AWARD NUMBER: W81XWH-19-1-0505

TITLE: Understanding and Targeting Pulmonary Arteriovenous Malformations Using Repurposed Drugs

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CONTRACTING ORGANIZATION: Stanford University, Stanford, CA

REPORT DATE: August 2021

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2021	Annual Report	01Aug2020-31Jul2021

4. TITLE AND SUBTITLE

Understanding and Targeting Pulmonary Arteriovenous Malformations Using Repurposed Drugs

5a. CONTRACT NUMBER
W81XWH-19-1-0505

5b. GRANT NUMBER
GRANT12711633, PR181774

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER
1

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)
Year 2 Technical Report

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Hereditary hemorrhagic telangiectasia (HHT) is a genetic disease characterized by multiple **arteriovenous malformations (AVMs)** which are direct connections between arteries and veins, bypassing the capillary bed. Severe epistaxis (nosebleeds) is the most common symptom, yet visceral AVMs in the brain (1-10%), lung (15-45%), liver and gastrointestinal tract cause *significant morbidity and mortality due to embolic stroke, cerebral abscess, migraines, hemorrhagic stroke, seizures and life-threatening bleeding complications*. *In order to reduce the morbidity and mortality associated with HHT, we need a better understanding of HHT development and novel treatment approaches*. Our aims are: Aim 1: To understand the cellular and molecular mechanisms of PAVM development in mice and to identify the cell behaviors and populations that give rise to PAVMs. Aim 2: To identify and target pathological downstream signaling in endothelial cells derived from iPSCs (iPSC-ECs) from HHT patients with visceral AVMs. Aim 3: To target pathological downstream signaling with repurposed drugs to *prevent* and *reverse* PAVMs in the mouse model. The ***short-term impact*** will be a better understanding of how AVMs form in the lung and potentially in other organs. The ***long-term impact*** will be the identification of potential novel treatments for AVMs.

15. SUBJECT TERMS
Arteriovenous malformations, vascular malformations, epistaxis, Hereditary hemorrhagic telangiectasia, HHT, induced pluripotent stem cells, AVMs, Endoglin, Alk-1, Smad-4

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	20	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

Hereditary hemorrhagic telangiectasia (HHT) is a genetic disease characterized by multiple **arteriovenous malformations (AVMs)** which are direct connections between arteries and veins, bypassing the capillary bed. Severe epistaxis (nosebleeds) is the most common symptom, yet visceral AVMs in the brain (1-10%), lung (15-45%), liver and gastrointestinal tract cause *significant morbidity and mortality due to embolic stroke, cerebral abscess, migraines, hemorrhagic stroke, seizures and life-threatening bleeding complications. In order to reduce the morbidity and mortality associated with HHT, we need a better understanding of HHT development and novel treatment approaches*. HHT causing mutations in *ENDOGLIN,* ALK1 and SMAD4 are heterozygous loss-of function mutations resulting in haplo-insufficiency and are responsible for the development of HHT in 85% of patients. *We still do not know precisely how AVMs develop, whether they are congenital or acquired, and how to prevent or even reverse them*. We **hypothesized** that *understanding the cellular and molecular mechanisms that govern the development of vascular malformations paired with the identification of* ***clinically relevant,*** *pathological signaling abnormalities and endothelial cell behaviors will allow us to develop and test novel therapeutic approaches that prevent and potentially reverse disease.* We have the following specific aims: **Aim 1:** To understand the cellular and molecular mechanisms of PAVM development in mice and to identify the cell behaviors and populations that give rise to PAVMs. **Aim 2:** To identify and target pathological downstream signaling in endothelial cells derived from iPSCs (iPSC-ECs) from HHT patients with visceral AVMs. **Aim 3:** To target pathological downstream signaling with repurposed drugs to *prevent* and *reverse* PAVMs in the mouse model of PAVMs. The ***short-term impact*** will be a better understanding of how AVMs form in the lung and potentially in other organs (brain, liver GI tract). The ***long-term impact*** will be the identification of potential novel treatments for AVMs.

2. KEYWORDS:

Arteriovenous malformations (AVM), hereditary hemorrhagic telangiectasia (HHT), epistaxis, pulmonary AVMs, Endoglin, Alk-1, Smad-4, induced pluripotent stem cells.

3. ACCOMPLISHMENTS:

○ What were the major goals of the project?

Research-Specific Tasks:

Aim 1: To understand the cellular and molecular mechanisms of PAVM development in mice and to identify the cell behaviors and populations that give rise to PAVMs		
Major Task 1: Develop a mouse model of pulmonary AVMs by deleting HHT genes in specific subtypes of endothelial cells		
Aim 1a. *To localize HHT genes in endothelial cells of the embryonic, postnatal, and adult mouse lung using multiplex single-molecule fluorescence in situ hybridization to determine which anatomic and molecularly defined cell populations co-express them* *Transgenic mice used: C57 BL6 mice (Jackson Laboratory)* *80% completion*	1-6	Dr. Metzger/Dr. Gillich
Aim 1b. *To determine in which endothelial cell type the HHT genes mediate PAVM development by deleting Smad4 as well as Alk1 in specific endothelial cell populations in the embryonic, postnatal, and adult mouse lung and applying additional stimuli to facilitate PAVM development such as increased shear stress and flow by left pulmonary artery banding.* *Transgenic mice used: floxed Smad4 mice, floxed Alk1 mice, Apj-CrER and Cx40-CreER mice (available in Dr. Metzgers Lab)* *50% completion. We have deleted Smad 4 as well as Alk1 in Apj expressing endothelial cells, which mark the pulmonary capillaries at different time-points: Early post-natally (deletion with tamoxifen P2, harvest15 days postnatally as well as injection with tamoxifen P34, harvest 18 days after).*	1-6	Dr. Metzger/Dr. Gillich
Aim 1c: *To elucidate the cellular events underlying PAVM development and growth, and define the cell behaviors that give rise to PAVMs using multicolor labeling and high-resolution 3-D imaging* *Transgenic mice used: floxed Smad4 mice, floxed Alk1 mice, Apj-CrER and Cx40-CreER mice, Confetti mice (available in Dr. Metzgers Lab)*	1-12	Dr. Metzger/Dr. Gillich

10% completion. We are using the confetti mice (multicolor labeling) yet have not optimized the mouse model of pAVMs to really benefit from the Confetti mice yet, to determine clonality when assessing cell proliferation.		
Milestone(s) Achieved: 1. *Identification of the specific endothelial cell type responsible for AVM formation* 2. *Deletion of Smad4 and ALK1 in the endothelial subtype resulting in vascular pathology and AVM formation*	12	Dr. Metzger/Dr. Gillich/Dr. Spiekerkoetter
Aim 2: To identify and target pathological downstream signaling in endothelial cells derived from induced pluripotent stem cells (iPSCs) from HHT patients with visceral AVMs		
Major Task 2: To use cells from HHT patients to unravel common downstream pathways of ALK1, Endoglin and SMAD4 and to test repurposed drugs for their therapeutic potential.		
Aim 2a. *To identify common and unique downstream targets related to ALK1, ENG and SMAD4 mutations in iPSC-derived ECs and their CRISPR corrected isogenic controls derived from PBMCs from HHT patients with visceral AVMs (pulmonary, cerebral, hepatic) using RNA sequencing.* *<u>Cells used:</u> Peripheral blood mononuclear cells (PBMCs) from HHT patients at Stanford University. Generation of iPSCs from patient PBMCs. Reprogramming of iPSCs – to iPSC-Endothelial cells. Genome editing using CRISPR-Cas9.* *The Stanford IRB/SCRO is approved, yet due to COVID-19 the clinical research and patient blood sampling at Stanford to collect PBMCs and make iPSCs has been extremely slowed down and we were not able to invite patients for study purposes until earlier this year. To identify common and unique downstream targets of ALK1, ENG and SMAD4 and to move the project forward we have deleted the above genes in commercially available pulmonary artery endothelial cells using siRNA and have performed RNA sequencing in the presence and absence of the ligand BMP9. We have identified several common and unique downstream signals, and are currently confirming the candidates by QPCR. We will submit the Stanford IRB to the DoD to get the approval to start those experiments.*	1-13	Dr. Spiekerkoetter/ Dr. Metzger
Aim 2b. *To test whether existing drugs already in clinical trials (Avastin), novel drugs identified in high throughput drug screens for activators of BMPR2-ALK1 such as FK506 and Enzastaurin, as well as drugs predicted in silico can reverse the pathological downstream signaling in iPSC-derived endothelial cells and reverse abnormal endothelial cell behaviors and function (tube formation, migration, apoptosis, proliferation).* *<u>Cells used:</u> Peripheral blood mononuclear cells (PBMCs) from HHT patients at Stanford University. Generation of iPSCs from patient PBMCs. Reprogramming of iPSCs – to iPSC-endothelial cells. Genome editing using CRISPR-Cas.* *Planned for the next 6 months:* *Until iPSCs will become available, we will continue to use PAECs +/- siRNA for ENG, ALK1 and SMAD4 with Enzastaurin or FK506 and perform again RNA seq to determine whether those drugs are able to reverse the gene expression signature as well as function of the dysfunctional cells.*	13-24	Dr. Spiekerkoetter
Milestone(s) Achieved: 1. *Generating iPSC derived ECs from HHT patients and their isogenic CRISPR controls, which are unique cell pairs that allow us to identify novel canonical and non-canonical treatment targets* 2. *Identifying some novel common downstream target of all 3 HHT genes in addition to Id1*	24	Dr. Spiekerkoetter

3. *Testing previously identified repurposed drugs (FK506, Enzastaurin) and novel drugs on iPSC-EC to see whether they reverse gene expression and function*		
Aim 3: To target pathological downstream signaling with repurposed drugs to *prevent, inhibit growth* and potentially *reduce the size of* pulmonary AVMs.		
Major Task 3: To test AVM prevention and reversal strategies in HHT mouse models		
Aim 3a. *To test whether known repurposed drugs (FK506, Enzastaurin) as well as novel drugs identified and tested in Aim 2* ***prevent*** *the formation of PAVMs in mouse models with deletion of Smad4 in specific subsets of endothelial cells generated in Aim 1.* *Transgenic mice used: Same as in Aim1* *We have not been successful yet in creating a robust animal model of PAVMs to test the prevention of AVM formation with drugs.*	12-28	Drs. Metzger, Gillich, Spiekerkoetter
Aim 3b. *To test whether FK506, Enzastaurin or other repurposed drugs identified in Aim 2 can* ***inhibit growth and potentially reduce*** *established PAVMs.* *Transgenic mice used: Same as in Aim1* *We have not been successful yet in creating a robust animal model of PAVMs to test the growth inhibition and reversal of AVM formation with drugs.*	28-36	Drs. Metzger, Gillich, Spiekerkoetter
Milestone(s) Achieved: 1. *Testing of novel drugs that might inhibit AVM development in HHT* 2. *Testing of novel drugs that might inhibit AVM growth and induce regression in HHT* 3. *Publication of 2 peer reviewed papers*	36	Drs. Metzger, Gillich, Spiekerkoetter

- **What was accomplished under these goals?**

1.) Major activities:

A.) We have bred transgenic mice to get a larger colony: *floxed Smad4* and *Alk-1* mice with *Apj-CreER* as well as *Cx40CreER and Apelin-CreER mice.*

B.) We have deleted *smad4* in capillary endothelial cells at P2 (post-natal day 2) and have harvested the lungs d15 post injection (P17) using APJCreER$^{tg/+}$; *Smad4*$^{fl/+}$; Confetti$^{tg/tg}$ x *Smad4*$^{fl/fl}$ with the goal to create pulmonary AVMs when the lung is still growing / in development.

C.) We have deleted *Alk1* in capillary endothelial cells in the adult lung (P34) and harvested lungs 18 days later using APJCreER$^{tg/+}$; *Alk1*$^{fl/+}$; Confetti$^{tg/+}$ x *Alk1*$^{fl/fl}$
We hypothezised that a deletion in a subgroup of capillary endothelial cells might result in a less severe phenoptype than previously described when Alk1 as deleted in all endothelial cells. We hypothesized that we would be able to create PAVMs instead of pulmonary hemorrhage.

D.) We have looked in our single cell RNA dataset, whether HHT genes (Eng, Alk1, Smad4) and downstream signaling (Id1-3) are differentially expressed in aCap, gCap or plexus cells (see results below).

E.) For Aim2: As the generation of iPSCs is slowed down due to COVID-19 related reduced clinical research activities at Stanford (only currently actively recruiting trials are exempt and those patients/subjects can be seen in clinic for research purposes), we have modified our aim 2 slightly. Since recently we are able to make appointments for patients for research biomarker studies and we will get IRB approval from the DoD, get consent from HHT patients to harvest PBMCs and start creating iPSCs with our collaborator Joseph Wu, Stanford.

Until then we used a different method to identify common and unique downstream signaling genes and pathways of HHT genes: We downregulated HHT genes ENG, ALK-1 and SMAD4 in commertially available control microvascular pulmonary artery endothelial cells (PAECs) using siRNA – thereby mimicking the loss of function effect of mutations in these genes. We performed a time course of downregulation and BMP9 stimulation, have collected the RNA at 2 h and 24 h after BMP9 stimulation and performed RNAsequencing. We have idenfified common and unique downstream genes without stimulation, at 2h and 24h (see below).
These set of experiments will be very valuable once we move to PBMC derived iPSCs from HHT patients with specific mutations as we will be able to compare the downstream targets after complete deletion of the HHT genes (by siRNA) with the downregulation in the setting of HHT gene mutations, which leads to haploinsufficiency.

2.) Specific objectives

Creating a robust mouse model of pulmonary AVMs by deleting Smad4 and Alk1 in capillary endothelial cells in the "infant, still growing lung" as well as the adult lung (A-C). This woud be important for the detailed characterization of endothelial subtypes involved in AVM formation (arterial, capillary, venous).

Developing a technique to screen lungs for the presence of pulmonary AVMs (whole mount staining and assessment by stereoscope, Latex blue injection to differentiate between pulmonary AVMs and hemorrhage, deep tissue 3-D imaging

Determine the importance of different subgroups of capillary endothelial cells in AVM formation in the lung

Determine the common and unique downstream signaling pathway of the 3 HHT genes by knocking down those genes in healthy PAECs. Treating those cells with commonly used HHT drugs (VEGF-R blocker Avastin, Thalidomide, Pazopanib, Pomalidomide, Doxycyclin, itraconazole) as well as BMP activating drugs such as Enzastaurin and FK506 and assessment wether the signaling and function can be rescued.

3.) Significant results of key outcomes/major outcomes/Conclusions (pos/neg):

A-C.)
Recent attempts to generate a mouse model of PAVMs by using endothelial cell specific *Alk1* deletions have amounted to a phenotype with severe hemorrhages in the lung and 100% lethality. To produce a mouse model that recapitulates clinical characteristics of AVMs in the lung, and to determine which specific endothelial cell type gives rise to PAVMs *in vivo*, we generated a tamoxifen (TM)-inducible Cre-lox mouse model in which *Alk1* or *Smad4* can be deleted exclusively in a sub-population of endothelial cells. Our co-investigators Drs. Astrid Gillich and Ross Metzger from the Department of Biochemistry and Howard Hughes Medical Institute Stanford have discovered very recently that alveolar capillaries are comprised of two distinct cell types that they termed "aerocytes" and "general capillaries". Based on the identification of a marker that is specific for general capillaries, we are currently driving Cre recombination in the developing and maturing plexus, and in the general capillaries of adult mouse lungs, but not in arteries or veins using an Apj-CreER driver. We induced pulmonary capillary specific deletion of either *Alk1* or *Smad4* at different time-points in the developing or maturing lung. TM was administered via intragastric injection in P2 old mouse pups, or via intraperitoneal injection in P12 or P34 old mice **(Figure 1A)**. Intriguingly, 10 days post TM injection, macroscopic red dots were visible in the perfused and inflated mouse lungs of both *Alk1* or *Smad4* mutants, but not Cre-negative control lungs, implying there might be locally different blood circulation **(Figure 1B).**

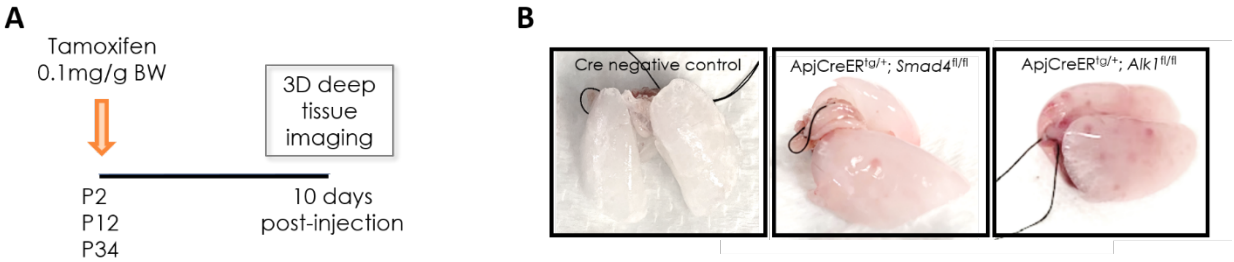


Figure 1: Generation of a novel mouse model of PAVMs. A) Study design for the generation of a mouse model of PAVMs. *Alk1* or *Smad4* were deleted in capillary endothelial cells using tamoxifen. Ten days after the deletion, **B)** lungs were explanted for subsequent visualization of PAVMs.

To determine when and where PAVMs develop, our newly developed 3D deep confocal imaging technique was performed. Subsequently, we will use a Cx40-CreER line to drive deletion of *Alk1* or *Smad4* specifically in endothelial cells of arteries in the developing, maturing and adult lung.

We faced obstabcles though as APJCreER$^{tg/+}$; *Smad4*$^{fl/+}$; Confetti$^{tg/tg}$ x *Smad4*$^{fl/fl}$ mice became sick about 15-20 days after Tamoxifen dosing. When animals were sacrified, we discovered that the mutant animals had significantly enlarged hearts.

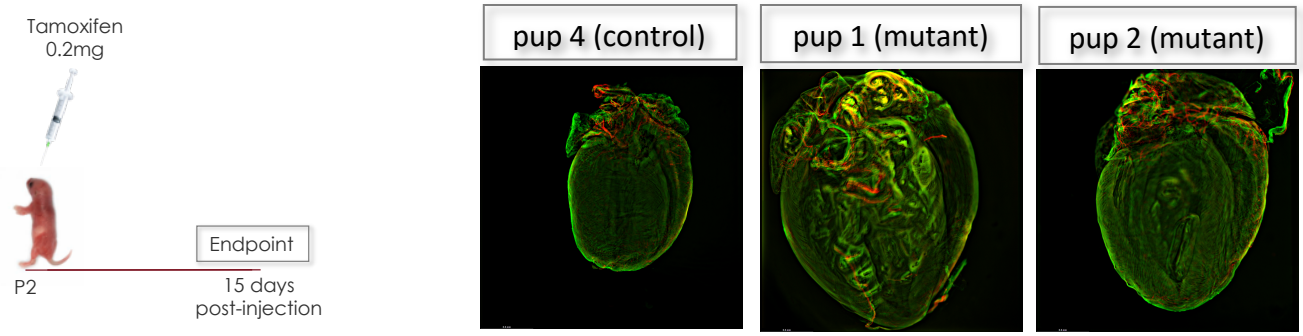


Figure 2: Injection of Tamoxifen in APJCreER$^{tg/+}$; *Smad4*$^{fl/+}$; Confetti$^{tg/tg}$ x *Smad4*$^{fl/fl}$ at P2 and harvest on day 15 leads to a signlificantly enlarged heart in the mutant mice.

While our goal was to delete smad4 **selectively** in capillary endothelial cells of the lung by targeting APJ positive cells, we discovered that APJ was expressed also in the heart and in particular cardiomyocytes (Pope et al, Peptide 2011). Targeted deletion of smad 4 in cardiomyocytes in particular has been previously described to lead to cardiac hypertrophy and heart failure (Wang et al. Circulation Research 2005). Therefore it might be difficult to use the APJ endothelial driver to knock-out *smad4* as this would invariably also reduce smad expression in cardiomyocytes and might limit the time-course of experiments we would need to detect and characterize pulmonary AVMs, as mice would die earlier due to heart failure unrelated to the pulmonary AVMs.
While *smad4* is abundantly expressed in cardioyocytes in mice, *alk1* is less abundantly expressed. Therefore, the APJ-mediated deletion of *alk1* in cardiomyocytes might not have the same deleterious effect with regards to hypertrophy and heart failure.

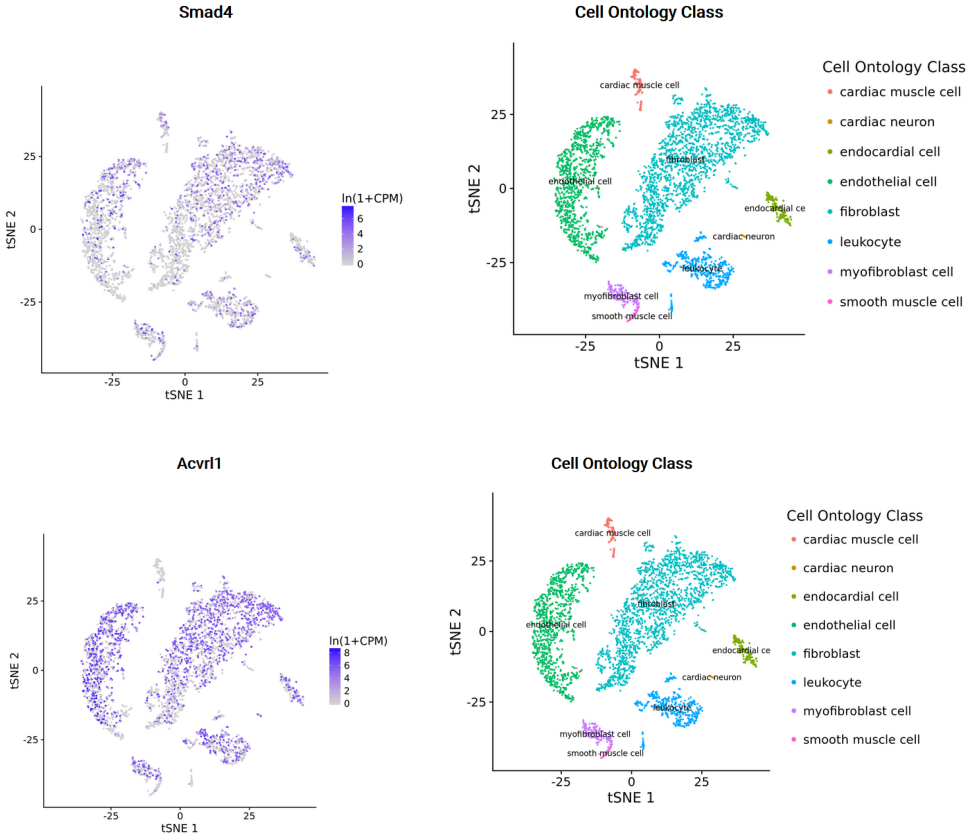


Figure 3: ***Smad4 is abundantly expressed in cardiomyocytes, whereas Alk1 is less abundantly expressed.*** *https://tabula-muris.ds.czbiohub.org/*

We will therefore focus on the APJCreER$^{tg/+}$; *Alk1*$^{fl/+}$; Confetti$^{tg/+}$ x *Alk1*$^{fl/fl}$ mice. While we have developed the deep tissue 3-D imaging technique to identify and characterize pulmonary AVMs, this technique is not suitable to screen a lung for pulmonaryAVMs. We will perform latex blue starining of vessels and then determine under the stereoscope whether we can identify pulmonary AVMs or alternatively pulmonary hemorrhage, with leakage of latex blue dye into the lung tissue.

D.)

To determine which lung endothelial cell types co-express HHT genes, we made use of single-cell RNA-sequencing datasets for adult mouse and human lung (Tabula Muris Consortium, 2018; Gillich et al., Nature, 2020; Travaglini et al., Nature, 2020). Our analysis of the expression patterns of HHT genes (*Acvrl1*/ALK1, *Eng*/endoglin, *Smad4*) and downstream targets of ALK1/BMPR2 signaling (*Id1, Id3*) revealed that ***Acvrl1*, *Eng*, *Smad4*, *Id1*, and *Id3* are co-expressed by subsets of artery, vein, and capillary cells of both types (aerocytes and gCap cells) in the mouse and human lung (Figures 1 and 2).** We also examined the expression patterns of the genes in single-cell RNA-sequencing data for developing mouse lung (Cohen et al. 2018). **We found that the HHT genes are expressed by subsets of plexus and capillary cells at all embryonic and postnatal stages (Figure 3)**. The fraction of cells that co-express *Acvrl1* and *Eng* increases with developmental time. We are now planning to localize the cells that co-express some or all HHT genes and their interaction partners in vessels of the developing, postnatal, and adult lung using single-molecule fluorescence in situ hybridization to reveal their abundance and distribution in different vessel types and to determine if ALK1/BMPR2 signaling is activated in the cells.

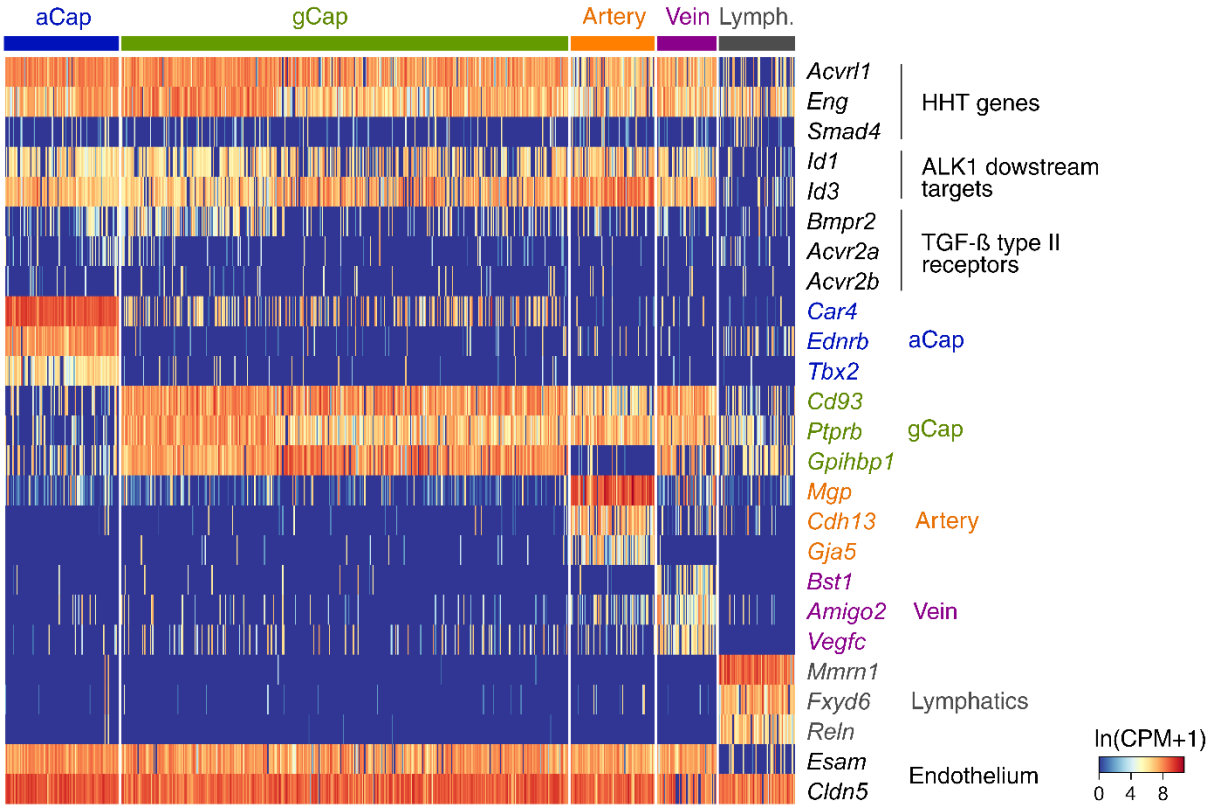


Figure 4. Expression of HHT genes in mouse lung endothelial cell types. Heatmap showing expression of HHT genes (*Acvrl1*/ALK1, *Eng*/endoglin, *Smad4*), ALK1/BMPR2 downstream targets (*Id1*, *Id3*) and markers for endothelial cell types (aerocytes (aCap), general capillary cells (gCap), artery, vein, lymphatics) in adult mouse lung.

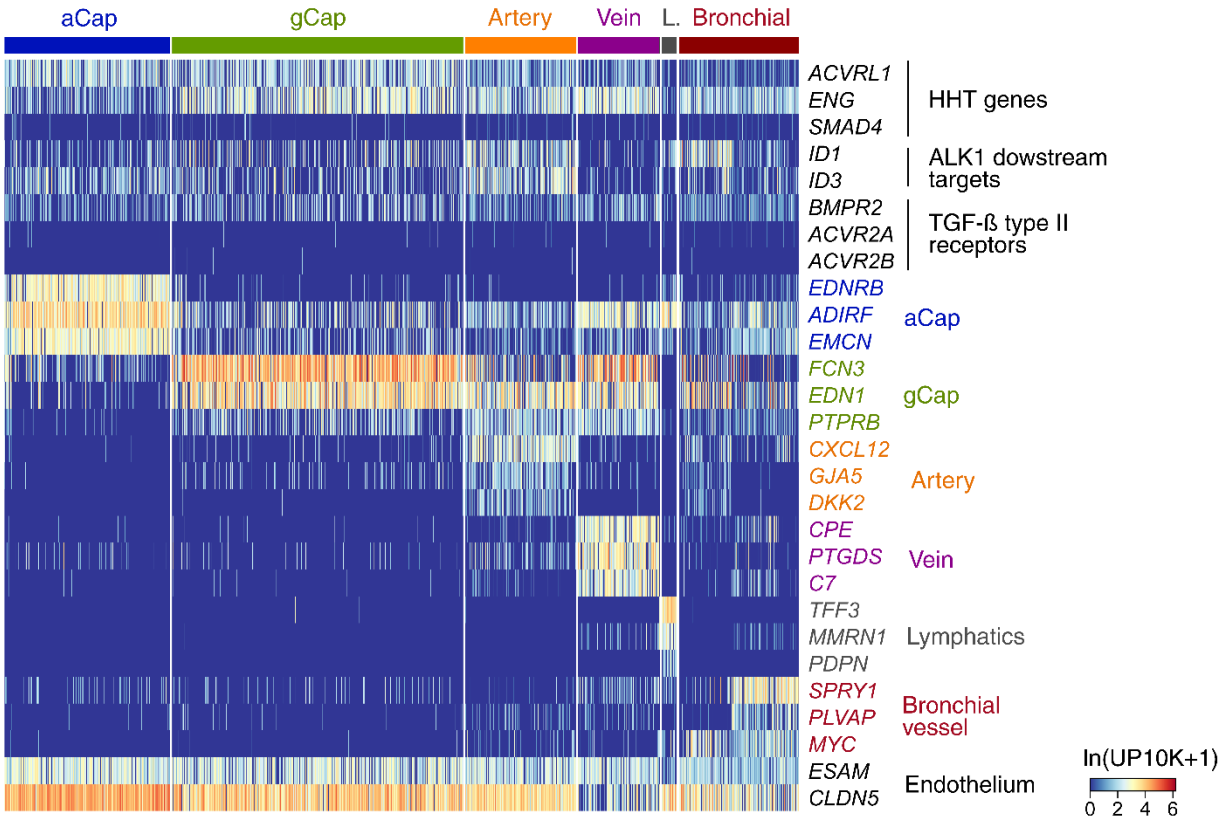


Figure 5. Expression of HHT genes in human lung endothelial cell types. Heatmap showing expression of HHT genes (*ACVRL1*/ALK1, *ENG*/endoglin, SMAD4), ALK1/BMPR2 downstream targets (*ID1*, *ID3*) and markers for endothelial cell types (aerocytes (aCap), general capillary cells (gCap), artery, vein, lymphatics, bronchial vessels) in adult human lung.

What was evident though was that alk1 (=acvrl1) was much more abundantly expressed in endothelial cells than smad4, another reason to focus more on alk1 deletion in endothelial cells than smad4.

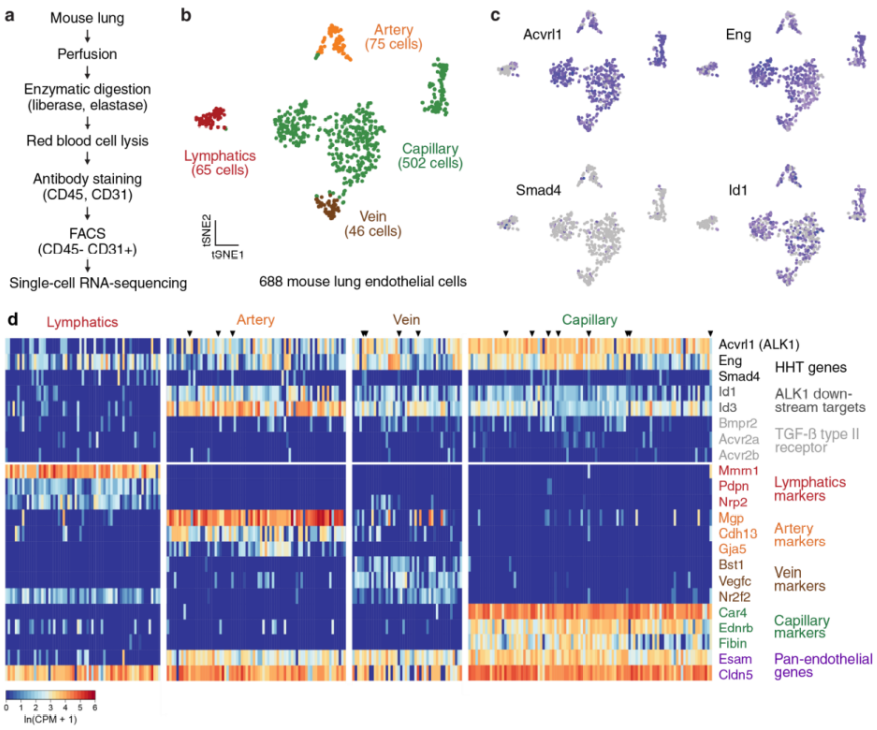
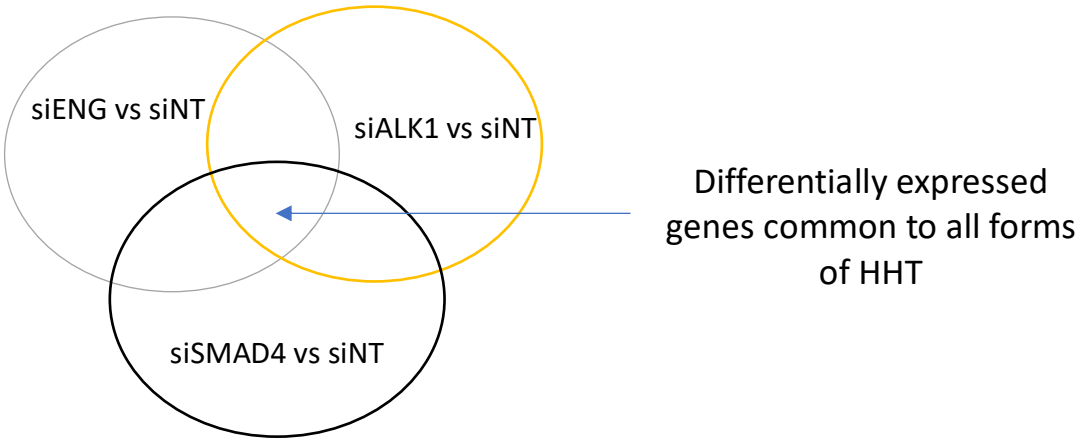


Figure 6. Expression of HHT genes in capillary cells of mouse lung. Heatmap showing expression of HHT genes, ALK1/BMPR2 downstream targets, and markers for lymphatics, artery, vain and capillary cells.

E.) Goal: Identifying common and unique downstream targets of all known HHT gene mutations.



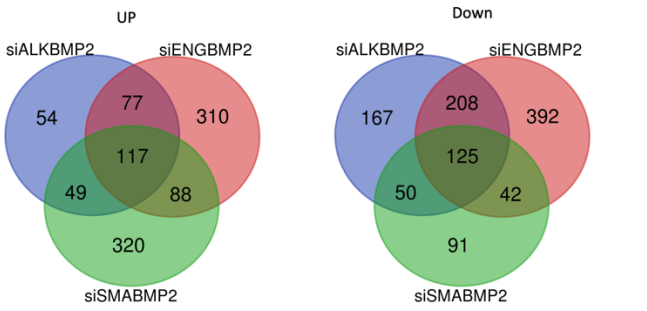
Deleting ENG, ALK1, SMAD4 in human PA endothelial cells with siRNA: Human pulmonary microvascular endothelial cells of passages 4-6 were seeded at 150K cells/well onto 6-well plates and incubated at 37ºC in a humidified 5% CO_2 atmosphere. Next day, cells were washed with PBS and transfected with 50nM siRNAs against non-target controls, ACVRL1 (ALK1), ENG or SMAD4 (Thermo Fisher Scientific, Waltham, MA) and 2ul of Lipofectamine RNAiMAX in a total 1ml of OPTIMEM media. After 5 hours of transfection, medium was replaced with normal complete growth media. Following day, starvation medium was added and incubated for 16hours. Cells were then stimulated with 20ng/ml of BMP9 for 2 or 24hours and harvested for RNA isolation.

RNAseq
RNA was isolated using RNeasy Plus Kits (Qiagen, Gaithersburg, MD) as per manufacturer's instructions. 52 RNA samples were sent to the Novogene Corporation (Sacramento, CA) where the following steps were carried out:
Quality control: Quality and integrity of total RNA were controlled on Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany).
Library construction: The RNA sequencing library was constructed using NEBNext® UltraII RNA Library Prep Kit (New England Biolabs) according to the manufacturer's protocols.
Library quality control: Library concentration was quantified using a Qubit 2.0 fluorometer (Life Technologies), and then diluted to 1ng/ul before checking insert size on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). The library was then quantified to greater accuracy by quantitative PCR (qPCR).
Sequencing: 30 million paired reads for each sample were acquired with the Illumina NovaSeq 6000 system.

RNA-seq data analysis
The quality of the RNA-seq data was examined by base sequence quality plots using FastQC. The trimming of sequence reads will be performed by TrimGalore. Then, the RNA-seq reads were aligned to the human genome (hg19) using the STAR software, and a gene database was constructed from Genecode v19. Differentially expressed genes (DEG) between groups will be quantified using DESeq2 R package. DEG was used to identify common and unique downstream targets of the HHT causing genes. To identify potential common pathways, DEGs were analyzed for biological process and pathway enrichment using DAVID, PANTHER and STRING.

We looked at different conditions:
Genes upregulated or down-regulated 2h OR 24h without stimulation and ENG, SMAD4 and ALK1 knockdown
Genes upregulated or down-regulated 2h OR 24h after BMP9 stimulation and ENG, SMAD4 and ALK1 knockdown
Genes upregulated or down-regulated 2h AND 24h after BMP9 stimulation and ENG, SMAD4 and ALK1 knockdown
Genes upregulated or down-regulated 2h AND/OR 24h after BMP9 stimulation and ENG and ALK1 knockdown, as the two genes most commonly mutated in HHT.
Below we list some of the results and some promising downstream targets when all 3 genes are knocked-out:

Venn diagrams of the HHT causing genes knockdown conditions (under BMP9 stimulation-2h-common genes)

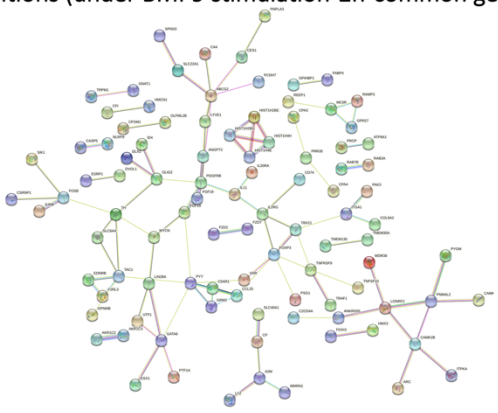


Log2 FC >2, P adjusted value < 0.05

Log2 FC < -2, P adjusted value < 0.05

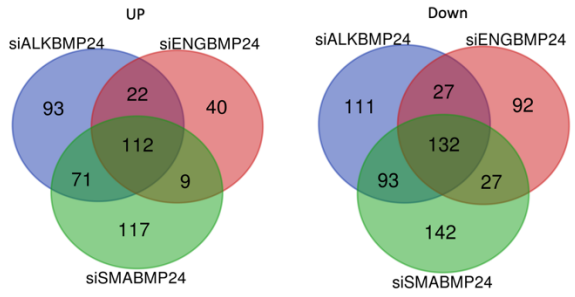
http://bioinformatics.psb.ugent.be/webtools/Venn/
http://www.nemates.org/MA/progs/Compare.html

STRING network of the HHT causing genes knockdown conditions (under BMP9 stimulation-2h-common genes)



minimum required interaction score: medium confidence (0.400)

Venn diagrams of the HHT causing genes knockdown conditions (under BMP9 stimulation-24h-common genes)



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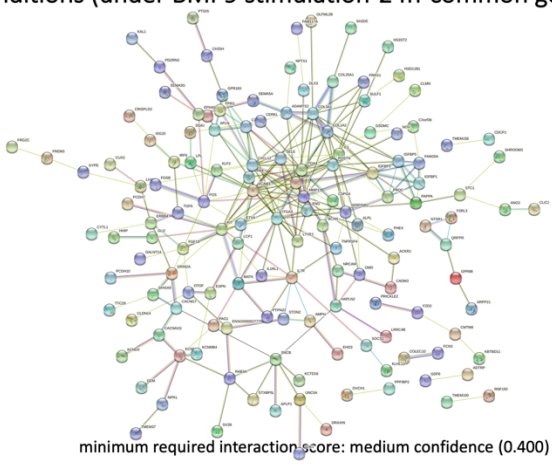
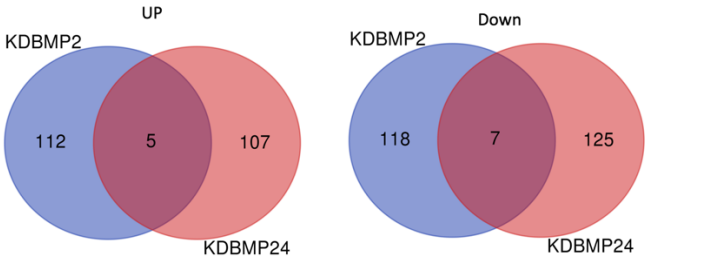
http://www.nemates.org/MA/progs/Compare.html

STRING network of the HHT causing genes knockdown conditions (under BMP9 stimulation-24h-common genes)



minimum required interaction score: medium confidence (0.400)

Common persistent genes of the HHT causing genes knockdown conditions (under BMP9 stimulation 2 and 24h)



HS3ST2 GPNMB
ANKRD33 RRAGD LYVE1

CPA4 SLC25A47 SHISA9 HIST1H2BE
MC5R FGF19 FRG2C

Log2 FC >2, P adjusted value < 0.05

Log2 FC < -2, P adjusted value < 0.05

http://bioinformatics.psb.ugent.be/webtools/Venn/
http://www.nemates.org/MA/progs/Compare.html

Common persistent genes of the HHT causing genes knockdown conditions (under BMP9 stimulation 2 and 24h)

Common persistently upregulated genes: 5
Heparan Sulfate-Glucosamine 3-Sulfotransferase 2 (HS3ST2)
Glycoprotein Nmb (GPNMB)
Ankyrin Repeat Domain 33 (ANKRD33)
Ras-related GTP-binding protein D (RRAGD)
Lymphatic Vessel Endothelial Hyaluronan Receptor (LYVE1)

Common persistently downregulated genes: 7
Carboxypeptidase A4 (CPA4)
Solute Carrier Family 25 Member 47 (SLC25A47)
Shisa Family Member 9 (SHISA9)
Histone cluster 1 H2B family member e (HIST1H2BE)
Melanocortin 5 Receptor (MC5R)
Fibroblast Growth Factor 19 (FGF19)
FSHD Region Gene 2 Family Member C (FRG2C)

The persistently down or upregulated genes under knockdown conditions of all 3 HHT causing gene mutations will be the basis to **a)** predict drugs that target these genes (LINCS database), (b) be the read-out to test drugs currently used for HHT treatment such as Avastin, thalidomide, pazopanib, Pomelidomide etc as well as BMP activating medications such as FK506 (Tacrolimus) and Enzastaurin, identified in our lab.

4.) Other achievements (including a discussion of stated goals not met):

Given also the limited excess to patients and collection of blood to isolate PBMCs and create iPSCs from HHT patients with the different disease causing mutations (due to research restrictions related to the COVID-19 pandemic), we have become creative and have used control PAECs, knocked down the 3 different HHT genes and performed RNA sequencing

on them. These experiments already provide valuable insight into common and unique downstream targets of the 3 HHT genes and will be in particular very helpful as preliminary data for the planned iPSC experiments of PBMCs of HHT patients.

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

Due to the restrictions during the current COVID-19 pandemic (shelter in place and travel restrictions) the participation in workshops, conferences and seminars has been minimal. Even 1:1 teaching of our postdoctoral fellow at the confocal microscope has been difficult given our strict policy at Stanford University of not having 2 people in the same room in close proximetry. We expect more opportunities for training and professional development.

- **How were the results disseminated to communities of interest?**

We have used the time to write a review article about the pathogenesis of AVMs with members of the HHT Center of Excellence at Stanford University:

Arteriovenous Malformations-Current Understanding of the Pathogenesis with Implications for Treatment. Schimmel K, Ali MK, Tan SY, Teng J, Do HM, Steinberg GK, Stevenson DA, **Spiekerkoetter E.** Int J Mol Sci. 2021 Aug 21;22(16):9037

- **What do you plan to do during the next reporting period to accomplish the goals?**

In normal C57Bl6 mice, we are planning to localize the cells that co-express some or all HHT genes and their interaction partners in vessels of the developing, postnatal, and adult lung using single-molecule fluorescence in situ hybridization (RNAscope) to reveal their abundance and distribution in different vessel types and to determine if ALK1/BMPR2 signaling is activated in the cells.

We will focus predominantely on deleting Alk1 in endothelial cells (instead of Smad4) to avoid an adverse effect of APJ-mediated changes in cardiomyoctes

We will use deep tissue 3-D imaging as well as Latex blue injection to better screen, visualize and to examine the pulmonary vasculature under these conditions.

We will analyze the "common HHT disease signature" as well as "unique HHT signatures for ALK, ENG and SMAD" and will test whether drugs that are currently tried as medical therapy in HHT such as Avastin, Tamoxifen, Doxycyclin, Itraconazole as well as experimental drugs such as FK506, Enzastaurin or Sirolimus are capable of reversing the HHT gene expression signature.

We will also obtain PBMCs from HHT patients with an ENG (n=3) and ALK1 (n=3) mutation as well as a SMAD4 (n=1) mutation and will, in collaboration with Dr. Wu's lab to generate iPSCs and then repeat the RNAseq experiments on those cells.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

"Nothing to report yet"

- **What was the impact on other disciplines?**

"Nothing to report yet"

- **What was the impact on technology transfer?**

"Nothing to report yet"

- **What was the impact on society beyond science and technology?**

"Nothing to report yet"

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

Given the limited excess to patients and collection of blood to isolate PBMCs and create iPSCs from HHT patients with the different mutations as a result of the reduced clinical activities at Stanford University and the lockdown of clinical research due yo COVID-19, we have become creative and have used control PAECs, knocked down the 3 different HHT genes and performed RNA sequencing on them. These experiments already provide valuable insight into common and unique downstream targets of the 3 HHT genes and will be in particular very helpful as preliminary data for the planned iPSC experiments of PBMCs of HHT patients.

I do not believe that these experiments present a significant change from the proposal. We are still determined to create iPSCs from HHT patients in the second year.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Getting all protocols approved (IRB, SCRO) - via DoD to start collecting blood.

COVID-19 related partial closure of bench experiments

See above for our proposed solutions

- **Changes that had a significant impact on expenditures**

none

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

I am attaching the approval notices at the end of the report:

Institutional IRB -54091 approval: April 30 2021

Institutional SCRO approval: last approval May 2020

Institutional animal protocol approval – APLAC 33492: 12/4/2020 – 11/18/22

- **Significant changes in use or care of human subjects** No
- **Significant changes in use or care of vertebrate animals.** No
- **Significant changes in use of biohazards and/or select agents** No

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award. **Journal publications.**

  Arteriovenous Malformations-Current Understanding of the Pathogenesis with Implications for Treatment. Schimmel K, Ali MK, Tan SY, Teng J, Do HM, Steinberg GK, Stevenson DA, **Spiekerkoetter E.** Int J Mol Sci. 2021 Aug 21;22(16):9037

  - **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?**

Edda Spiekerkoetter, MD
PI
Nearest Person Month worked: 12
Contribution to Project: design of cell culture and mouse experiments
Funding support: Wall Center for Pulmonary Vascular Disease Stanford, R01 HL128734-01A1, 20% FTE this grant

Ross Metzger, PhD
Collaborator / Co-investigator
Nearest Person Month worked: 9
Contribution to Project: breeding of transgenic mice, deletion of genes at different time points in developemt and imaging of mouse embryos
Funding support: Wall Center for Pulmonary Vascular Disease Stanford, 10% FTE on this grant

Astrid Gillich, PhD
Collaborator / Co-investigator
Nearest Person Month worked: 9
Contribution to Project: single cell RNA seq
Funding support: Salary as Research associate in Dr. Krasnow's laboratory, 10% FTE on this grant

MD Khadem Ali, PhD
Postdoctoral Research fellow
Nearest Person Month worked: 12
Contribution to Project: cell culture experiments with PAECS, RNA seq
Funding support: 100% FTE this grant

Astrid Schimmel, PhD
Nearest Person Month worked: 12
Contribution to Project: cell culture experiments with PAECS, RNA seq
Funding support: 100% FTE this grant

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Dr. Astrid Gillich stopped working at Stanford University and took a job in industry at the end of 6/2021. Our co-investigator, also a trained developmental biologist, is fully capable of replacing Dr. Gillich and has agreed to do so. I have informed the DoD about this change already.

- **What other organizations were involved as partners?**

"Nothing to Report."

8. **SPECIAL REPORTING REQUIREMENTS**
    - **COLLABORATIVE AWARDS: N/A**
    - **QUAD CHARTS: N/A**
9. **APPENDICES:**

STANFORD UNIVERSITY

Stanford, CA 94305 [Mail Code 5579]

David Spiegel, M.D. (650) 724-9815
CHAIR, PANEL ON MEDICAL HUMAN SUBJECTS (650) 725-6766

Certification of Human Subjects Approvals

Date: April 30, 2021

To: Edda Spiekerkoetter, MD, Medicine - Med/Pulmonary and Critical Care Medicine
Xuefei Tian MD,MS, Ross Metzger PhD, Sheetal Hanish Vaghela RN BSN, Joseph Wu M.D., Ph.D.

From: David Spiegel, M.D., Administrative Panel on Human Subjects in Medical Research

eProtocol Identifying Common and Unique Downstream Signaling Pathways in Cells from Patients with Hereditary Hemorrhagic Telangiectasia (HHT) and Different Disease Defining Mutations

eProtocol #: 54091 **IRB 3 (Registration** 350)

The IRB approved human subjects involvement in your research project on 04/30/2021. **'Prior to subject recruitment and enrollment, if this is: a Cancer-related study, you must obtain Cancer Center Scientific Review Committee (SRC) approval;** a CTRU study, you must obtain CTRU approval; a VA study, you must obtain VA R and D Committee approval; and **if a contract is involved, it must be signed.**'

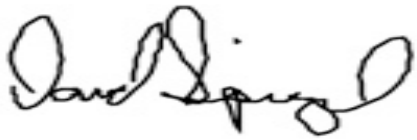
The expiration date of this approval is 04/30/2022 at Midnight. If this research is to continue beyond that date, it is your responsibility to submit a Continuing Review application in eProtocol. Research activities must be reviewed and re-approved on or before midnight of the expiration date. The approval period may be less than one year if so determined by the IRB. Proposed changes to approved research must be reviewed and approved prospectively by the IRB. No changes may be initiated without prior approval by the IRB, except where necessary to eliminate apparent immediate hazards to subjects. (Any such exceptions must be reported to the IRB within 10 working days.) Unanticipated problems involving risks to participants or others and other events or information, as defined and listed in the Report Form, must be submitted promptly to the IRB. (See Events and Information that Require Prompt Reporting to the IRB at http://humansubjects.stanford.edu.) Upon completion, you must report to the IRB within 30 days.

Please remember that all data, including all signed consent form documents, must be retained for a minimum of three years past the completion of this research. Additional requirements may be imposed by your funding agency, your department, HIPAA, or other entities. (See Policy 1.9 on Retention of and Access to Research Data at http://doresearch.stanford.edu/policies/research-policy-handbook)

This institution is in compliance with requirements for protection of human subjects, including 45 CFR 46, 21 CFR 50 and 56, and 38 CFR 16.

Includes: no amendments have been made

Waiver of Individual Authorization for recruitment under 45 CFR 164.512(i)(2)(ii)(A),(B),(C), pursuant to information provided in the HIPAA section of the protocol application.



David Spiegel, M.D., Chair

Approval Period: 04/30/2021 - 04/30/2022
Review Type: REGULAR - CONTINUING REVIEW
Funding: Department Of Defense - Grant: PR181774, SPO: 138858

Assurance #: FWA00000935 (SU), FWA00000934 (SHC)

STANFORD UNIVERSITY

Stanford, CA 94305 [Mail Code 5579]

David Spiegel, M.D.
Chair, Institutional Review Board/Stem Cell Research Oversight Panel

Phone: (650) 725-4133
Fax: (650) 725-6766

c/o Research Compliance Office

Acknowledgement of IRB/SCRO Notification

Institutional Review Board/Stem Cell Research Oversight Panel

Date: May 6, 2020

To: Edda Spiekerkoetter, MD, PhD, Medicine - Med/Pulmonary and Critical Care Medicine
Katelyn Elizabeth Black NA, Joseph Wu MD, PhD, Xuefei Tian MD, Yan Zhuge

From: David Spiegel, M.D., Chair, IRB/SCRO Panel

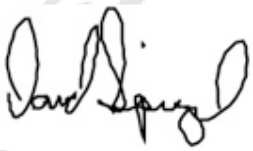
SCRO Protocol Title: Generating induced pluripotent stem cells (iPSCs) from peripheral blood mononuclear cells (PBMCs) from patients with hereditary hemorrhagic telangiectasia (HHT)

SCRO Protocol Number: 772

This letter is to acknowledge that IRB/SCRO has received written notification of your research project titled Generating induced pluripotent stem cells (iPSCs) from peripheral blood mononuclear cells (PBMCs) from patients with hereditary hemorrhagic telangiectasia (HHT). After review, the IRB/SCRO determined your research falls under the categories which require written notification according to the regulations at 17 CCR Sec.100070 or the CDPH Guidelines for Human Stem Cell Research, Sec.5(a)(3). You must promptly inform the IRB/SCRO of any significant changes to the research.

Prior to starting the study, if this is a human subjects-related study, you must obtain IRB approval; an animal-related study, you must obtain APLAC approval; and if a contract is involved, it must be signed.

Please remember that all data must be retained for a minimum of three years past the completion of this research. Additional requirements may be imposed by your funding agency, your department, or other entities. (See Policy on Retention of and Access to Research Data at http://stanford.edu/dept/DoR/rph/2-10.html).



David Spiegel, M.D., Chair

Funding: Department of Defense - SPO: 138858 - Grant: PR181774 - Understanding and targeting pulmonary arteriovenous malformations using repurposed drugs

Stanford University
Stanford, CA 94305 [Mail Code 5579]

Michael E. Moseley, Ph.D

IACUC Chair

null

VERIFICATION OF

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) APPROVAL

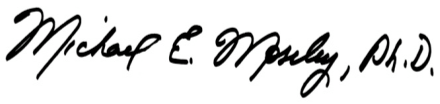
Date:	December 04, 2020
To:	Edda Spiekerkoetter, School of Medicine
	Ross Metzger, Xuefei Tian, Fan Zhang
Protocol Title:	"Understanding and Targeting Pulmonary Arteriovenous Malformations"
Funding:	Understanding and Targeting Pulmonary Arteriovenous Malformations Using Repurposed Drugs, Department Of Defense, SPO: 138858, Grant: PR181774, PI: Edda Spiekerkoetter
Assurance Number:	A3213-01
Approval Period:	12/04/2020 THROUGH 11/18/2022
Review Type:	REVISION
Protocol ID:	33492

The IACUC approved this protocol transaction on 12/04/2020. Prior to initiation of animal studies, if this study involves biohazardous or radioactive agents, you must obtain Biosafety Panel or Radiological Safety Panel approval.

The expiration date of this approval is 11/18/2022 at Midnight. If this project is to continue past that date, you must submit an updated protocol (renewal) in advance for IACUC re-approval. Proposed changes to approved research must be reviewed and approved prospectively by the IACUC. No changes may be initiated without prior approval by the IACUC, except where deemed necessary by veterinary staff. (Any such exceptions must be reported to the IACUC within 10 working days). The PD must notify the IACUC promptly of any complications that occur (see http://labanimals.stanford.edu/protocols/index.html for information on reporting complications).

All continuing protocols (renewals) must be reviewed and re-approved before the expiration date. It is your responsibility to resubmit the protocol to the IACUC as required.

Please remember that protocol records related to this study must be retained for a minimum of three years past the completion of this research. (See Policy on Retention of and Access to Research Data, Research Policy Handbook, http://stanford.edu/dept/DoR/rph/2-10.html.)



Michael E. Moseley, Ph.D, IACUC Chair.