2 and 37C) and media was changed every 1-2 days. Cells were passaged when the plate is 60-80% confluent or when individual colonies are large. Representative microscopic images were captured under 4x (left panel), 100x (middle panel), and 200x magnification (righ panel).

We first used iPS12-10 cell line to optimize and establish protocols for maintaining iPSCs in Dr. Kim's lab. Drs. Webber (Co-I) helped prepare reagents and provided protocols and know-how for iPSC culture and maintenance. We achieved in growing iPS12-10 cells with normal morphology (**Figure 5**) by troubleshooting issues during the culture (**Figure 6**). Optimization of protocols for BYS-0110 cells is underway.

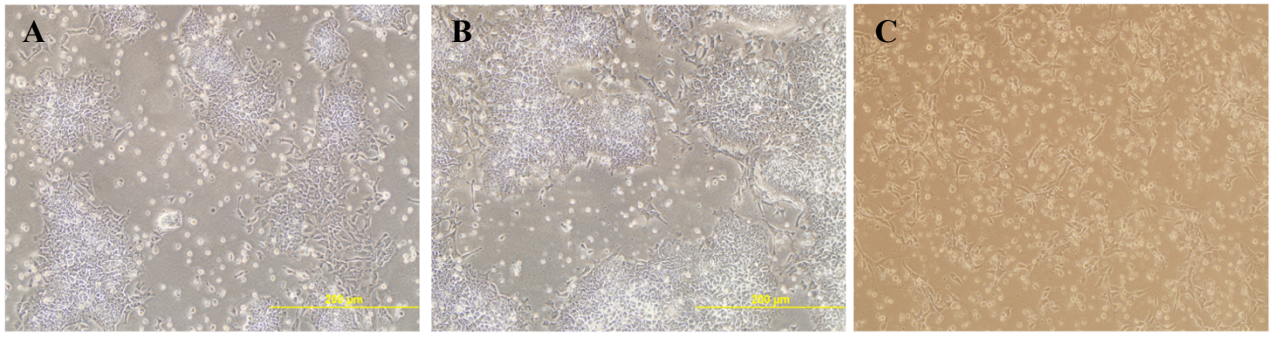

Figure 6. Representative microphotographs of undesirable cell morphology. iPSCs can undergo spontaneous differentiation showing a spindle-shaped morphology stretched from colonies (A and B; 100x magnification). The spindle-shaped cells that grow individually without forming colonies may also be seen in case with excess ROCK inhibitor (C; 100x magnification).

Subtask 2: Engineering gene mutation (*TP53, PIK3CA, TP53/PIK3CA*) in iPSCs

- Partially completed (25% of completion) and ongoing

TP53 mutation was induced in iPS12-10 cells by Dr. Webber lab, and we optimized protocols to compare cell behavior, growth pattern, and cellular morphology between wild-type iPS12-10 cells and *TP53* mutant cells. We found that *TP53* mutant cells show comparable morphology with wild-type cells (**Figure 7**) as seen in Figure 5. It seemed that *TP53* mutant cells grew relatively slower than wild-type cells for first 1-2 days after splitting (**Figure 8**), probably having different growth kinetics; however, no significance was observed during hemangioblast differentiation as described in the following section. We will continue to evaluate molecular and behavioral difference between them.

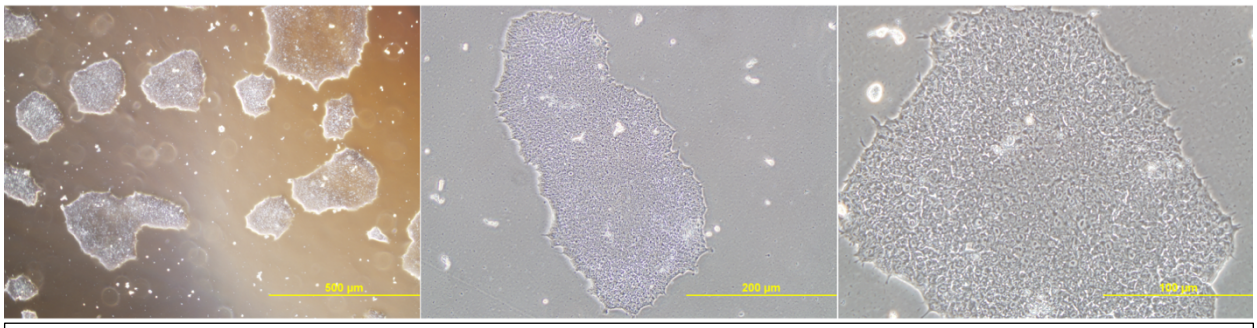

Figure 7. Morphology and growth of *TP53* mutant iPSC. Representative images present that *TP53* mutant iPSCs show virtually identical morphology with that of wild-type iPSCs.

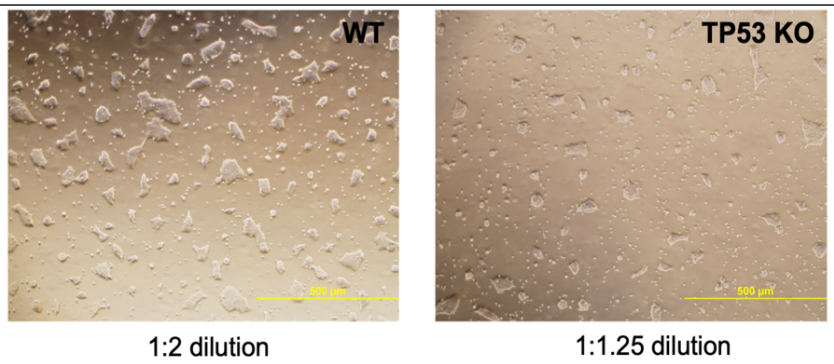

Figure 8. Morphology and growth pattern of TP53 mutant iPSC. Representative images show that *TP53* mutant iPSCs (right panel) has smaller and less colonies than lower wild-type (WT; left panel) cells, although higher number of TP53 mutant cells were plated. Images were captured on day 2 after splitting cells.

Subtask 3: Differentiation of hemangioblasts from engineered iPSCs

- Partially completed (50% of completion) and ongoing

We adapted protocol of iPSC differentiation into hemogenic endothelium or hemangioblast from previous study (**Figure 9A**; obtained from a previous study, Cao et al., 2019; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6565887), and we modified the protocol to differentiate hemangioblasts into endothelial cell lineage. Specifically, iPSCs were maintained to reach 80% confluence, and cultured by replacing respective media as described in Figure 9A until day 5. At day 5, media was replaced with endothelial growth media (EGM) and cells were maintained in EGM for endothelial differentiation.

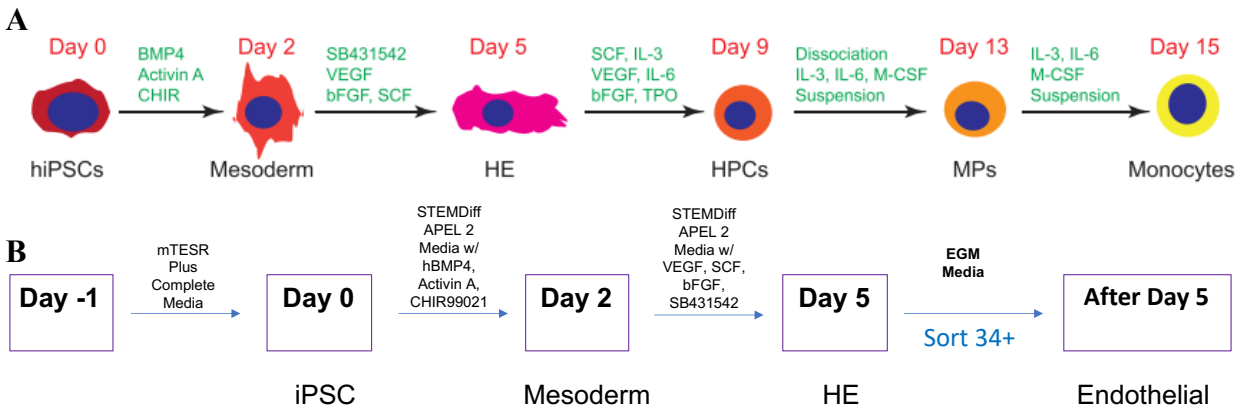

Figure 9. Protocol overview of hemangioblast differentiation from iPSCs. (A) Schematic diagram illustrates experimental steps for iPSC differentiation to hemogenic endothelium that subsequently differentiates into hematopoietic progenitor cells (HPCs) (adapted from Cao et al., 2019). (B) The protocol was modified to develop approaches to differentiate hemangioblast and endothelial cells. HE = Hemogenic endothelium; HPC = hematopoietic progenitor cell; MP = myeloid progenitor.

We achieved differentiation of WT and *TP53* KO (or mutant) iPSCs into sequential developmental stages as described above. Our data showed that no difference in morphology and growth pattern was found between WT and *TP53* cells during differentiation (**Figure 10**).

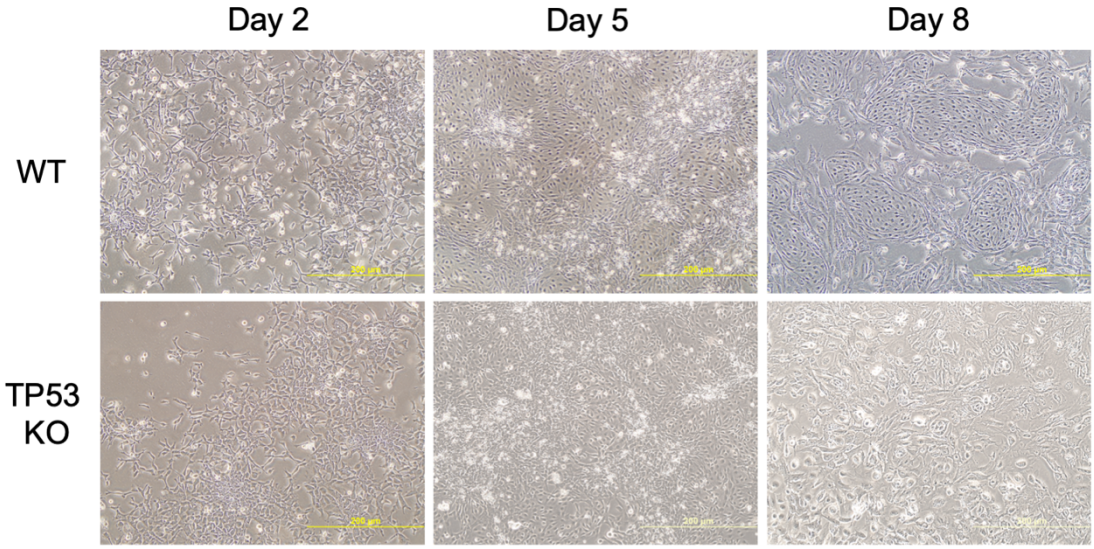

Figure 10. Differentiation from iPSCs into mesoderm, hemangioblast, and endothelial cells. WT and *TP53* KO iPSCs were differentiated over time (day 2, 5, and 8) as described in Figure 9. Representative phase contrast images were captured at 100x magnification.

<u>Subtask 4: Functional validation of engineered cells</u>

- Partially completed (25% of completion) and ongoing

We were able to identify CD34+/CD43- cells, putative hemangioblasts differentiated from iPSCs. The cells were characterized by flow cytometry at day 8 since differentiation initiated. Our data show that the number of CD34+ cells was 32% of total population grown continuously

in stem cell differentiation (SCD) condition after day 5, while 20% of cells were CD34+ positive when they were switched to grow in endothelial growth media (EGM) (**Figure 10A and B**). We also found that cells did not form tube-like structure efficiently in SCD media assessed by Matrigel tube formation assay, whereas cells grown in EGM achieved the tube formation (**Figure 10C and D**). These data suggest that cells presumed as hemangioblasts have capacity to differentiate into endothelial cell lineage. We also harvested both WT and *TP53* cells from each differentiation stage at day 2, 5, and 8, ran flow cytometry to evaluate expression of surface markers, and extracted RNA for gene expression analysis. Data analyses are ongoing.

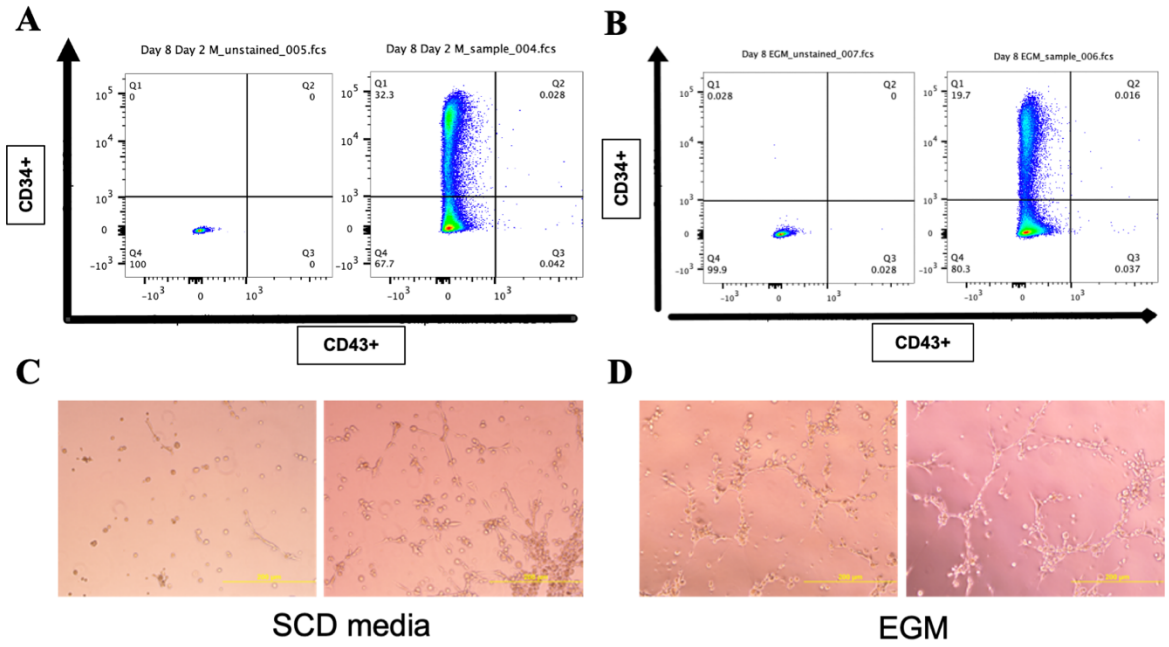

Figure 10. Endothelial cells differentiated from iPSC-derived CD34+ hemangioblast. Representative scatter plots of flow cytometry data show CD34+ population grown in stem cell differentiation (SCD) media (A) and in endothelial growth media (EGM) (B) measured at day 8. Microscopic images present tube-forming capability of CD34+ cells cultured in SCD media (C) and EGM (D). Two representative areas of each condition were captured under microscope at 100x magnification.

<u>Subtask 5: Cell line authentication and Mycoplasma screening (iPSCs-derived cells, HUVEC, fibroblasts, AS5, ISO-HAS)</u>

- Partially completed (20% of completion) and ongoing

We have done cell line authentication for AS5 as shown above. We will complete authentication of iPSC-derived cells in next 3 months. We have not grown other cell types in this period, and we will continue to do cell authentication when new batches of cells are recovered from frozen inventory and maintained in culture.

<u>Subtask 6: Project meetings</u>

- Completed (100% of completion) for this period

Subtask 7: Career development for Dr. Kim (acquisition of new experimental skills for iPSCs generation and genome engineering)

- Partially completed (25% of completion) and ongoing

Dr. Kim closely worked with Ms. Sophia Wenthe (research scientist) in his lab and Dr. Webber lab to learn iPSC maintenance and hemangioblast differentiation. He obtained background knowledge of the stem cell experiments, optimized the protocol, and compared data with literature. He also worked with Dr. Brian Ruis (research scientist) in the the University of Minnesota Genome Engineering Shared Resource (GESR) co-led by Dr. Moriarity (co-I for this project). He acquired knowledge of CRISPR/Cad9 system and learned about gRNA designation for inducing *PIK3CA* H1047R point mutation. Briefly, a 90-nucleotide (nt) single-stranded oligo DNA nucleotide (ssODN) was designed for single-base substitutions to create H1047R homozygous mutations in *PIK3CA* gene. Editing efficiency of the single guide RNA (sgRNA) at generating a single nucleotide insertion is being determined by Tracking of Indels by Decomposition (TIDE) analysis. Dr. Kim is coordinating the work to induce *PIK3CA* mutation in AS5 cells and will apply this approach for gene editing in iPSCs in collaboration with Dr. Webber group.

Subtask 8: Career development for Dr. Kim (starting development of strategies to secure funding)

- Partially completed (25% of completion) and ongoing

Dr. Kim started to develop strategies to secure internal and external funding during the project Year-1. For instance, he prepared multiple grant proposals including DoD (FY20 RCRP and PRCRP; Idea Award) and NIH (NIBBIB R21) applications by developing new projects related to use of iPSCs. Dr. Kim initiated a new collaboration with Dr. Daeha Joung, faculty in physics at the Virginia Commonwealth University to develop a novel 3D bioprinting model for brain cancers, where he applied knowledge and skills to use iPSCs and genomic analysis. Their DoD pre-application has been invited for full application to FY21 DoD PRCRP, and he will contribute to iPSC biology and bioinformatic analyses for the project, as a sub-awarded, principal investigator at the University of Minnesota. He also developed experimental plan to use canine iPSCs to create a tumor model for canine hemangiosarcoma. From this approach, he seeks for funding opportunities to continue projects to identify convergent oncogenic mechanisms of human angiosarcoma and canine hemangiosarcoma. He is preparing an NIH Director's New Innovator Award (RFA-RM-21-016) due in Aug, 2021 and an R01 due in Oct, 2021 or Feb, 2022.

What opportunities for training and professional development has the project provided?

Training development: Dr. Kim advanced experimental skills in stem cell biology and practical experience in bioinformatics data analysis as described above. These activities were done by scientific discussion and collaboration with other individuals as well as by self-learning through online tutorial and manual.

Professional development: Dr. Kim presented his work in the institutional seminar series including the Comparative Oncology and Immunology Seminar (Jul 9, 2020) and Genetic Mechanisms of Cancer (GMOC) seminar (Apr 26, 2021). He attended two virtual conferences, 2021 The Evolving Tumor Microenvironment in Cancer Progression (Jan 11-12, 2021) with poster presentation and 2021 AACR Virtual Meeting: Sarcomas (June 28-29, 2021). From these activities, he practiced data organization and presentation to improve scientific communication

skills. It also provided him to have opportunities to increase knowledge and research trend in a broader range of cancer research.

How were the results disseminated to communities of interest?
Some results of the project were disseminated through presentations at AACR virtual international conference "Role of PI3K Pathway in Reprogramming the Tumor Niche of Angiosarcoma. 2021 The Evolving Tumor Microenvironment in Cancer Progression held on Jan 11-12, 2021) and at internal seminar series "GMOC seminar" in the Masonic Cancer Center, on Apr 26, 2021.

What do you plan to do during the next reporting period to accomplish the goals?
As described above, we began with tasks for specific aim 2 in Year-1, while some of tasks for specific aim 1 were postponed until Year-2. We will continue to perform experiments for next-generation sequencing from human tissues and data analysis and will complete xenograft experiment of human angiosarcoma cells as planned for specific aim 1. Besides, we will follow the milestones as addressed in Year-2. We will move forward with iPSC-derived hemangioblast differentiation and functional characterization from another iPSC line, BYS-0110. Dr. Kim's career development plan including preparation of applications for federal grants will also be continued.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?
We had designed this project to determine convergent molecular programs that define the pathogenesis of angiosarcoma, and in particular, genomic complexity and heterogeneity of the tumor and their role in revealing angiosarcoma-specific phenotype. However, it would be challenging to achieve this goal for this rare type of cancer when a reliable model does not exist. We offer a novel experimental approach using human induced pluripotent stem cells along with genomic analysis. During first project year, we have established and optimized protocols that solidify our experimental strategies and help troubleshooting. The data from this project have shown feasibility, reproducibility, and reliability for the tumor modeling using the stem cells. In addition, the data have shown developmental behavior of stem cells that differentiate into a specific cell type, as putative angiosarcoma progenitor cell. Since precise origin of angiosarcoma is unknown, the data have gained our understanding of the cellular property and mechanisms that give rise to the tumor.

What was the impact on other disciplines?
ATAC-seq analysis becomes popular in biomedical research for a variety of diseases to understand genomic and epi-genomic features. However, optimization of protocols for sample preparation, quality control process, and bioinformatic pipeline for ATAC-seq data have not been standardized and need further development. Our work has shown early success of ATAC-seq generation and quality control as well as has identified a series of bioinformatic pipelines. Our ongoing work will help in applying this technique to other cell types and medical conditions that reveal complex genomic features. Furthermore, data from hemangioblast differentiation of iPSCs will impact on modeling strategies for other types of tumor, vascular diseases, and inflammatory diseases.

What was the impact on technology transfer?
- Nothing to Report

What was the impact on society beyond science and technology?
- Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
The project was activated to start as of July 1st, 2020 during the COVID-19 pandemic. The pandemic had a significant impact on our capacity to initiate laboratory work at the beginning of the project due to lab hibernation and limited activities ordered and regulated by the University of Minnesota and the State of Minnesota. Specifically, the University of Minnesota announced all in-person event cancellation, remote work, and research lab hibernation as of March 17, 2020 (for more information: COVID-19 Announcement Archive; https://safe-campus.umn.edu/return-campus/covid-19-updates). Then, the University of Minnesota Return to Work Sunrise Plan was initiated as of May 18 (for more information: https://safe-campus.umn.edu/sunrise-plan). It required submission of the "Request for Return to On-Site Work Authorization" form by describing justification of on-site work in details. The Sunrise plan consists of three steps: 50% of on-site work is allowed in Step 1, 75% in Step 2, and 100% in Step 3. The request for return to my own lab (50% of on-site work) was approved by the authorized department on July 28, 2020. The Sunrise Plans was active until June 30, 2021, which is a period of the project Year-1.

Some of facilities and services resumed in July of 2020, but COVID-19-related work was prioritized to conduct, and this continued to have negative effect on our plan during the project Year-1. The University of Minnesota Genome Center (UMGC) deferred sequencing work for non-COVID-19 testing and the subsequent backlog in sequencing order caused the delay of our work. Limited access to the research Animal Resources (RAR) and shortage of PPE in the animal facility also impacted on animal work until 2020.

Actual or anticipated problems or delays and actions or plans to resolve them
Based on the pandemic impact, we switched some of our work between Year-1 and -2 since those tasks were independent: Specifically, we deferred work to generate sequencing data and to conduct animal experiment; instead, we focused on bioinformatic analysis and cell culture work including iPSC maintenance and differentiation during Year-1.

Despite the impact on mostly timeline of the project during the pandemic, we believe that we made considerable progress towards the stated goals and task of the project as reported.

Changes that had a significant impact on expenditures
- Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
- Mouse experiments using mice planned to perform during Year-1 was not done due to the COVID-19 pandemic. There is no significant change in use or care of animals; instead, we changed timeline to perform the animal experiment in Year-2.

Significant changes in use or care of human subjects
- Nothing to Report

Significant changes in use or care of vertebrate animals
- Nothing to Report

Significant changes in use of biohazards and/or select agents
- Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Sophia Wenthe, Ashley J. Schulte, Mathew G. Angelos, Dan S. Kaufman, Jaime F. Modiano, Jong Hyuk Kim. *Role of PI3K Pathway in Reprogramming the Tumor Niche of Angiosarcoma.* 2021 The Evolving Tumor Microenvironment in Cancer Progression (Virtual conference; Jan 11-12, 2021)

  Journal publications.
  - Nothing to Report

  Books or other non-periodical, one-time publications.
  - Nothing to Report

  Other publications, conference papers and presentations.
  - Nothing to Report

- **Website(s) or other Internet site(s)**
  - Nothing to Report

- **Technologies or techniques**
  - Nothing to Report

- **Inventions, patent applications, and/or licenses**
  - Nothing to Report

- **Other Products**
  - Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

There is no significant change from original SOW.

Name:	*Jong Hyuk Kim*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-1645-0036 (ORCID ID)*
Nearest person month worked:	*7.8*

Contribution to Project:	*Dr. Kim has performed work in the area of bioinforamtics and iPSC-derived hemangioblast differentiationg.*
Funding Support:	*DoD Career Development Award*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Completed grants and new grants changed during period of project Year-1 are listed below.

Completed Grants (2020 – 2021)

a) Title of project: Biosensors for Early Disease Detection
b) Funding agency: Boston Scientific
c) Goals of the project: *The goal is to develop a sensor to detect diagnostic molecular profiles for cancer and other chronic conditions*
d) Specific aims/tasks:
Specific Aim 1: Develop cell culture models for breath sensor analysis.
Specific Aim 2: Develop machine learning models for predictive data analysis.
e) Estimated start and end date: 01/01/19 – 06/30/20 (NCE)
f) Level (%) of effort: 50% effort (6.0 person-calendar months)
g) Point of contact at the funding agency:
Greg Sherwood
Email: Gregory.Sherwood@bsci.com
h) Overlap: None

a) Title of project: Mechanisms of Resistance to Immunotherapy in Osteosarcoma
b) Funding agency: Department of Defense
c) Goals of the project: *The goal of this project is to identify microRNAs that impair T cell activation at the tumor site and in the draining lymph node, creating immunologically barren tumors.*
d) Specific aims/tasks:
Specific Aim 1: We will determine the prevalence of CD28-targeting microRNAs in osteosarcoma and osteosarcoma-derived exosomes using a multi-species comparative approach.
Specific Aim 2: We will establish mechanisms of CD28 silencing by osteosarcoma-derived exosomes.
Specific Aim 3: We will define the relationship between CD28 silencing and resistance to immune checkpoint blockade in pre-clinical osteosarcoma mouse models.
e) Estimated start and end date: 09/01/18 - 07/31/20
f) Level (%) of effort: 10% effort (1.2 person-calendar months)
g) Point of contact at the funding agency:
Emilee Senkevitch, PhD, PMP
Science Officer
Email:
emilee.r.senkevitch.ctr@mail.mil
h) Overlap: None

a) Title of project: eBAT as a Modulator of the Myeloid Immune Checkpoint in Cancer
b) Funding agency: Rein In Sarcoma Foundation
c) Goals of the project: *The goal of this project is to determine the effect of immunosuppressive myeloid cell depletion or persistence in the therapeutic efficacy of eBAT against sarcomas.*
d) Specific aims/tasks:
Specific Aim: To determine the effect of immunosuppressive myeloid cell depletion or persistence in the therapeutic efficacy of eBAT against sarcomas.
e) Estimated start and end date: 03/01/20 - 02/28/21
f) Level (%) of effort: 5% effort (0.6 person-calendar months)
g) Point of contact at the funding agency:
Jessica Raines-Jones
Email:
mccgrant@umn.edu
h) Overlap: None

New Grants (2020 – 2021)

a) Title of project: Novel Immunotherapy for Metastatic Osteosarcoma
b) Funding agency: CVM Comparative Signature Program
c) Goals of the project: *The goal of this project is to develop a novel immunotherapeutic strategy for metastatic osteosarcoma using eBAT and ONIx.*
d) Specific aims/tasks:
Specific Aim 1: To characterize the mechanisms of action of eBAT and ONIx to remodel the metastatic osteosarcoma microenvironment and promote anti-tumor immunity.
Specific Aim 2: To establish safety and efficacy of combination of eBAT and ONIx in a model of metastatic osteosarcoma.
e) Estimated start and end date: 06/01/21 - 05/31/23
f) Level (%) of effort: 1% effort; cost shared (0.12 person-calendar months)
g) Point of contact at the funding agency:
Kersten Warren
Email: kdanders@umn.edu
Phone:
h) Overlap: None

What other organizations were involved as partners?
- Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
- Not applicable

QUAD CHARTS:
- Not applicable

9. APPENDICES:

Abstract presented at the virtual conference, "2021 The Evolving Tumor Microenvironment in Cancer Progression" on Jan 11-12, 2021.

Role of PI3K Pathway in Reprogramming the Tumor Niche of Angiosarcoma

Sophia Wenthe,[1,2,3] Ashley J. Schulte,[1,2,3] Mathew G. Angelos,[4] Dan S. Kaufman,[5] Jaime F. Modiano,[1,2,3] Jong Hyuk Kim[1,2,3]

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[2]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN
[3]Masonic Cancer Center, University of Minnesota, Minneapolis, MN
[4]Internal Medicine, Hospital of the University of Pennsylvania, Philadelphia, PA
[5]Division of Regenerative Medicine, Department of Medicine, University of California-San Diego, La Jolla, CA

Angiosarcoma (AS) is a highly aggressive, rare soft-tissue sarcoma that forms malignant blood vessels. Approximately half of patients have metastatic or unresectable disease with a median overall survival of less than 6 months, and tumor-related mortality is high. Inflamed tissue is frequently seen in angiosarcomas, and immune cells are key components that contribute to the landscape of the tumor microenvironment. Hemangiosarcoma (HSA) occurs commonly in companion dogs, and it shares clinical and morphological features with human AS. Recurrent mutations in *TP53* and genes involved in PI3K pathway such as *PIK3CA*, and *PIK3R1* were observed in both human AS and canine HSA, and the mutational landscape was associated with distinct molecular tumor subtypes; in particular, differing immunophenotypes. Recently, we have shown that human AS and canine HSA establish convergent transcriptional programs driven by angiogenic fusion genes and *TP53* mutation. However, these vascular tumors are genomically complex, and molecular mechanisms that establish the tumor microenvironment are incompletely known. In this study, we analyzed RNA-seq transcriptomic data generated from human AS tissues (n=13) and canine HSA tissues (n=76) and HSA cell lines (n=11). The data showed that the tumors of both species enriched gene signatures associated with maintenance of hematopoietic stem cells. Then, we found that canine HSA cells supported expansion and differentiation of human CD34+ umbilical cord blood cells using long-term culture-initiating cell and colony-forming unit assays. We also developed xenograft models of canine HSA that facilitate myelopoiesis and macrophage infiltration in the tumor tissue. Our data showed that gene signatures of monocytes and naïve macrophages were detectable in canine HSA cells. In human ASs, macrophage-associated genes, in particular M2 macrophage signatures were significantly enriched, compared to normal tissues. Collectively, our data suggest that human AS and canine HSA have cell-autonomous capacity to govern hematopoietic progenitors and immune cells, potentially establishing the tumor immune niche. We engineered HSA cells to induce homozygous H1047R mutations in *PIK3CA* gene using CRISPR-Cas9 system. Our ongoing work is to determine if regulating PI3K pathway in the tumor cells contributes to the molecular programs that create the tumor niche of the vascular malignancy.