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PRINCIPAL INVESTIGATOR: Edda Spiekerkoetter

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6. AUTHOR(S)

Edda Spiekerkoetter, MD
Ross Metzger, PhD, Astrid Gillich, PhD, David Stevenson, MD

E-Mail:eddas@stanford.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Stanford University
450 SERRA MALL
STANFORD CA 94305-2004

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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

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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)* *20% completion (see below)*	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)* *20% completion (see below)*	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)* *10% completion (see below)*	1-12	Dr. Metzger/Dr. Gillich

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.* *Cells used: 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. To identify common and unique downstream targets of ALK1, ENG and SMAD4 and to move the project forward we have therefore deleted the above genes in commercially available pulmonary artery endothelial cells using siRNA and have sent RNA for RNA sequencing.*	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).* *Cells used: 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.* *Not started.* *Until iPSCs will become available, we will treat 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 created by deleting these genes*	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* 3. *Testing previously identified repurposed drugs (FK506, Enzastaurin) and novel drugs on iPSC-EC to see whether they reverse gene expression and function*	24	Dr. Spiekerkoetter
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* *Not started*	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* *Not started*	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.) Breeding transgenic mice to get a larger colony: *floxed Smad4* and *Alk-1* mice with *Apj-CreER* as well as *Cx40CreER and Apelin-CreER mice.* We are starting with floxed smad4 mice.

B.) Deleting *smad4* in endothelial cells in the developing and maturing lung plexus (at E9.5 or E11.5) using *Apj-CreER* mice with the goal to create pulmonary AVMs in development.

C.) Deleting *smad4* in arterial endothelial cells in the adult lung using *Cx40CreER* mice after birth to create pulmonary AVMs in the adult lung. If early deletion, potential of pulmonary hemorrhage.

D.) Deleting *smad4* in endothelial cells in the adult lung in 2 different capillary cell types *aerocytes (aCap) and gCap cells* using *Apelin-CreER* mice and *Apj-CreER* to thereby create pulmonary AVMs in the adult lung. A deletion in a subgroup of capillary endothelial cells might result in a less severe phenoptype and therefore result in PAVMs instead of pulmonary hemorrhage.

E.) 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).

F.) 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.
Until we are able to generate iPSCs from PBMCs from HHT patients (hopefully in the next 6 months), we are using 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. As seen below in our results, we have performed a time course of downregulation and BMP9 stimulation and have collected the RNA and have sent the samples for RNAsequencing.
These set of experiments will be very valuable once we move to PBMC derived iPSCs from HHT patients, as we will have already determined the optimal experimental conditions for stimulation experiments.

2.) Specific objectives

Breed enough mice to delete Smad4 and Alk1 in endothelial cells of the developing plexus, as well as in different endothelial cells (arterial and capillary) in the adult lung (A-D).

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.

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

A-D.)
To determine in which endothelial cell type mutations in HHT genes mediate pulmonary AVM development (Aim 1b) we have **been breeding mice** carrying floxed alleles of either *Smad4* or *Alk1* with conditional endothelial Cre driver mice in order to delete HHT genes in specific endothelial populations first in embryonic, then in postnatal, and adult mouse lung. **Our progress has been severely limited by the restriction on animal breeding during Stanford's Covid-19 shutdown.** Despite this, we have generated male mice carrying both *Apj-CreER* and floxed *Smad4* (1 male) or *Alk1* (4 males), as well as female mice homozygous for either floxed *Smad4* or *Alk1*. *Apj-CreER* drives Cre-mediated recombination in endothelial cells in the developing and maturing lung plexus. By breeding these mice, we have generated mice that carry *Apj-CreER* and are homozygous for either floxed *Smad4* or *Alk1*, and have begun to examine the effects of deleting the genes by administering tamoxifen, starting at the time the lung bud emerges (E9.5). *Apj-CreER; Smad4*$^{fl/fl}$ embryos from pregnant females administered high doses of tamoxifen at either E9.5 or E11.5 (collected at E13.5) were not viable, demonstrating an early requirement for *Smad4*, likely—judging from the severity of the phenotype—outside the lung. As animals become available, we will continue to delete the genes at successively later embryonic time points.

In order to delete the HHT genes in specific endothelial cell populations of the lung after birth, we will breed homozygous floxed *Smad4* and *Alk1* female mice to male mice currently in our colony carrying the *Cx40CreER* allele, in order to drive recombination in artery endothelial cells and in connection cells. In addition, Drs. Gillich and Metzger (co-investigators on this grant) have recently discovered that there are two distinct, intermingled capillary cell types in the postnatal and adult lung ("Capillary cell type specialization in the alveolus," *Nature*, in press) which they have named aerocytes (aCap) and gCap cells. To delete the HHT genes specifically in emerging and mature aerocytes, we will breed homozygous floxed *Smad4* and *Alk1* female mice to *Apelin-CreER* mice currently in our colony. To delete the HHT genes specifically in gCap cells, because after birth *Apj-CreER* is expressed in gCap cells but not aerocytes, we will take advantage of the mice we have generated that carry both *Apj-CreER* and floxed *Smad4* or *Alk1*.

E.)
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, in press; Travaglini et al., Nature, in press). 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.

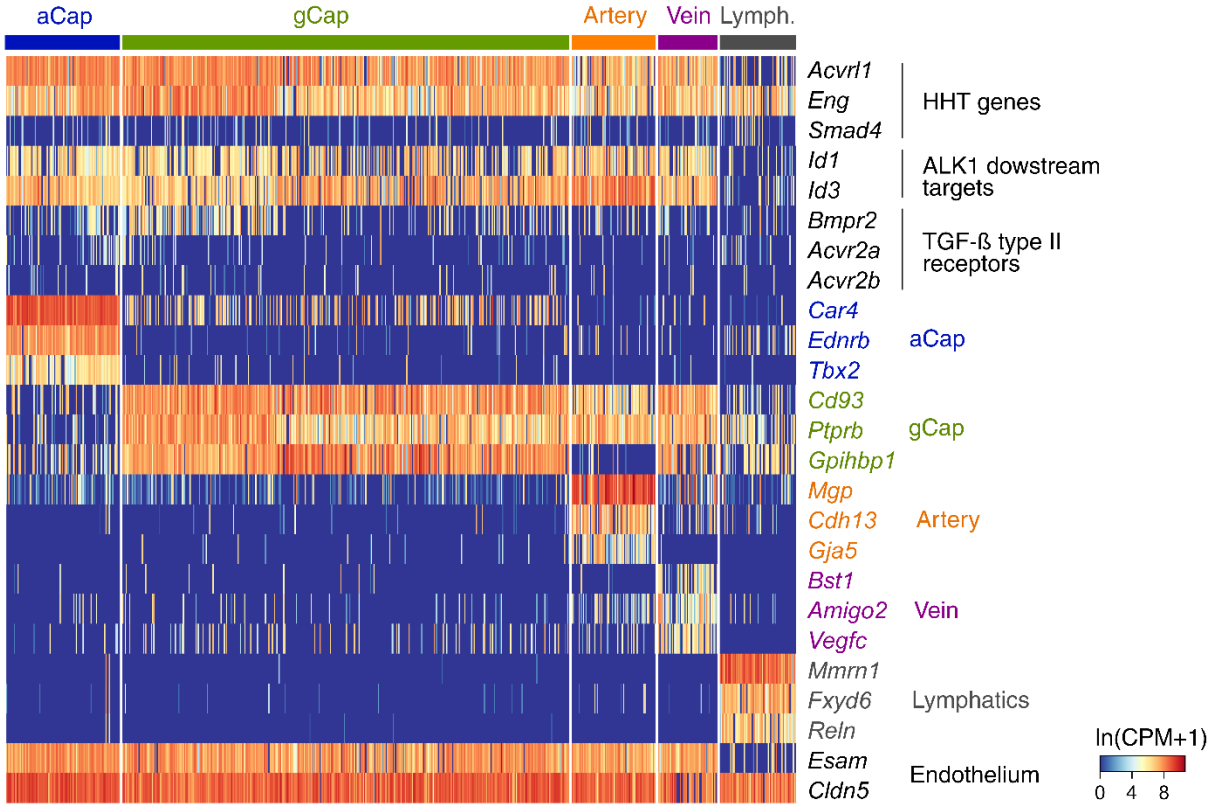


Figure 1. Expression of HHT genes in mouse lung endothelial cell types. Heatmap showing expression of HHT genes (*Acvrl1*/ALK1, *Eng*/endoglin, *Smad4*), ALK1/BMPR2 downstream targets (*Id1*, *Id3*) and markers for endothelial cell types (aerocytes (aCap), general capillary cells (gCap), artery, vein, lymphatics) in adult mouse lung.

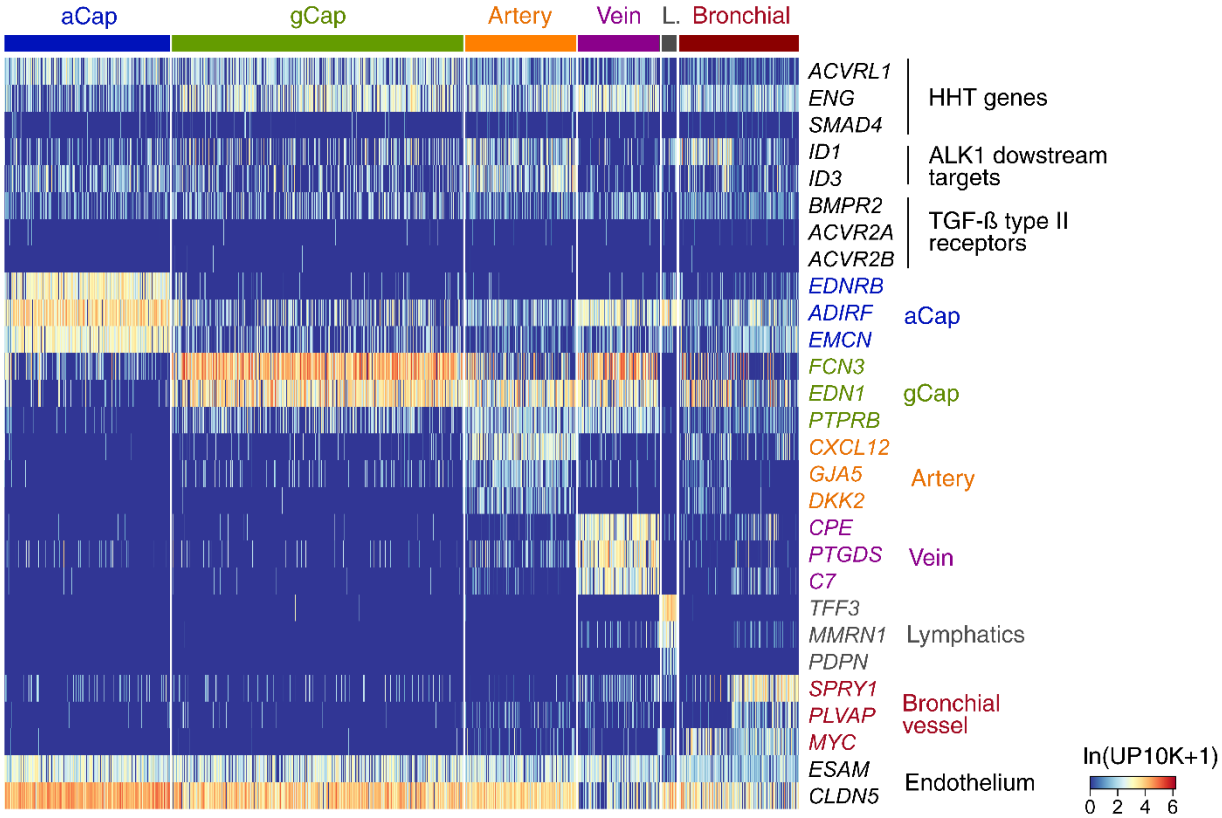


Figure 2. Expression of HHT genes in human lung endothelial cell types. Heatmap showing expression of HHT genes (*ACVRL1*/ALK1, *ENG*/endoglin, SMAD4), ALK1/BMPR2 downstream targets (*ID1*, *ID3*) and markers for endothelial cell types (aerocytes (aCap), general capillary cells (gCap), artery, vein, lymphatics, bronchial vessels) in adult human lung.

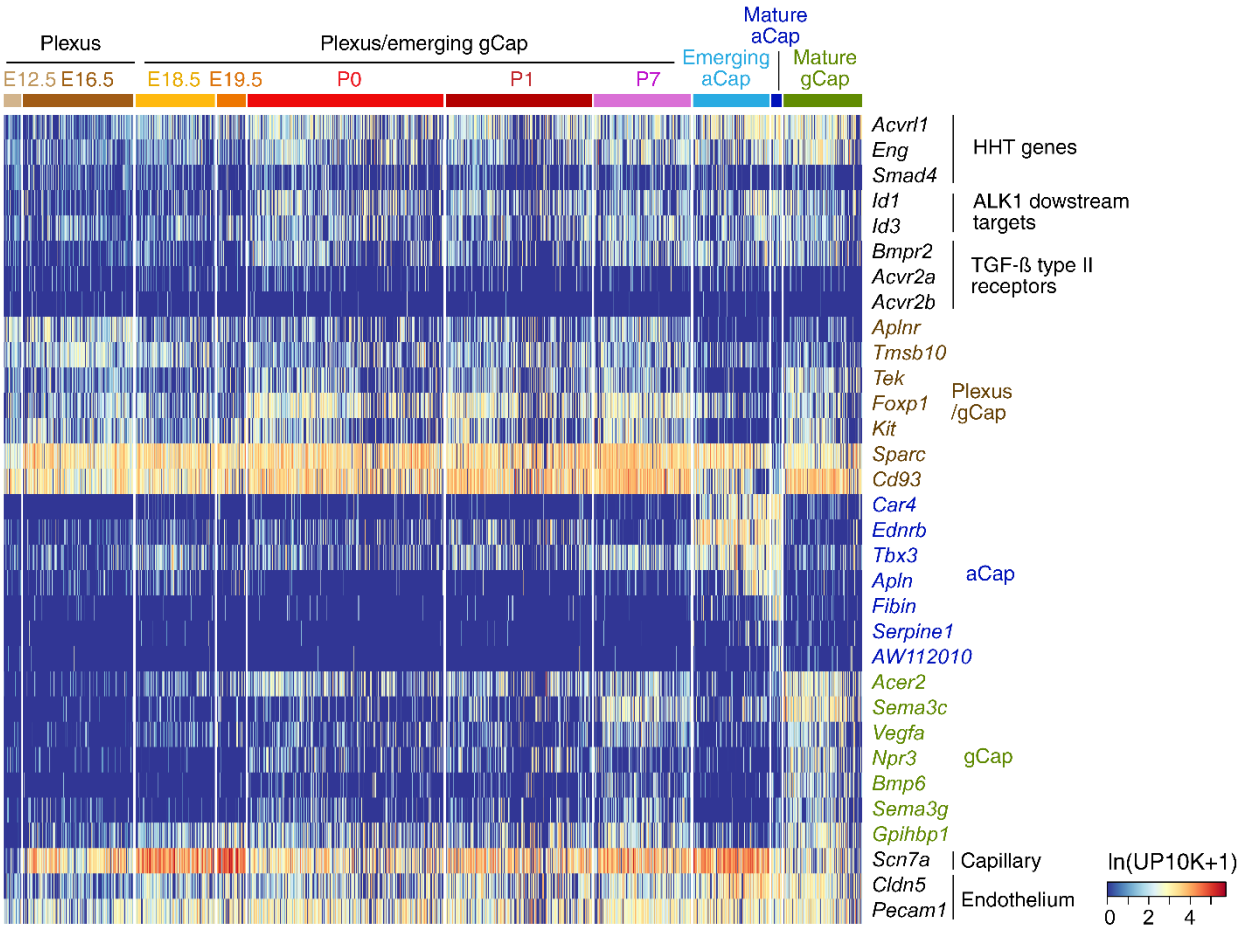


Figure 3. Expression of HHT genes in plexus and capillary cells of developing mouse lung. Heatmap showing expression of HHT genes, ALK1/BMPR2 downstream targets, and markers for plexus, emerging and mature capillary cell types in embryonic (E12.5-E19.5) and postnatal (P0-adult) mouse lung.

F.)

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, 10 or 24hours and harvested for RNA isolation.

As seen in **Figure 4,** we achieved a knockdown of over 80% for all three genes, ALK1, ENG and SMAD4 using siRNA. Stimulation with the ligand BMP9 did not increase the gene expresssion of ALK1, ENG and SMAD4.

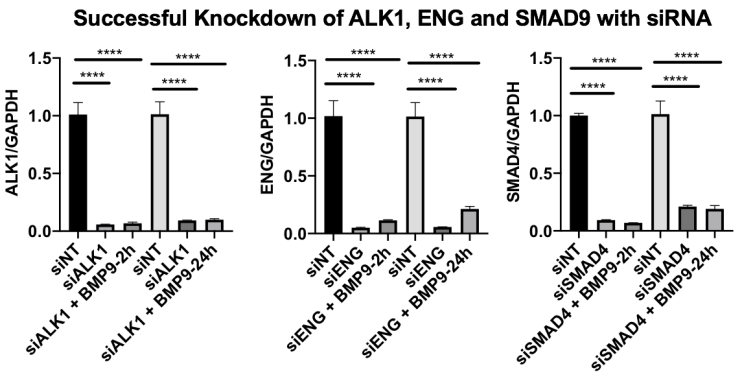


Figure 4: Timecourse and efficiency of siRNA mediated knockdown of ALK1, ENG and SMAD4. With and without stimulation with BMP9 20 ng/ml.

We then looked at one of the known downstream targets of the BMPR2/ALK1/ENG/SMAD4 pathway, inhibitor of differentiation (Id) **(Figure 5)** and whether the expression of Id1 is reduced by knocking down the 3 HHT genes. Interestingly, while BMP9 induced Id1 expression is blocked by knocking down ALK1 and SMAD4, a reduction in ENG does not affect BMP9 inducted Id1 expression. Thereby Id1 is NOT the common downstream target of all 3 HHT genes, but is specific for ALK1 and SMAD4 mediated signaling. A very important finding when it comes to identifying common downstream targets and subsequently predicting perpurposed drugs that might increase the signaling.

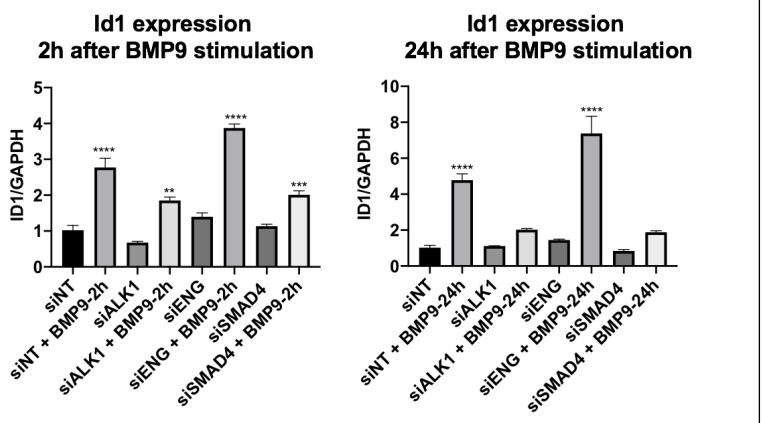


Figure 5: BMP9 induced Id1 expression after siRNA mediated knockdown of ALK1, ENG and SMAD4

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 - ongoing

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

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

After the animal protocol was approved, the part of the funding period was used to set up the breeding experiments of the multiple transgenic animals. Unfortunately our breeding efforts were limited as for about 4 months we were not allowed to continue to expand our breeding colonies due to the shut-down of the laboratories at Stanford in the setting if the COVID-19 pandemic. We were only allowed to perform essential experiments and to keep our breeding strains alive, yet not to expand the colonies. We are now in Stage 2 of the re-opening phase and have increased our breeding capacities. I expect that we will have significantly more results from our animal experiments in YR 2 of the grant.

Given also the limited excess to patients and collection of blood to isolate PBMCs and create iPSCs from HHT patients with the different mutations, 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?**

"Nothing to report"

- **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 expand our mouse colony and will delete smad4 and Alk1 in development and in adult mice in different endothelial subtypes. We will use deep tissue 3-D imaging to examine the pulmonary vasculature under these conditions.

We will perform the RNAseq analysis of the PAECs samples in which ALK1, ENG and SMAD4 were deleted with siRNA and will identify common and unique downstream targets of the 3 different HHT genes. We will create a "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 mediacl 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 generate iPSCs.

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 for at least 2 months 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)

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 approval: May 6 2020

Institutional SCRO approval: May 6 2020

Institutional animal protocol approval: Jan 15 2020

- **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:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

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

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

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

"Nothing to Report."

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

"Nothing to Report."

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from **BOTH** the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ebrap.org for each unique award.*

  N/A

- **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

- **N/A**

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***

Stanford University
Stanford, CA 94305 [Mail Code 5579]

Michael E. Moseley, Ph.D

IACUC Chair

650-724-0465

VERIFICATION OF

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) APPROVAL

Date:	January 15, 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:	01/15/2020 THROUGH 11/18/2022
Review Type:	NEW
Protocol ID:	33492

The IACUC approved this protocol transaction on 01/15/2020. 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

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

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: May 6, 2020

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)

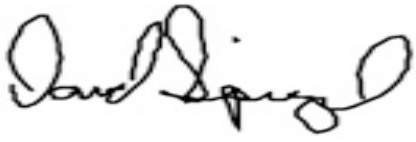
The IRB approved human subjects involvement in your research project on 05/06/2020. **'Prior to subject recruitment and enrollment, if this is: a Cancer-related study, you must obtain Cancer Center Scientific Review Committee (SRC) approval;** a CTRU study, you must obtain CTRU approval; a VA study, 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 05/06/2021 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.

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: 05/06/2020 - 05/06/2021
Review Type: REGULAR - NEW
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

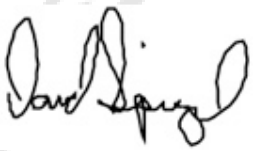
SCRO Protocol Title: Generating induced pluripotent stem cells (iPSCs) from peripheral blood mononuclear cells (PBMCs) from patients with hereditary hemorrhagic telangiectasia (HHT)

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