THE PROGRESSION OF DNA DAMAGE IN PULMONARY ARTERIAL ENDOTHELIAL CELLS INTO PULMONARY ARTERIAL HYPERTENSION

by

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A THESIS

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As one of the categories of pulmonary hypertension, pulmonary arterial hypertension is a disease that results in high blood pressure in the blood vessels of the lungs. Although there are several treatments available, there is currently no cure to prevent the worsening of the disease over time, and it can be life-threatening from the dangerous accumulation of pressure in the pulmonary arteries. Research from the Rabinovitch lab at Stanford School of Medicine highlights the crucial involvement of DNA damage in pulmonary arterial endothelial cells in the development of pulmonary arterial hypertension. They found that deletion of nuclear receptor PPARγ gene in endothelial cells results in unrepaired DNA damage and worsened pulmonary vasculature characteristic to the disease. As a follow-up, my research attempts to uncover a significant connection between unrepaired DNA damage and the progression of pulmonary arterial hypertension: I propose that the accumulation of unrepaired DNA damage leads to a worsened disease phenotype. To test this, endothelial cell-specific deletion of a DNA damage sensing gene Mre11 in transgenic mice was utilized to induce a condition susceptible to DNA damage. Both wild-type and knock-out mice with endothelial cell-specific deletion of Mre11 were first placed in hypoxic chambers

of 8% oxygen for 10 days. I then compared the two groups in the following categories to characterize differences in DNA damage levels and disease severity: hemodynamic measurements of right ventricular systolic pressure (RVSP), left ventricular end diastolic pressure (LVEDP), complete blood count (CBC); quantification of DNA damage; and the number of (muscularized) small pulmonary arteries. Contrary to my expectations, I found a higher trend of RVSP in the wild-type mice, a greater amount of DNA damage in the knock-out mice that was only slightly significant, no significant difference in the number of small pulmonary arteries between the two groups of mice, and a significantly greater amount of fully muscularized small pulmonary arteries in the wild-type mice. These results do not support the notion that DNA damage in pulmonary arterial endothelial cells is a causal factor of the progression of pulmonary arterial hypertension, but the limitations of the experimental design and potential improvements must be considered before coming to a definite conclusion.

Acknowledgements

I would like to thank first and foremost Dr. Marlene Rabinovitch, MD, at Stanford School of Medicine for giving me the valuable opportunity to learn and grow in her lab and helping me receive a grant from the American Heart Association for this research. I am also very grateful to Caiyun Grace Li, PhD, for her guidance and persistence in teaching and helping me carry out the research for this thesis, all the while monitoring my progress in understanding and improving my skills in the lab with much care. In addition, I would like to extend my gratitude to Dr. Kryn Stankunas, PhD, Dr. Vincent Pat Lombardi, PhD, and Professor Monique Balbuena, PhD, for their kind support and thoughtful suggestions throughout the writing process that have expanded my perception on my research from both a scientific and layman approach. Lastly, it brings me much joy to acknowledge my family, especially my parents, who have been my sturdy foundation and source of encouragement during the difficulties I faced while carrying out this study. They never stopped believing in my potential, and thanks to their love and patience I have completed my four years of undergraduate college and my thesis with success.

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Significance

Pulmonary Arterial Hypertension

Pulmonary hypertension is a form of high blood pressure that affects pulmonary arteries, or the arteries in the lungs, of which pulmonary arterial hypertension is a subset. It can be clinically diagnosed by an elevation of mean pulmonary arterial pressure above 25 mmHg at rest or 30 mmHg with exercise; according to the American Heart Association, normal pulmonary arterial pressure is about 14 mmHg (*12*). Symptoms during the initial phase, however, are not unique to the disease, such as fatigue and difficulty breathing, so the disease is usually not detected in patients until more severe symptoms are manifest, such as chest pain and palpitations. This range in severity is described by the four functional classes of the disease, from Class I with patients showing no symptoms during physical activity to Class IV with patients suffering from fatigue or shortness of breath from right heart failure even while resting – this last class has the highest mortality rate out of the four (*16*). Although there are treatments available, there is currently no cure.

The pathology of pulmonary arterial hypertension has been characterized in considerable detail. The development of the disease contributes to changes in the pulmonary arteries that cause high resistance to blood flow from the heart to the lungs. Specific pathological effects include the following (see Figure 1): muscularization of pulmonary arteries, medial hypertrophy of muscularized arteries, obliterative neointimal lesions, loss of small pre-capillary arteries, and plexiform lesions (*12*). The muscularization of the pulmonary arteries occurs as a result of the differentiation of pericytes, or contractile cells that envelop endothelial cells lining the inner blood vessel,

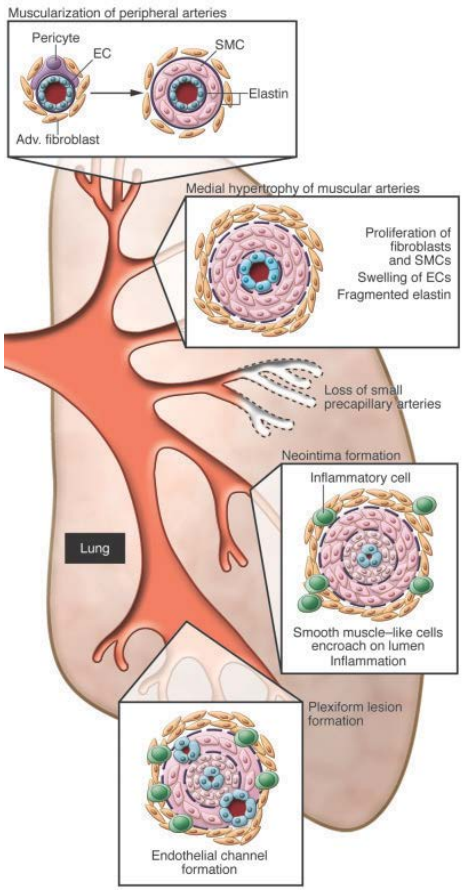


Figure 1. An illustrative overview of pulmonary arterial hypertension pathology. Vascular abnormalities that occur as a result of the disease are shown (*12*).

into smooth muscle cells that then proliferate. The continuous proliferation and migration of smooth muscle cells lead to two serious pathological effects of the disease by thickening the layer of the arteries: medial hypertrophy of muscularized arteries and neointimal lesions. Neointima refers to the new layer formed in the innermost lining of blood vessels that results from inflammatory cell signaling and injured endothelial cells that lead to the proliferation of smooth muscle-like cells, or cells resembling smooth muscle cells. Small pre-capillary arteries are lost through apoptosis, or programmed cell death, of endothelial cells and/or pericytes, while plexiform lesions, a marker of severe pulmonary arterial hypertension in the form of blocked pulmonary arteries with

abnormal channels, are due to the eventual, unregulated proliferation of endothelial cells.

Genetics has a crucial role in the pathology of pulmonary arterial hypertension. A significant genetic cause of pulmonary arterial hypertension in 70% or more of patients with hereditary pulmonary arterial hypertension and 10-20% of patients with idiopathic pulmonary arterial hypertension has been identified as a haploinsufficiency, or a condition of having one functional copy out of two for a particular gene, of the bone morphogenetic protein receptor 2 (BMPR2) gene or defective bone morphogenetic protein (BMP) signaling (*12*). BMPR2 is a protein belonging to the TGF-β family of growth factor receptors and normally signals through multiple pathways. This haploinsufficiency or defective signaling leads to endothelial cell dysfunction, which explains both the apoptosis responsible for the loss of pulmonary arterial micro-vessels and the failure of pulmonary arterial endothelial cells to control the proliferation and migration of smooth muscle-like cells that lead to lumen occlusion and vascular resistance. Under normal conditions, BMP stimulation promotes endothelial cell survival through downstream activation of a receptor in the cell nucleus called peroxisome proliferator-activated receptor-γ (PPARγ) that is crucial for the maintenance of vascular stability (see Figure 2). The formation of a complex in the nucleus between PPARγ and another protein called β-catenin initiates transcription, or the first step to the conversion of a gene into a protein, of a pro-survival gene called apelin. This promotes the necessary endothelial cell proliferation and migration for angiogenesis and inhibits proliferation of pulmonary arterial smooth muscle cells (*1*). The other pathway beginning from BMPR2 depends on PPARγ as a transcriptional activator of apoE, a

gene that inhibits PDGF-BB-mediated smooth muscle cell proliferation and migration (*9*). Without BMPR2 function or BMP signaling, these pathways do not occur, thus leading to the characteristics of the disease.

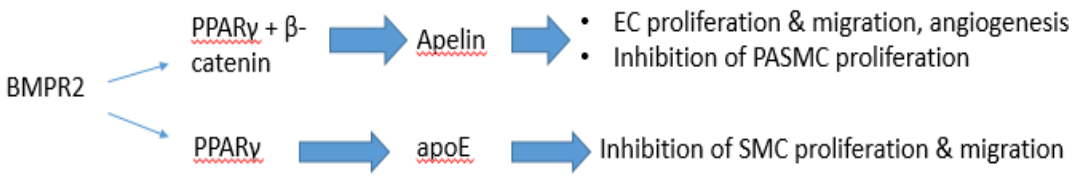


Figure 2. Overview of the BMPR2 pathway. Downstream, the transcription of genes necessary for angiogenesis and inhibition of smooth muscle cell proliferation and migration occurs.

Question and Hypothesis

Further evidence centered on PPARγ have shown a connection between the regulation of DNA stability and the pathology of pulmonary arterial hypertension. In lung tissues of human patients with pulmonary arterial hypertension, studies have shown a reduced expression of PPARγ, which is highly expressed in normal pulmonary arterial endothelial cells (*4*). Rabinovitch et al. have looked into this finding and found two significant effects of this reduction in PPARγ: extensive unrepaired DNA damage and a worsened disease phenotype. They have discovered significantly more unrepaired DNA damage in the pulmonary arterial endothelial cells of patients than in those of donor controls using an experimental technique called the comet assay, which allows for the measurement of DNA breaks in eukaryotic cells. This can be explained by current studies in the lab that are uncovering interactions between PPARγ and DNA damage sensing proteins, one of which is the MRN complex (see Figure 3), and by further studies that have shown through the comet assay that PPARγ knock-down impairs the DNA Damage Response (DDR) activation and reduces DNA repair capacity in pulmonary arterial endothelial cells.

A separate study found significant differences in the severity of pulmonary arterial hypertension between mice with endothelial cell-specific deletion of PPARγ and wild-type mice after recovery from hypoxia, a condition of oxygen deficiency (*8*). In short, the absence of PPARγ results in both significant unrepaired DNA damage and a worsened disease phenotype, which leads to the following question: Does an accumulation of DNA damage in pulmonary arterial endothelial cells contribute to the progression of pulmonary arterial hypertension?

To investigate into the posed question, I propose that extensive, unrepaired DNA damage in pulmonary arterial endothelial cells leads to endothelial cell dysfunction and worsening pulmonary arterial hypertension.

Specific Aims

Specific Aim 1

Determine if endothelial cell-specific deletion of Mre11 in a transgenic mouse model leads to persistent DNA damage.

Rationale

In more than half of nine examined human patients within a study, somatic chromosomal mutations in pulmonary arterial endothelial cells have been identified, signifying a link between genomic instability and the disease (*2*). In addition, as previously mentioned, studies done in the Rabinovitch lab showing unrepaired DNA damage in pulmonary arterial endothelial cells with PPARγ knock-down point to the same conclusion. As a result, it is clear that the DDR pathway is crucial within the model of pulmonary arterial hypertension.

A crucial component of this study is the MRN complex, which functions as a DNA damage sensor. Consisting of proteins called Meiotic Recombination 11

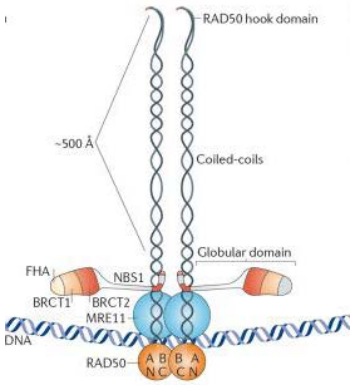


Figure 3. Depiction of the MRN complex, consisting of MRE11, NBS1, and RAD50. MRE11 directly binds to the DNA to carry out its functions (*13*).

(MRE11), Radiation sensitive 50 (RAD50), and Nijmegen breakage syndrome 1 (NBS1), the complex physically localizes to sites of damage quickly once it senses DNA damage (see Figure 3). The other significant functions of the complex (see Figure 4) include governing the activation of ATM, a kinase–a phosphorylating protein–that primarily controls responses to double-stranded DNA breaks, and ATR, a kinase primarily recruited to single-stranded DNA breaks (*5*,*13*). ATM and ATR then phosphorylate and activate downstream factors, such as CHK1, CHK2 and H2AX (*5*). Activated CHK1 or CHK2 then dissociates from the DNA and phosphorylates p53, a protein regulator of the cell cycle that functions as a tumor suppressor. This leads to transcription of p21, a strong regulator of the cell cycle progression that arrests the cell cycle to allow repair of DNA damage. γH2AX, or H2AX phosphorylated by ATM and ATR, forms the focus for recruitment of DNA repair proteins, such as BRCA.

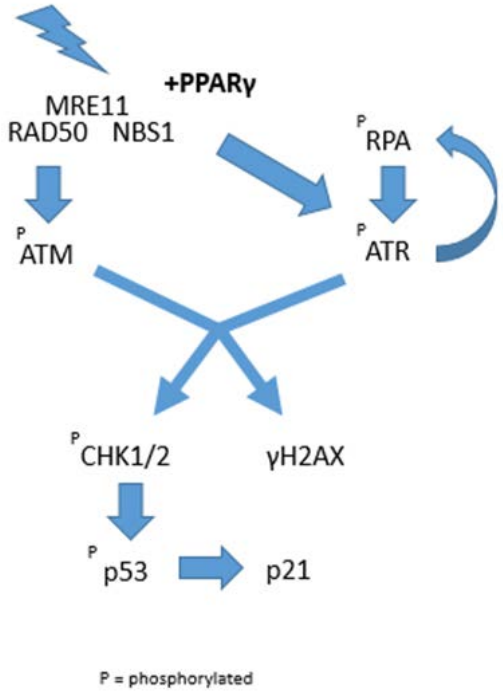


Figure 4. Overview of the DNA Damage Response (DDR) pathway. The lightning symbol represents DNA damage, from which the MRN complex activates downstream effectors of DNA damage repair. Studies have discovered interactions between PPARγ and the MRN complex.

This study will focus on MRE11, one of the three proteins of the MRN complex (see Figure 3). Within the DDR pathway, it functions both as an endonuclease that cuts single-stranded DNA and an exonuclease that cuts double-stranded DNA in one direction. Like the other two components of the MRN complex, MRE11 is essential for viability, as verified by the early deaths during embryogenesis of transgenic mice, or mice with modified genomes, whose Mre11, Rad50, or Nbs1 gene had been deleted (*13*). The purpose of this specific aim is to generate and verify the accumulation of DNA damage through endothelial cell-specific deletion of Mre11 in transgenic mice in order to investigate the role of DNA damage in the pathology of pulmonary arterial hypertension.

Experimental Design

A) Mice

The transgenic mice used in this study have been engineered to express a tamoxifen-inducible protein called CreER recombinase, whose expression is driven by the 5' endothelial enhancer of the stem cell leukemia (SCL) locus. For the purpose of this experiment, the SCL 5' endothelial enhancer was used to direct the expression of CreER recombinase to endothelial cells, so that the protein would function only in endothelial cells (*7*). Attached to the CreER recombinase is a ligand-binding domain of estrogen receptor (ER) that binds to tamoxifen, an estrogen antagonist, resulting in the entry into the nucleus where the recombinase functions. Genetic crosses between two breeds of mice created the "knock-out" progeny we used: one breed consisted of a transgene with the 5' endothelial enhancer region of the SCL locus genetically engineered with the CreER recombinase gene and the ligand-binding domain of ER, so that upon injection of tamoxifen, CreER recombinase enters the nuclei of endothelial cells, and the other breed consisted of a transgene with Mre11 flanked by two LoxP sites that are recognized by CreER for deletion (see Figure 5). In this way, the Mre11 is conditionally inactivated in endothelial cells in the transgenic knock-out mice upon tamoxifen delivery at the desired age. The gene containing Mre11 in the wild-type mice, on the other hand, does not contain the LoxP sites, which explains the Mre11 expression regardless of the type of cell (see Figure 6). Five male wild-type mice with the genotype SCL-Cre-Mre11$^{+/+}$ and five male knock-out mice with the genotype SCL-Cre-Mre11$^{cond/cond}$ were used for this study.

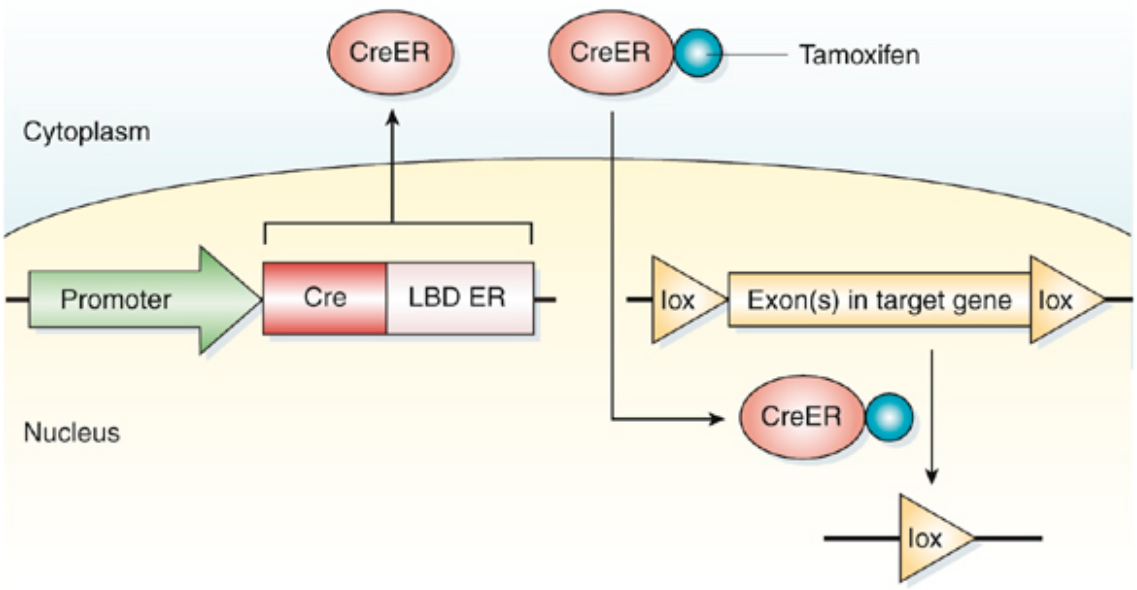


Figure 5. A depiction of Cre-lox recombination using CreER. CreER recombinase expression occurs in specific cells to where the fused promoter drives expression, and after binding to tamoxifen, the protein enters the nucleus where it deletes genes flanked by two loxP sites, resulting in a specific knock-out (*10*).

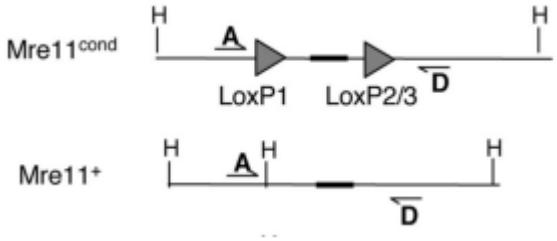


Figure 6. Depiction of the SCL-Cre-Mre11 transgenes for knock-out and wild-type mice. The two LoxP sites are shown in the Mre11cond knock-out, where the CreER recombinase binds to upon its activation by tamoxifen, resulting in the excision of the area enclosed by the LoxP sites and the consequent deletion of Mre11, as shown by the dark region. The Mre11+ wild-type gene does not include LoxP sites (*5*).

For two weeks on a daily basis before the mice were used, tamoxifen solution (10 mg/mL dissolved in corn oil) was injected into all mice when they were between 10 and 13 weeks old in order to activate the cell-specific gene expression of CreER recombinase in the cells. Then the mice were placed under conditions of hypoxia (8% O_2) for ten days to induce DNA damage instead of other damaging agents since

moderately hypoxic environments are characteristic of pulmonary arterial hypertension. In addition, it is known that moderate hypoxia activates the DDR pathway in endothelial cells (*11*).

B) DDR Pathway Evaluation

After the mice were euthanized, a total of 3 thin samples from the distal, middle, and proximal sections of lung tissues were placed on a microscope slide for each mouse, from which lung histology was carried out to analyze evidence of DNA damage in the pulmonary arteries. The indirect method of immunohistochemistry was used on the lung tissues to identify certain tissue components through two sets of interactions: one between a primary antibody and its target antigen and another between a secondary antibody conjugated with a fluorescent tag and the primary antibody (see Figure 7). In this part of the study, a fluorescent blue stain called DAPI for nuclei was used, as well as two sets of primary and secondary antibodies to identify specific antigens: rabbit von Willebrand Factor (vWF) and green-tagged secondary anti-rabbit antibody for the endothelial cells, and mouse γH2AX and red-tagged anti-mouse antibody for DNA damage. The antibodies vWF and γH2AX were used because vWF is a blood clotting protein made within endothelial cells and γH2AX is a histone variant that is phosphorylated upon the generation of double-stranded breaks, a form of DNA damage. Pictures of the prepared tissue samples were taken using a fluorescence microscope in the lab.

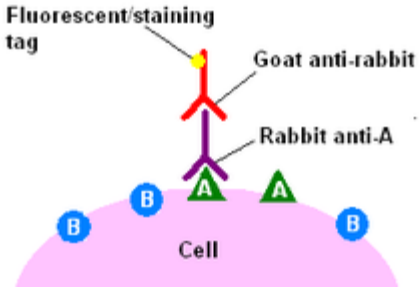


Figure 7. An example depicting the indirect method of detecting target antigen in immunohistochemistry (*14*).

To quantify DNA damage, small pulmonary arteries were first identified from the pictures taken with the microscope, using a systematic approach blinded to whether the mouse was wild-type or knock-out. On a program called ImageJ that is typically used for visual analysis of fluorescently marked cells, merging the fluorescent blue DAPI, green vWF, and red γH2AX markers allowed for the counting of small pulmonary arteries with DNA damage within the size range of a 20-50μm diameter and located below the respiratory bronchioles level and along the alveolar ducts (see Figure 8). The inclusion criteria for a small pulmonary artery with DNA damage required at least having 2-3 nuclei of endothelial cells and at least one red-tagged γH2AX marker located within an endothelial cell nucleus. Results from wild-type and knock-out mice were analyzed using an unpaired t-test via a computer program called Prism.

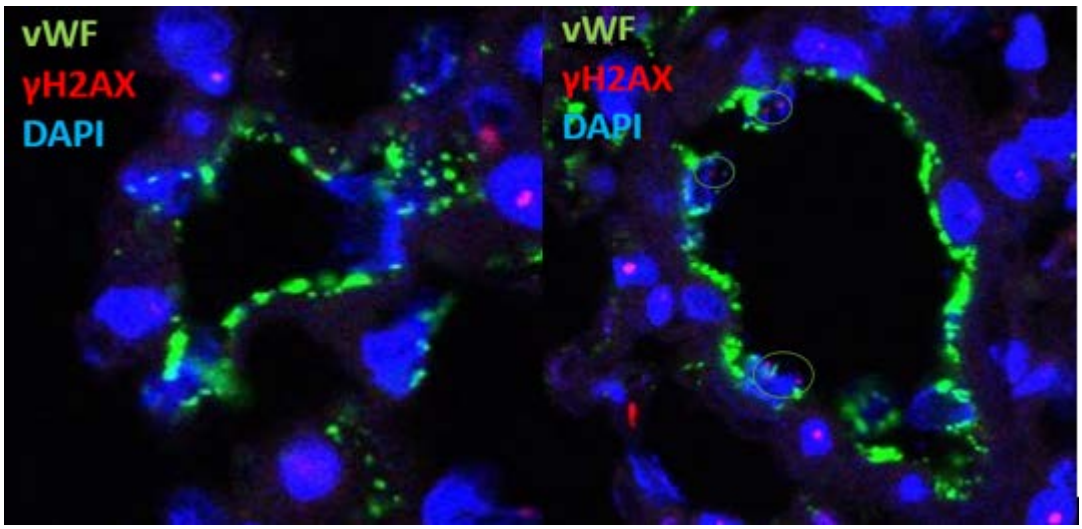
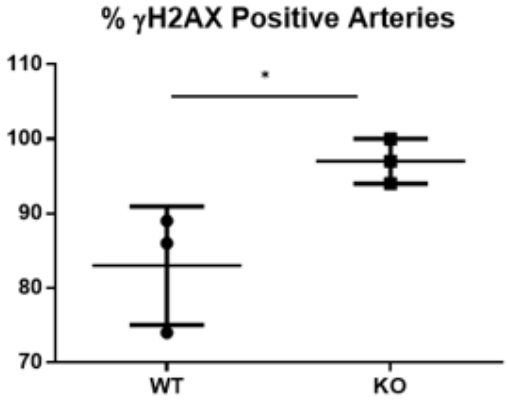


Figure 8. Sample pictures of fluorescently marked lung tissues taken on the fluorescence microscope using the markers vWF, γH2AX, and DAPI. The picture on the left represents an artery with no DNA damage, as indicated by the lack of the red marker, and the picture on the right represents an artery with DNA damage, as indicated by the circles surrounding red markers in the nuclei. Both arteries shown have at least 3 nuclei of endothelial cells (blue DAPI flanked by green vWF).

Results and Discussion

As expected, there were significantly more pulmonary arteries with DNA damage in the knock-out mice than in the wild-type mice (see Figure 9). Only 3 wild-type and 3 knock-out mice were used for the counting due to time constraints associated with taking pictures of the lung tissues using the fluorescence microscope. There was greater consistency in the amount of DNA damage in the knock-out mice than in the wild-type mice, but the fact that the difference in the amounts of DNA damage between the two groups of mice is only slightly significant must be considered. The p-value from the unpaired t-test was 0.0344, which is only slightly less than 0.05. We had expected the Mre11-knock-out mice to have a substantially greater amount of DNA damage than the wild-type mice due to their loss of a crucial DNA damage sensing protein, but our

unexpected result can be explained for by the possibility that the hypoxic conditions of 8% oxygen may have been too severe for all mice–in other studies, 10% was used (*8*).



Mouse	# Positive Arteries	# Negative Arteries	% Positive γH2AX
WT 1	23	7	77%
WT 2	28	12	70%
WT 3	31	4	89%
KO 1	33	2	94%
KO 2	18	0	100%
KO 3	38	1	97%

Figure 9. Results of DNA damage amounts in wild-type (WT) and knock-out (KO) mice in percentage are shown in a graph–γH2AX positive arteries refer to arteries with DNA damage. There were significantly more pulmonary arteries with DNA damage in the knock-out mice than in the wild-type mice with a p-value of 0.0344 from the unpaired t-test done by Prism. The raw data are shown in a table.

This could have led to similarly extensive DNA damage across all mice, regardless of genotype. Our results can be alternately explained for by the small total sample size of 6 mice or by inaccurate counting of γH2AX-positive arteries. It is unlikely that the SCL-CreER system failed to activate the specific deletion of Mre11, since we have obtained consistently high amounts of DNA damage in the knock-out mice.

Specific Aim 2

Characterize the effects of endothelial cell-specific deletion of Mre11 on pulmonary vasculature to observe induced symptoms of pulmonary arterial hypertension.

Rationale

This specific aim lies at the heart of this research, for its primary purpose is to identify whether accumulation of DNA damage results in worsened pulmonary vasculature, or pulmonary arterial hypertension. As mentioned previously, findings from the Rabinovitch lab that uncover two potential correlations between reduced PPARγ expression and unrepaired damaged DNA and between reduced PPARγ expression and pulmonary arterial hypertension support this aim.

Experimental Approach

A) Mice

The same transgenic mice from Specific Aim 1 were used for this aim.

B) Hemodynamic Measurements

The following hemodynamic measurements were taken of the mice: right ventricular systolic pressure (RVSP), left ventricular end diastolic pressure (LVEDP), and a complete blood count (CBC). RVSP, or the pressure in the right ventricle when the heart contracts, was measured for the mice because it is generally increased in patients with pulmonary arterial hypertension. LVEDP, or the pressure in the left ventricle at the end of the heart's resting period between contractions, was also measured in order to rule out contributions from the left ventricle when analyzing the outcomes of the mice. RVSP and LVEDP measurements were obtained simultaneously

by a closed-chest technique that took measurements on a computer program via a catheter in the jugular vein of the mice under anesthesia. The CBC was carried out by a laboratory within the Stanford facilities that analyzed blood samples–the purpose of this was to measure the effects of hypoxia on the mice that can be then further analyzed and understood. This included three groups: red blood cells, white blood cells, and platelets. To analyze how the mice responded to reduced oxygen, the measurements of red blood cells included red blood cells, hemoglobin (oxygen-carrying protein in red blood cells), hematocrit (the ratio of the volume of red blood cells to the total volume of blood), and absolute reticulocyte (immature red blood cells). For the purpose of gauging the health of the mice, the measurements of white blood cells included white blood cells, absolute neutrophil, absolute lymphocyte, and absolute monocyte. Platelet count was merely included in the CBC without relevance to the study.

C) Lung Histology

Indirect immunohistochemistry was the primary method used for the counting of (muscularized) small pulmonary arteries to assess the loss and the muscularization of small pulmonary arteries. In this part of the study, fluorescent blue DAPI for nuclei was used, as well as two sets of primary and secondary antibodies to identify specific antigens: rabbit vWF and green-tagged secondary anti-rabbit antibody for the endothelial cells, and mouse α-smooth muscle actin (SMA) and red-tagged anti-mouse antibody for the muscularized cells. Pictures of the prepared tissue samples were taken using a fluorescence microscope in the lab.

The numbers of small pulmonary arteries and of muscularized small pulmonary arteries were counted from the images taken with the microscope, using a systematic

approach blinded to whether the mouse was wild-type or mutant. First, a total of five respiratory zones consisting of respiratory bronchioles, alveolar ducts, and alveoli were located for each mouse (see Figure 10). Next, to maintain consistency for each tissue

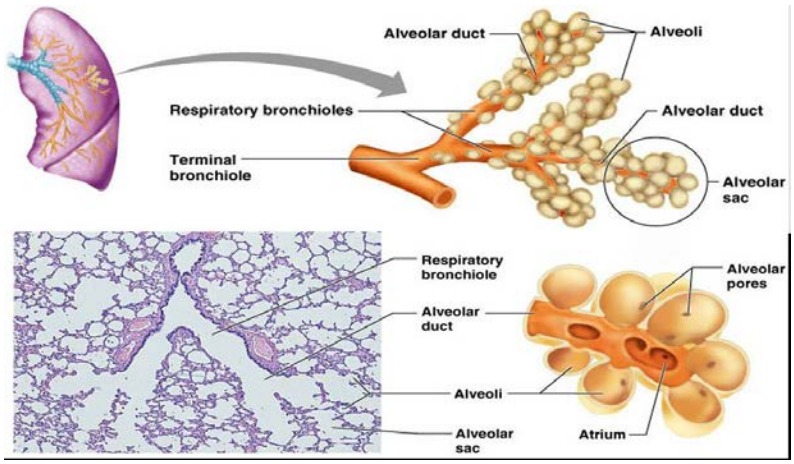


Figure 10. Depiction of a respiratory zone and its components, including respiratory bronchioles, alveolar duct, and alveoli. An image of a mouse lung tissue section is shown (*15*).

portion, a section enclosing 100 alveoli surrounding alveolar ducts was determined for each respiratory zone, from which the small pulmonary arteries were counted. The alveoli were counted by converting the images into black-and-white to facilitate the counting process (see Figure 11). Then with the use of the ImageJ program, small pulmonary arteries marked by the green vWF marker for endothelial cells within a 20-50µm diameter and located below the respiratory bronchioles level and along the alveolar ducts were then counted. An average was taken for each mouse. Out of the small pulmonary arteries that were counted, the muscularized ones were counted by merging the green vWF and red SMA markers on ImageJ, with the level of muscularization ranging from none to partially to fully muscularized. No

muscularization would be indicated by a lack of red, partial muscularization by an incomplete red lining of the artery, and full muscularization by a complete red lining of the artery. Finally, the differences between the counts of small pulmonary arteries for wild-type and for knock-out mice were analyzed with an unpaired t-test, and the differences between the counts of fully muscularized small pulmonary arteries for wild-type and for knock-out mice and between the counts of partially muscularized small pulmonary arteries for wild-type and for knock-out mice were analyzed with one-way ANOVA via the Prism program.

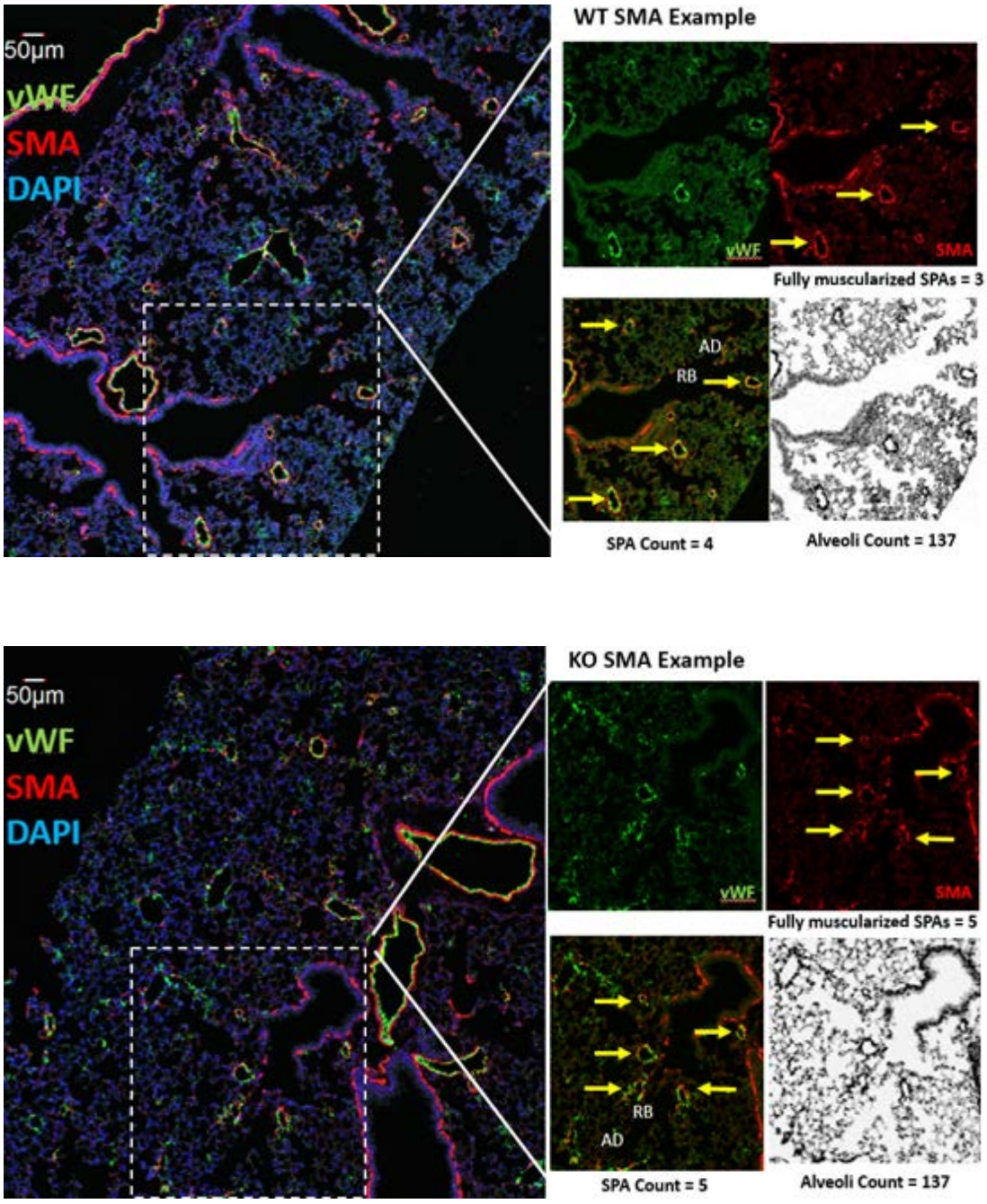


Figure 11. Sample pictures of fluorescently marked lung tissues taken on the fluorescence microscope using the markers vWF, SMA, and DAPI. Each of the two groups of images shown include a merged picture of all markers on the left and 4 pictures on the right that display how the (muscularized) small pulmonary arteries and alveoli were counted. The top group of images is from a wild-type mouse, and the bottom group from a knock-out mouse.

Results and Discussion

A) Hemodynamic Measurements

1. RVSP and LVEDP – No significant differences were observed in both the RVSP and LVEDP measurements between the wild-type and knock-out mice, but general trends were noted (see Figure 12). RVSP measurements of only 4 wild-type and 4 knock-out mice rather than 5 for each group were taken due to complications with one wild-type mouse during the measuring procedure and the death of one knock-out mouse after 10 days of hypoxia. Contrary to our expectations, the average of the RVSPs of the wild-type mice was higher than that of the knock-out mice. We had expected greater

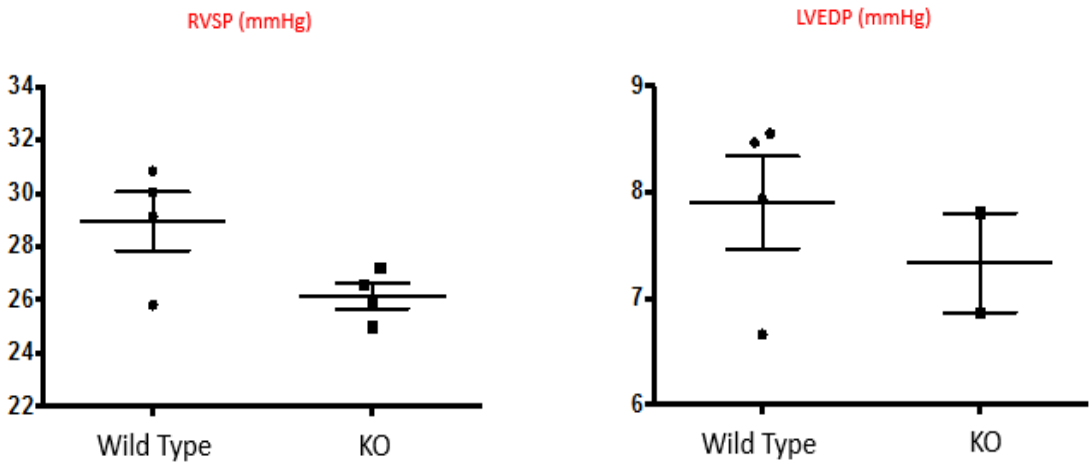


Figure 12. Graphs of RVSP and LVEDP measurements with averages of each group shown. There were no significant differences between wild-type and knock-out mice for RVSP and LVEDP. Normal range of RVSP is below 25 mmHg during rest and of LVEDP is between 0-10 mmHg. There were 4 wild-type mice used for both RVSP and LVEDP measurements, while for knock-out mice, 4 were used for RVSP and 2 for LVEDP measurements.

RVSPs in the knock-out mice than in the wild-type mice, since we anticipated that unrepaired DNA damage caused by lack of Mre11 would make the mice more susceptible to characteristics of pulmonary arterial hypertension, such as an elevated

RVSP. In the knock-out mice, the deletion of Mre11 in endocardial cells could have influenced the results somehow. Both groups of mice, however, had average RVSPs greater than the normal 25 mmHg, suggesting that the hypoxic conditions did affect the pulmonary vasculature. For the LVEDP measurements, only 4 wild-type and 2 knock-out mice were used due to unexpected deaths during the procedure. The average LVEDP measurements for both groups of mice, however, did not differ very much and were within the normal range of LVEDP of 0-10 mmHg, suggesting that the left ventricle was not greatly affected by hypoxia.

ii. CBC – To analyze further how the mice responded to hypoxia, we took blood samples from 4 wild-type and 4 knock-out mice and processed a complete blood count for each mouse. Within the group of results pertaining to red blood cells–red blood cells, hemoglobin, hematocrit, platelet count, and absolute reticulocyte–the wild-type mice generally had higher averages than the knock-out mice, except for red blood cells which both groups had an average of around the same amount (see Figure 13). The wild-type mice had significantly greater averages of hematocrit and absolute reticulocyte than the knock-out mice. The hematocrit results could indicate that the wild-type mice generally had less blood volume than the knock-out mice because the red blood cell averages are similar for both groups, and the absolute reticulocyte results could indicate that the wild-type mice responded more severely than the knock-out mice to hypoxia by producing red blood cells more quickly. At the time the blood samples were taken, it is possible that this difference in rate of red blood cell production was not translated into a difference in red blood cell count because the reticulocytes had yet to mature. Another possible explanation can be made with the fact that the Scl-CreER

system functions in hematopoietic cells in addition to endothelial cells, which could have negatively affected the amount of reticulocytes in the knock-out mice. Despite their differences, both groups of mice had amounts of red blood cells, hemoglobin, and hematocrit higher than the normal range, suggesting that they responded to the low-oxygen environment by making more red blood cells than they would have under normal oxygen levels. This supports the sufficiency of the 10-day long hypoxia period in initiating a cellular response in the mice.

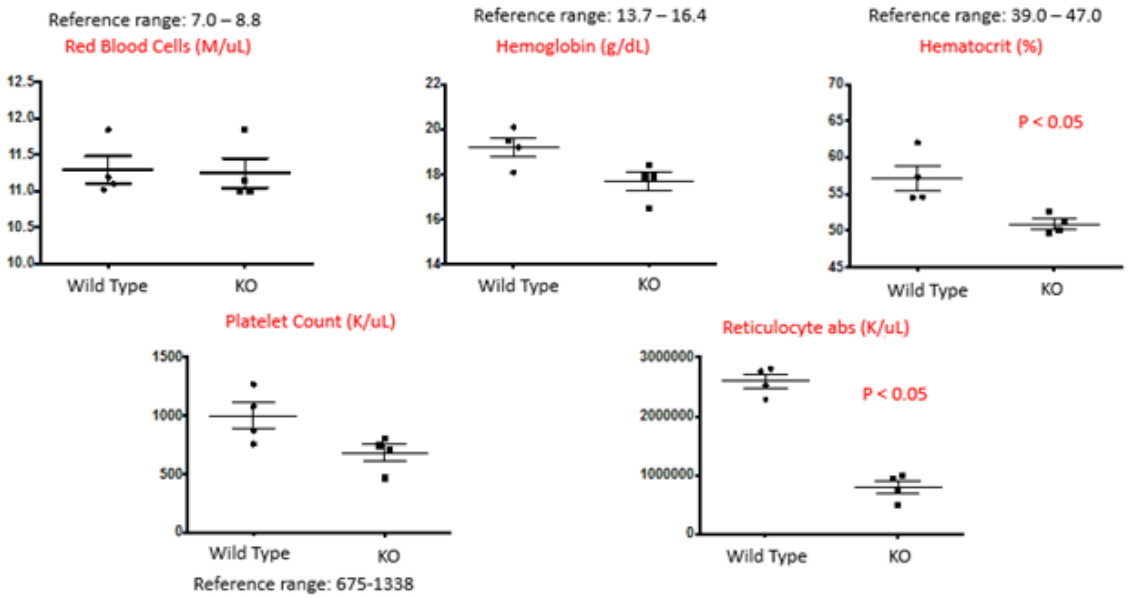


Figure 13. Graphs of CBC results for 4 wild-type and 4 knock-out mice. Here shown are red blood cell, hemoglobin, hematocrit, platelet count, and absolute reticulocyte results, all representative of red blood cells. Reference ranges are shown for each group except for absolute reticulocyte, for none was given. Wild-type mice had averages of hematocrit and absolute reticulocyte significantly greater than those of knock-out mice with a p-value less than 0.05. For both wild-type and knock-out mice, amounts of red blood cells, hemoglobin, and hematocrit were higher than the normal range.

Within the group of results pertaining to white blood cells–white blood cells, absolute neutrophil, absolute lymphocyte, and absolute monocyte–only the absolute neutrophil count was significantly greater in the knock-out mice than in the wild-type mice (see Figure 14). This is due to the abnormal low amount of neutrophils in the wild-

type mice. For both mice, white blood cell and absolute lymphocyte counts were noticeably lower than the normal range, suggesting either that the mice became infected by some means that led to the destruction of white blood cells or that they were merely susceptible to infection. Wild-type mice seem to have been more at risk of infection and unable to protect themselves efficiently in comparison to knock-out mice.

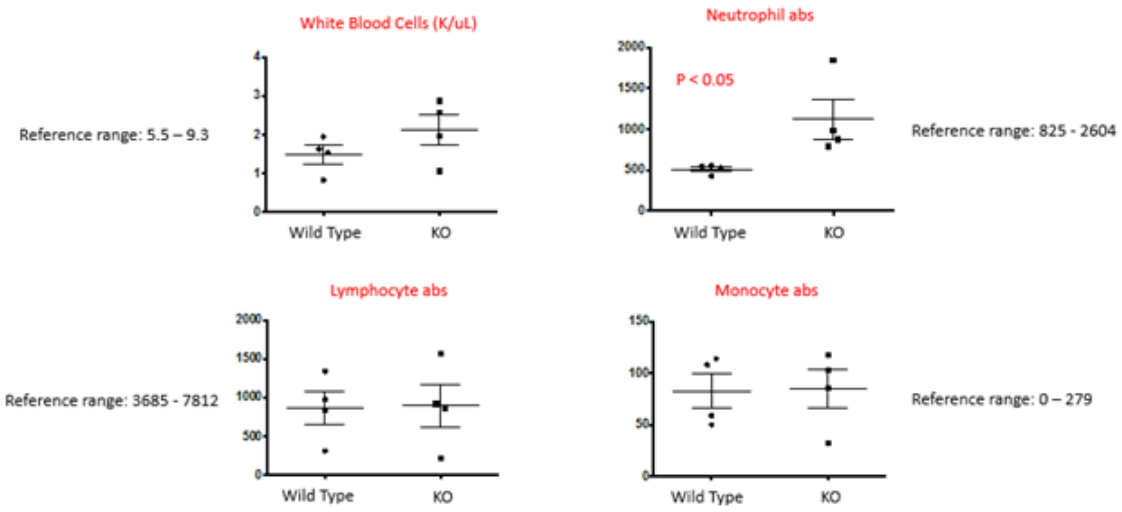


Figure 14. Graphs of more CBC results of 4 wild-type and 4 knock-out mice. Here shown are white blood cell, absolute neutrophil, absolute lymphocyte, and absolute monocyte results, all representative of white blood cells. Reference ranges are shown for each group. Knock-out mice had a significantly higher average of absolute neutrophil than that of wild-type mice with a p-value less than 0.05. White blood cell and absolute lymphocyte counts were lower than the normal range for both groups of mice, while absolute neutrophil counts were lower than the normal range for only wild-type mice.

B) Lung Histology

After counting was finished, the number of small pulmonary arteries and the number of fully and partially muscularized small pulmonary arteries respectively for wild-type and knock-out mice were compared (see Figure 15). There was no significant difference between the averages of small pulmonary arteries in wild-type and knock-out mice–we had expected there to be more small pulmonary arteries in the wild-type than

in the knock-out mice, or for the disease phenotype to be more severe in knock-out than in wild-type mice. Our results could be due to the short 10-day period of hypoxia or due

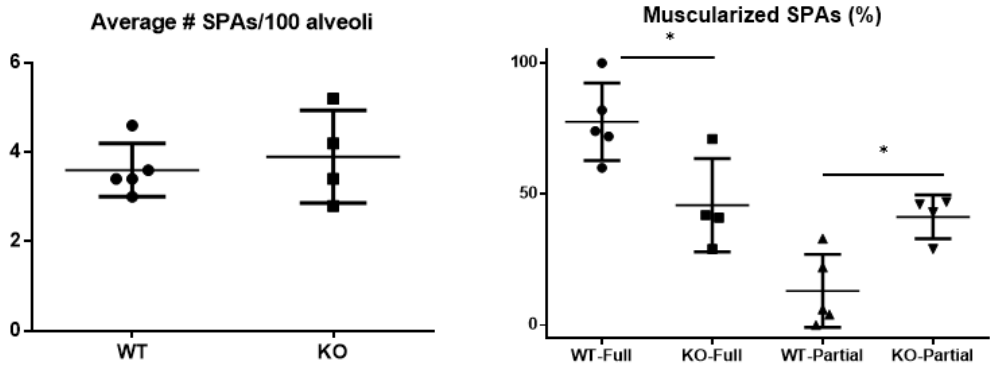


Figure 15. Graphs of results from counting small pulmonary arteries and muscularized small pulmonary arteries for 5 wild-type mice and 4 knock-out mice. The averages of small pulmonary arteries in wild-type and knock-out mice were very similar. Wild-type mice had significantly more fully muscularized small pulmonary arteries than knock-out mice, while knock-out mice had significantly more partially muscularized small pulmonary arteries than wild-type mice.

to our method of choosing 5 respiratory zones as opposed to using every respiratory zone for each mouse. As for the muscularized small pulmonary arteries, the average of fully muscularized small pulmonary arteries in the wild-type mice was significantly greater than the average in the knock-out mice contrary to what we expected, and the average of partially muscularized small pulmonary arteries in the knock-out mice was significantly greater than the average in the wild-type mice as we expected. The results for fully muscularized small pulmonary arteries align with the RVSP results, however: it is possible that the greater muscularization of the small pulmonary arteries in the wild-type mice is correlated with the higher RVSP trend. The muscularization signifies more effort than normal in pumping the blood through the arteries, which would be connected with a high RVSP since the right ventricle pumps blood through the

pulmonary arteries. The reason for the greater amount of full muscularization in the wild-type mice than in the knock-out mice can only be speculated; the knock-out mice seemed to have a slower physiological response than the wild-type mice. One thing for certain, however, is that the results representative of red blood cells cannot serve as an explanation, for a study has shown that in hypoxic mice the increased muscularity of pulmonary arteries is independent of elevation of hematocrit; in other words, vascular remodeling models are not correlated with blood results (*5*). The wild-type mice must have fared more poorly in hypoxic conditions than the knock-out mice, but why this is so is uncertain.

Conclusion

In conclusion, my results did not support my hypothesis, or more specifically, that accumulation of DNA damage leads to worsened pulmonary arterial hypertension. As expected, there was significantly more DNA damage as indicated by the γH2AX marker in the pulmonary arterial endothelial cells of the knock-out mice than of the wild-type mice, but the significance was small. The RVSPs of the wild-type mice were generally higher than that of the knock-out mice, but for both groups the values were higher than normal suggesting that some vascular remodeling took place in response to the 10 days of hypoxia. The LVEDP values were similar and within the normal range, suggesting that the remodeling did not affect the left side of the heart in both groups. With a general trend of higher amounts in the wild-type mice than in the knock-out mice, the counts representative of red blood cells were significantly different for hematocrit and absolute reticulocyte, perhaps meaning that the blood volume was less and that red blood cell production was faster in the wild-type mice than in the knock-out mice. Overall, however, both groups of mice responded to hypoxia by increasing red blood cell production to more than what is considered normal. On the contrary, the counts representative of white blood cells showed a significant difference in absolute neutrophil amounts between both groups, wild-type mice having an abnormally low amount and knock-out mice having a normal but significantly greater amount than the wild-type mice. Both groups had abnormally low amounts of white blood cells and lymphocytes, suggesting that the mice were susceptible to infection. Whether this occurred from the hypoxic conditions or from an infection that the mice caught by some means remains to be known. There was no significant difference in the amounts of

small pulmonary arteries; the disease phenotype of loss of pulmonary arteries was not observed in the knock-out mice. The wild-type mice had significantly more fully muscularized arteries than the knock-out mice, but the knock-out mice had significantly more partially muscularized arteries. These results suggest that the wild-type mice developed the disease characteristic of muscularized arteries more so than the knock-out mice, which correlates with the RVSP values. Although these findings seem to disprove my hypothesis, further research is required to confirm what I have found.

There were a number of limitations to this study that may have caused unexpected results. First, the period of 10 days of hypoxia may have been too short for the DNA damage to translate into the expected resulting pathology in the knock-out mice–other studies have used a period of hypoxia of 3 weeks (*6, 8*). This could result in a lack of a significant difference between the wild-type and knock-out mice in the amounts of DNA damage and severity of the pathology. Ten days was chosen, however, because of the unexpected death of one of the knock-out mice and the uncertainty over additional following deaths. Second, no measurements of the mice were taken before hypoxia to set a baseline to which the results from the hypoxic period could be compared. Third, a period of re-oxygenation was not included after hypoxia, which could have confirmed a difference between the wild-type and knock-out mice in the way that they recover from the DNA damage induced by hypoxia. Fourth, the sample size of this study may have been too small for various parts, ranging from 6 to 9 mice throughout. Fifth, the SCL-CreER system used to knock-out the Mre11 gene may not have been specific enough to endothelial cells, since the SCL 5' endothelial enhancer region also directs gene expression to early hematopoietic progenitor cells (*7*). This

inability to restrict the conditional inactivation of Mre11 to endothelial cells must be taken into consideration, for it could have led to unforeseen effects by inducing more stress upon the mice than had been intended. It is possible that knock-out of Mre11 in early hematopoietic progenitor cells could be related in some way to the significantly fewer amount of absolute reticulocyte measured in the knock-out mice than in the wild-type mice. Sixth, human error is always a possibility for unexpected results: the counting of (muscularized) small pulmonary arteries and DNA damage in the endothelial cells was not entirely fool-proof and could be considered subjective by the counter. There was only one person who did the counting, so no confirmation could be made of the results.

With these limitations in mind, a future study can be designed differently to test the same hypothesis for more reliable results. Rather than using the SCL-CreER transgenic line to delete the Mre11 gene in vascular endothelial cells, this study can take on a different method utilizing the vascular endothelium cadherin (VE-cadherin) promoter, which Alva et al. support by asserting that, in contrast to other endothelial cell-specific transgenic lines such as Tie-2-Cre and SCL-CreER, the use of VE-cadherin allows for expression of CreER-recombinase in endothelial cells that have become quiescent, or inactive, thus targeting adult endothelium (*3*). The structure of the study should include two sets of experiments: one set of 5 wild-type mice and 5 knock-out mice at 12 weeks of age in room air and another set of 5 wild-type and 5 knock-out mice in hypoxia at 10% O_2 for 3 weeks and then in room air for 4 weeks of recovery, as was done in the study done by Guignabert et al. These additions serve the purpose of setting a baseline for the measurements taken in hypoxia and of observing whether

differences between the wild-type and knock-out mice remain manifest in the recovery period in the form of differences in amounts of DNA damage and/or worsened pulmonary vasculature. As part of the subsequent lung histology, a TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay typically used to assess apoptosis directly and DNA damage indirectly by labeling fragmented DNA can be carried out alongside with the immunohistochemistry identifying endothelial cells with DNA damage for confirmation of the quantified results I had obtained by counting endothelial cells with DNA damage using the γH2AX marker. For the analysis of the immunohistochemistry stainings and, if included, of the TUNEL assay results, there should be at least 3 people counting, and the results should be averaged. With these edits made to the study, it is possible that different results will be obtained to elucidate more clearly the progression of pulmonary arterial hypertension from DNA damage in endothelial cells to worsened pulmonary vasculature characteristic of the disease.

Bibliography

1. Alastalo, Tero-Pekka, Molong Li, Vinicio de Jesus Perez, David Pham, Hirofumi Sawada, Jordon K. Wang, Minna Koskenvuo, Lingli Wang, Bruce A. Freeman, Howard Y. Chang, and Marlene Rabinovitch. "Disruption of PPARγ/β-catenin-mediated regulation of apelin impairs BMP-induced mouse and human pulmonary arterial EC survival." *The Journal of Clinical Investigation* 121, issue 9 (2011): 3735-3746. doi:10.1172/JCI43382.

The importance of the PPAR-γ and β-catenin complex in the BMP-2-mediated EC survival is highlighted within this paper, including the importance of apelin, the transcriptional target of the PPAR-γ/β-catenin complex. The researchers ran experiments that revealed the pro-survival effects of apelin, the results showing that a loss of apelin worsens hypoxia-induced PAH via a decreased number and an increased muscularization of peripheral arteries; that the presence of apelin prevents EC apoptosis, promotes regeneration of precapillary arteries, and inhibits proliferation of and promotes apoptosis in PASMCs through paracrine signaling; and that treating Tie2Cre PPAR-$\gamma^{floxed/floxed}$ mice with apelin for 14 days leads to the reversal of PAH effects on RVSP, RVH (right ventricular hypertrophy), and muscularization of alveolar wall pulmonary arteries. The deletion of the PPAR-γ gene in the transgenic mice that they used in their experiments utilized the Tie2 promoter.

2. Aldred, Micheala A., Suzy A. Comhair, Marileila Varella-Garcia, Kewal Asosingh, Weiling Xu, George P. Noon, Patricia A. Thistlethwaite, Rubin M. Tuder, Serpil C. Erzurum, Mark W. Geraci, and Christopher D. Coldren. "Somatic Chromosome Abnormalities in the Lungs of Patients with Pulmonary Arterial Hypertension." *American Journal of Respiratory and Critical Care Medicine* 182, issue 9 (2010): 1153-1160. doi:10.1164/rccm.201003-0491OC.

This study contributes to the insufficient body of knowledge on the genetic basis of PAH with its results. With an objective to look for a larger-scale genomic instability associated with the disease, the researchers have found mosaic chromosomal abnormalities in PAEC cultures from five out of nine PAH lungs and a patient with a BMPR2 mutation and a somatic loss of chromosome-13. The results that they have found point towards the frequent presence of genetic abnormalities within PAH lung vessels, more so in ECs than in SMCs, which they speculate may lead to a growth advantage and contribute to the progression of the disease. This study presents the first evidence on the molecular level of a second genetic hit from the germline BMPR2 mutation as well as fresh support of somatic mutations in PAH lungs, which suggests that there may be a genetic characteristic common to the various forms of the disease.

3. Alva, Jackelyn A., Ann C. Zovein, Arnaud Monvoisin, Thomas Murphy, Anthony Salazar, Natasha L. Harvey, Peter Carmeliet, and M. Luisa Iruela-Arispe. "VE-Cadherin-Cre-Recombinase Transgenic Mouse: A Tool for Lineage Analysis and Gene Deletion in Endothelial Cells." *Developmental Dynamics* 235 (2006): 759-767. Doi: 10.1002/dvdy.20643.

Understanding the limits of the available transgenic lines used for endothelium-specific knock-out of genes, the authors of this study focused on the VE-Cadherin promoter as a more specific, constitutive method of gene deletion in endothelium in both embryos and adults. They created VE-Cadherin-Cre transgenic mice and analyzed in which cells Cre-recombinase was expressed. This study proves to be useful in offering a new, alternative method of gene deletion that may be more reliable than others in targeting a greater portion of endothelial cells, including mature, quiescent ones.

4. Ameshima, Shingo, Heiko Golpon, Carlyne D. Cool, Daniel Chan, R. William Vandivier, Shyra J. Gardai, Marilee Wick, Raphael A. Nemenoff, Mark W. Geraci, and Norbert F. Voelkel. "Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) Expression Is Decreased in Pulmonary Hypertension and Affects Endothelial Cell Growth." *Circulation Research* 92 (2003): 1162-1169. doi: 10.1161/01.RES.0000073585.50092.14.

Emphasizing the significant role of PPARγ in regulating PAH, the results of this study provide evidence of a prevailing PPARγ expression in normal lungs and a reduced PPARγ expression in the lungs or, more specifically, in the muscularized precapillary arterioles and plexiform lesions from patients with severe PAH through quantitative PCR analysis and IHC of lung tissue samples. The researchers confirmed this finding with further evidence of a reduced expression of PPARγ protein in the lung vascular lesions of rats induced with severe PAH and exposed to hypoxia. They carried out additional experiments and found that fluid shear stress on ECs in cell culture reduced PPARγ expression, that dominant-negative PPARγ (DN-PPARγ) ECs injected into the tail veins of mice lead to the formation of lung vascular lesions, and that DN-PPARγ protects against apoptosis.

5. Buis, Jeffrey, Yipin Wu, Yibin Deng, Jennifer Leddon, Gerwin Westfield, Mark Eckersdorff, JoAnn M. Sekiguchi, Sandy Chang, and David O. Ferguson. "Mre11 Nuclease Activity has Essential Roles in DNA Repair and Genomic Stability Distinct from ATM Activation." *Cell* 135, issue 1 (2008): 85-96. doi:10.1016/j.cell.2008.08.015.

This paper builds off from "The Mre11 complex: starting from the ends" review paper by beginning with a discussion of the MRN complex and moving into the significance of the nuclease activity of Mre11 through a series of results from experiments using $Mre11^{H129N/\Delta}$ mutated mice. Mre11 is known to have two important functions: a double-stranded 3'-5' exonuclease activity and a single-

stranded endonuclease activity on 5' overhangs, 3' flaps, 3' branches, and closed hairpins. An Mre11 mutation causes similar effects as an ATM mutation, but while MRN null alleles result in embryonic lethality, ATM inactivation does not defer the viability of humans and mice. A nuclease-deficient Mre11 phenotype was found to be similar to a loss-of-MRN complex phenotype, showing the necessity of the nuclease function, and $Mre11^{H129N/\Delta}$ mice were found to be hypersensitive to IR damage and replication stress.

6. Fried, Ruthellen, Barbara Meyrick, Marlene Rabinovitch, and Lynne Reid. "Polycythemia and the acute hypoxic response in awake rats following chronic hypoxia." *Journal of Applied Physiology* 55, no. 4 (1983): 1167-1172.

In this study, 22 rats were used to study the acute hypoxic pressor response, or more specifically, to see the effects of hypoxia on pulmonary arterial pressure and pulmonary vascular resistance while comparing normocytic and polycythemic rats. These two measurements were increased in all groups of rats in 10% oxygen; more specifically, the values were greater in rats under hypoxia than in the controls and were greater in polycythemic than normocytic rats. They found that the pulmonary arterial circulation was remodeled after 10 days of hypoxia and that pulmonary arterial pressure was independent of hematocrit.

7. Göthert, Joachim R., Sonja E. Gustin, J. Anke M. van Eekelen, Uli Schmidt, Mark A. Hall, Stephen M. Jane, Anthony R. Green, Berthold Göttgens, David J. Izon, and C. Glenn Begley. "Genetically tagging endothelial cells in vivo: bone marrow derived cells do not contribute to tumor endothelium." *Blood* 104, issue 6 (2004): 1769-1777. doi:10.1182/blood-2003-11-3952.

This study provides evidence against the controversial issue on whether tumor endothelium is derived from bone marrow (BM)-derived cells. The researchers used a transgenic mouse model with a tamoxifen-inducible recombinase Cre-ER driven by SCL enhancers and showed that tumor endothelium does not come from BM cells. They were able to tag ECs *in vivo* by crossing these transgenic mice with Cre reporter strains through which β-galactosidase (LacZ) or enhanced yellow fluorescent protein (EYFP) were expressed upon recombination directed by Cre. They found that in new tumor endothelium Cre-mediated recombination occurred whereas in adult BM cells it occurred at a reduced amount, suggesting that tumor endothelium is not derived from BM cells.

8. Guignabert, C., C.M. Alvira, T.-P. Alastalo, H. Sawada, G. Hansmann, M. Zhao, L. Wang, N. El-Bizri, and M. Rabinovitch. "Tie2-mediated loss of peroxisome proliferator-activated receptor-γ in mice causes PDGF receptor-β-dependent pulmonary arterial muscularization." *American Journal of Physiology – Lung Cellular and Molecular Physiology* 297, no. 6 (2009): L1082-L1090. doi:10.1152/ajplung.00199.2009.

Knowing that PPAR-γ expression is reduced in patients with PAH, the researchers of this study set out to find out more about the sequence of effects of loss of PPAR-γ in PAECs. They reproduced a similar situation as in PAH patients by knocking out PPAR-γ by using PPAR-$\gamma^{flox/flox}$ transgenic mice induced by the Tie2 promoter. They placed Tie2 PPAR-$\gamma^{-/-}$ mice and WT mice under conditions of room air, hypoxia for 3 weeks, or hypoxia and a consequent period of recovery in room air for 4 weeks. The purpose of the period of recovery in room air is to observe how loss of PPAR-γ affects the ECs' ability to recover from the damage incurred by the hypoxic conditions, and the researchers found that after the 4 weeks of recovery, Tie2- PPAR-$\gamma^{-/-}$ mice had a significantly higher residual RVSP than the WT mice, suggesting an impaired ability to recover. To measure the severity of PAH in the mice, the researchers analyzed their results of RVSP, RVH, and muscularized PAs in room air, after chronic hypoxia, and after recovery for four weeks.

9. Hansmann, Georg, Vinicio A. de Jesus Perez, Tero-Pekka Alastalo, Cristina M. Alvira, Christophe Guignabert, Janine M. Bekker, Stefan Schellong, Takashi Urashima, Lingli Wang, Nicholas W. Morrell, and Marlene Rabinovitch. "An antiproliferative BMP-2/PPARγ/apoE axis in human and murine SMCs and its role in pulmonary hypertension." *The Journal of Clinical Investigation* 118, issue 5 (2008): 1846-1857. doi:10.1172/JCI32503.

This study contributes fresh knowledge to what is known about PAH at the molecular level: it reports the new findings of two potential downstream effectors of BMPR2 signaling which are PPAR-γ and apoE and of the linkage of BMP-2, PPAR-γ, and apoE. Several key results from this paper's experiments are the double negative feedback loop involving BMP-2 by way of PPAR-γ and PDGF-BB; the inhibition of PDGF-BB signaling in human PASMCs by BMP-2 and a PPAR-γ agonist called rosiglitazone by increasing PPAR-γ DNA binding; rosiglitazone prevented the PDGF-BB induced proliferation found in BMPR2 mutant cells and WT cells, showing that PPAR-γ agonists can rescue BMP-2 effects in PASMCs that have nonfunctional BMPR2; and without PPAR-γ in PASMCs PAH develops. Mice with deletion of the PPAR-γ gene via Cre were used, and the SM22α Cre promoter was used to induce the recombination.

10. Kohan, Donald E. "Progress in gene targeting: using mutant mice to study renal function and disease." *Kidney International* 74 (2008): 427-437. doi: 10.1038/ki.2008.146.

This paper discussed genetic engineering techniques and had a useful diagram explaining how the Cre-lox system works with CreER recombinase.

11. Olcina, Monica, Philip S. Lecane, and Ester M. Hammond. "Targeting Hypoxic Cells through the DNA Damage Response." *Clinical Cancer Research* 16 (2010): 5624. doi: 10.1158/1078-0432.CCR-10-0286.

Focusing on hypoxic cells in the context of cancer, the authors of this review paper discuss the relevance of the DDR pathway and the potential effectiveness of DDR inhibitors in combination with other agents in clinical treatment. The background information in this paper supports the use of hypoxia in my study as a way of inducing DNA damage when one of the DDR components is lost, for normally the DDR is activated in endothelial cells that experience moderate levels of hypoxia.

12. Rabinovitch, Marlene. "Molecular pathogenesis of pulmonary arterial hypertension." *The Journal of Clinical Investigation* 122, issue 12 (2012): 4306-4313. doi:10.1172/JCI60658.

Dr. Rabinovitch's review article extensively covers the existing knowledge of the progression of the disease PAH at the molecular level. Beginning with the defining characteristics of the pathology, the article addresses the genetic contributions to the disease involving BMPR2, as well as additional key features such as contributions of inflammation and serotonin. Interspersed throughout the paper are therapeutic suggestions for patients with the disease that are based on the evidence suggesting a convergence of pathways in the disease's pathology.

13. Stracker, Travis H. and John H. J. Petrini. "The MRE11 complex: starting from the ends." *Nature Reviews Molecular Cell Biology* 12, issue 2 (2011): 90-103. doi:10.1038/nrm3047.

This review paper covers all that is known about a DDR sensor protein called the MRN complex (or the MRE11 complex as the authors call it in the paper). Consisting of three crucial components – MRE11, RAD50, and NBS1 – the highly conserved MRN complex senses double strand breaks (DSBs) and controls the DDR pathway by regulating the activation of ATM. It regulates DSB repair through HDR (Homology Directed Repair) and NHEJ (Non-Homologous End Joining), and it has both a structural and enzymatic function in DNA repair. MRE11 has both 3'-5' exonuclease activity and ssDNA endonuclease activity, which do not require NBS1 or RAD50 but are enhanced within the complex. MRE11 mouse models were studied, showing that null mouse mutants of MRE11, RAD50, and NBS1 do not survive and that mouse mutants with homozygous nuclease dead allele of MRE11 leads to embryonic lethality.

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16. “Assessing the severity of PAH.” *PAH Info.* Accessed December 11, 2014. http://www.pah-info.com/Assessing-the-severity-of-PAH.

This website provides extensive information on pulmonary arterial hypertension. This particular site was chosen because it describes in detail how the severity of the disease is assessed and provides extensive information on the different characteristic classes. Additional information includes other parameters used to assess the severity: exercise capacity, cardiopulmonary exercise testing, hemodynamic parameters, and biochemical markers.