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An Investigation into Y Chromosome Protection and Therapeutic Potential in Pulmonary Hypertension

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An Investigation into Y Chromosome Protection and Therapeutic Potential in Pulmonary Hypertension

A dissertation submitted in partial satisfaction of the

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by

Christine Marie Cunningham

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ABSTRACT OF THE DISSERTATION

An Investigation into Y Chromosome Protection and Therapeutic Potential in Pulmonary Hypertension

by

Christine Marie Cunningham

Doctor of Philosophy in Molecular, Cellular & Integrative Physiology

University of California, Los Angeles, 2021

Professor Mansoureh Eghbali, Chair

Pulmonary arterial hypertension is a rare but terminal pulmonary vascular disease characterized by elevated pressure in the pulmonary circulation coupled with right ventricular hypertrophy and failure. This disease is up to four times more common in women—a striking sex difference that cannot be explained by the contributions of sex hormones alone. This dissertation is a compilation of the first work investigating the sex biasing contributions of sex chromosomes in pulmonary arterial hypertension.

In **Chapter 2**, we use powerful mouse models to identify the male-specific Y chromosome confers protection against the development of experimental pulmonary hypertension. In **Chapter 3**, we use a combination of experimental models and bioinformatic approaches to identify the gene on the Y chromosome that protects males against the development of pulmonary hypertension and its downstream effector genes. We found the Y

chromosome gene Uty protects against the development of pulmonary hypertension by attenuating a proinflammatory lung response. Loss or lack of Uty gene expression results in an elevated inflammatory response that contributes detrimentally to disease severity. We identified two chemokines, Cxcl9 and Cxcl10, that are downstream of and inversely related to Uty as particularly harmful in pulmonary hypertension. We show that blocking their activity is sufficient to extend Y chromosome protection to females in a novel, preclinical therapy. In **Chapter 4**, we expand on our findings of downstream Uty effector genes and identify a sex-specific upregulation of Endothelin-2 that is linked to loss of Uty expression and elevated Cxcl9 and Cxcl10 levels. While the role of Endothelin-1 is known in pulmonary hypertension, the role of Endothelin-2, a proinflammatory and vasoconstrictive peptide, has not yet been studied. Our findings regarding Endothelin-2 help explain a clinical sex difference in the efficacy of endothelin receptor antagonist therapies for pulmonary hypertension where females are more responsive to treatment.

Taken together, this dissertation offers evidence of a powerful sex-biasing Y chromosome effect in pulmonary hypertension, elucidates novel therapeutic approaches for the treatment of this terminal disease, and serves as an example of the translational relevance in investigating sex differences in disease. While the studies presented provide the first insight into the role of sex chromosomes in pulmonary hypertension, they also provide a foundation for further examination which are highlighted here as future directions.

The dissertation of Christine Marie Cunningham is approved.

Arthur P. Arnold

Thomas Vondriska

Xia Yang

Mansoureh Eghbali, Committee Chair

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2021

This dissertation is dedicated to my family whose love and support are unconditional and unwavering.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

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This dissertation highlights some of the scientific findings I've been lucky enough to have helped discover throughout my graduate studies; however, it does not encompass the self-discovery that has happened parallel to my scientific endeavors. The pursuit of a PhD is a tremendously humbling experience, but is also one of self-discovery and maturity as well. Throughout these six years, I've overcome many of my fears, insecurities, and self-doubts. I've learned that in order to be able to trust in the scientific process, you must trust yourself. While the data I show in the subsequent pages represent my passion for and impact on science, I am most proud of the intrapersonal achievements I've made. The confidence, tenacity, and independence I've gained through this process are the most important skills that I take with me to ensure successful future scientific endeavors. It's through applying these skills that I will be able to continue scientific pursuits and, with some luck, contribute to meaningful discoveries. While some months and years were more productive than others, I know now that no time is wasted as long as there is personal growth.

I also know that these personal and professional triumphs were the direct result of overcoming challenges, and that I could not have overcome the many challenges I faced throughout my PhD alone. For that, I would like to acknowledge all of those who have supported me during this time.

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CHAPTER 1: Introduction

This chapter includes a portion of text adapted from *Cunningham CM, Eghbali M. An Introduction to Epigenetics in Cardiovascular Development, Disease, and Sexualization. Adv Exp Med Biol 2018;1065:31–47.*

1. Pulmonary circulation

The mammalian heart is composed of four chambers: two atria which act as reservoirs to receive blood entering the heart, and two ventricles which act as muscularized pumps to distribute blood out from the heart (**Figure 1**). The ventricles, which are separated longitudinally by an intraventricular septum (IVS), largely vary in their size and appearance due to the physiological demands imposed by the body. While the larger, muscularized left ventricle (LV) pumps oxygenated blood throughout the body via the high pressured systemic circulatory system, the much smaller, thin-walled right ventricle (RV) pumps deoxygenated blood throughout the lungs via the low pressured pulmonary circulatory system to allow for gas exchange to occur. Typically, the RV fills and contracts without significant dilation or impact on the overall pressure of the circulatory system as to not impede LV filling or function (1–3). To accomplish this, the RV maintains its shape and pressure while adapting to dynamic changes in the blood volume it receives from the vena cava. Since venous return is largely dependent on the metabolic demands of the body, the volume of blood the RV receives and overall cardiac output can change rapidly as a response to stress and exercise (4). The RV is able to comply with rapid changes in venous return and increased cardiac output without fluctuations in pressure pulmonary artery (PA) pressure due to adequate vascular compliance and pulmonary vascular resistance (PVR) (4, 5). Vascular compliance refers to the ability of an artery, in this case the PA, to expand and constrict to compensate for the change in the pressure and volume of blood. PVR, a measure of RV afterload, refers to the pressure gradient across the lungs as determined by the quantity and elasticity of the lung vasculature (1). In healthy circulation, pulmonary vascular compliance is relatively high while PVR is low allowing for

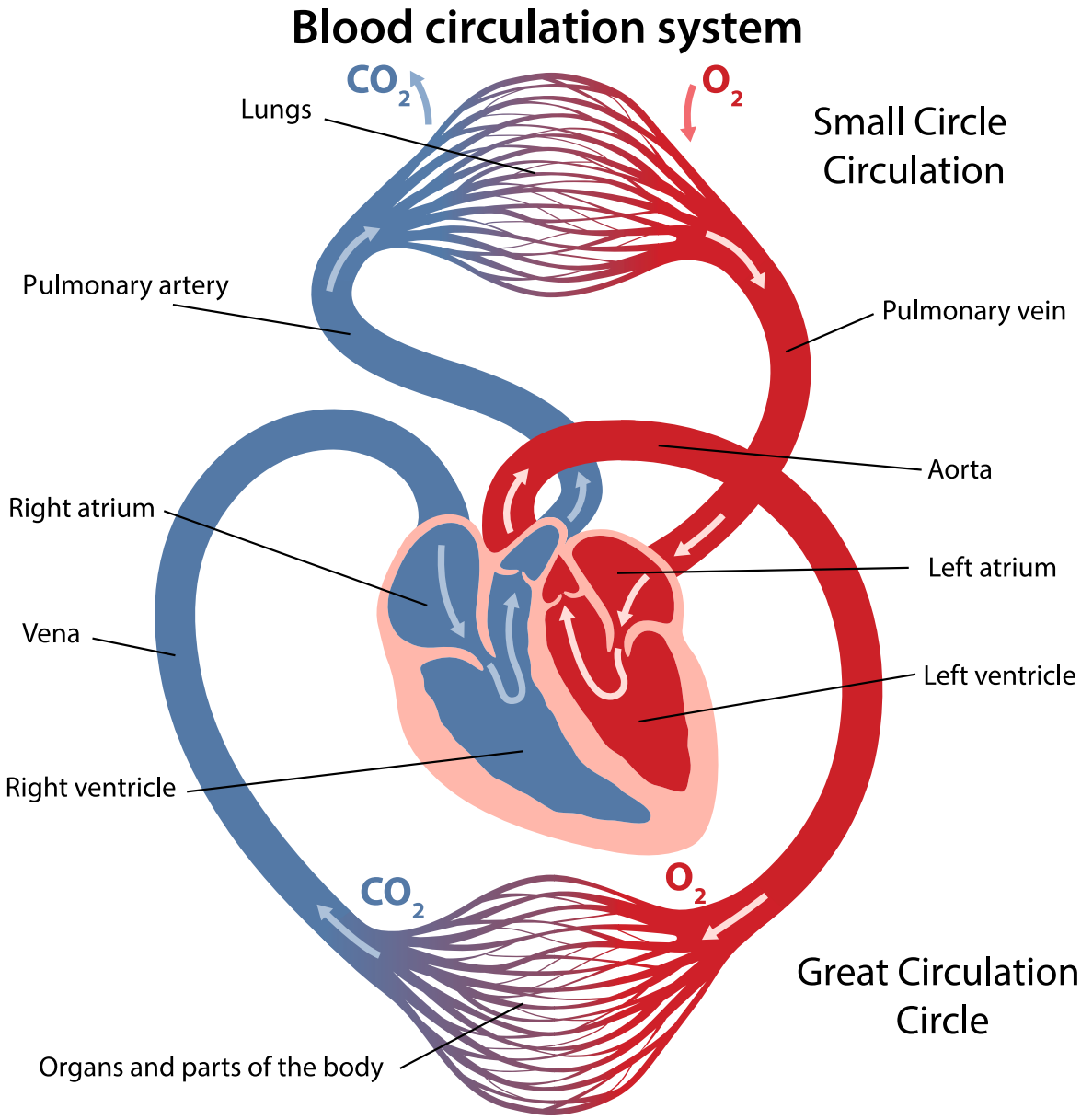

Figure 1. Schematic of pulmonary (small circulation) and systemic (great circulation) in the mammalian heart. *Reprint from Shutterstock.*

low pressure gas exchange to occur despite fluctuations in blood volume due to metabolic demands.

Changes in pulmonary vascular compliance and PVR can result in increased PA pressure (PAP). Since the thin-walled RV is optimized for a low-pressure system, chronic increase in PA pressure leads to persistent increased RV afterload which can quickly progress to RV failure (5).

2. Pulmonary hypertension

Overview and prevalence

PH is a rare but terminal disease that is increasing in prevalence worldwide (6). PH refers to an overarching classification of cardio-pulmonary diseases characterized by an increase in mean PAP (mPAP) above 20 mmHg at rest (7). Chronic increase in mPAP, as seen in patients with PH, results in sustained increased RV pressure which leads to compensatory RV hypertrophy (**Figure 2**). Rather quickly, the RV is unable to maintain increased pressures and decompensatory heart failure ensues resulting in RV dilation and failure. The diagnosis of PH includes right ventricular catheterization to measure pressure, although parameters of PH can also be estimated via noninvasive echocardiography.

The classification of pulmonary hypertension is split into five groups based on shared pathophysiology, hemodynamics, and treatment strategies. Group 1 PH includes pulmonary arterial hypertension (PAH), Group 2 includes PH due to left heart disease, Group 3 includes PH due to lung disease and/or hypoxia, Group 4 includes PH due to

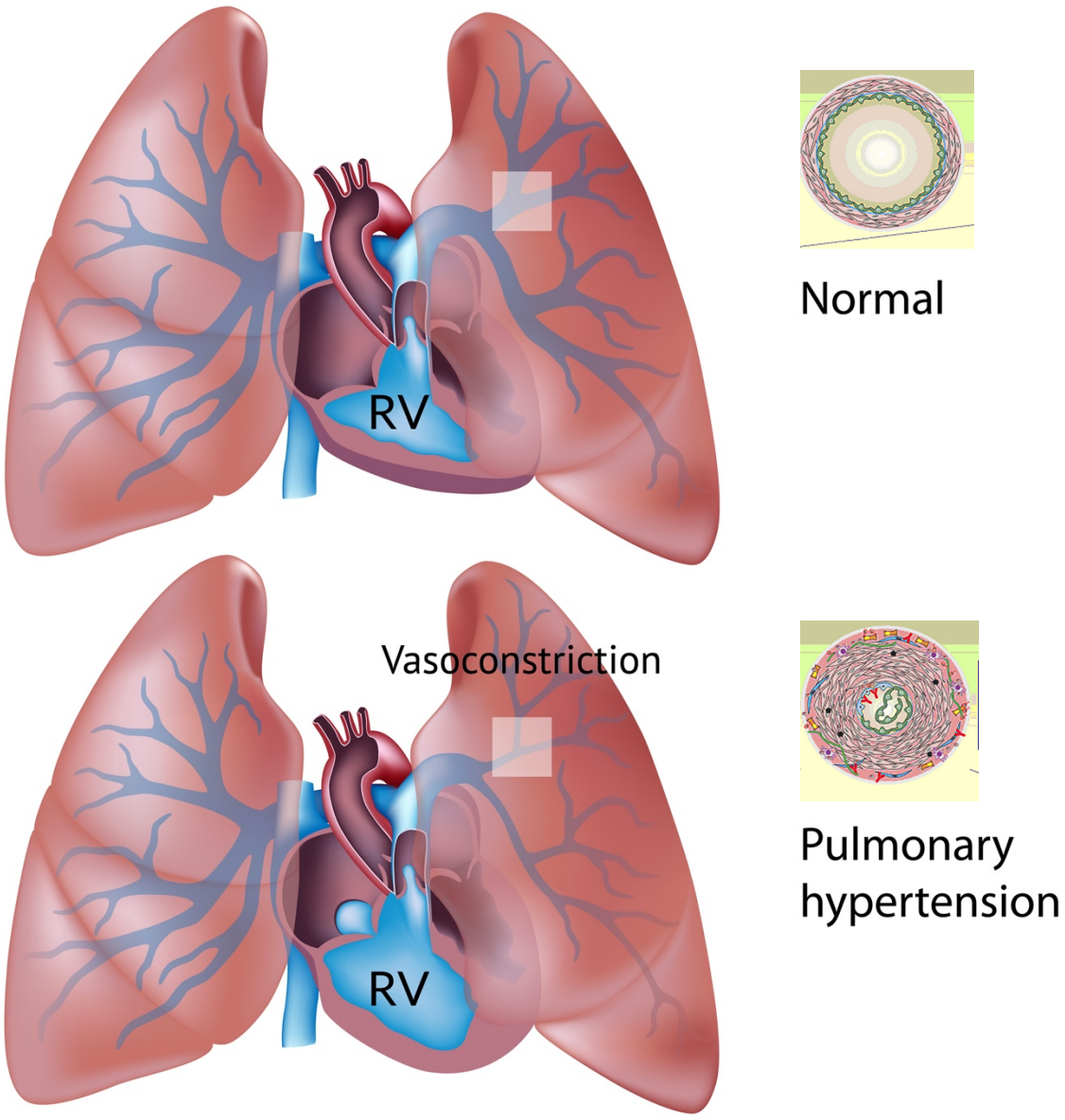

Figure 2. Overview of normal pulmonary circulation compared to pulmonary hypertension. Pulmonary hypertension is characterized by aberrant vasoconstriction of the pulmonary arterioles and increased pressure, hypertrophy, and failure of the right ventricle (RV). *Adapted from Shutterstock.*

pulmonary artery obstructions, and Group 5 includes PH due to unclear or multifactorial problems (7). The main focus of this dissertation is PAH which is classified as Group 1 PH.

Pulmonary arterial hypertension

PAH is a pre-capillary form of PH hemodynamically characterized by an increase in mPAP > 20 mm Hg without elevation in a PA wedge pressure (PAWP), indicating high mPAP in the absence of elevated left atrial pressures (8). Thus, the increased mPAP indicative of PAH is primarily due to increased PVR within the lungs. Increased PVR associated with PAH is caused by vascular abnormalities mainly in the medial and distal arteries in the lungs (9). The source of these vascular abnormalities further subdivides PAH into groups based on pathogenesis. These groups include primary forms of PAH (idiopathic, heritable, drug/toxin induced PAH) as well as secondary forms of PAH (7).

Unfortunately, there are between 500-1000 new cases of PAH diagnosed annually, and the median survival time is five to seven years following diagnosis (10, 11). Taken together, it is clear that investigation into the pathogenesis and treatment of PAH is of utmost importance.

Histological and molecular signatures

PAH is histologically characterized by aberrant vascular remodeling within the pulmonary arterioles. Types of vascular remodeling found in PAH patients varies and can include medial hypertrophy, neointimal formation, complex lesions, and loss or muscularization of distal arterioles (9). These histological processes result in stiffening, occlusion, and loss of pulmonary vasculature which contribute to the increased PVR and mPAP

indicative of PAH (**Figure 2**). The pathogenic vascular changes in PAH are triggered by a multitude of molecular processes that include, but are not limited to, vascular cell dysfunction and inflammation (**Figure 3**).

While many cell types contribute to the healthy homeostasis of the pulmonary vasculature, the most common vascular cell types implicated in PAH include smooth muscle cells (SMCs) and endothelial cells (ECs). SMCs are muscularized cells found in the medial layer of arteries which provide vascular integrity and ability to modulate the diameter of vessels through vasoconstriction and vasodilation (**Figure 2**). SMCs exhibit excessive proliferation and hypertrophy resulting in thickening and stiffening of medial pulmonary arteries which are normally relatively thin-walled and compliant (9). Dysfunctional and hyperproliferative SMCs also migrate toward the intimal layer of the vessels in a process called neointimal formation which contributes to the narrowing and occlusion found in PAH (9). Dysfunctional ECs, which comprise the innermost intimal later within vessels, can also contribute to the aberrant muscularization and thickening within the distal pulmonary arterioles (9, 12). While distal arterioles normally lack a muscular SMC layer, dysfunctional ECs in PAH patients transition to a contractile SMC phenotype in a process called endothelial-to-mesenchymal transition which, in addition to EC swelling, contribute to narrowing of vessels (13, 14). Dysfunctional ECs found in PAH patient lung vasculature also contribute to disease severity through plexiform lesion formation as well as impaired angiogenesis and altered production of vasoconstrictive agents (**Figure 2**). Diseased ECs initially exhibit increased apoptosis which contributes to a reduced density of distal pulmonary arterioles within patients' lung and an impaired angiogenic response to inefficient oxygen exchange. However, it is known that ECs that

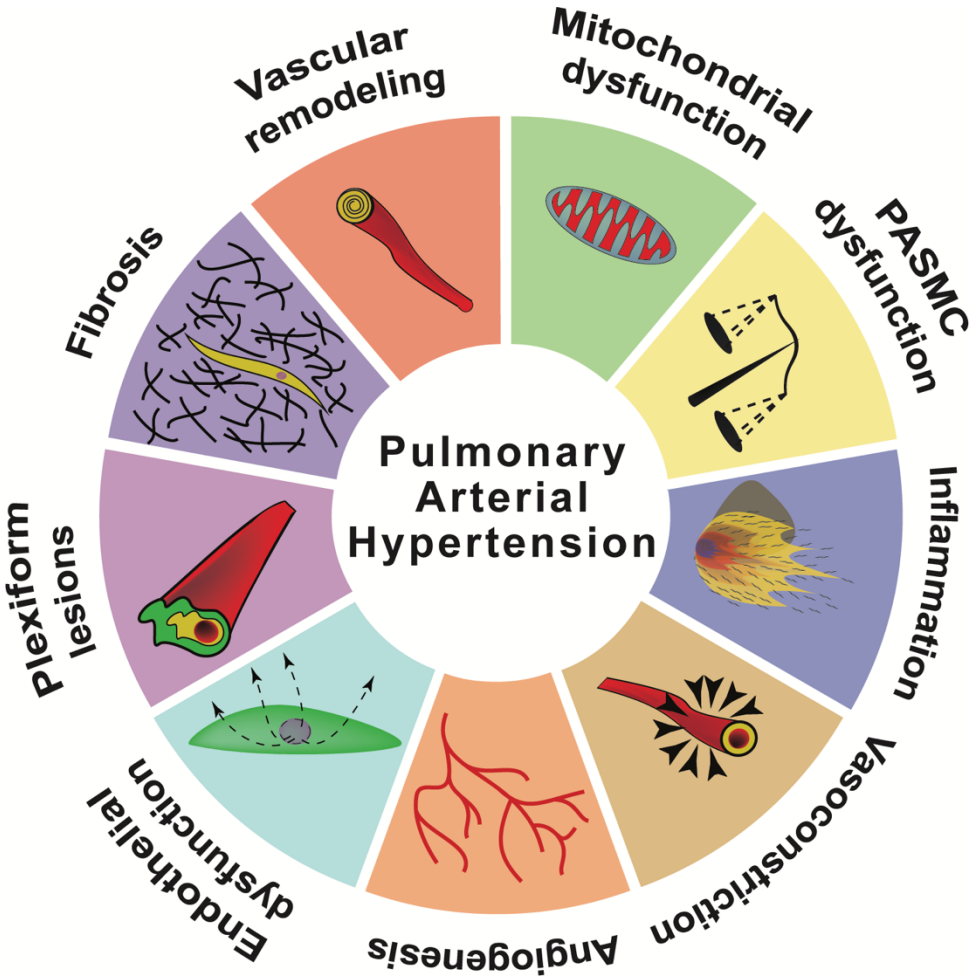

Figure 3: Overview of the various pathological processes known to be associated with PAH development. *Adapted from Sharma, et al., Pulm Circ. 2016* (15).

resist apoptosis in response to PAH are dysfunctional which results in increased expression of the potent vasoconstrictive peptide Endothelin-1 (ET-1) and the formation of plexiform lesions which are complex channels within the vascular lumen that impede blood flow (9, 12, 16–18).

Taken together, the hyperproliferative state of SMCs and muscularization of ECs in contributes to increased PVR and increased PAP in PAH patients' lungs through occlusion and stiffening of the medial and distal pulmonary vasculature. This is further compounded by increased expression of vasoconstrictive agents, such as ET-1, which are released by injured ECs. EC apoptosis and dysfunction also contribute to an overall loss of vascular density within the lungs of PAH patients and turbulent and disordered flow through the vasculature.

PAH patients also exhibit dysregulation of inflammatory pathways, and lung inflammation is now thought to play a causal role in PAH development (19). Complex plexiform lesions, which are highly disorganized and remodeled vessels within PAH patient lungs, exhibit perivascular inflammation and increased infiltrate of immune cells particularly lymphocytes and macrophages (19–21). Regulatory T-lymphocytes and $Cd68^{+}$ macrophages are known to be necessary for maintenance of pulmonary vascular homeostasis, and alterations in the function or balance between these cells has been linked to aberrant vascular remodeling seen in PAH (20). Further studies report that degree of perivascular inflammation is positively correlated with vascular wall thickening and elevated RV pressure indicating a causal role of increased inflammation in PAH development (19). Experimentally, studies confirm that inflammation is a driving force for PAH development since aberrant inflammation triggers vascular remodeling and elevated

RV pressure in various animal models of PH—particularly in the monocrotaline (MCT)-induced and oxidized lipid-induced models of PH (20). In the MCT model, MCT toxin is injected into rats which causes endothelial cell injury followed by a large infiltration of immune cells to the injured vasculature (22). MCT treated animals develop severe PH within 4 weeks of exposure to MCT toxin. Likewise, multiple reports demonstrate a causal role of increased oxidized lipid exposure, which triggers elevated lung inflammation, in PH development in rodent models (23, 24).

Because PAH patients exhibit elevated lung inflammation, components of proinflammatory signaling pathways have been linked to PAH and identified as biomarkers for PAH development. Signaling peptides, called chemokines, or chemotactic cytokines, play a large role in cell migration and function (25, 26). This includes recruiting inflammatory cells to the site of injured vasculature through forming a chemokine gradient by adhering to the vascular endothelium. Chemokines are secreted by various inflammatory cells in the lung as well as vascular cell types. They act by eliciting a proinflammatory response and/or alter cellular function through biding to their respective receptor. Circulating levels of chemokines have been associated with disease severity and serve as biomarkers and prognostics for PAH. Furthermore, dysregulation of chemokines and their receptors within the lungs have been associated with PAH development (26).

Inflammation, vascular injury, and vascular remodeling are all histological and molecular signatures of PAH that are intimately connected and perpetuated by one another. While current PAH therapies target symptoms of disease, the future direction of PAH

therapeutics lies in understanding and targeting these underlying factors that contribute to PAH pathogenesis.

Therapeutic strategies

While there is no cure for PAH, there are a handful of drugs regularly prescribed to PAH patients that can improve patient quality of life, hemodynamics, and functional outcome. The majority of therapies used primarily target vasoconstriction through the endothelin, nitric oxide (NO), and prostacyclin pathways (27, 28). Targeting these pathways in combination is commonplace and has been shown to have an additive benefit compared to treatment with a single therapy (29).

Endothelin receptor antagonists (ERAs), blocking one or both endothelin receptor, are often prescribed to block the vasoconstrictive and proliferative effects of ET-1. ET-1, which is upregulated by injured ECs and known to be elevated in PAH patient lungs, promotes contraction and proliferation of vascular SMCs resulting in increased PVR (30). ERAs effectively increase exercise capacity, pulmonary hemodynamics, symptoms, and right-ventricular function (31). They have also been found to increase overall patient survival in cases of advanced idiopathic PAH (32). Interestingly, treatment with ERAs exhibit a marked sex difference which is described further below (33).

Conversely, NO is a powerful vasodilator secreted by the lung endothelium that also inhibits SMC proliferation (34, 35). Inhaled NO is used as a therapy for PH patients, particularly in pediatric cases; however, there are substantial concerns regarding cost, feasibility, and efficacy of this treatment (36). Since NO imparts its vasodilatory effects though cyclic guanylate monophosphate (cGMP), therapeutics that inhibit the

degradation of cGMP by phosphodiesterases (PDEs) are commonly prescribed to enhance and prolong the effects of NO in the lung (36). PDE inhibitors, which are mainstay treatment for PAH patients, have been shown to significantly improve the functional capacity and hemodynamics in PAH patients (37, 38). Compounds that stimulate and sensitize NO pathway activation though guanylate-cyclase are used as a therapeutic as well (39).

Finally, the most effective form of PAH treatment currently available include prostacyclin (PGI_2) analogs. PGI_2 is another vasodilator that is known to exhibit reduced expression in PAH lungs. In addition to vasodilation, PGI_2 analogs have been shown to inhibit platelet aggregation and reduce EC dysfunction in the lungs of PAH patients (40). Continual infusion of a PGI_2 analog has been shown to reduce symptoms, increase exercise tolerance, and has the most significant effect on enhancing overall PAH patient survival (41, 42).

Despite the variety of commercial drugs available to target the above pathways, treatment efficacy remains suboptimal and patient survival remains poor at around 60% at five years (43). While these available treatments target vasoconstriction in the lungs of PAH patients, no current PAH therapies target the underlying cellular dysfunction and inflammation that are known to be central to PAH pathogenesis. This dissertation proposes a novel therapeutic strategy that targets proinflammatory chemokines upregulated in PAH patient lungs in a sex-specific manner.

3. Sex differences in PAH

Overview

Striking and complicated sex differences exist within the prevalence, progression, and prognosis of PAH. According to a composite of national and international PAH registries between 1981 and 2013, females are between two to four times more likely to be diagnosed with PAH (44–48). This marked female predominance holds true for all subtypes of PAH with the exception of HIV-associated and portopulmonary PH, which are both more common in males (49). Paradoxically, while females are more likely to be diagnosed with PAH, they are more responsive to therapies and have a better overall prognosis than their male counterparts (50). On average, females with PAH have a lower mPAP and increased survival compared to their male counterparts (50). Additionally, they exhibit better functional improvement with treated with ERA therapies as indicated by a higher 6-minute walk score (33). Taken together, this indicates that the underlying basis for the sex differences in PAH are complex and likely a result of multiple sex-biasing factors including sex hormones and sex chromosomes.

Sex Hormones

Decades of research have been devoted to uncovering the sex disparity in PAH with an emphasis on the role of sex hormones—namely estrogens. While the role of estrogens has been well studied in the context of PAH pathogenesis, the data surrounding the effects of estrogens in PAH are paradoxical and controversial. Since estrogen is the main circulating sex hormone in premenopausal females, it was initially hypothesized that estrogen and its metabolites would have a largely deleterious effect in PAH. Surprisingly, *in vivo* studies found estrogens to be protective against PAH since they were shown to

prevent and rescue disease development in animal models of PH, particularly through binding estrogen receptor β (51–54). On a molecular basis, estrogens have been shown to protect against PH through mediating RV compensation to pressure overload (55, 56). Since RV compensation and function is a marker for survival, perhaps it is the protective effects of estrogens in the RV that accounts for the better prognosis in female patients.

Conversely, estrogens have also been shown to predispose females to PAH development through their interaction with bone morphogenic protein 2 (BMPR2). BMPR2 is a known heritable loss-of-function mutation found in PAH patients, and reduced expression of BMPR2 is associated with PAH in non-heritable forms of PAH as well (57). Estrogens were found to reduce expression of BMPR2 (58, 59). Regardless, the penetrance of the heritable BMPR2 mutation is only around 20%, indicating that the development of PAH may require a “two-hit” phenomenon where it takes a combination of multiple pathogenic stimuli to trigger the onset of disease. It seems one of these pathogenic factors may even include female sex, since BMPR2 mutant carrying females have a significantly higher risk of developing PAH (43%) compared to BMPR2 mutant carrying males (14%) (60, 61). Within BMPR2 mutant carrying females, levels of estrogen and its metabolites are significant predictors of increased risk of PAH development (62).

Other sex hormones have also been studied in the context of PAH, but none to the degree of estrogens. Briefly, the female-specific gonadal hormones progesterone and follicle-stimulating hormone were linked to PAH severity in reproductive-aged women. High levels of follicle-stimulating hormone and low levels of progesterone were associated with increased PAH severity (63). Testosterone, the main androgenic steroid hormones which is typically elevated in males compared to females, is known to be associated with

cardiovascular disease. Studies show testosterone contributes to worse RV hypertrophy and fibrosis following pressure overload in mice (64). Since RV function is the main indicator of PAH patient survival (65), elevated testosterone levels in male compared to female patients could contribute to the worse prognosis of male PAH patients.

The PAH field is saturated with studies examining sex biasing hormones, their metabolites, and receptors. Taken together, these data indicate that sex hormones undoubtably influence PAH development and prognosis and play a role in the profound sex differences associated with PAH. However, our understanding of sex hormones remains paradoxical—indicating other sex biasing factors are at play.

Sex Chromosomes

Sex hormones are not the only sex basing factor that contribute to disease susceptibility and severity. Males and females differ in their sex chromosomes, and genes encoded by the sex chromosomes have been linked to sex-specific differences and disease. Outlined in this dissertation are the first studies to investigate sex chromosome effects in the context of PAH.

1. Overview

Mammalian sex is genetically determined by the combination of two sex chromosomes: X and Y. Typically, each female cell contains two X-chromosomes (XX) while each male cell contains one X-chromosome and one Y-chromosome (XY). While the Y-chromosome encodes for the sex determining *Sry* gene, it is much less rich in genomic data when compared to the X-chromosome or autosomes. To reduce the genetic density in female

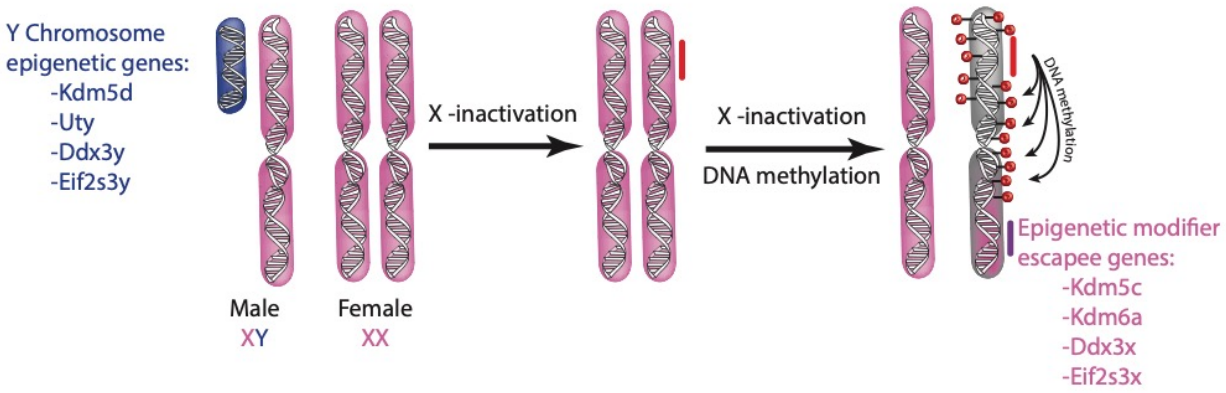

Figure 4. The genetics behind sex chromosome biasing effects. The X chromosome *(pink)* and Y chromosome *(blue)* encode genes *(listed)* that are expressed in the heart and lungs which exhibit sex-specific expression patterns. Y chromosome genes are exclusive to males (XY) and are absent in female (XX) tissue. Incomplete X-inactivation of the second chromosome in females, through DNA hypermethylation and chromosome condensation, results in a subset of genes that that escape inactivation and are expressed at higher levels in females than males. *Adapted from Cunningham, Eghbali, Adv Exp Med Bio. 2018* (66).

cells to match that of males, one X-chromosome in females becomes largely inactivated in a finely-tuned process orchestrated by a series of epigenetic modifiers (**Figure 4**). The condensed, X-inactivated state of one X chromosome is maintained throughout all subsequent cell divisions in females (67).

2. X-chromosome effects

While X-inactivation largely silences one X-chromosome in female cells to match the X-chromosome gene expression levels in male cells, this inactivation does not include all genes. In fact, it is thought that between 12-20% of the protein coding genes on the inactivated X-chromosome are expressed (68). These genes, which escape X-inactivation, are commonly referred to as X-escapee genes.

While it is not entirely clear why certain X-chromosome genes escape activation, studies have shown that X-chromosome dosage (one X-chromosome versus two) imparts sex differences in disease progression and severity. Most notably, the **Four Core Genotype** (FCG) mouse model allows researchers to study sex differences due to sex chromosomes independent of gonads or gonadal hormones (69). In the FCG mouse model, the testis-determining *Sry* gene is removed from the Y-chromosome and expressed by an autosome. The autosomal *Sry* gene segregates independently from the Y-chromosome allowing for the generation of gonadal males and females with both XX and XY genotypes. Thus, mice with four genotypes are created: XY males, XX males, XY females and XX females (**Figure 5A**) (69).

Studies using FCG mice reveal that sex chromosomes are implicated in various pathologies that exhibit sex differences, including cardiovascular diseases (70–72). The

majority of these studies demonstrate that having one X-chromosome is better than having two (70–73). In the context of ischemia/reperfusion heart injury, our lab found that gonadectomized male and female mice with two X-chromosomes have a larger area of infarct, lower hemodynamic functional recovery post reperfusion, and greater mitochondrial dysfunction than male and female mice with one X chromosome (72).

X-escapee genes likely play a role in these X-chromosome dosage-dependent sex differences. Genes that have been regularly demonstrated to escape X-inactivation in various tissues, including the heart and lung, include: *Kdm5c, Kdm6a, Ddx3x* and *Eif2s3x* (73). Interestingly, these genes affect widespread gene transcription through direct or indirect epigenetic mechanisms as *Kdm5c* and *Kdm6a* are histone demethylases, *Ddx3y* is an RNA helicase involved in RNA splicing, transcription and translation, and *Eif2s3x* is a translation initiation factor. All of these genes are capable of driving changes to normal homeostasis and have been implicated in a disease or developmental process (74–78).

Protein coding genes are not the only possible source of X-chromosome determined sex differences. In fact, the X-chromosome is densely packed with microRNAs (miRNAs) and contains approximately double the number of miRNAs found on autosomes in both mice and humans (79). In contrast, the Y-chromosome is not enriched with miRNAs and encodes little to no miRNAs (80). The degree to which X-chromosome linked miRNAs escape X-inactivation continues to be investigated, although it is known that X-chromosome encoded miRNAs undergo mutation much more rapidly than autosomal miRNAs. These X-encoded miRNAs could be a source of sex differences in tissues and diseases (79, 81).

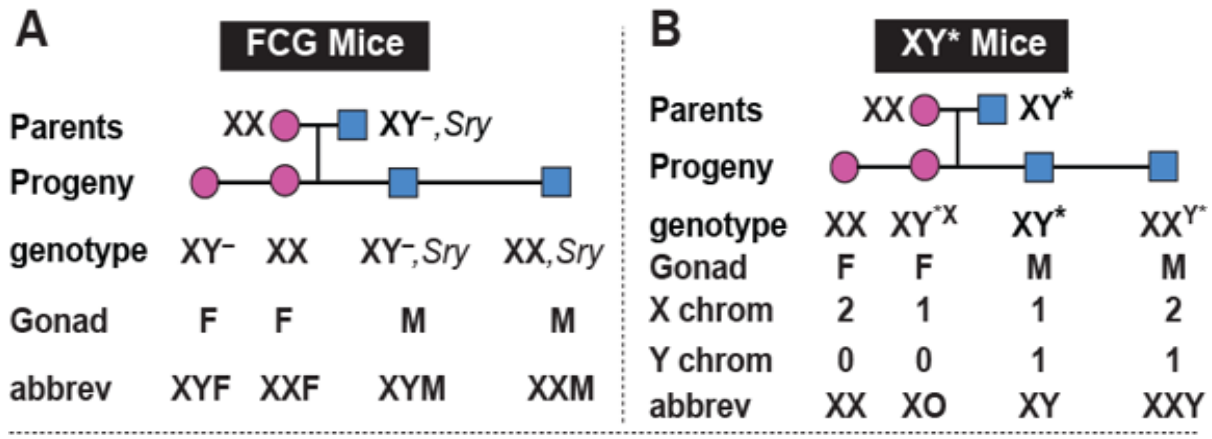

Figure 5. An overview of the FCG and XY* mouse models. A. The Four Core Genotypes (FCG) mouse model produces gonadal males and females with both XX and XY genotypes to test the effects of sex chromosomes and gonadal sex hormones separately. B. The XY* mouse model produces near-equivalent XX and XO gonadal females and XY and XXY gonadal males to compare the effect of the number of X-chromosomes or the presence of a Y-chromosome. *Adapted from Li et al., Cardiovasc Res. 2014* (72).

3. Y-chromosome effects

In the FCG mouse model, the number of X-chromosomes is not the sole sex chromosome effect that could influence disease risk and/or pathogenesis. In fact, the differences identified between XX and XY mice of both sexes could be due to the number of X-chromosomes or the presence of a Y-chromosome. As mentioned earlier, the Y-chromosome is much less dense than autosomes and its X-chromosome counterpart. Unlike the X-chromosome, the Y-chromosome is not necessary for proper development; however, while it contains the genes necessary for proper male gonad formation and spermatogenesis, it also retained a subset of ancestral genes during its rapid evolution from an autosome beginning millions of years ago (82). These ancestral genes, expressed only on the Y-chromosome, provide another source of sex difference between males who have a Y-chromosome and females who do not.

A handful of Y-chromosome genes are known to be expressed in heart and lung tissue (83). While difficult to examine in human populations, Y-chromosome effects in cardiovascular and cardiopulmonary diseases have been documented. Single nucleotide polymorphisms within the Y-chromosome may be associated with increased risk of cardiovascular disease in men. A large study found British men inheriting the Y-chromosome haplogroup I had a 50% greater risk of developing coronary artery disease (CAD) than British men with other haplogroups (84). Interestingly, a separate study found that macrophages with Y-chromosome haplogroup I had decreased expression of the Y-chromosome genes *UTY* and *PRKY* (85). As macrophages play an integral role in the development of CAD and atherosclerosis, these Y-chromosome genes could be play a role in attenuating inflammation in response to CAD (86). Notably, the studies included in

this dissertation also identified loss of *Uty* expression was associated with elevated inflammation and increased disease severity in PH.

To investigate the role of the Y-chromosome in disease experimentally, the FCG mouse model can be used in combination with the **XY* mouse model**. While the FCG mouse model allows researchers to test the effects of sex chromosomes independent from gonadal sex (**Figure 5A**), the XY* mouse model tests whether the sex differences found are due to the number of X-chromosomes or the presence of a Y-chromosome. The XY* mouse model produces near-equivalent XX and XO gonadal females and XY and XXY gonadal males (**Figure 5B**). Comparing mice with one X-chromosome (XO, XY) to those with two X-chromosomes (XX, XXY) reveals an effect of X-chromosome number, whereas comparing mice with a Y-chromosome (XY, XXY) to those without a Y-chromosome (XO, XX) shows the effect of the presence/absence of the Y-chromosome.

4. Summary

This dissertation is a collection of the first studies investigating sex chromosome effects in PAH, a pulmonary vascular disease that affects the RV and is up to four times more common in females. In **Chapter 2**, we used the FCG and XY* mouse models to investigate the role of the X and Y chromosomes in the absence of gonadal hormones. In this study, we found that the presence of a Y-chromosome was protective against the development of experimental PH. Gonadal male and female mice with a Y-chromosome exhibited lower RV pressure and less severe vascular remodeling due to experimental PH, regardless of the number of X-chromosomes (83). We hypothesized that genes

encoded by the Y-chromosome that are expressed in the heart and lung, *Kdm5d*, *Uty*, *Ddx3y* and *Eif2s3y*, confer protection against the development of PH, and we tested each of these Y-chromosome candidate genes in the context of PH in **Chapter 3**. We found that *Uty* is the sole Y chromosome gene responsible for conferring protection against experimental PH. Loss of *Uty* expression in macrophages resulted in an increase in proinflammatory chemokines Cxcl9 and Cxcl10 which resulted in endothelial dysfunction and more severe PH. Blocking the activity of Cxcl9 and Cxcl10 was sufficient to rescue PH development in female rats indicating that targeting autosomal genes downstream of *Uty* can extend Y chromosome protection against PH to females. In **Chapter 4** we examined *Endothelin-2* (ET-2), another autosomal gene downstream of *Uty* that may contribute to PH development and explain the sex differences observed in patient response to ERAs where females are more responsive to treatment. We found ET-2 was upregulated in the lungs of *Uty* knockdown mice with PH as well as in female patients with PAH but not in the lungs of male PAH patients. ET-2 is a lesser-known member of the endothelin family of potent vasoconstrictive peptides. While ET-1 is known to be upregulated in the lungs of PAH patients, we were the first to identify the sex-specific upregulation of ET-2 in females. Future experiments based on this study will further investigate the role of ET-2 in the development and treatment of PAH.

In summary, the experiments that included in this dissertation make up the first comprehensive investigation into sex chromosome effects in PH. The following chapters **1)** elucidate the protective effect of the male-specific Y chromosome in PH, **2)** uncover the molecular mechanism by which the Y chromosome exerts its protective effect in males, **3)** highlight the involvement of proinflammatory chemokines in PAH pathogenesis,

4) present a novel preclinical treatment strategy using a small molecular inhibitor already approved by the Food and Drug Administration, and **5)** provide a molecular explanation to the sex-specific disparity in response to ERA therapies for the treatment of PH. Together, this work exemplifies how thoroughly investigating sex as a biological variable can lead to novel insight into disease pathogenesis and shed light on innovative treatment strategies.

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Chapter 2: The Y Chromosome Plays a Protective Role in Experimental Hypoxic Pulmonary Hypertension

Umar S, Cunningham CM, Itoh Y, Moazeni S, Vaillancourt M, Sarji S, Centala A, Arnold AP, Eghbali M. The Y Chromosome Plays a Protective Role in Experimental Hypoxic Pulmonary Hypertension. Am J Respir Crit Care Med. 2018 Apr 1;197(7):952-955.

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Abstract

Rationale

The incidence of pulmonary arterial hypertension (PAH) is markedly higher in female patients than in males. Research investigating the role of sex hormones in PAH has yielded paradoxical results indicating that other sex biasing factors could be involved.

Objectives

We investigated the role of sex chromosomes, independent of gonadal hormones, in the development of hypoxia-induced pulmonary hypertension.

Methods

We used two powerful mouse models to study the role of sex chromosomes in susceptibility to pulmonary hypertension. The Four Core Genotypes model allowed us to compare the effect of chromosome complement (XX or XY). The XY* model allowed us to determine if differences are due to the presence of a Y chromosome or the number of X chromosomes. Mice were gonadectomized to remove gonadal hormones and placed in hypoxia (10% oxygen) for three weeks. Hemodynamic parameters were measured terminally via direct cardiac catheterization. Pulmonary vascular remodeling and fibrosis were quantified.

Measurements and Main Results

Hypoxic XY mice, irrespective of gonadal sex, developed less severe pulmonary hypertension than XX mice indicated by lower right ventricular systolic pressure (RVSP) and less vascular remodeling and fibrosis than XX mice. Protection of XY mice was due

to the presence of a Y chromosome but not the number of X chromosomes. Analysis of online microarray data revealed three Y chromosome genes are expressed in both mouse and human heart and lung tissue.

Conclusions

The Y chromosome is protective against development of hypoxia-induced PH in mice in the absence of gonadal hormones.

Introduction

Pulmonary arterial hypertension (PAH) is a chronic pulmonary vascular disease characterized by elevated pulmonary arterial pressure and vascular remodeling leading to right ventricular failure (1, 2). Patients with PAH exhibit narrowing of the main pulmonary artery with constriction and stiffening of the distal pulmonary arterioles primarily due to aberrant smooth muscle cell proliferation and muscularization, vascular endothelial cell dysfunction, and intimal and adventitial fibrosis (1–3). PAH in general, and its idiopathic form in particular, has long been considered to be a disease of young women (4, 5) with female sex being a risk factor (6). Surprisingly, being female was found to protect against the development of PH in various animal models (7–9). Because this effect has largely been attributed to the protective effects of estradiol (7, 10–13), the phenomenon is known as the ‘estrogen paradox’ of PH (14, 15). One resolution of the paradox is that sex hormones alone are not sufficient to explain the marked sex bias observed in PAH in humans.

Sex differences between males and females are not solely attributed to the effects of gonadal hormones. Sex chromosomes are inherently different between males and females from the time of zygote formation. This determination of sex by chromosome complement precedes the formation of the gonads. Together, sex chromosomes and sex hormones act directly on tissues to produce sex differences (14, 16, 17). Mouse studies in which chromosome complement, the number and type of sex chromosomes, is independent of gonadal sex indicate that sex chromosome complement influences sex bias in autoimmune disease, metabolic dysfunction, neurodegeneration, and ischemia/reperfusion injury in the heart (18–21). Therefore, genes encoded on the sex

chromosomes have already been shown to have strong effects contributing to sex differences in phenotype including disease susceptibility and progression.

In the current study, we investigate for the first time the role of sex chromosomes, in the absence of gonadal hormones, in the development of hypoxia-induced PH using the powerful FCG and XY* mouse models. In addition, we identify Y chromosome genes expressed the in male heart and lung tissue and compare Y chromosome gene expression levels between male PAH patients versus healthy control males.

Materials and methods

Mouse models

We used two powerful mouse models, the four core genotypes (FCG) and XY* models, to study the role of sex chromosomes in the susceptibility to PH (22) (**Figure 1**). The FCG model produces mice in which sex chromosome complement (XX vs. XY) is independent of gonadal sex (female vs. male). This model allows us to assess the effect of sex chromosomes independent of gonadal sex (**Figure 1A**).

The XY* model consists of female and male mice with one or two X chromosomes (females: XO or XX, males: XY or XXY) (**Figure 1B**). This model allows us to determine if the XX-XY difference found in the FCG model is a result of i) the presence or absence of a Y chromosome (comparing mice with or without a Y chromosome),

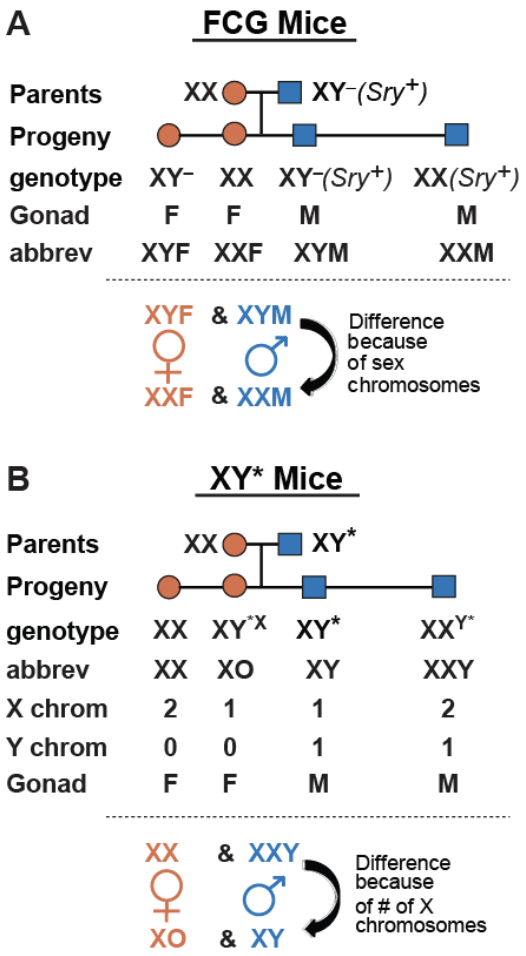

Figure 1. Overview of the FCG and XY* mouse models. A. The FCG model produces mice in which gonadal sex is independent of sex chromosomes. The FCG model allows us to compare gonadal female mice with XX and XY chromosomes and gonadal male mice with XX and XY chromosomes. B. The XY* model produces mice with one or two X chromosomes (female XO and XX, male XY and XXY). This model allows us to determine if the XX-XY difference found in the FCG model is caused by the presence or absence of the Y chromosome or the number of X chromosomes.

or ii) the number of X chromosomes (comparing mice with one or two X chromosomes, **Figure 1B**).

Gonadectomy surgery and hypoxia protocol

All protocols described in the methods received institutional review and committee approval. This investigation conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

Mice were gonadectomized (GDX) at day 75 after birth to remove potential group differences in the levels of gonadal hormones during testing. Thirty days after GDX, mice were placed in a hypoxia chamber (10% oxygen) for three weeks (n=4-6 per group for FCG mice and n=5 per group for XY* mice). For the FCG mouse model, some FCG mice (n=5 per group) were kept under normoxic conditions for 3 weeks.

Cardiopulmonary hemodynamic monitoring

Direct cardiac catheterization was performed terminally to measure RV systolic pressure (RVSP) by inserting a catheter into the RV immediately prior to sacrifice. The mice were anesthetized with a mixture of Ketamine (80 mg/kg) and Xylazine (8 mg/kg) administered *via* intraperitoneal injection. The animals were placed on a controlled warming pad to keep the body temperature constant at 37°C. After a tracheostomy was performed, a cannula was inserted, and the animals were mechanically ventilated using a rodent ventilator (Harvard Apparatus, Canada). Following ventilation, mice were placed under a stereomicroscope (Zeiss, Hamburg, Germany), and a pressure-conductance catheter (model 1.4F Millar SPR-671) was introduced via the apex into the RV and positioned towards the pulmonary valve. The catheter was connected to a pressure transducer

(Power Lab, ADInstruments) and RV pressures were recorded digitally. After recording the RVSP, lungs were removed rapidly under deep anesthesia for preservation of protein integrity.

Histopathologic analysis, immunohistochemistry, and imaging

Lungs were weighed, perfused and/or snap frozen for further histological and molecular investigation. Briefly, whole lungs were isolated and inflated manually using a syringe by perfusing 4% paraformaldehyde in 0.1 M Na_2HPO_4 and 23 mM NaH_2PO_4 (pH 7.4) through the trachea. Isolated perfused lungs were fixed in 4% paraformaldehyde on ice overnight. Following fixation, the tissue was immersed in ice-cold 20% sucrose overnight and embedded using optimum cutting temperature compound. Five μm lung tissue sections were obtained with a cryostat (Microm HM525, Thermo Scientific). Standard Masson Trichrome (Sigma) staining was performed according to the manufacturer's protocol, and images were acquired with a confocal microscope (Nikon). Stitched images of entire lung sections were obtained using 10x objective on a high-resolution confocal microscope (Nikon). Pulmonary vascular remodeling was assessed by quantifying percent occlusion of peripheral lung arterioles (<100 um diameter) from a total of 392 arterioles, n=4-6 mice per group and ~20 arterioles per mouse. Pulmonary fibrosis was assessed from the stitched images as percent fibrosis using a grid (n=4-6 mice per group).

Immunofluorescence staining

Lung sections (5μm) were fixed in acetone for 15 minutes at –20°C. The sections were then washed with PBS+0.1% Triton three times and incubated with 10% normal goat serum in PBS+0.1% Triton for 30 minutes to block the background. Following blocking,

the sections were incubated with primary antibodies against alpha-smooth muscle actin and von Willebrand Factor in PBS+0.1% Triton+1% normal goat serum at 4°C overnight. Sections were then washed with PBS+0.1% Triton three times, incubated with the appropriate secondary antibodies in PBS+0.1% Triton+1% normal goat serum at room temperature for one hour. After washing the secondary antibodies with PBS+0.1% Triton three times, the sections were incubated with DAPI and then mounted for imaging using Prolong gold (Molecular Probes). Images were acquired with a confocal microscope (Nikon).

Analysis of online gene expression databases

For analysis of gene expression from online RNA-Seq data (GSE29278: male mouse heart and lung, GSE49417: male human heart and lung), we measured the expression levels by calculating RPKM (Reads Per Kilobase of transcript per Million mapped reads). R package “QuasR” was used for the read alignment to the human (hg19) or mouse (mm10) genome, followed by the counting of reads at the gene level and calculation of RPKM(23). In **Table 1**, we report protein-coding genes, pseudogenes and non-coding RNAs with RPKM >1. For the microarray analysis of gene expression in **Table 2** (GSE15197: human lung, control and PAH), we quantile normalized gene expression levels and compared the expression of Y chromosome genes in PAH and Ctrl lungs using a one-way ANOVA followed by False Discovery Rate (FDR) analysis (23).

Reagents

Primary antibodies used include: anti-smooth muscle actin (Sigma (A2547), 1:250 dilution) and anti-von Willebrand Factor (Abcam (ab6994), 1:200 dilution). Secondary

antibodies used include: Goat anti-Mouse IgG (H+L), Alexa Fluor 488 (ThermoFisher (A-11001), 1:1000 dilution and Goat anti-Rabbit IgG (H+L), Alexa Fluor 594 (ThermoFisher (A-11012), 1:1000).

Statistical analysis

Two-way ANOVAs were used to compare experimental groups. For comparison of FCG mice, the factors were sex chromosome complement (XX vs. XY), and gonadal sex (male vs. female). For studies of XY* mice, the factors were numbers of X Chromosomes (one vs. two) and presence of Y Chromosome (present vs. absent, same as gonadal sex). $P<0.05$ was considered statistically significant. Values were expressed as Mean± SEM.

Results

XY mice, irrespective of their gonadal sex, develop less severe PH than XX mice.

We examined the role of sex chromosome complement on susceptibility to PH development in the absence of sex hormones in GDX FCG mice. Under normoxic conditions, the RV pressure was not significantly different between XX vs XY mice regardless of their gonads (male or female) (**Figure 2**). When mice were subjected to hypoxic insult for three weeks, we found male and female XY mice developed less severe PH than male and female XX mice. RV pressure was significantly lower in XY mice than XX mice (RVSP: 43.31± 5.72 mmHg in XX females and 46.51± 4.52 in XX males *vs.* 33.17± 2.36 in XY females and 40.4± 2.31 in XY males; $p<0.05$, n=4-6 per group, **Figure 2**).

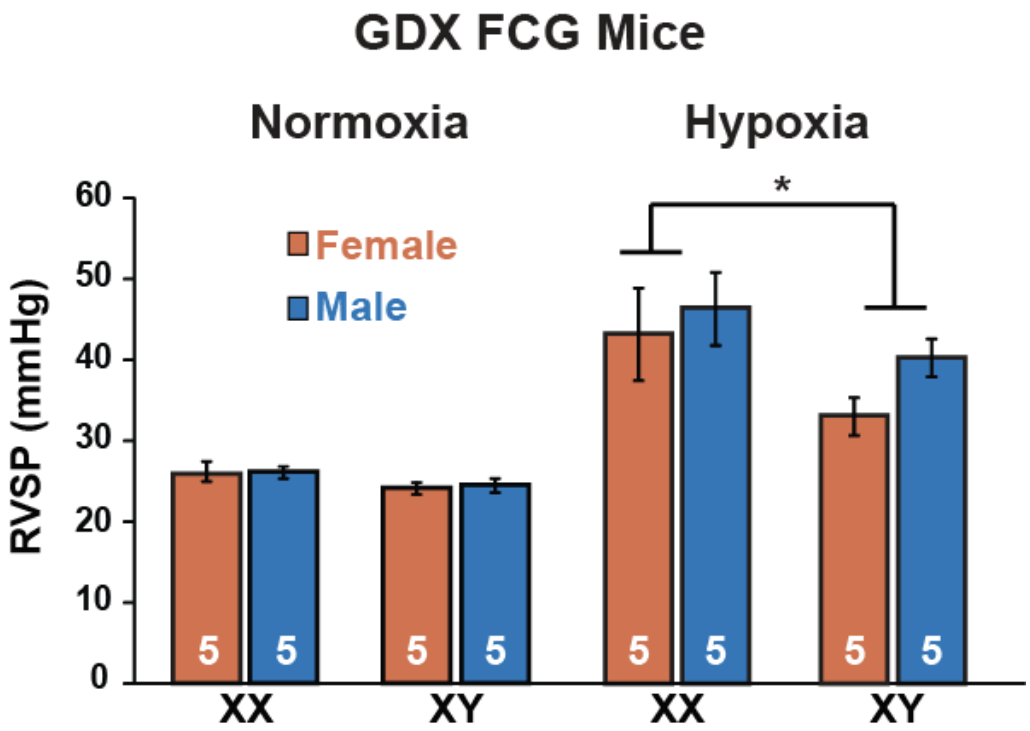

Figure 2. Gonadectomized male and female XY mice develop less severe PH compared to male and female XX mice. Bar graphs showing RV systolic pressure (RVSP) in normoxia or hypoxia for GDX FCG mice. Female XX and XY mice (white bars) are plotted against male (black bars) XX and XY mice. *p<0.05; for normoxia experiment n=5 mice/group; for hypoxia experiment XX females n=4; XX males n=6; XY females n=5; XY males n=5.

Male and female XY mice have less pulmonary vascular remodeling compared to male and female XX mice.

Consistent with the lower RV pressures found in XY mice, PH protection in XY mice was also accompanied by less severe pulmonary vascular remodeling as measured by percent occlusion of pulmonary arterioles (XY female: 23.84± 3.14; XY male: 22.47± 3.15; XX female: 58.8± 7.95; XX male: 44.56± 11.68) (**Figure 3**). These results further indicate that GDX male and female mice with XY chromosome complement develop less severe PH compared to the male and female mice with XX chromosome complement.

PH protection in XY mice is due to the presence of a Y chromosome rather than the number of X chromosomes.

To explore whether the less severe PH in XY mice compared to XX mice is due to the number of X chromosomes or the presence of the Y chromosome, we used mice from the XY* mouse model (XO, XX, XY XXY) and compared PH severity. RV pressures from XY (33.49±0.60 mm Hg) and XXY (32.03±2.17 mm Hg) mice were significantly lower than RV pressures measured in XO (38.72±2.44 mm Hg) and XX mice (41.56±3.84 mm Hg) (**Figure 4**). We did not observe any significant change in RV pressure between mice with one or two X chromosomes (XO and XX compared to XY and XXY). These results indicate that the lower susceptibility of XY mice to hypoxic insult is due to the presence of the Y chromosome rather than the number of X chromosomes.

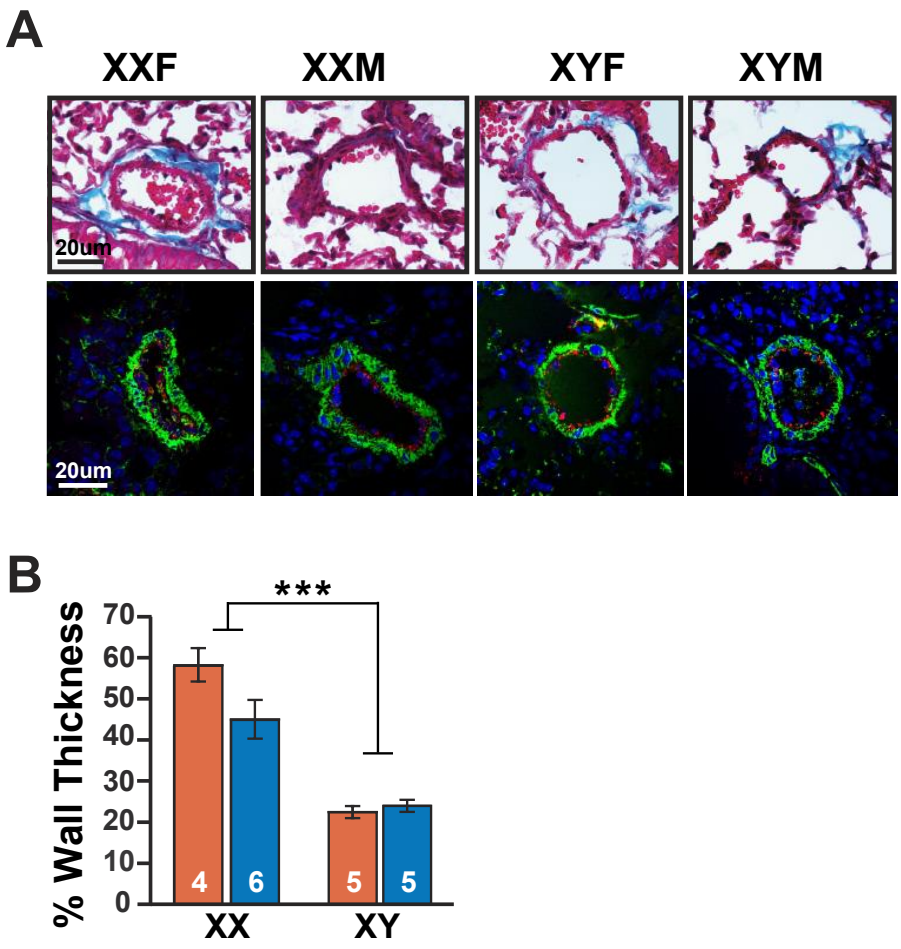

Figure 3. Hypoxic gonadectomized male and female XX mice develop more severe pulmonary vascular remodeling compared to male and female XY mice. A. Representative Masson trichrome (upper panel) and immunofluorescence staining (lower panel) for a-smooth muscle actin (green), von Willebrand Factor (red) and DAPI (blue) of lung sections from gonadectomized FCG mice kept in hypoxia for 3 weeks showing thicker pulmonary arterioles in XX mice compared to XY mice regardless of male (XXM, XYM) or female (XXF, XYF) gonads. B. Quantification of percent occlusion of the arterioles in the lung sections from XX and XY male and female mice. XX females n=4, XX males n=6, XY females n=5, XY males n=5, *p<0.05.

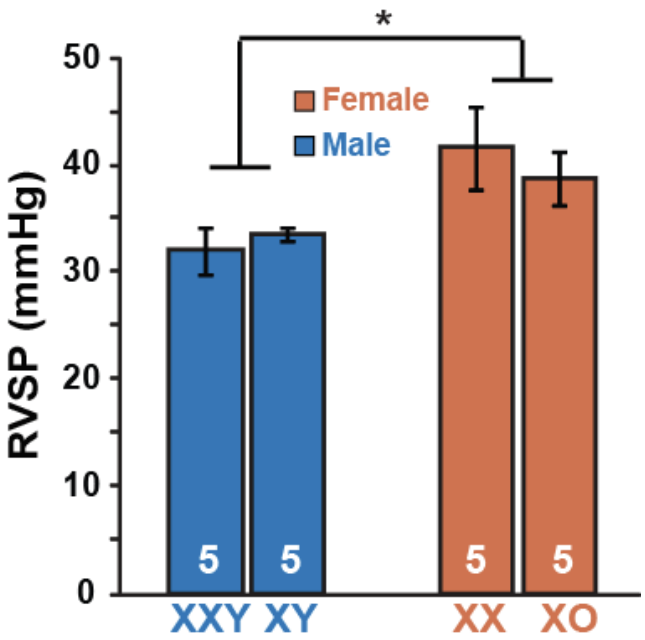

Figure 4. Hypoxic gonadectomized mice with a Y chromosome develop less severe PH compared to mice without a Y chromosome. Bar graphs showing RV systolic pressure (RVSP, mmHg) in hypoxic gonadectomized XY* mice. Female XX and XO mice (orange bars) are plotted against male XXY and XY mice (blue bars). *p<0.05, n=5 mice/group.

Y chromosome genes are expressed in mouse and human lung and heart tissues.

We infer from the above results that genes encoded by the Y chromosome that are expressed in the lung or heart tissues confer protection against PH. To discover which Y chromosome genes are expressed in the lungs and heart, we analyzed several datasets in the GEO databases. In **Table 1**, we summarize Y chromosome genes showing at least 1 RPKM in male tissues. In mice, four Y chromosome protein-coding genes are expressed in the lungs and heart: *Ddx3y*, *Eif2s3y*, *Kdm5d* and *Uty*. These represent the top candidates to explain the protective effect of the Y chromosome. The same genes are also expressed in human heart and lung with the exception of *Eif2s3y*, which is not conserved in humans. In **Table 2**, we analyzed a GEO microarray dataset and compared the expression of Y chromosome genes in the lungs of male PAH patients versus healthy male controls. We found that *KDM5D* and *UTY* expression is downregulated in PAH lungs ($p<0.05$) whereas *DDX3Y* is not significantly altered.

Mouse			
Lung	**RPKM**	**Heart**	**RPKM**
Ddx3y	9.8	***Ddx3y***	7.5
Eif2s3y	9.6	*Eif2s3y*	6.5
Kdm5d	4.6	***Kdm5d***	2.0
Uty	2.4	***Uty***	1.4

Human			
Lung	**RPKM**	**Heart**	**RPKM**
*CD24P4**	94.4	*PSMA6P1**	33.8
*PSMA6P1**	61.2	*EIF1AY*	11.8
*EIF4A1P2**	38.3	***DDX3Y***	10.1
DDX3Y	32.4	*EIF4A1P2**	6.8
RPS4Y1	6.0	*VDAC1P6**	5.5
*RNU6-941P**	4.7	*USP9Y*	4.0
EIF1AY	4.0	*RPS4Y1*	3.5
*RPL26P37**	3.3	*ZFY*	3.3
KDM5D	3.2	***KDM5D***	2.2
USP9Y	2.8	*TTTY14***	1.5
*TXLNGY**	2.6	*TXLNGY**	1.2
ZFY	2.6	***UTY***	1.0
*PRKY**	2.3		
*TTTY15***	2.0		
UTY	1.8		
*VDAC1P6**	1.5		
*KRT18P10**	1.2		

Table 1. Y chromosome genes expressed in lung and heart tissues. RNA-seq analysis of Y chromosome gene expression (GSE29278: mouse heart and lung, GSE49417: human heart and lung). RPKM (Reads Per Kilobase of transcript per Million mapped reads) values were calculated using R package “QuasR.” Only genes with RPKM > 1 are listed. Genes found in both mouse and human tissues are bolded. *pseudogene, **ncRNA.

Human Lung			
Gene	**p-Value**	**FDR**	**PAH/Ctrl**
KDM5D	**0.00001**	**0.04**	**0.52**
USP9Y	0.001	0.11	0.13
*TXLNGY**	0.002	0.13	0.16
ZFY	0.005	0.14	0.64
UTY	**0.018**	**0.19**	**0.40**
EIF1AY	0.110	0.35	0.55
DDX3Y	**0.151**	**0.40**	**0.70**
RPS4Y1	0.779	0.89	1.07

Table 2. Change in Y chromosome gene expression between male healthy and PAH lung tissue. Microarray analysis showing the change in Y chromosome gene expression between PAH lungs and control (Ctrl) lungs (GSE15197: human lungs, PAH and Ctrl). We quantile normalized gene expression levels and compared PAH and Ctrl expression using a one-way ANOVA followed by False Discovery Rate (FDR) analysis (24). Only genes with RPKM > 1 in lung tissue via RNAseq analysis from Table 1 are listed. Genes found in both mouse and human tissues are bolded. *pseudogene, **ncRNA.

Discussion

In this study, using two informative mouse models that elucidate sex chromosome differences in disease, we investigated for the first time the effect of sex chromosomes in hypoxia-induced PH. In FCG mice, we found that GDX male and female mice with XY chromosomes developed less severe hypoxia-induced PH and exhibited reduced vascular remodeling and pulmonary fibrosis than male and female mice with XX chromosomes. Using XY* mice, we found that the lower susceptibility of XY mice to hypoxic insult is not due to the number of X chromosomes, but is instead due to the presence of a Y chromosome. We found only four protein-coding genes are expressed in male mouse heart and lung tissue (**Table 1**) (25). These four genes are prime candidates to explain the protective effect of the Y chromosome in our experiments. Three of these Y chromosome encoded genes are conserved in humans and may contribute to the sex differences found in PAH (26). The protective effect of the Y chromosome helps explain why male sex is the single best deterrent for developing PAH.

There are complex sex differences in PH susceptibility and prognosis which has led to two important paradoxical findings (4, 14, 15): **i)** higher incidence of PAH in women (up to 4:1 ratio in some forms of PAH) whereas wild type female rodents are protected against some forms of experimental PH by estrogens; and **ii)** although female patients are more susceptible to PAH than men, they respond better to currently available therapies and have increased survival compared to men (27). Taken together, these data indicate that a single sex-biasing factor is not sufficient to explain the complex sex differences in PAH. This study adds dimension to the current literature by exploring the influence of sex chromosomes on the development of PH. Our findings indicate that the Y chromosome,

in the absence of sex hormones, is a protective factor against hypoxia-induced PH in mice.

The FCG and XY* mouse models, which allow investigators to highlight sex differences in disease driven by both the type and number of sex chromosomes (22) have uncovered effects of sex chromosomes on various pathologies including autoimmune disease, metabolic dysfunction, neurodegeneration and, in our lab, ischemic heart disease (18–21). The findings from the aforementioned studies indicate that the presence of two X chromosomes can increase susceptibility to various diseases (16). Since females have two X chromosomes, compared to one in males, X chromosome genes that escape inactivation result in increased expression in females. These X-escapee genes, which are expressed higher in females, may explain the harmful effects of X chromosome dosage in female tissues (18–21). Interestingly, none of the preceding studies found a significant effect, either protective or harmful, due to the presence of a Y chromosome. Many X genes that escape inactivation are similar to a paralogous “partner” gene encoded by the Y chromosome, which evolved from a common autosomal orthologue. Although the X and Y gene pairs may retain common functions, in some cases the Y chromosome paralogue gene has evolved novel functions due to different evolutionary pressure and a high rate of mutation (26, 28, 29). These Y genes are therefore interesting candidates to explain the protective effect of the Y chromosome reported here. Four of these Y chromosome genes—*Ddx3y*, *Kdm5d, Uty* and *Eif2x3y*— are expressed in the heart and lung tissue in males (**Table 1**) (25). Although information is limited, a growing body of evidence suggests that these protein-coding candidate genes have potential to impact fundamental cellular processes affecting proliferation, apoptosis, inflammation, and

epigenetic regulation (26, 30). Dysregulation of proliferation, apoptosis, and inflammation are hallmarks of PAH pathogenesis, and epigenetic regulation has recently emerged as a promising direction for PAH research (2, 31–33).

KDM5D, a histone demethylase, modulates gene expression by removing methyl groups from the trimethylation mark on histone 3 lysine 4 (H3K4me3) which generally represses the transcription of specific genes that may be implicated in PAH (34, 35). As an epigenetic modifier, it has potential to elicit widespread changes in gene expression and has recently been found to influence the development and metastasis of prostate cancer through regulation of androgen receptor synthesis and signaling (36, 37).

UTY is a paralog of the more characterized X chromosome gene UTX (KDM6A)—a histone demethylase that activates expression by removing methyl groups from H3K27me3 and is required for proper cardiac development. UTY retains some overlapping functions with UTX since it was shown that *Uty* expression is sufficient to prevent improper cardiac development and reduce mid-gestation lethality resulting from loss of *Utx.* Interestingly, UTY appears to lack histone demethylase activity, so its mechanism of action overlaps incompletely with the effect of UTX (29, 38). Mutations in both *Uty* and *Utx* have been implicated in the development of various cancers, illuminating their role as powerful regulators of disease (39–41). Furthermore, expression of UTY in macrophages has been linked atherosclerosis risk in men (42).

Ddx3y encodes an RNA helicase involved in ribosome synthesis and metabolism that was recently linked to the development of pulmonary disease. Using protein-protein interactive network analysis, Yang *et al.* determined that DDX3Y is not only upregulated

in chronic obstructive pulmonary disease, but also acts as a regulatory hub gene in the network (43).

EIF2S3Y, a translation initiation factor, has been shown to be dramatically upregulated in parallel with regulation of apoptosis in endothelial cells (44). Endothelial cell dysfunction within the walls of pulmonary vasculature is symptomatic of PH and is the target of many therapies currently used for PH in patients (45).

Our study demonstrates for the first time the protective effect of the Y chromosome in the development of PH and represents an important paradigm shift in the approach towards understanding the marked sex differences in PH. In attempts to uncover the molecular mechanism by which the Y chromosome confers protection, further investigation into the four Y chromosome candidate genes—*Ddx3y, Kdm5d, Uty,* and *Eif2x3y*—is needed.

Analysis of online GEO datasets indicates that three of the identified Y chromosome candidate genes, *KDM5D*, *UTY* and *DDX3Y*, are also expressed in human lung and heart tissue (**Table 1**). If one or more of these genes is responsible for the protection against PH in mice, it could have exciting implications for human PAH and give rise to novel therapeutic strategies. Notably, analysis of online human PAH and control lung microarray datasets indicates that *KDM5D* and *UTY* expression is significantly downregulated in PAH (**Table 2**). This finding further highlights the potential role of Y chromosome genes, namely KDM5D and UTY, in PAH pathogenesis and protection.

Additionally, this study identifies the effects of sex chromosomes in the absence of sex hormones; however, future studies are needed to examine the complex relationship between PH development and sex chromosomes within the influential hormonal milieu.

Subsequent studies investigating these genes may uncover a much-needed novel strategy for the treatment of PAH in patients.

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Chapter 3: Y-Chromosome gene, *Uty*, protects against pulmonary hypertension by reducing proinflammatory chemokines

Abstract

Rationale: Idiopathic pulmonary arterial hypertension (PAH) is a terminal pulmonary vascular disease characterized by increased pressure, right ventricular failure and death. PAH exhibits a striking sex bias and is up to 4x more prevalent in females. Understanding the molecular basis behind this sex difference is paramount to elucidating PAH pathophysiology and uncovering novel therapies.

Objectives: We previously discovered the Y-Chromosome is protective against hypoxia-induced PH which explains the reduced PAH prevalence in males. Here, we identify the gene responsible for Y-Chromosome protection, investigate key downstream autosomal genes, and demonstrate a novel therapy.

Methods, Measurements and Main Results: To test the effect of Y-Chromosome genes on PH development, we knocked-down each Y-Chromosome gene expressed in the lung via intratracheal instillation of siRNA in gonadectomized male mice exposed to hypoxia. Knockdown of Y-Chromosome gene *Uty* resulted in more severe PH measured by increased right ventricle pressure and decreased pulmonary artery acceleration time. RNAsequencing revealed an increase in proinflammatory chemokines *Cxcl9* and *Cxcl10* as a result of *Uty* knockdown. We found *CXCL9 and CXCL10* significantly upregulated in human PAH lungs, with more robust upregulation in PAH females. Treatment of human pulmonary artery endothelial cells with CXCL9 and CXCL10 triggered apoptosis. Inhibition of CXCL9 and CXCL10 activity in female PH rats significantly reduced PH severity.

Conclusions: ChrY gene, *Uty*, is protective against PH. Reduction of *Uty* expression results in increased expression of proinflammatory cytokines CXCL9 and CXCL10 which

trigger endothelial cell death and PH. Inhibition of CXCL9 and CXCL10 activity rescues PH development.

Introduction

Pulmonary arterial hypertension (PAH) is a pulmonary vascular lung disease characterized by increased pressure in the pulmonary arteries leading to right ventricular (RV) hypertrophy, RV failure, and death. Pressure overload begins in the distal pulmonary arteries which undergo vascular remodeling largely characterized by vascular endothelial cell (EC) dysfunction and aberrant smooth muscle cell proliferation (1). Inflammation may play a key role in this vascular remodeling, since it is known that PAH is concomitant with a proinflammatory lung milieu and inflammatory regulators are associated with PAH disease progression and severity (1–3).

The idiopathic form of PAH exhibits striking sex differences where females are up to 4x more likely to be diagnosed than men (4). As female sex is a risk factor for developing PAH (5), the interplay between sex hormones and PAH pathogenesis has been actively investigated with particular attention to estrogens. Estrogens have been implicated in PAH pathogenesis and shown to reduce expression of bone morphogenic protein 2 (BMPR2), a mutation whose loss is associated with PAH (6, 7). However, studies have also demonstrated that circulating estrogens are protective against PH severity, and estrogen treatment has been shown to prevent and rescue PH in animal models (8–10) through mediating RV compensation to pressure overload (11, 12). The culmination of these powerful yet paradoxical studies indicates that other sex-biasing factors contribute to the sex differences observed in PAH.

Our group was the first to examine the influence of sex chromosomes in PH where we found, in the absence of circulating gonadal hormones, the male-specific Y chromosome (ChrY) is protective against experimental pulmonary hypertension (PH) (13). While this

study helps explain why males are more protected against developing PAH, the mechanism by which ChrY protection is achieved has yet to be discovered and may lead to a greater understanding around the pathogenesis of PAH and elucidate novel therapeutic strategies. ChrY genes are known to have widespread effects on autosomal gene regulation (14) and have been implicated in systemic diseases (15, 16).

In the present study, we investigated the role of the four ChrY genes that are expressed in lung tissue for their potential involvement in PH protection. We identified *Uty* as the protective ChrY gene, because reduced *Uty* expression in hypoxic (Hx) mouse lungs was sufficient to eliminate ChrY protection. We also identified two proinflammatory chemokines, CXCL9 and CXCL10, downstream of *Uty* that contribute to PH pathogenesis in a sex-specific manner and targeted these cytokines as a novel therapeutic approach. We found that inhibiting the shared CXCL9 and CXCL10 receptor significantly rescues PH development in female rats, suggesting that targeting autosomal genes downstream of *Uty* is sufficient to extend ChrY protection to females with PH.

Methods

Models of experimental PH and treatments

All experimental protocols received institutional review and Animal Research Committee approval.

siRNA knockdown in hypoxia-induced PH: Male C57BL/6J mice aged 6-8 weeks were purchased from Jackson Laboratories. Mice underwent gonadectomy (GDX) surgery under isoflurane anesthesia to remove gonads. After 30 days, mice were randomly divided into two groups where they received recurring intratracheal instillation of either short interfering RNA (siRNA) targeting a ChrY gene of interest or a scrambled siRNA every 5 days (1nmol/instillation, *Dharmacon Accell mouse SMARTpool:* Si-Kdm5d: E-054675-00-0005; Si-Ddx3y: E-043317-00-0005; Si-Eif2s3y: E-046339-00-0005; Si-Uty: E-046843-00-0010; Si-Scrm: siGENOME Non-Targeting siRNA Pool #2, D-001206-14-05). Mice were housed in hypoxic (Hx, 10% oxygen) conditions for three weeks while receiving their instillations (**Fig. 1A**).

Cxcr3 inhibition in monocrotaline-induced PH: Young, female intact Sprague Dawley rats (~200g) were purchased from Charles Rivers laboratories. At day 0, all animals received a subcutaneous injection of monocrotaline (MCT, 60mg/kg, Sigma). At day 14, animals were randomly divided into control or treatment groups (n=8/group). Rats were injected subcutaneously 2x/day at 12-hour intervals with small-molecule CXCR3 inhibitor AMG487 (Tocaris, cat# 4487) at a dose of 1.5mg/kg. AMG487 was dissolved in sterile 20% hydroxypropyl-β-cyclodextrin (Sigma) prior to injection for a final volume of 250ul/injection. Rats in the control group were injected twice daily with vehicle (250ul sterile 20% hydroxypropyl-β-cyclodextrin). Injections were performed on familiarized rats

without the use of anesthesia and continued 2x daily for the remainder of the MCT protocol (2 weeks, **Fig. 5A**). Prior to the termination of the experiment, rats underwent echocardiography followed by open-chested catheterization.

Echocardiography

Noninvasive echocardiography was performed for all animals using Vevo 2100 (Visualsonics) at baseline, before treatment, and prior to terminal catheterization under isoflurane anesthesia. Two-dimensional doppler echocardiography and long axis B-mode recordings were used to evaluate and quantify pulmonary artery acceleration time (PAAT) and RV fractional area changes (RVFAC), respectively. All quantifications were performed on blinded samples.

Hemodynamics and gross histological evaluation

Animals were anesthetized with isoflurane, placed on a heating pad to keep body temperature of ~37^{o}C and mechanically ventilated via tracheostomy. RV systolic pressure (RVSP) was measured via direct, open-chested catherization (Millar SPR-671) of the RV, which was recorded via a pressure transducer (Power Lab, ADInstruments). RVSP measurements were obtained blindly and a RV pressure recording was recorded for 5 mins/animal. Following RV catheterization, the catheter was inserted into the left ventricle (LV) and LVSP was recorded as a control for potential blood loss. The heart was excised, dissected into components of RV wall, LV wall and interventricular septum (IVS), and weighed to calculate the Fulton index of RV hypertrophy, a ratio of RV weight to LV plus IVS weight ([RV/(LV+IVS)]).

Tissue preparation, staining and imaging

All excised murine tissues were immediately fixed in 4% paraformaldehyde, soaked in 20% sucrose solution, and embedded in OCT compound (Tissue-Tek).

For RNAscope, lungs were sectioned at 7um and pretreated and stained following protocols outlined by ACD Bio RNAscope assay (*Uty validation and quantification*: RNAscope® 2.5 HD-RED, Mm-Uty cat# 451741; *Colocalization:* RNAscope® Multiplex Fluorescent Detection Kit cat# v2323110, Mm-Uty cat# 451741; Mm-Cxcl9-C2 cat# 489341-C2, Mm-Cxcl10-C2 cat# 408921-C2, Mm-Cd68-C3 cat# 316611-C3, Hs-UTY cat# 420851, Hs-CD68-C2 cat# 560591-C2, Hs-CXCL9-C3 440161-C3, Hs-CXCL10-C3 cat# 311851-C3). Images were acquired using a Nikon Confocal (Nikon Eclipse Ti, A1R MP) at 40-60X magnification using Z-stack capture and compressed and analyzed using ImageJ. For images acquired for quantification, at least 5 images were randomly taken per animal per slide. Quantification of RNA transcripts was performed on stained sections as RNAscope is demonstrated to have single-transcript resolution (17). All quantification was performed by two separate, blinded individuals on at least five images per animal.

For immunofluorescence, rat lung samples were prepared as described above and sectioned at 5μm. The sections were washed (PBS+0.1%TWEEN (Sigma) three times for 5 minutes) and incubated with 10% fetal bovine serum in PBS+0.1% TWEEN for 30 min to block the background. The sections were incubated with cleaved-caspase3 antibody (1:400, Cell Signaling Technology, cat# 9661S) and CD31 antibody (10ug/mL, Novus Biotech, cat# NB100-2284) in PBS+0.1% Triton+ 3% fetal bovine serum at 4^{o}C overnight. The sections were washed and incubated with the secondary antibody (1:500, Alexa Fluor 488, Thermo Fisher, cat# A-11055) in PBS+0.1% TWEEN + 3% fetal bovine serum at room temperature for 1 hour. The sections were washed and incubated with

secondary antibody (1:1000, Alexa Fluor 594, Thermo Fisher, cat# A-11012) in PBS+0.1% TWEEN + 3% fetal bovine serum at room temperature for 1 hour. After washing, slides were mounted using Prolong gold with Dapi (Thermo Fisher) for imaging. Images were acquired using a Nikon Confocal (Nikon Eclipse Ti, A1R MP) at 40X magnification and analyzed using ImageJ. For images acquired for quantification, at least 5 images were randomly taken per animal per slide and cleaved caspase-3 expressing nuclei proximal to positive CD31 expression were considered positive. Quantification was obtained blindly.

Human lung samples were obtained from Pulmonary Hypertension Breakthrough Initiative repository as fixed, paraffin-embedded sections. Slides were deparaffinized, underwent target retrieval using boiling Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) prior to staining for immunofluorescence. Slides were washed (PBS, 5 minutes, 3 times) and then blocked with PBS+ 5% normal donkey serum at room temperature for 1 hour. Slides were then incubated at 4^{o}C overnight in PBS+5% normal donkey serum primary antibody+ primary antibody (10μg/mL, R&D Systems, CXCL9 cat# AF392-SP, CXCL10 cat# AF-266-SP). Slides were washed and then incubated at room temperature for 30 minutes with PBS+5% normal donkey serum+ secondary antibody (1:500, Alexa Fluor 488, Thermo Fisher, cat# A-11055). After washing, slides were mounted using Prolong gold with Dapi (Thermo Fisher) for imaging. Images were acquired using a Nikon Confocal (Nikon Eclipse Ti, A1R MP) at 40X magnification

Real-time qPCR

For *in vivo* experiments, total lung RNA was isolated using Trizol (Thermo Scientific), and for *in vitro* experiments, RNA was isolated from cells using Trizol followed by RNeasy

Micro Kit (Qiagen). Mouse and human RNA were reverse transcribed with polydT primers using Omniscript reverse transcription kit (Qiagen), except rat RNA was reverse transcribed using gene specific primers. Real-time quantitative PCR was performed on polyA+ cDNA with primers for using iTaq Universal SYBR® (Bio-Rad). *Actb* (mouse), *Gapdh* (rat) and *RPLP0* (human) were used as housekeeping genes. See **Supplementary Table E1** for primer sequences used.

Bioinformatic Analysis

Total lung RNA was isolated using Trizol extraction and purified using RNeasy Mini Kit (Qiagen). Libraries for RNA-seq were prepared by the Technology Center for Genomics & Bioinformatics at UCLA and sequenced using paired-end Hiseq 3000 (Illumina). HISAT2 version 2.1.0 was used to align RNA-seq reads to the Mus musculus genome (mm10, Ensembl 84). StringTie version 1.3.3b was used to assemble RNA-seq alignments into transcripts and estimate expression levels of all genes detected. Differential expression analysis was performed using DESeq2 R package version 1.25.16. Differently expressed genes (DEGs) with false discovery rate <0.1 were considered statistically significant. Pathway enrichment analysis and clustering was performed using Cytoscape software (18). Publicly available human microarray data (19) was analyzed using the limma package for R to define a list of inflammatory genes expressed higher in female PAH patient lungs vs male PAH lungs.

Cell studies

PAEC experiments: PAEC from a healthy, nonsmoking male patient (Lonza) were cultured in tissue culture plates or on cover slips and spiked with recombinant CXCL9 [2 μM, R&D Systems], CXCL10 [5 μM, R&D Systems] or vehicle (medium) for 24 hours for

EC apoptosis assay measured via cleaved caspase-3 immunofluorescence (1:250, Cell Signaling Technology, cat# 9661S) and 72 hours for cell viability and proliferation via CCK-8 assay (Dojindo Molecular Technology). For the viability assay, 3-4 separate experiments were performed with 5-6 technical replicates per experiment. For the apoptosis assay, 4 separate experiments were performed with 2 slides per experimental group in each experiment. Images were captured with using a Nikon Confocal (Nikon Eclipse Ti, A1R MP) at and analyzed using ImageJ. 400-500 cells were counted per slide to determine the percent apoptotic cells. In both studies, experimental replicates were averaged and the value was considered one experimental data point.

Bone marrow-derived macrophage experiments: We compared WT and *Uty*-KO mice (B6.129P2(CD1)-Uty$^{Gt(XS0378)Wtsi}$/Mmnc) at 8-10 weeks old (Shpargel, et al (20), Mutant Mouse Resource & Research Centers strain #37420). WT and KO mice had comparable genetic backgrounds (8 generations of backcross from CD1, with a strain 129 Y chromosome). To generate these groups, a C57BL/6 mouse (male for WT group, female for *Uty*-KO group) was bred with another mouse (female for WT group, KO male for the *Uty*-KO group) that derived from the strain #37420 and was backcrossed from CD1 to C57BL/6 for 7 generations. Bone marrow (BM) was flushed from the tibia and fibula of mice using sterile PBS with 1% Antibiotic-Antimycotic (Thermo Fisher) through a sterile needle and incubated on ice with ACK lysing buffer (Thermo Fisher, cat# A1049201) followed by wash steps. BM was incubated with colony stimulating factor (20ng/mL, Sigma) for six days to induce macrophage differentiation. Once adherent, cells were treated with interferon gamma (IFNg, 10ng/mL, Thermo Fisher) for four hours. *Cxcl9* and *Cxcl10* expression was determined via RT-qPCR on isolated BM-derived macrophages

as described above. Macrophage identity and polarization were confirmed using primers for *Cd68* and *MhcII* (See **Supplemental Table E1** for primer sequences).

Lung Macrophage Isolation

Fresh lung tissue from age-matched WT and *Uty*-KO male mice (as described above) was minced with scissors prior to enzyme dissociation. Tissue was incubated in enzymatic solution (DMEM, Thermo Fisher, cat# 11965-092), Liberase (400 ug/mL, Sigma), Elastase (100 ug/mL, Worthington Bio), and DNase (20 units/mL, Sigma) at 37^{o}C with continuous horizontal agitation (300 rpm). After incubation, dissociated tissues were sequentially passed through 70- and 40-micron cell strainers with centrifugation at 300g for 5 minutes. Cells were treated with ACK lysing buffer (Thermo Fisher, cat# A1049201) for 3 minutes at room temperature and quenched with PBS and pelleted. The cell pellet was incubated with viability dye for 20 minutes (0.1 uL/test, Thermo Fisher, cat# L34965) and Fc block (Cd16 antibody, 0.5uL/test, Thermo Fisher, cat# 14-0161-82) for 20 minutes on ice in the dark. After being washed and spun at 300g for 3 minutes, the cell pellet was stained with CD64 antibody (0.4 µg/test, Thermo Fisher, cat# 46-0641-80) and then washed and pelleted. Pellets were kept on ice and in the dark in PBS with 5% fetal bovine serum until cell sorting. Fluorescent activated cell sorting was carried out by the UCLA Flow Cytometry Core Facility on a BD FACSAriaIII. The cell population obtained for Western blot experiments consisted of live (viability dye negative) $CD64^{+}$ cells (**Supplemental Fig. E3C**).

Protein isolation, SDS-PAGE and Western Blotting

Whole cell protein lysates were prepared from whole lung tissue and isolated lung macrophages using RIPA lysis buffer (50 mM NaCl, 50 mM Tris pH 8, 1% NP-40, 0.5%

sodium deoxycholate, and 0.1% SDS, all from Sigma) containing protease and phosphatase inhibitors (Roche #04-906-845-001 and #118-3615-3001). Proteins (whole lung: 75 ug/well, isolated macrophages: 5 ug/well) were diluted in 4x Laemmli sample buffer (BioRad #161-0747), boiled, separated on 20% gels by SDS-PAGE and subsequently transferred onto nitrocellulose membranes (Biorad #170-4270) using semi-dry blotting (TransBlot Turbo System, BioRad). After transfer, membranes were blocked with 5% bovine serum albumin (Sigma #A9647) and incubated with antibodies directed against Histone H3K27 (1:300, Abcam, cat#ab6002) and Histone H3 (1:500, Abcam, cat#ab1791). IRDye-conjugated secondary antibodies (1:10.000, LI-COR #32210 and #68070) were used for detection and blots were scanned using the LI-COR Odyssey Infrared Imaging System. Band intensity was quantified using Image Studio Lite.

Statistics

For comparisons of two normally distributed, independent groups, we used an unpaired t-test. For the in vitro cell studies using PAEC, a paired t-test was used to compare viability and apoptosis between control and experimental groups within the same experimental batch. For a comparison between two nonparametric groups, we used a Mann-Whitney test. A significance level less than 5% ($p<0.05$) was deemed statistically significant. Analyses were made with Graph Pad Prism v.7 software. Values are expressed as mean ± SEM.

Results

Lung-specific knockdown of Uty*, but not other ChrY genes expressed in the lung, eliminates ChrY protection against Hx-induced PH.*

Our previously published work demonstrated that ChrY is protective against Hx-induced PH in mice (13). We identified only four protein coding ChrY genes expressed in mouse lung tissue, *Ddx3y* [Y-linked DEAD-box helicase 3], *Eif2s3y* [eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked], *Kdm5d* [lysine demethylase 5D], and *Uty* [ubiquitously transcribed tetratricopeptide repeat containing, Y-linked] (13). Three of these genes, *Ddx3y*, *Kdm5d* and *Uty*, are conserved in humans, and *KDM5D* and *UTY* were both found to be downregulated in male PAH patient lungs when compared to healthy patient lungs (13).

To determine which ChrY gene is responsible for PH protection, we individually knocked down (KD) each ChrY candidate gene in the lungs of GDX Hx mice and measured the effect on PH development (**Fig. 1A**). We found no significant change in the RVSP of mice with KD of *Kdm5d* (**Fig. 1B**), *Ddx3Y* (**Fig. 1C**), or *Eif2s3y* (**Fig. 1D**). However, KD of *Uty* resulted in significantly elevated RVSP compared to the Si-Scrm control group in two separate experiments (**Fig. 1E**). Mice that received Si-Uty demonstrated a significantly lower PAAT (**Fig. 1F**). We found *Uty* expression in Si-Uty treated lungs was reduced by ~50% compared to Si-Scrm lungs (**Fig. 1G**).

These data indicate that *Uty* is likely the sole ChrY gene that confers protection because reduction of *Uty*, but not the other ChrY genes expressed in the lung, is sufficient to exacerbate PH.

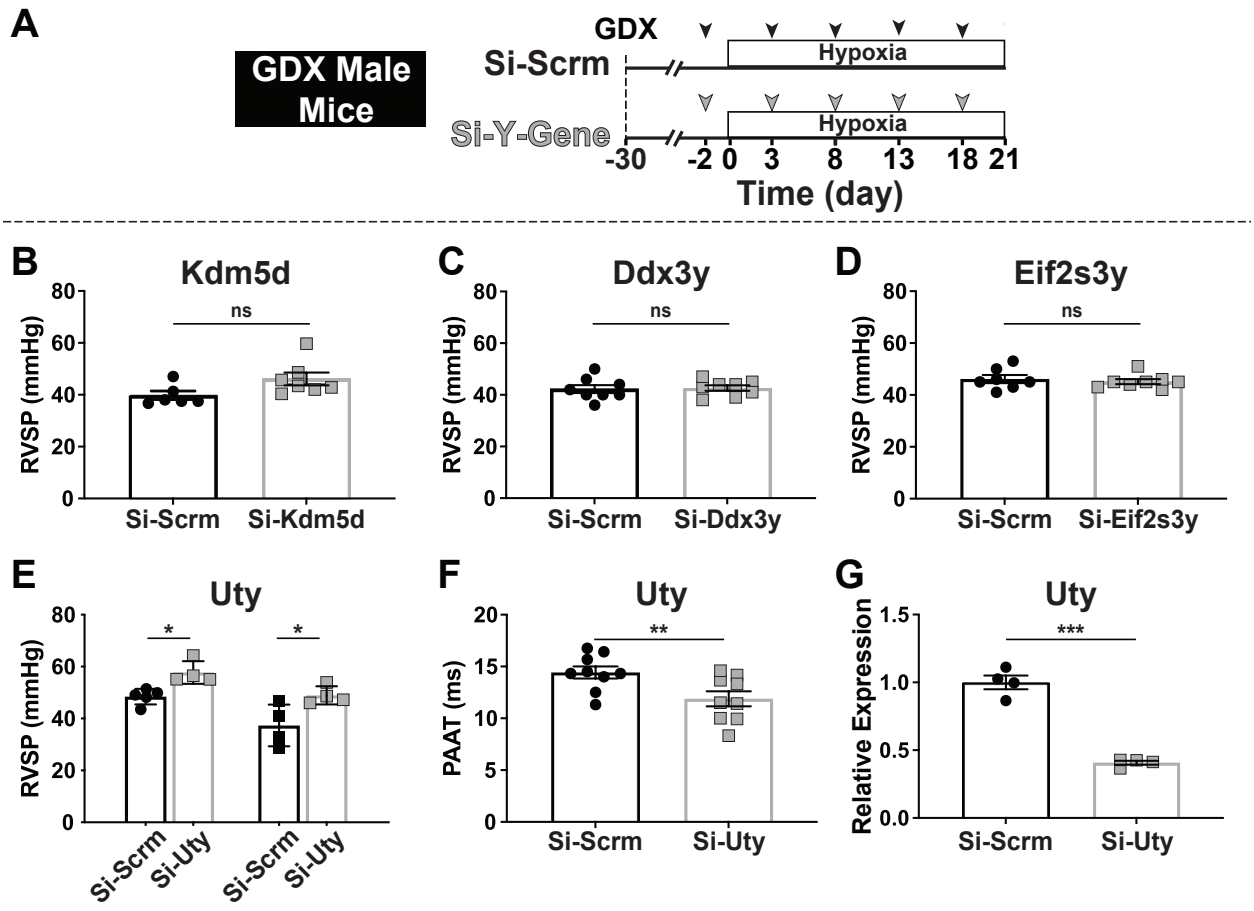

Fig. 1. Lung-specific knockdown of *Uty*, but not other ChrY genes expressed in the lung, eliminates ChrY protection against Hx-induced PH. A. Experimental protocol. 30 days after gonadectomy (GDX), Male mice receive scramble Si-RNA or Si-RNA targeting ChrY genes every 5 days and are placed in hypoxia chamber for 3 weeks. **B-E.** RVSP measured by direct RV catheterization. **F.** PAAT measured by echocardiography. **G.** Quantification of *Uty* transcripts in lung tissue via RNAscope in situ hybridization (normalized to Si-Scrm). **p<0.05, **p<0.01, ***p<0.001.*

Proinflammatory chemokines CXCL9 and CXCL10 are autosomal downstream deterrents of* Uty *protection and are upregulated in PAH patient lungs in a sex-specific manner.

To unravel the molecular mechanism underlying *Uty* protection in PH, we performed RNAseq on Hx Si-Scrm and Si-Uty mouse lung tissue and identified 523 DEGs (**Fig. 2A**). Pathway enrichment analysis highlighted five main pathways including cell signaling, extracellular matrix, transcription, translation, and inflammation (which was particularly enriched, **Fig. 2B**). Because inflammation is implicated in PAH pathogenesis and severity (2), we focused on DEGs localized in inflammatory pathways. We cross-referenced our inflammatory DEG set with an online microarray from PAH patient lung tissue (19) and searched for genes that were elevated in both Si-Uty compared to Si-Scrm, and female PAH samples compared to male, since females do not have the *UTY* gene. This analysis yielded two top genes of interest: proinflammatory chemokines *CXCL9* and *CXCL10* (**Fig. 2C**), both of which we validated to be upregulated in male and female PAH lungs with a more robust upregulation in female patients (**Fig. 2D-G**) as represented by immunofluorescence images of human healthy and PAH lungs (**Fig. 2F,G**).

Taken together, we found that downregulation of *Uty* is associated with upregulation of *Cxcl9* and *Cxcl10* in *Uty*-KD mouse lungs (**Fig. E1** of Supplement) and that *CXCL9* and *CXCL10* expression levels are upregulated in human PAH lungs in a sex-specific manner.

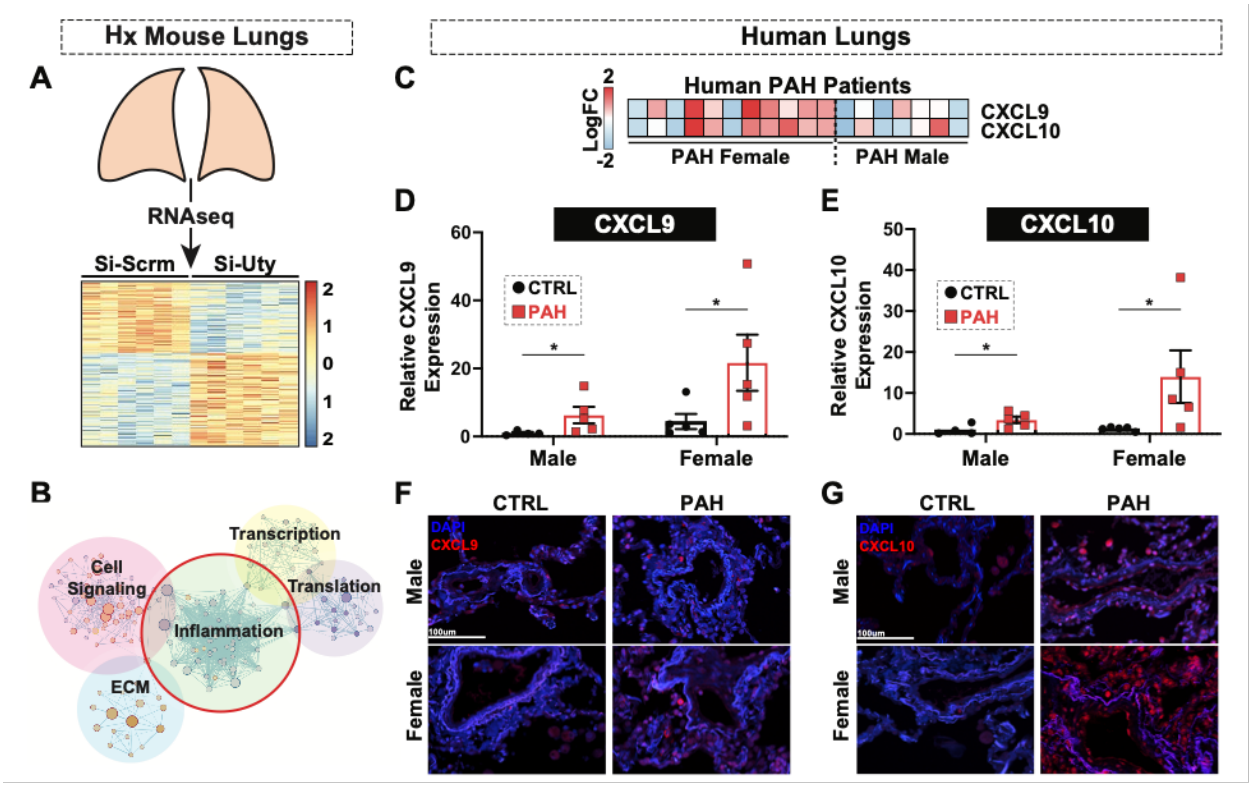

Fig. 2. Proinflammatory chemokines CXCL9 and CXCL10 are autosomal downstream deterrents of *Uty* protection and are upregulated in PAH patient lungs in a sex-specific manner. A. Comparison of RNAseq data from Si-Scrm and Si-Uty Hx mouse lungs revealed 523 differently expressed genes (DEGs). **B.** Pathways enriched with DEGs. **C.** Integration of RNAseq data with an online microarray dataset of male and female human PAH lung samples revealed *Cxcl9/CXCL9* and *Cxcl10/CXCL10* as upregulated in PAH females (vs PAH males) and Si-Uty mice (vs Si-Scrm). Relative expression of CXCL9 (**D**) and CXCL10 (**E**) in human lungs as measured by RT-qPCR. *p<0.05. Representative immunofluorescence staining of CXCL9 (**F**) and CXCL10 (**G**) in male and female lung tissue from healthy (CTRL) and diseased (PAH) patients. **p<0.05*

Uty*, Cxcl9 and Cxcl10 are co-localized in macrophages and* Uty *expression is inversely related to* Cxcl9 *and* Cxcl10 *expression.*

Given the importance of macrophages in inflammation and their known expression of CXCL9 and CXCL10 (21), we tested for *Uty* expression in macrophages. Stained tissue sections from male mouse and human lungs confirmed *Uty* expression within *Cd68*$^{+}$ macrophages (**Fig. E2A** of Supplement). Although we found *Uty* inversely related to chemokines *Cxcl9* and *Cxcl10*, we examined male mouse and human lung tissue for colocalization within macrophages and found *Uty* transcripts colocalized with *Cxcl9* (**Fig. 3A,C**) and *Cxcl10* (**Fig. 3 B,D**).

To identify whether *Uty* expression in macrophages directly influences *Cxcl9* and *Cxcl10* production, we measured *Cxcl9* and *Cxcl10* expression in BMDM from male WT and *Uty*-KO mice (**Fig. 3E**). BMDM from *Uty*-KO mice expressed significantly more *Cxcl9* and *Cxcl10* compared to BMDM extracted from WT mice (**Fig. 3 F,G**).

Since UTY is a member of the Jumonji family of histone demethylases and its role as an H3K27 demethylase is contested in literature (20, 22–24), we examined whether the effects of UTY in the lung were mediated through demethylation. We found that UTY expression in whole lung and isolated lung macrophages did not influence levels of H3K27 tri-methylated protein as measured by western blot (**Fig. E3** of Supplement).

In summary, we found *Uty*, which colocalizes with *Cxcl9* and *Cxcl10* in lung macrophages, regulates *Cxcl9* and *Cxcl10* expression, but has no apparent effect on H3K27 demethylation.

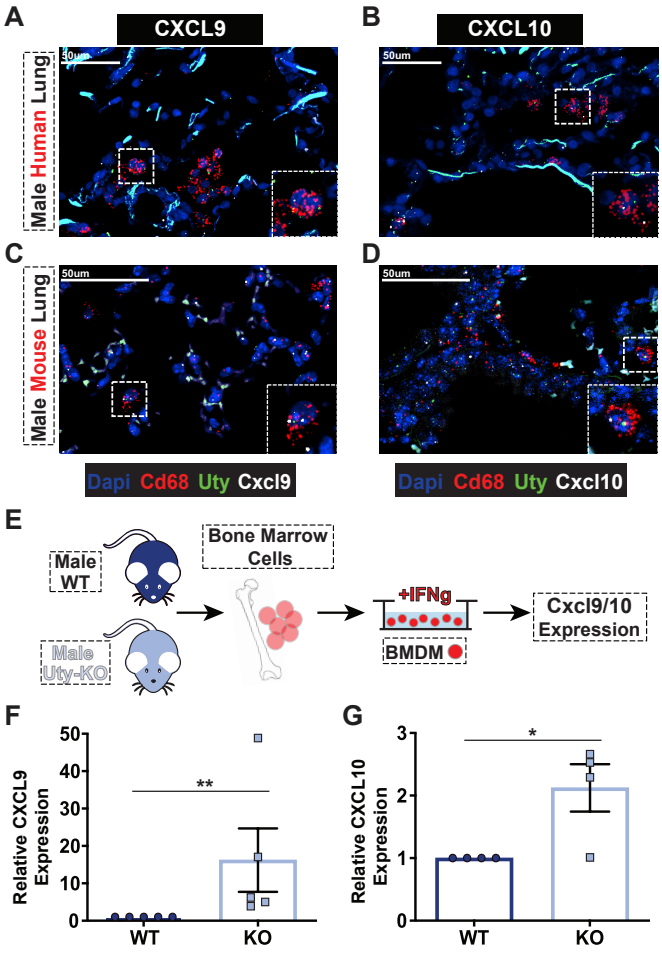

Fig. 3. *Uty*, *Cxcl9* and *Cxcl10* are co-localized in macrophages and *Uty* expression is inversely related to *Cxcl9* and *Cxcl10* expression. Representative images depicting colocalization of in situ probes labeling *Cd68/CD68* (red), *Uty/UTY* (green) and *Cxcl9/CXCL9* (white) in mouse (**A**) and human (**C**) lung sections. Representative images depicting colocalization of *in situ* probes labeling *Cd68/CD68* (red), *Uty/UTY* (green) and *Cxcl10/CXCL10* (white) in mouse (**B**) and human (**D**) lung sections. Schematic of bone marrow derived macrophage (BMDM) in vitro experiments (**E**). Relative expression of *Cxcl9* (**F**) and *Cxcl10* (**G**) as measured by RT-qPCR in BMDM isolated from wildtype (WT) and Uty knockout (Uty-KO) mice. *$p<0.05$, **$p<0.01$

CXCL9 and CXCL10 expression triggers pulmonary artery endothelial cell dysfunction.

To identify the mechanism by which *Uty* downstream autosomal genes *Cxcl9* and *Cxcl10* promote PH severity, we performed *in vitro* cell studies using human PAEC, which express the shared CXCL9 and CXCL10 receptor and are known to contribute to vascular remodeling in PAH. We incubated human PAEC with exogenous recombinant human CXCL9 and CXCL10 protein and measured the effect on EC dysfunction, a hallmark of PAH pathophysiology (**Fig. 4A**). Treatment of PAEC with either CXCL9 or CXCL10 resulted in decreased PAEC viability as measured by CCK-8 assay (**Fig. 4B,C**) and increased PAEC apoptosis as measured by quantification of cleaved caspase-3 staining (**Fig. 4D,E,F**).

These data demonstrate that proinflammatory chemokines CXCL9 and CXCL10, which are upregulated in the absence of *Uty*, induce PAEC dysfunction *in vitro*.

Blocking the activity of CXCL9 and CXCL10 is sufficient to rescue PH in female MCT rats.

Since we identified that *Uty* expression regulates *Cxcl9* and *Cxcl10*, we hypothesized that *Uty* protection against PH is mediated through reduced levels of proinflammatory chemokines CXCL9 and CXCL10. We tested whether we could extend the mechanism of *Uty* protection to females with PH by blocking the effects of CXCL9 and CXCL10 using a small-molecular inhibitor (AMG487) targeting their shared receptor, CXCR3 (**Fig. 5A)**.

To better highlight protective effects, we used a more severe model of PH which was induced by MCT injection in rats. We tested our preclinical treatment strategy on gonad-

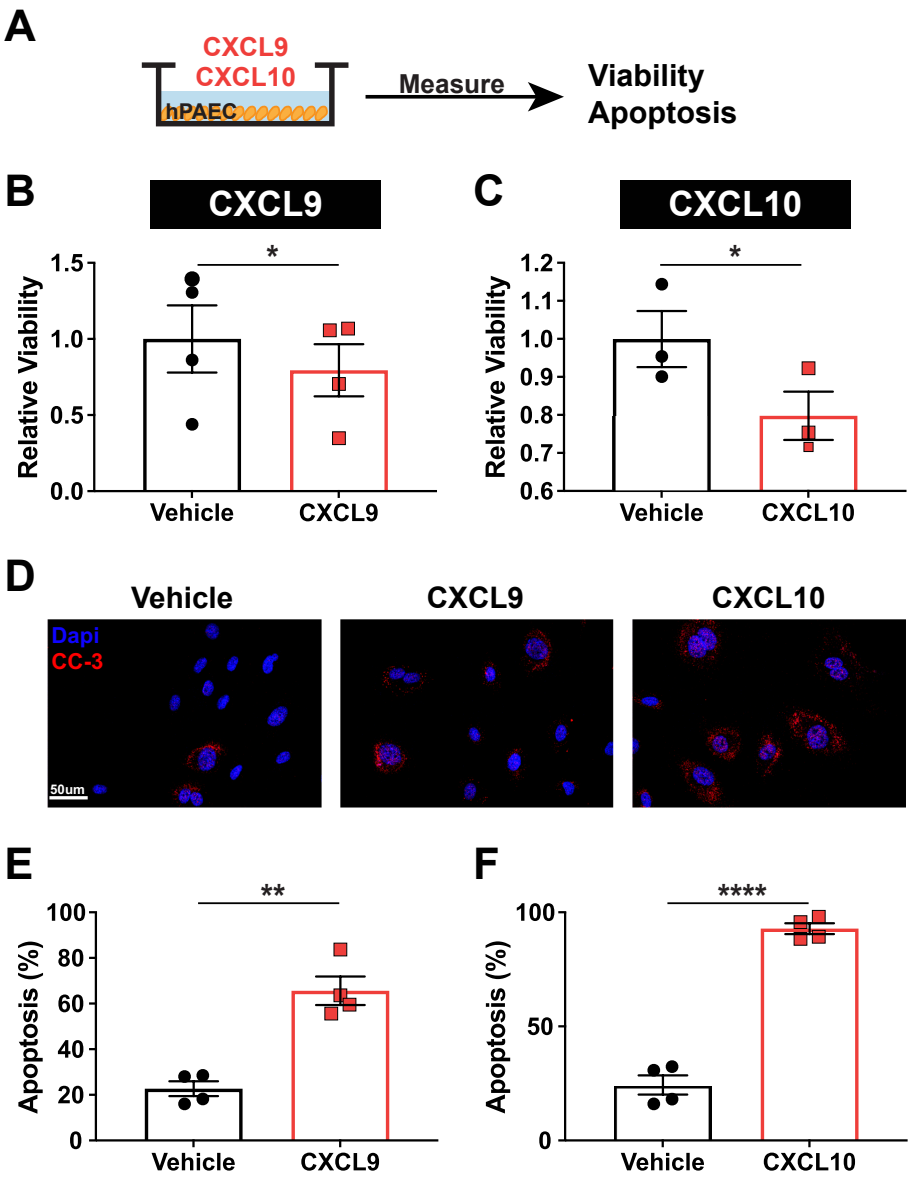

Fig. 4. CXCL9 and CXCL10 recombinant protein triggers pulmonary artery endothelial cell dysfunction. Schematic of human PA endothelial cell (PAEC) experiments (**A**). Relative viability of PAEC treated with CXCL9 (**B**) or CXCL10 (**C**) as measured by CCK-8 assay. Representative images of cleaved caspase-3 (CC-3, red) immunofluorescence in vehicle, CXCL9 and CXCL10 treated cells (**D**). Quantification of percent apoptosis measured in PAEC treated with CXCL9 (**E**) or CXCL0 (**F**) as measured by CC-3 immunofluorescence. **p<0.05, **p<0.01, ****p<0.0001*

intact females to determine if targeting CXCL9 and CXCL0 would be a viable treatment strategy for the most at-risk PAH population. We found that AMG487 sufficiently rescued PH development as treated rats had significantly lower RVSP (**Fig. 5B**). Fulton index measurements revealed that reduced RV pressure was concomitant with a reduction in RV hypertrophy (**Fig. 5C**). AMG487 treated rats exhibited increased PAAT (**Fig. 5D**) and RV fractional area change (RCFAC, **Fig. 5E**) indicative of lower PA pressure and improved RV function, respectively.

Since CXCL9 and CXCL10 induce PAEC apoptosis and reduce viability *in vitro* (**Fig. 4**), we aimed to delineate whether this cellular mechanism underlies the PH protection conferred by AMG487 treatment in rats. We found reduced EC apoptosis in the lungs of AMG487 treated rats, as observed by less cleaved caspase-3 expression in EC, indicating that blocking the actions of CXCL9 and CXCL10 reduced pulmonary vascular EC dysfunction (**Fig. 5F,G**).

Taken together, we demonstrate that inhibiting the shared CXCL9 and CXCL10 receptor in gonad-intact female rats is a viable preclinical treatment strategy as it reduced PH severity and lung vascular EC dysfunction.

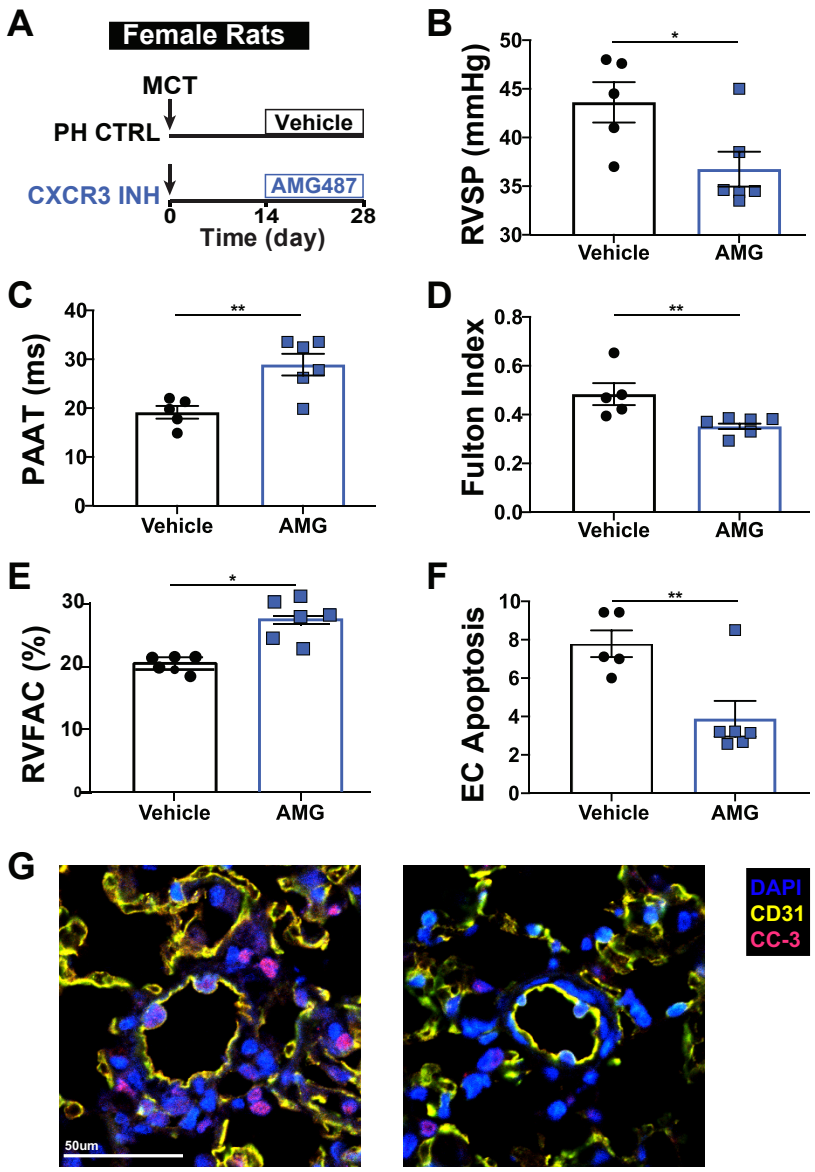

Fig. 5. Blocking the activity of CXCLl9 and CXCL10 is sufficient to rescue PH in female MCT rats. Schematic of in vivo experiments in female rats (**A**). RV systolic pressure (**B**), PA acceleration time (**C**), Fulton index (RV/LV+IVS) (**D**); and RV fractional area change (**E**) measured in AMG487 treated rats compared to vehicle controls. Quantification (**F**) and representative images (**G**) of apoptotic EC cells in CD31 (green) and cleaved-caspase 3 (CC3, pink) labeled sections from vehicle and AMG treated lungs**.** **p<0.05, **p<0.01*

Discussion

We recently demonstrated that ChrY is protective against experimental PH (13), and this study is the first to investigate the role of each ChrY gene expressed in the lung in protecting against PH. We found that KD of *Uty*, but not ChrY genes *Eif2s3y*, *Ddx3y* or *Kdm5d*, within the lung tissue of GDX male mice exposed to Hx increased PH severity thereby eliminating ChrY protection (**Fig. 1**). We examined *Uty* in the context of PH pathogenesis and found that loss of *Uty* expression in PH lungs resulted in an upregulation of proinflammatory chemokines *Cxcl9* and *Cxcl10* (Supplemental **Fig. E1**). Interestingly, *CXCL9* and *CXCL10* are upregulated in PAH patient lungs in a sex-specific manner with a more robust upregulation in female patients compared to males (**Fig. 2**). We found that stimulating human PAEC with exogenous CXCL9 or CXCL10 was sufficient to trigger PAEC dysfunction, a hallmark of PAH pathogenies (**Fig. 4**). Furthermore, blocking CXCL9 and CXCL10 action is a novel strategy for the treatment of PH because it reduced EC apoptosis and PH severity in female rats (**Fig. 5**). **Figure 6** summarizes our findings.

UTY is a member of the Jumonji family of H3K27 histone demethylases and is the ChrY homolog of the ChrX gene *Kdm6a (25)*. Although KDM6A is a known H3K27 demethylase (26), the demethylase activity of UTY is contested (20, 23, 24). While structurally very similar to KDM6A, UTY has undergone biochemical changes and is known to have unique functions (15, 20, 27). While Walport *et al* demonstrated residual demethylase activity in the human UTY enzyme (22), Lan *et al*, Hong et al, and Shpargel *et al* found no UTY enzymatic activity (20, 23, 24). We investigated whether UTY expression influences the H3K27 histone residue and found no significant difference in H3K27 methylation between

male WT and *Uty*-KO in whole lung tissue or isolated lung macrophages (**Supplemental Fig. E3).** Our data suggest that Uty induced protection is not mediated through epigenetic regulation of H3K27.

Regardless of its loss of catalytic activity, *Uty* expression was recently demonstrated to have widespread effects on autosomal gene expression (14) and is known to be involved in cardiac development (20). Dysregulation of *UTY* is implicated in bladder cancer (28) and its expression within macrophages is associated with increased atherosclerosis risk in men (15, 29, 30). Our study, however, is the first to identify and examine the role of *Uty* in the context of PH pathogenesis and its contribution to ChrY protection through mediating the expression of proinflammatory chemokines *Cxcl9* and *Cxcl10*.

Since *Uty* expression was previously found in macrophages (30) and CXCL9 and CXCL10 are proinflammatory chemokines secreted by immune cells (21), we confirmed colocalization of *Uty* with *Cxcl9* and *Cxcl10* in lung macrophages using RNAscope *in situ* hybridization (**Fig. 3**). *In situ* probes were used after we determined the commercially available UTY antibodies were not specific to UTY as they also recognized KDM6A which is expressed in both male and female lung tissue compared to UTY which is exclusive to males. We believe our RNAscope images are the first to depict *Uty*, but not *Kdm6a*, expression in mouse and human lung tissue (**Fig. E2B** of Supplement).

As *Uty* expression is colocalized with *Cxcl9* and *Cxcl10* in lung macrophages, we tested whether *Uty* expression in macrophages directly influences *Cxcl9* and *Cxcl10* production. We found M1 macrophages derived from *Uty*-KO BM have higher expression of *Cxcl9* and *Cxcl10* indicating that *Uty* expression in macrophages regulates *Cxcl9* and *Cxcl10* production (**Fig. 3**).

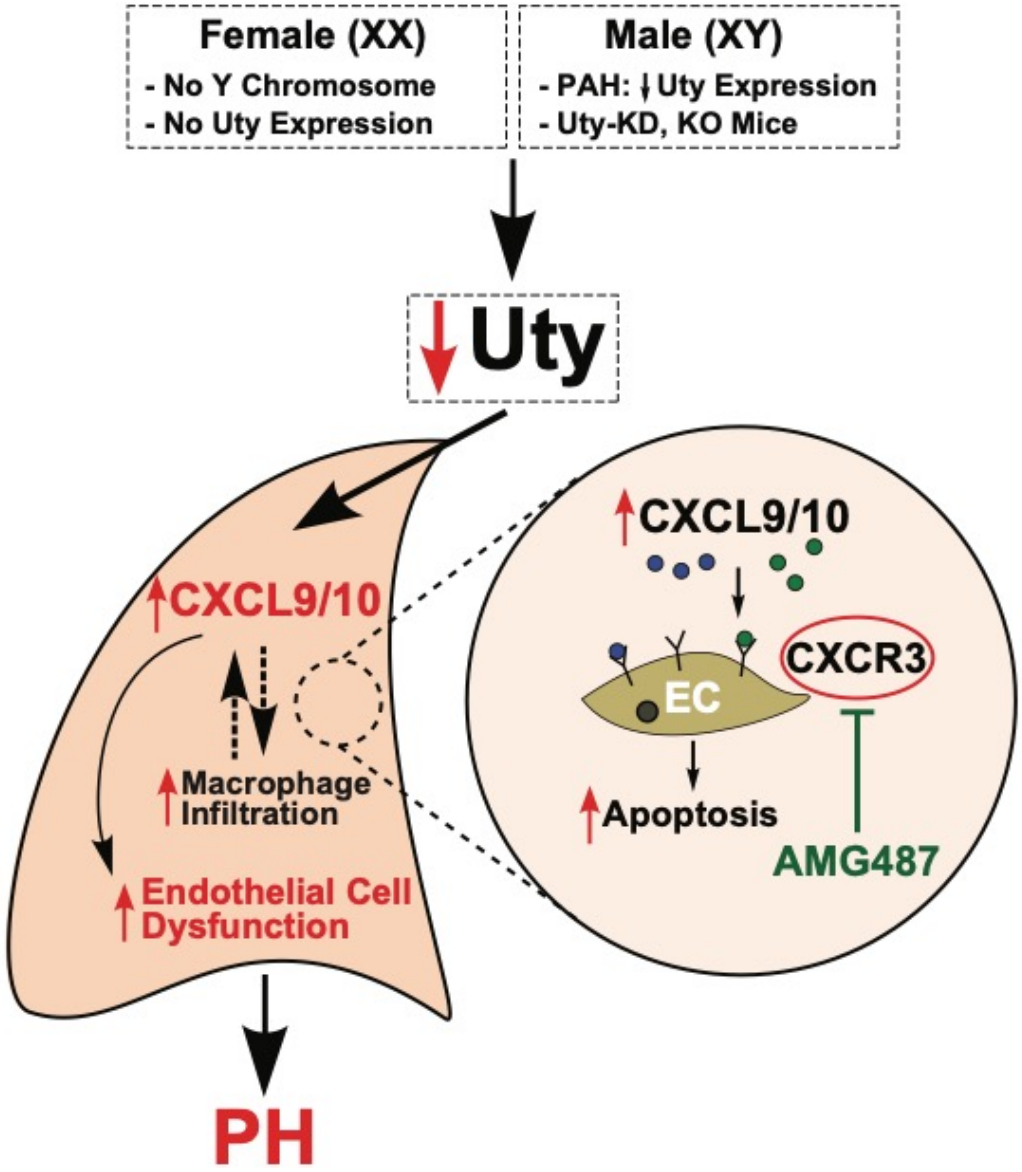

Fig. 6. Proposed mechanism of *Uty/Cxcl9/10* axis in PH pathogenesis. *Uty/UTY* absence (in females) or reduced expression (males with PAH or *Uty*-KD or KO mice) results in an upregulation of proinflammatory chemokines CXCL9 and CXCL10 in the lung. CXCL9 and CXCL10 trigger vascular EC dysfunction resulting in increased PH severity. Blocking CXCL9 and CXCL10 activity by pharmacologically inhibiting their shared receptor, CXCR3, is a novel treatment strategy to rescue PH development.

CXCL9 and CXCL10 are proinflammatory chemokines that act through the same G protein-coupled receptor, CXCR3 (21). Dysregulation of these small proteins, which are known to promote chemotaxis and immune cell differentiation, is associated with a myriad of systemic inflammatory diseases including cancers, scleroderma, pulmonary fibrosis and even PAH (31–35). Prior to this study, CXCL9 and CXCL10 were associated with PAH severity, and plasma levels of CXCL10 were found to be upregulated in PAH patients compared to healthy controls (33, 34). However, sex differences in *CXCL9* and *CXCL0* expression in PAH patients and the mechanism by which they promote PAH development were not known. We found that *CXCL9* and *CXCL10* are upregulated in human PAH lung tissues with a more robust upregulation in female patients compared to males (**Fig. 2**). This finding provides further evidence that complex sex differences exist within the immune response to PAH which could impact susceptibility, disease severity and treatment strategies between male and female patients (36, 37).

Previous studies found that upregulation of CXCR3 expression and the CXCL9 and CXCL10 ligands promote EC apoptosis (38–40). As pulmonary EC are one of the cell types most implicated in PAH, we investigated whether CXCL9 and CXCL10 recombinant protein directly influence PAH pathogenesis through PAEC. We found treating human PAEC with exogenous CXCL9 or CXCL10 was sufficient to trigger PAEC dysfunction as measured by decreased PAEC viability concomitant with increased apoptosis (**Fig. 4**). PAEC dysfunction, a hallmark of PAH pathogenies, is known to increase PH severity through dysregulation of angiogenesis, altered secretion of vasoactive agents and increasing vascular permeability (41).

Considering females, who lack ChrY, are particularly susceptible to PAH and that males with PAH have reduced expression of *UTY*, we tested whether blocking the activity of the downstream effector genes *Cxcl9* and *Cxcl10* could reduce PH severity akin to *Uty* protection. We found that blocking the activity CXCL9 and CXCL10 by inhibiting their shared receptor is a promising treatment strategy as it significantly reduced RV pressure and PH severity in female rats (**Fig. 5**). We also found *in vivo* inhibition of CXCL9 and CXCL10 reduced vascular EC apoptosis (**Fig. 5**).

We chose small-molecule inhibitor AMG487 for our preclinical *in vivo* studies for its efficacy and clinical relevance. Since *CXCL9* and *CXCL10* are upregulated in PAH patients and contribute to PH pathophysiology, blocking the activity of both through inhibiting their shared receptor provides an efficient treatment strategy. Furthermore, AMG487 compound has been tested as stable, specific, and safe in humans (42). Our preclinical experiments demonstrate that identifying and targeting Uty downstream autosomal effector genes is a promising and powerful way to extend ChrY protection against PAH.

We believe our current study presents the first and only data to directly explain ChrY protection against PH. A recent study from Yan, *et al* noted a connection between the testes determining ChrY gene *Sry* and the PAH-related gene *Bmpr2* in dermal fibroblasts (43); however, we do not believe this connection is sufficient to explain ChrY protection. Our previous study demonstrating ChrY protection utilized the Four Core Genotypes (FCG) mouse model which is well published and highlights striking sex chromosome effects in a variety of diseases (44). The FCG model allowed us to determine the effects of sex chromosomes, independent of gonadal effects, by producing gonadal male and

female mice with either XX or XY chromosomes (13). The model produces XY gonadal females lacking *Sry*, which we found to be protected against PH compared to XX females (13). Furthermore, *Sry/SRY* expression is not detected in lung tissue. *Uty* is therefore the only ChrY gene expressed in the lung tissue that we demonstrate to influence PH severity. Our study is the first to provide mechanistic insight into ChrY protection in PH and elucidates a novel therapeutic strategy targeting the activity of *Uty* downstream mediators upregulated in male and female PAH patients. We show that reduced expression of *Uty*, which is downregulated as a result of PAH in males and absent in females, upregulates proinflammatory chemokines *Cxcl9* and *Cxcl10* which promote PH severity through PAEC dysfunction and that targeting the activity of CXCL9 and CXCL10 reduces PH severity (**Fig. 6**). While these results explain why females, who lack *UTY*, are more likely to be diagnosed with PAH, they do not explain why males have worse prognoses and clinical outcomes once diagnosed (45). Since we show *Uty* expression is involved in mediating the immune response, we hypothesize that the reduction of *UTY* expression in male lungs, as a result of PAH, may trigger a harmful immune cascade and imbalance that causes severe PAH. As a future direction, we aim to characterize the immune system in WT and *Uty*-KO mice to identify additional downstream effector genes. Investigating additional *Uty* downstream genes and their connection to PAH pathogenesis, as demonstrated in this study, is a promising avenue of research that may provide additional insight and therapeutic approaches for this rare, terminal disease.

Supplemental Table E1: Primer sequences

Species	**Gene**	**Sequences** (5 ’to 3’)
Human	*RPLP0*	F: CAGGTGTTCGACAATGGCAG R: ACAAGGCCAGGACTCGTTTG
	CXCL9	F: GGTGTTCTTTTCCTCTTGGGC3 R: AACAGCGACCCTTTCTCACT
	CXCL10	F: GGTGTTCTTTTCCTCTTGGGC3 R: AACAGCGACCCTTTCTCACT
Mouse	*Actb*	F: ATGTGGATCAGCAAGCAGGA R: AAGGGTGTAAAACGCAGCTCA
	Uty	F: TGACCCTAATTTTTGCAGAGC R: TGAAACTGAATTTCGACACTGG
	Cxcl9	F: GGAGTTCGAGGAACCCTAGTG R: GGGATTTGTAGTGGATCGTGC
	Cxcl10	F: CCAAGTGCTGCCGTCATTTTC R: GGCTCGCAGGGATGATTTCAA
	MhcII	F: AAGGCATTTCGTGTACCAGTTC R: CCTCCCGGTTGTAGATGTATCTG
	Cd68	F: TGTCTGATCTTGCTAGGACCG R: GAGAGTAACGGCCTTTTTGTGA
Rat	*Gapdh*	F: GTGCCAGCCTCGTCTCATAG R: GGTAACCAGGCGTCCGATAC
	Uty	F: AGACGCTGTTGAACAAGGCA R: TTTGCTGCACCATGAGTTCCT
	Uty (Gene-specific RT)	AGGGGTCCTTCAGTCTCACA

Supplemental Figures

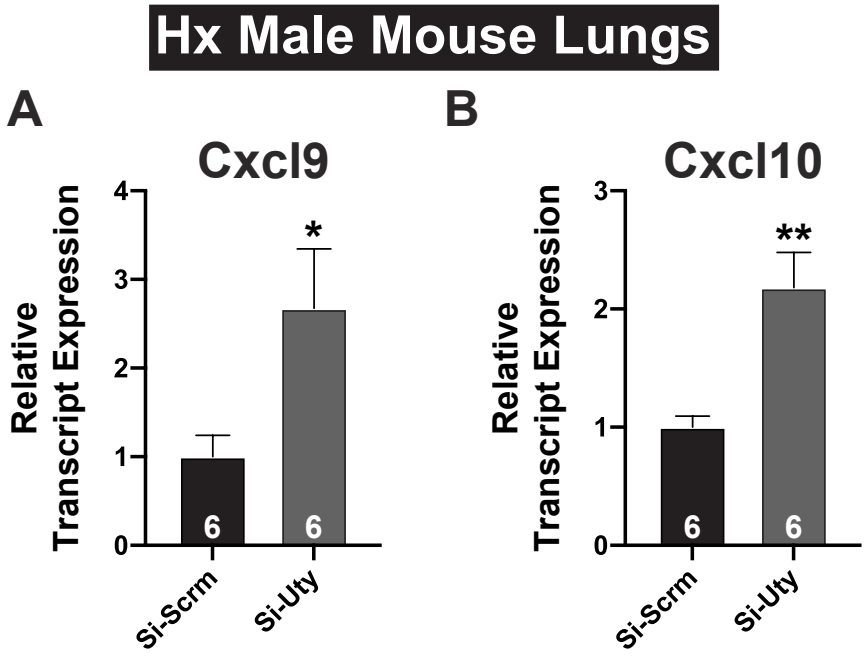

Fig. E1. Knockdown of *Uty* in Hx male mouse lungs results in increased *Cxcl9* and *Cxcl10* expression. Relative expression of (A) *Cxcl9* and (B) *Cxcl10* in GDX Hx mouse lungs that received intratracheal instillation of either Si-Scrm or Si-Uty. Analysis is based on RNAsequencing data from Si-Scrm and Si-Uty Hx mouse lungs. *p<0.05, **p<0.01

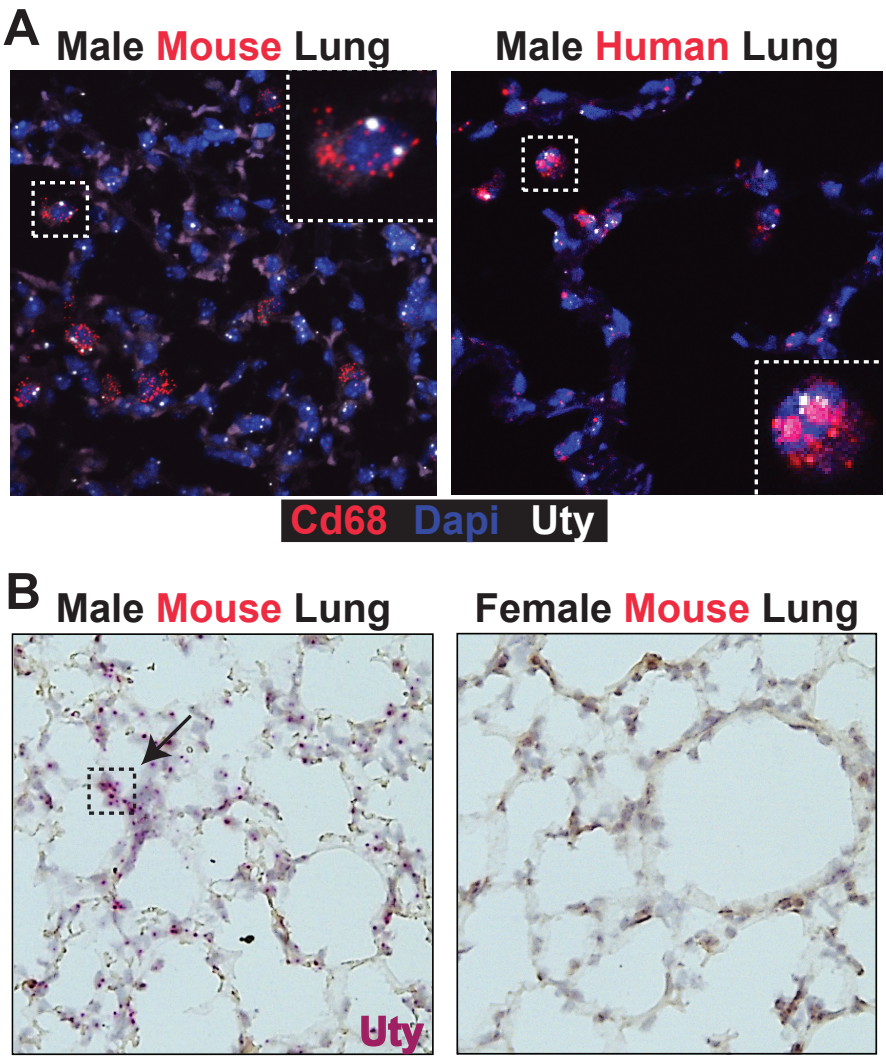

Fig. E2. *Uty* is expressed in macrophages in the lungs of male mice and humans. **A.** RNAscope fluorescent in situ duplex hybridization for *Uty/UTY* (white) and macrophage marker, *Cd68/CD68* (red) in mouse and human. Nuclei are stained with dapi (blue). Small dotted boxes are shown at higher magnification. **B.** RNAscope in situ hybridization shows *Uty* signal in male mouse lung (red dots) but not in female.

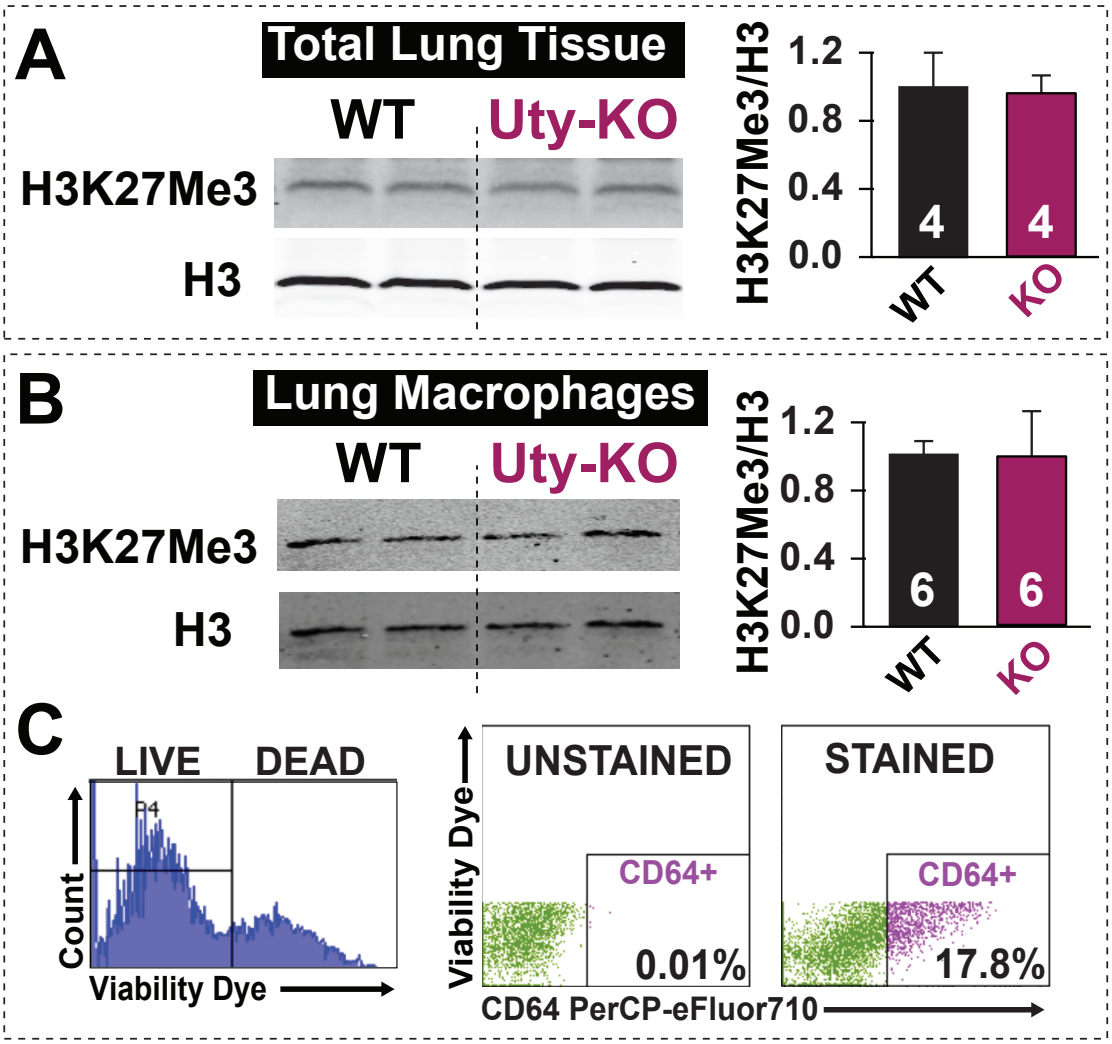

Fig. E3. *Uty* protection in PH is not mediated through an epigenetic modification. Western blot depicting relative expression of tri-methylated H3K27 normalized to H3 (n=4 or 6/group) in whole lung tissue (**A**) or lung macrophages (**B**) from *Uty*-KO and WT male mice. (**C**). Representative images of fluorescent activated cell sorting of live $CD64^{+}$ macrophages isolated from WT and UTY-KO lungs.

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Chapter 4: Upregulation of Endothelin-2 in pulmonary arterial hypertension in females could explain sexually dimorphic response to endothelin receptor antagonists

Introduction

Pulmonary arterial hypertension (PAH) is a pulmonary vascular disease caused by a decrease in pulmonary vascular compliance which leads to an increased pulmonary artery and right ventricular pressures. Reduced pulmonary vascular compliance is due to aberrant vasoconstriction and cellular dysfunction within the medial and distal pulmonary arterioles (1, 2). The idiopathic form of PAH exhibits complex sex differences where females are more likely to be diagnosed with PAH yet are more likely to respond to treatment (3, 4). While there is no cure for this rare but terminal disease, the mainstay of patient care, which include treatment with prostacyclin, nitric oxide and endothelial receptor antagonists (ERAs), largely focus on vasodilation and increase median patient lifespan from 5 to 7 years (5). Once diagnosed, females generally have a better prognosis and are more responsive to treatment with ERAs; however, the underlying cause of this sex disparity in ERA treatment remains unknown (3, 4).

We previously found that the male-specific Y chromosome gene *Uty* is protective against experimental pulmonary hypertension through attenuating the deleterious effects of proinflammatory chemokines Cxcl9 and Cxcl10 which trigger vascular endothelial cell (EC) dysfunction and more severe PH ((6), **Chapter 3**). In the current study, we found that ET-2, a member of the Endothelin family of potent vasoconstrictive peptides, is also upregulated in the lungs of mice with PH as a result of *Uty* knockdown. Additionally, we found CXCL9 and CXCL10 treatment was sufficient to trigger the secretion of ET-2 by dysfunctional vascular EC indicating that there is crosstalk between these downstream pathways that are regulated by *Uty* expression.

Endothelin-1 (ET-1), which shares the same ETA and ETB receptors, is known to be upregulated in PAH lungs and linked to pulmonary vasoconstriction and elevated PA pressure (5, 7); however, ET-2 has not been studied in the context of PAH. We found ET-2 is exclusively upregulated in the lungs of female PAH patients, but not male PAH patients, compared to healthy controls. We also found a sex-specific and PAH-specific expression of ET receptors, ETA and ETB. In healthy lung tissue, females exhibited increased expression of ETA compared to males; and in PAH lung tissue, females exhibited increased expression of ETB compared to males. Regardless, the expression of ET receptors seems to be upregulated in females compared to males, indicating that ET-2 signaling is increased in the lungs of PAH females compared to those of PAH males and healthy males and females.

Both ETA and ETB receptors are the targets of ERA therapies used to block the vasoconstrictive effects of ET-1 in PAH. It is known that females, while more likely to be diagnosed with PAH, exhibit a better prognosis and respond more strongly to ERA treatment. Sex hormones and sex chromosomes are known to contribute to the marked sex differences in PAH disease susceptibility; however, the underlying cause of sex-specific responses to ERA therapies is not known. Our findings are the first to suggest sex differences in response to ERA treatment in PAH patients may be due to increased ET-2 expression as well as higher expression of ET receptors in female PAH patients compared to male patients driven by lack of *UTY* expression.

Methods

Uty knockdown in mice

Uty knockdown experiments in mice with hypoxia-induced PH were performed in a previous study described in **Chapter 3**. Briefly, male C57BL/6J mice aged 6-8 weeks were purchased from Jackson Laboratories and underwent gonadectomy (GDX) surgery under isoflurane anesthesia to remove gonads. After 30 days, mice were randomly divided into two groups where they received recurring intratracheal instillation of either short interfering RNA (siRNA) targeting a Uty or a scrambled siRNA every 5 days (1nmol/instillation, *Dharmacon Accell mouse SMARTpool:* Si-Uty: E-046843-00-0010; Si-Scrm: siGENOME Non-Targeting siRNA Pool #2, D-001206-14-05). Mice were housed in hypoxic (Hx, 10% oxygen) conditions for three weeks while receiving instillations. Upon the termination of these experiments, mouse lungs were quickly excised, perfused with PBS, snap snap frozen in liquid nitrogen, and stored in -80^{o}C until use.

Bioinformatic Analysis

Total lung RNA was isolated using Trizol extraction and purified using RNeasy Mini Kit (Qiagen). Libraries for RNA-seq were prepared by the Technology Center for Genomics & Bioinformatics at UCLA and sequenced using paired-end Hiseq 3000 (Illumina). HISAT2 version 2.1.0 was used to align RNA-seq reads to the Mus musculus genome (mm10, Ensembl 84). StringTie version 1.3.3b was used to assemble RNA-seq alignments into transcripts and estimate expression levels of all genes detected. Differential expression analysis was performed using DESeq2 R package version 1.25.16. Differently expressed genes (DEGs) with false discovery rate <0.1 were

considered statistically significant. Pathway enrichment analysis and clustering was performed using Cytoscape software (8).

Human samples:

Human samples were obtained from UCLA lung transplant group or Pulmonary Hypertension Breakthrough Initiative. Control samples were collected from failed donor lung tissue or resected tissue during lung biopsy. Patients in control group do not exhibit PAH. PAH lung samples were collected from patients with a clinical PAH diagnosis at the time of lung transplantation. All tissue samples were snap frozen in liquid nitrogen and stored in -80^{o}C until use.

Real-time qPCR

RNA was isolated from tissue samples using Trizol reagent (ThermoFisher). Mouse and human RNA were reverse transcribed with polydT primers using Omniscript reverse transcription kit (Qiagen). Real-time quantitative PCR was performed on polyA+ cDNA with primers for using iTaq Universal SYBR® (Bio-Rad). *Actb* (mouse) and *RPLP0* (human) were used as housekeeping genes.

Cell studies

Human pulmonary artery EC from a healthy male were incubated at 37^{o}C with human recombinant CXCL9 or CXCL10 protein or vehicle. After 72 hours, ET-2 expression in the conditioned culture medium of treated EC was measured by ELISA (FineTest).

Statistics

For comparisons of two normally distributed, independent groups, we used an unpaired t-test. For comparisons of sex differences, we compared male and female values from each experimental group (healthy or PAH). For a comparison between two nonparametric groups, we used a Mann-Whitney test. A significance level less than 5% ($p<0.05$) was deemed statistically significant.

Results

Knockdown of Y chromosome gene *Uty* in the lungs of PH mice results in an upregulation of ET-2 expression.

We previously found that the male-specific Y chromosome is protective against PH development and that knocking down Y chromosome gene *Uty* within the lungs of PH mice is sufficient to eliminate this protection (**Chapters 2, 3**). We Integrated RNA sequencing data from *Uty* knockdown mouse lungs with an online microarray dataset of male and female human PAH lung samples which revealed Endothelin-2 (ET-2) is upregulated in both *Uty* knockdown mice compared to wildtype and PAH females compared to males (**Figure 1**). We validated that the lungs of mice with *Uty* knockdown exhibited elevated ET-2 expression compared to wildtype PH controls (**Figure 2A**). Expression of ET-1 and ET receptors ETA and ETB did not change as a result of Uty knockdown in PH mouse lungs (**Figure 2B**).

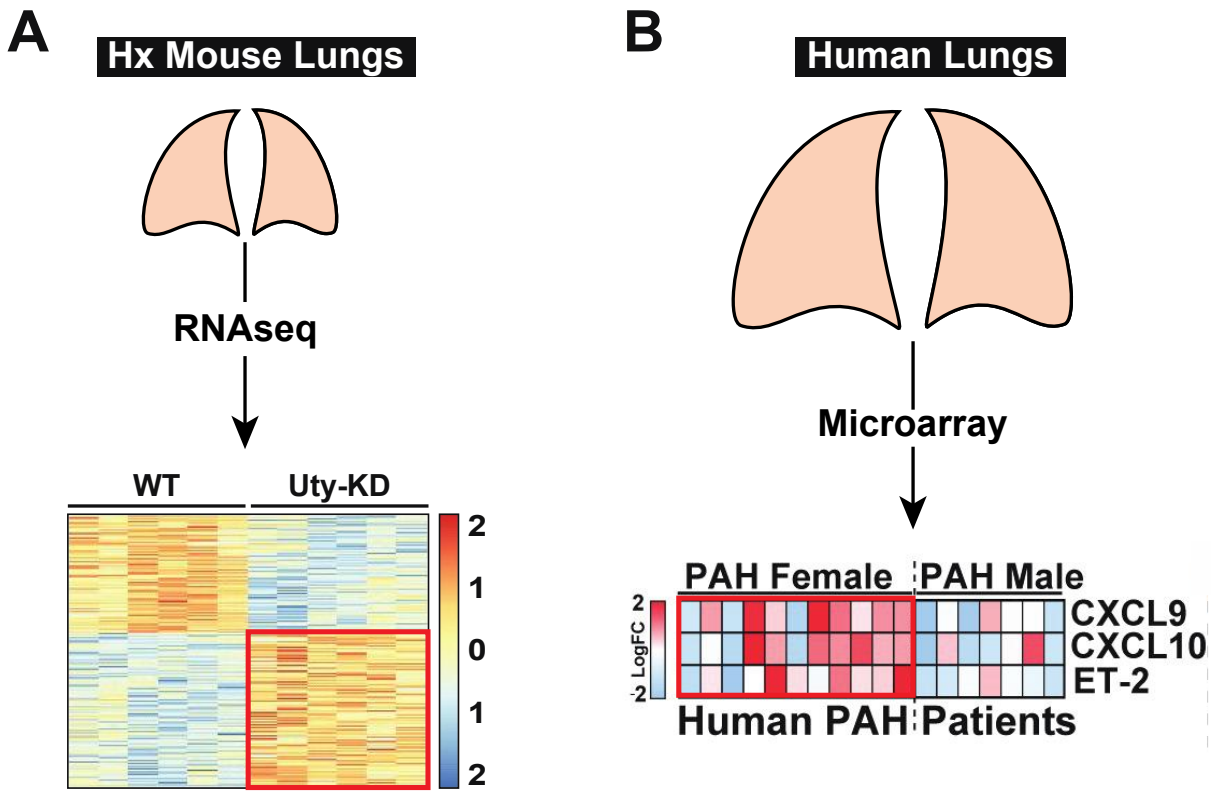

Figure 1. Schematic of bioinformatic analysis to identify Uty- and sex-specific candidate genes. A. A heatmap depicting 523 differently expressed genes as a result of Uty knockdown (Uty-KD) in hypoxic mouse lungs. **B.** A heatmap depicting genes that are expressed higher in both Uty-KD lungs (vs wildtype (WT)) and female PAH patients (vs male PAH patients) (*red boxes*).

ET-2 expression is upregulated in the lungs of PAH female patients but not male PAH patients.

After identifying the upregulation of ET-2 in PH mouse lungs as a result of reduced expression of the Y chromosome gene *Uty*, we examined the effect of sex on ET-2 expression in human PAH lung tissue. We found female PAH patients exhibited elevated ET-2 lung tissue expression compared to healthy female patients (**Figure 3**). ET-2 upregulation in PAH patient lungs was not observed in males (**Figure 3**). Thus, elevated ET-2 expression in PAH lung tissue is sex-specific and exclusive to female patients.

ET receptor expression is upregulated in healthy female lungs compared to healthy males and altered in PAH in a sex-specific manner.

Following the identification of a robust, female-specific elevation of ET-2 as a result of PAH, we examined the expression of ET receptors, ETA and ETB, in the lung tissue of males and females with and without PAH. We found that in healthy lung tissue, ET receptors are elevated in females compared to males with a significant upregulation of ETA and a trend toward upregulation of ETB (**Figure 4A**). We identified that the sex differences in ET receptor expression were also altered as a result of PAH. In male and female PAH lung tissue, female upregulation of ETA is no longer significant; however, both ETA and ETB trend toward elevated expression compared to males (**Figure 4B**).

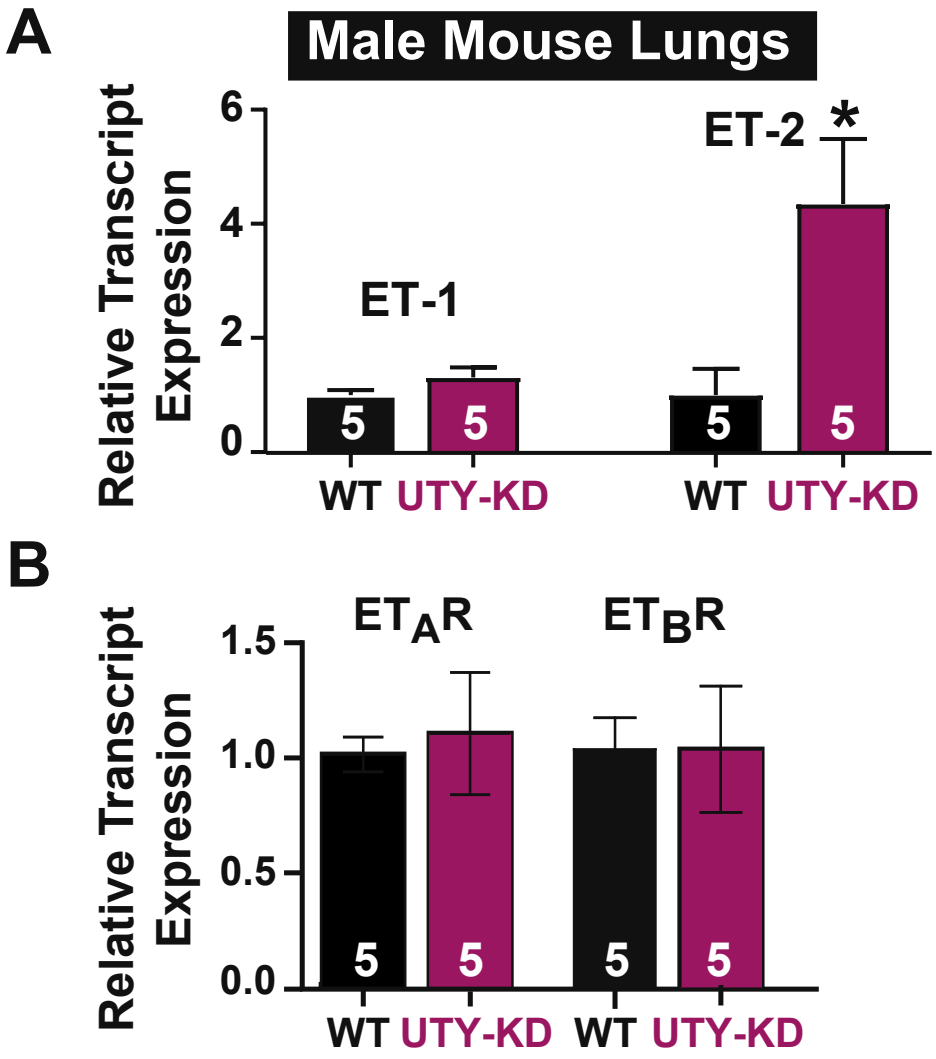

Figure 2. Knockdown of Y chromosome gene *Uty* in the lungs of PH mice results in an upregulation of ET-2 expression. A. Expression of ET-2, but not ET-1, is increased in the lungs of *Uty* knockdown mice vs. wildtype (WT) controls. *p<0.05. **B**. Expression of ETA and ETB receptors is not altered as a result of *Uty* knockdown in the lung.

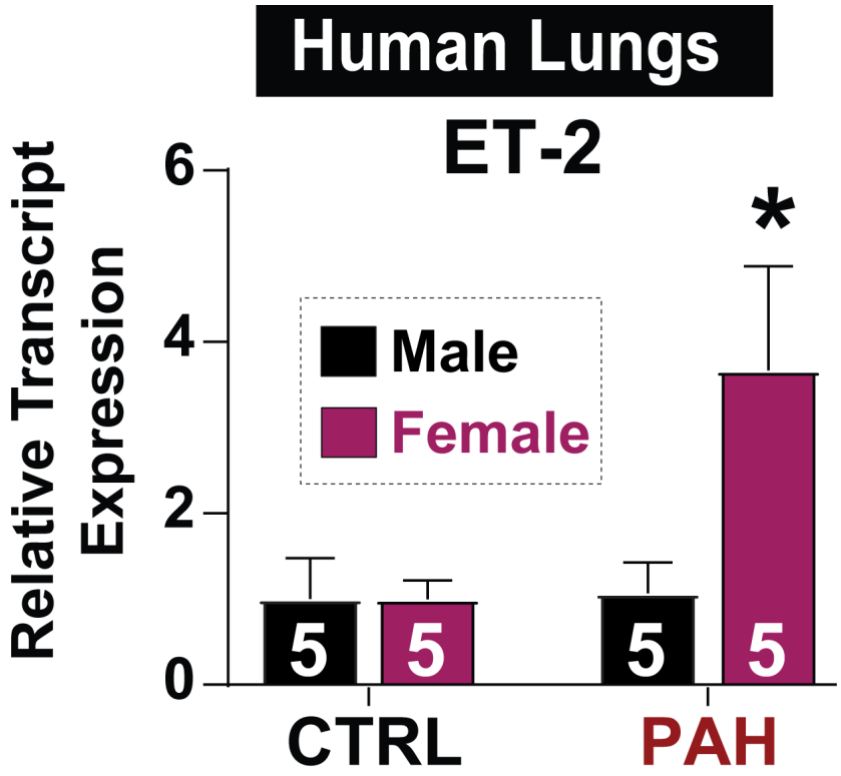

Figure 3. ET-2 expression is upregulated in the lungs of PAH female patients but not male PAH patients. Expression of ET-2 is increased exclusively in the lungs of PAH women, but not in men. Expression in females is normalized to corresponding males. *p<0.05.

Taken together, female healthy and PAH lung tissue have slightly elevated expression of ET receptors compared to males, and this sex difference in ET receptors is altered, but not eliminated, by PAH development.

Vascular endothelial cell dysfunction by treatment with Cxcl9 and Cxcl10 triggers ET-2 production.

EC dysfunction, which is characteristic of PAH, is known to contribute to expression of endothelins. Since we previously identified the proinflammatory chemokines CXCL9 and CXCL10 as a sex-specific source of EC dysfunction that are upregulated in PAH females compared to PAH males (**Chapter 3**), we hypothesized that CXCL9- and CXCL10-induced EC dysfunction could contribute to the increased ET-2 expression found in female PAH lungs. We measured the production of ET-2 by EC in the presence of elevated CXCL9 and CXCL10 and found human pulmonary artery EC incubated with exogeneous human CXCL9 and CXCL10 exhibited increased ET-2 secretion as measured by ELISA (**Figure 5**).

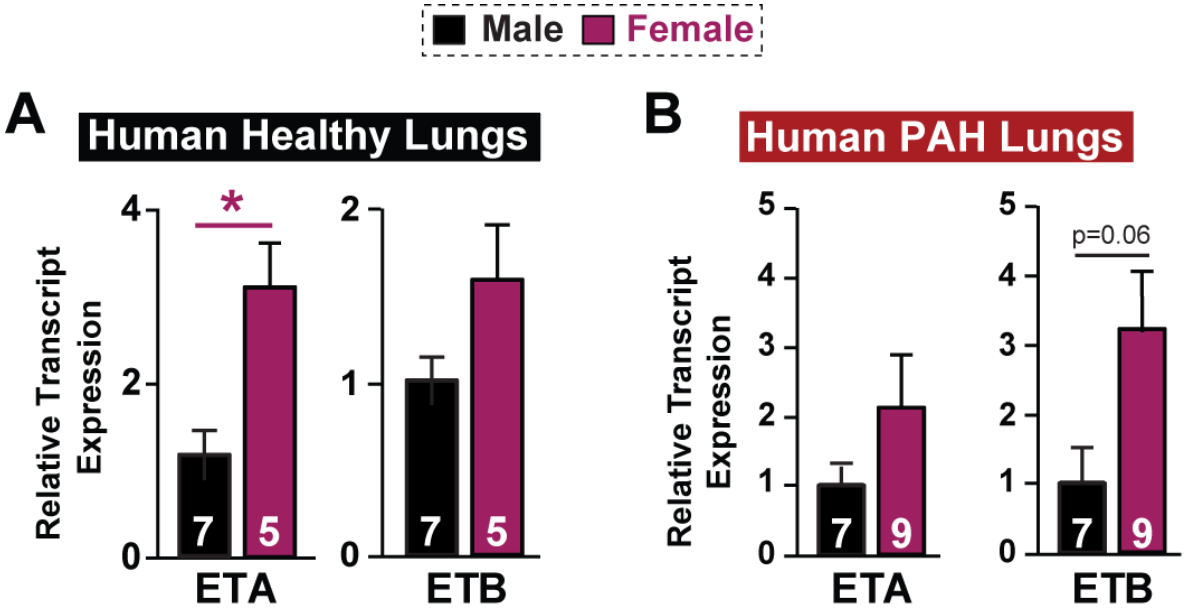

Figure 4. Endothelin receptor expression is upregulated in healthy female lungs compared to healthy males and altered in PAH in a sex-specific manner. A. In healthy human lungs, expression of ETA receptor is significantly higher in females compared to males (n=5-7/group). *p<0.05. **B.** In human PAH lungs, expression of ETB receptor is higher (though not significant, p=0.06) in PAH females compared to PAH males.

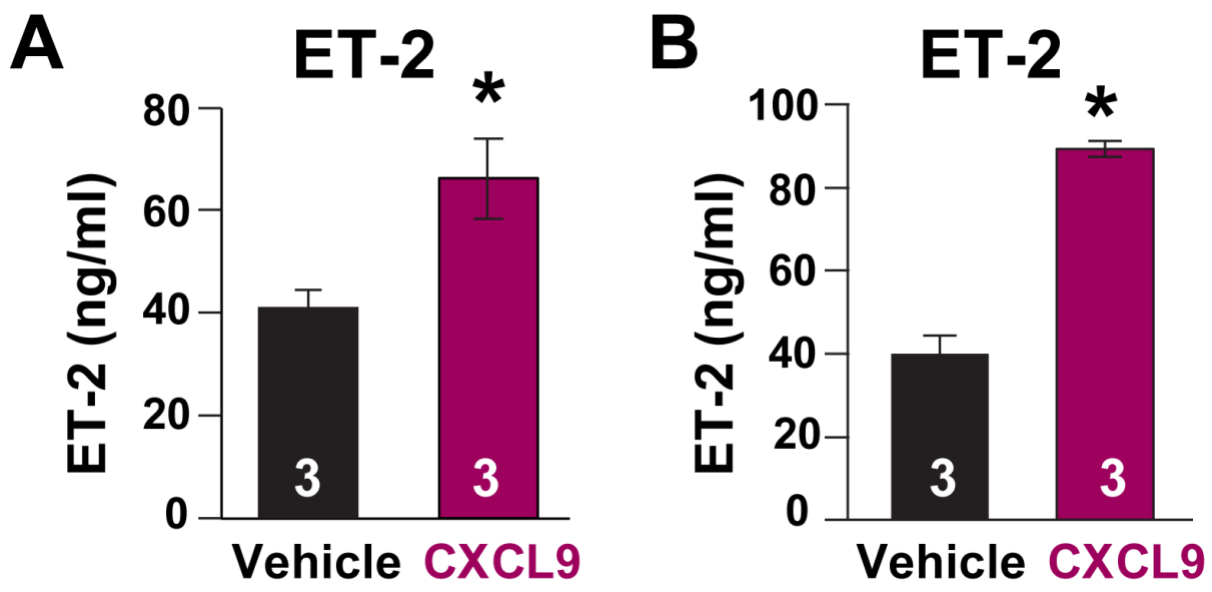

Figure 5. Human recombinant CXCL9 and CXCL10 chemokines promote ET-2 secretion by vascular endothelial cells. Quantification of ET-2 secretion in cultured medium of PAEC treated with Cxcl9 (**A**), or CXCL10 (**B**) as measured by ELISA. $p<0.05$ vs vehicle.

Discussion

This study identified a link between the expression of the protective Y chromosome gene Uty and the expression of ET-2 in the lung. Male mice with reduced *Uty* expression and female PAH patients with no *UTY* expression both exhibit elevated ET-2 expression in lung tissue. We found that both healthy and PAH females have increased expression of ET receptors compared to their male counterparts. This indicates that ET-2 signaling is increased in PAH females and may contribute sex-bias in disease susceptibility, progression, and response to treatment. Furthermore, we found crosstalk between the Cxcl9 and Cxcl10 inflammatory pathways (**Chapter 3**) and ET-2 upregulation, all of which we identified to be inversely related to Uty expression and correlated with PH disease severity.

While the powerful vasoconstrictive peptide ET-1 is known to be upregulated in male and female PAH patents, ET-2 upregulation in PAH female lungs has not been reported. Like ET-1, ET-2 signaling is also associated with vasoconstriction. In this way, we believe that elevated ET-2 signaling in female PAH lungs results in increased vasoconstriction in female patients. Previous studies also found additional cellular responses are altered as a result of ET-2 binding. ET-2 is known to inhibit EC proliferation and new vessel formation in the eye (9) and promote smooth muscle cell (SMC) proliferation in myometrium (10). ET-2 peptide, whose structure is similar to that of a CXC chemokine (11), has also been found to act as a proinflammatory signaling factor to recruit macrophages (12). All of these cellular pathways are known to contribute to PAH development. EC apoptosis, reduced angiogenesis, and aberrant SMC proliferation are hallmarks of the vascular dysfunction associated with PAH pathogenesis including formation of plexiform lesions, loss

pulmonary vascularization, and the muscularization and hypertrophy of distal arterioles (1, 13). Furthermore, elevated inflammation and altered macrophage ratio in the lung have been associated with, and even found to play a causal role in, PAH development (14, 15). Because of the overlap between ET-2 functions and PAH pathogenic pathways, we believe there is evidence to support a pathogenic role for ET-2 in PAH pathogenesis. We hope to continue to study the effects of ET-2 within the context of PAH and shed light on these important contributions to the disease.

Since ET-2 elevation is exclusive to females with PAH, alterations in ET-2-driven pathways are sex-specific. ET-2 shares the same receptors as ET-1, which are inhibited as a common therapy for PAH patients. Treatment with ERAs exhibit sex differences in efficacy since females are known to be more responsive to treatment than males (3, 4). We believe that our data help explain the molecular mechanism behind this sex difference. Since elevated ET-2 levels contribute to excess vasoconstriction and potentially other pathogenic pathways associated with PAH development, blocking the activity of ET-2 through ERA treatment attenuates these dysfunctional pathways which are excusive to females. In this way, female PAH patients report greater effects from ERA treatment since the benefit is multifactorial through the inhibition of both ET-1 and ET-2 driven pathogenic pathways.

Our data indicate that sex-specific elevation of ET-2 in PAH females is a result of sex differences imparted by sex chromosomes. ET-2, but not ET-1, is upregulated in male PH mice as a result of knockdown of the Y chromosome gene *Uty*. We previously found that Uty protects against PH and that loss of or no Uty expression results in elevated expression of proinflammatory chemokines Cxcl9 and Cxcl10 and increased vascular

endothelial cell dysfunction (**Chapter 3**). We found evidence of crosstalk between these pathways since incubation of EC with CXCL9 or CXCL10 was sufficient to increase ET-2 production by dysfunctional EC. Additionally, we previously found that Uty is expressed in the lung by macrophages, and proinflammatory macrophages lacking *Uty* produce increased levels of *Cxcl9* and *Cxcl10* (**Chapter 3**). Since ET-2 is a known macrophage chemoattractant, we believe lack of Uty in the lungs results in a proinflammatory positive feedback loop regulated by increased expression of Cxcl9, Cxcl10, and ET-2. In this way, we believe that a combination therapy blocking both the effects of Cxcl9, Cxcl10, and ET receptors could provide additive benefit to PAH patients compared to treatment with ERAs alone. Testing the potential synergistic effect between these two treatment strategies is another future direction for this project.

In summary, our working model is that Uty, a male-specific Y chromosome gene, protects against PH development. The absence of Uty in females results in more severe PH, in part, through increased expression of ET-2. Elevated ET-2 expression in female PAH patients contributes to PAH severity through increased vasoconstriction, vascular dysfunction, and inflammation. Blocking the action of ET-2 via ERAs reduces the severity of PH in a sex-specific manner rendering ERA treatment more effective in females (**Figure 6**). This work identifies a novel gene associated with PAH development, provides an explanation to the marked sex-disparities in ERA treatment, and highlights a potential combination treatment strategy for PAH patients.

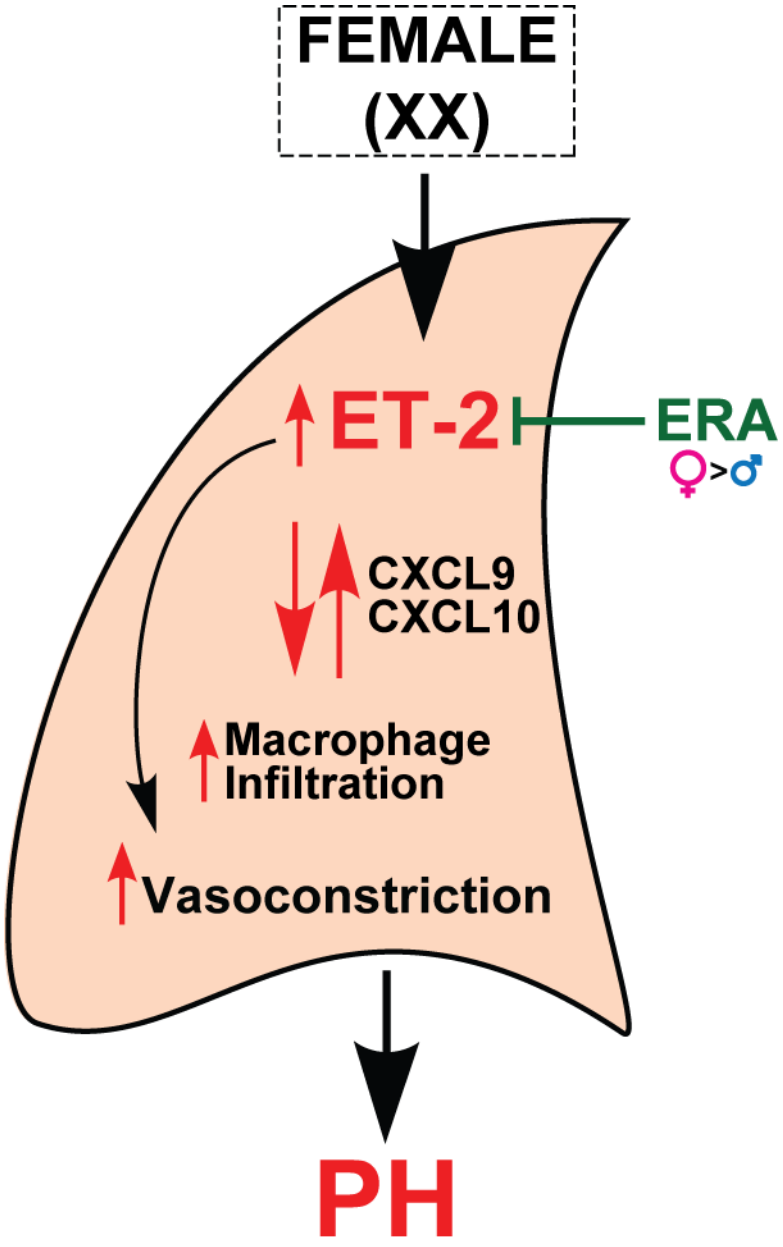

Figure 6. Schematic of ET-2 overexpression in female PAH lungs. Females with PAH exhibit elevated ET-2 expression in their lungs with contributes to worsening PAH through increased vasoconstriction and inflammation. Blocking the activity of ET-1 and ET-2 through treatment with endothelin receptor antagonists (ERAs) exhibits a greater response in female PAH patients compared to males.

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Chapter 5: Conclusions and Future Directions

Conclusions

PAH is a rare but terminal pulmonary vascular disease originating in the pulmonary arterioles that exhibits a marked female predominance. While PAH is subdivided based on shared or overlapping characteristics, the exact cellular mechanism by which pulmonary arteriole remodeling is triggered remains largely elusive. The work included in this dissertation presents a novel approach to study PAH pathological triggers using a hypothesis-based approach centered around the associated sex differences.

The original hypothesis explored in this dissertation was an attempt to better understand the complex sex differences found in PAH both clinically and experimentally. While females are up to 4x more likely to be diagnosed with PAH, they suffer from a milder form of the disease and are more responsive to treatment than males (1, 2). Experimental investigation into sex differences associated with PAH are paradoxical as well, and the "Estrogen Paradox in PAH" is a term coined to describe the culmination of decades of research being conducted on the role of estrogens in an attempt to understand these complex sex differences. In short, estrogens were found to be largely protective in animal models of PH despite female sex being a risk factor for the development of PAH. Estrogens have been shown to prevent and rescue PH development in animal models of PH by attenuating pulmonary vascular remodeling and reducing RV dilation and hypertrophy (3–6). Taken together, it is clear that other sex biasing factors influence PAH predisposition and disease severity, and this dissertation is a compilation of work highlighting the previously unknown sex chromosome contributions to this disease.

In the absence of circulating gonadal hormones, we found that the male-specific Y chromosome is protective against hypoxic PH in mice (7). This study was made possible

by the use of the Four Core Genotypes mouse model, which allowed us to parse apart the effects of sex chromosomes independent of gonadal sex, and the XY* mouse model, which allowed us to distinguish if our sex chromosome effect was due to the number of X chromosomes or the presence of a Y chromosome (**Chapters 1, 2**). Following gonadectomy, mice with XY chromosome complement exhibited reduced RV pressure and less pulmonary vascular muscularization compared to hypoxic gonadal male and female mice with XX chromosome complement. We then identified that this protective effect was due to the presence of the Y chromosome, and not a result of X chromosome dosage, since hypoxic gonadectomized mice with a Y chromosome (XY and XXY) exhibited decreased RV pressure compared to those without a Y chromosome (XO and XX) regardless of the number of X chromosomes (**Chapter 2**). This exciting finding led us to identify which genes encoded by the Y chromosome are expressed in the heart and lung tissue that could contribute to PH protection. We found four coding genes were expressed in these tissues (*Kdm5d, Uty, Ddx3y* and *Eif2s3y*), three of which were also conserved in human tissues (*KDM5D*, *UTY*, *DDX3Y*). Interestingly, our bioinformatic analysis of online human microarray data revealed two of these genes, *KDM5D* and *UTY*, were downregulated in lung tissue isolated from PAH males compared to healthy male lung tissue (**Chapter 2**).

By knocking down each Y chromosome gene expressed in gonadectomized hypoxic male mouse lungs via recurring intratracheal instillation of siRNA, we found that reduction of *Uty*, but not the other three Y chromosome encoded genes, was associated with loss of Y chromosome protection against PH (**Chapter 3**). In light of this evidence, we believe Uty to be the Y chromosome encoded gene responsible for Y chromosome protection.

While Uty is a member of the Jumonji family of histone demethylase enzymes which are known to remove methyl groups from histone 3 lysine residue 27, our studies did not find evidence of histone modification at this residue as a result of *Uty* expression. This finding is supported by the literature where multiple studies reported a loss of demethylase activity in the Uty gene (8–10), potentially as a result of the increased rate of mutation associated with Y chromosome genes due to the biological redundancy of X chromosome paralog genes and reduced evolutionary pressure (11). Understanding the mechanism of Uty gene action within the context of PH is a focus of future directions, but molecular pathways downstream of Uty expression that contribute to PH severity have already been elucidated by the studies in this dissertation.

We found that knockdown of Uty in hypoxic mouse lungs resulted in profound gene expression changes and inflammatory pathways being particularly perturbed (**Chapter 3**). Out of these genes, we identified the proinflammatory chemokines Cxcl9 and Cxcl10 as upregulated in both Uty knockdown mouse lungs and human female PAH patients, which have no Uty expression, compared to male patients. Indeed, the proinflammatory chemokines Cxcl9 and Cxcl10 are both upregulated in male and female PAH patient lungs compared to healthy control tissue with a more robust upregulation in female PAH lungs compared to male PAH lungs.

Using fluorescent *in situ* hybridization in mouse and human male lung tissue sections, we showed that *Uty* was not highly enriched in vascular cell types but was expressed by *Cd68*$^{+}$ lung macrophages. We further identified that *Uty*, *Cxcl9*, and *Cxcl10* transcripts colocalized in *Cd68* expressing macrophages in the lungs and found a *Uty* expression directly affects *Cxcl9* and *Cxcl10* production within macrophage cells. Proinflammatory

bone marrow derived macrophages from Uty total knockout mice exhibited increased *Cxcl9* and *Cxcl10* expression when compared to those isolated from age-matched wildtype mice with Uty expression (**Chapter 3**). Since CXCL9 and CXCL10 chemokines elicit a proinflammatory response in tissues through binding the shared CXCR3 receptor on cells, including immune cells, we believe increased production of these chemokines in Uty-null macrophages produces a positive feedback loop by recruiting more Uty-null macrophages.

In addition to inflammatory cells, endothelial cells are also known to express the CXCR3 receptor. We further linked increased Cxcl9 and Cxcl10 expression to PAH pathogenesis using an *in vitro* assay of vascular endothelial cell dysfunction. Exogenous CXCL9 and CXCL10 treatment in healthy human endothelial cells isolated from the pulmonary artery resulted in cellular dysfunction as characterized by decreased viability and increased apoptosis (**Chapter 3**). Damaged endothelial cells are a hallmark of PAH pathogenic response and also trigger a vascular inflammatory response by expressing proinflammatory chemokines in turn (12, 13). We believe this is a catalyst for vascular remodeling in the lung that is compounded by vascular inflammation as a result of reduced or no Uty expression.

We found that *in vivo* inhibition of the activity of the proinflammatory chemokines downstream of Uty was sufficient to extend Y chromosome protection to females in a preclinical animal model. Administration of the small-molecule CXCR3 antagonist, AMG487, twice a day was able to rescue PH development in intact female rats with monocrotaline-induced PH (**Chapter 3**). We determined that AMG487 treatment was able to recue PH development, at least in part, by blocking Cxcl9- and Cxcl10-induced

vascular endothelial cell dysfunction since histological sections from treated lungs exhibited less vascular endothelial cell apoptosis compared to vehicle treated control lungs. We believe that this already FDA approved compound has clinical potential which is discussed in the future directions section below.

We also found a Uty-specific and sex-specific upregulation of the gene that encodes the ET-2 peptide (**Chapter 4**). ET-2 is a member of the vasoconstrictive endothelin family of peptides which includes ET-1. ET-1 is known to be upregulated in the lungs of PAH patients and is a target of many mainstay PAH therapies used clinically; however, the role of ET-2 was not previously known studied in PAH. While the expression of ET-1 is unchanged in the lungs of hypoxic mice as a result of *Uty* knockdown, ET-2 is upregulated in *Uty* knockdown lungs versus experimental control lungs. We found ET-2 upregulation in female PAH lungs compared healthy female lungs; however, we did not see an increase of ET-2 in male PAH lungs compared to healthy male lungs. Interestingly, it is known that female PAH patients are more responsive to endothelin receptor antagonist treatment than male patients (2), but the source of this sexual dimorphic response is not known. We hypothesize that ET-2 plays a female-specific pathogenic role in PAH development, and that blocking the activity of ET-2 through administration of an endothelin receptor antagonist is more effective in females since it attenuates the activity of both ET-1 and ET-2 signaling by blocking their shared endothelin receptors. Vasoconstrictive agents, like ET-1 and ET-2, contribute to increased pressure in the pulmonary vasculature which worsens PAH severity (12, 14).

Interestingly, we found evidence of crosstalk between ET-2 expression and the Uty downstream proinflammatory chemokines, Cxcl9 and Cxcl10. CXCL9 and CXCL10

treated vascular endothelial cells express increased levels of ET-2 peptide (**Chapter 4**). The structure of ET-2 peptide is strikingly similar to the proinflammatory chemokines, and it is known that ET-2 elicits an inflammatory response as well (15, 16). We think this is further proof of a proinflammatory feedback loop in the lungs that is mitigated by Uty expression in healthy males and pathogenic in males or females with reduced or no Uty expression, respectively.

Taken together, this dissertation provides compelling evidence that the Y chromosome gene Uty influences the sexually dimorphic predisposition and clinical response in PAH and highlights a sex-specific immune response in PAH patients that influences disease development which can be targeted as a novel therapeutic pathway (**Figure 1**). Further investigation into the molecular mechanism of Uty action and its downstream pathways will likely lead to even greater understanding of the PAH disease process and associated sex differences. These future studies are outlined in the following section.

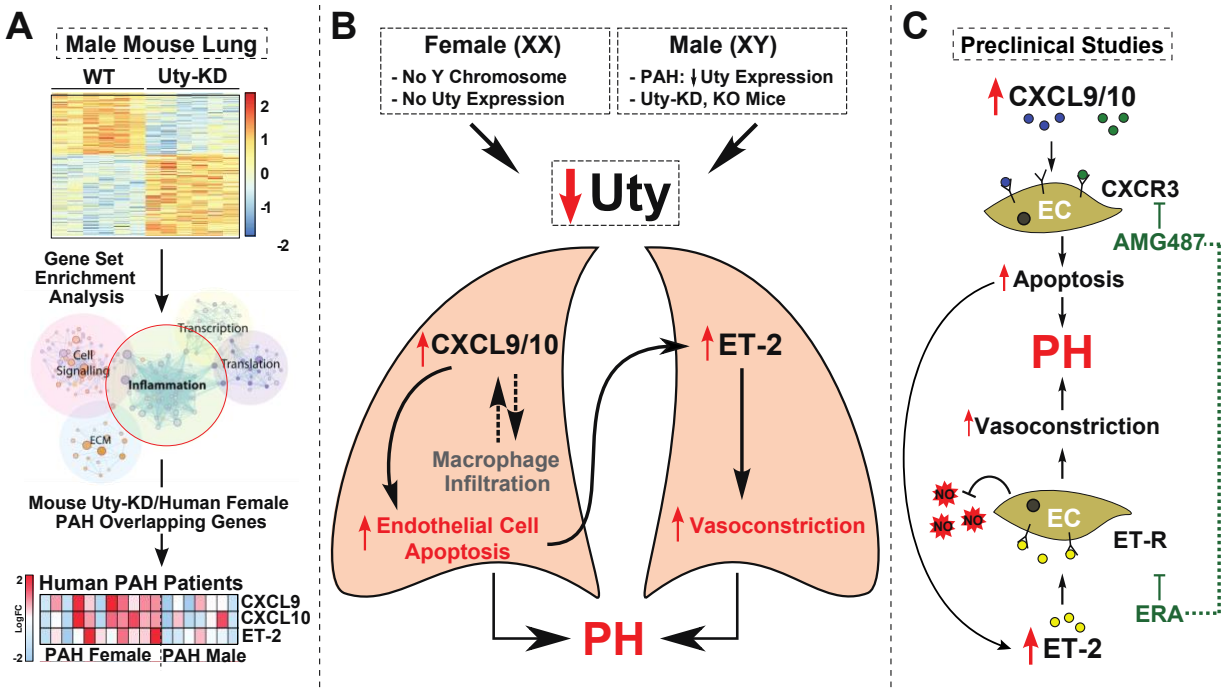

Fig. 1. Schematic of the proposed mechanism of Y chromosome gene, Uty, protection in PH through the downstream genes Cxcl9/10 and ET-2 and potential therapeutic targets. *Panel A*: Integration of our RNAseq analysis with an online microarray dataset of male and female human PAH lung samples revealed Cxcl9/10 and ET-2 as potential downstream effector genes in Uty mediated PH protection. *Panel B:* Proposed mechanism of Uty/Cxcl9/10 and Uty/ET-2 axes in PH pathogenesis. CXCL9/10 promote endothelial cell apoptosis and increase ET-2 secretion supporting a cross talk between CXCL9/10 and ET-2. Increased ET-2 expression results in increased vasoconstriction. Both downstream pathways contribute to increased PAH severity. *Panel C*: Overview of downstream Uty therapeutic targets via Cxcl9/10 and ET-2 which can be blocked using AMG487 alone or in combination with endothelin receptor antagonists (ERAs).

Future Directions

The experiments performed in this dissertation to identify the Y chromosome protective effect in experimental PH and further identify *Uty* as the Y chromosome protective gene were all carried out in the absence of circulating gonadal hormones. Gonadal hormones, including estrogens, progesterone, and testosterone, have been studied in the context of PH and found to have an effect in disease severity (17). We performed gonadectomy on our animals 30 days prior to the start of our experiments, which investigate the role of sex chromosomes and their genes, in order to eliminate gonadal hormone effects as a variable. While this was a viable strategy to identify the role of the Y chromosome and *Uty* gene within PH, it limits our understanding of Y chromosome and Uty protection within a gonadally intact environment. There is the possibility that Y chromosome protection through Uty interacts with gonadal hormones in a direct or indirect way, and sex chromosome-sex hormone interactions have been previous described in literature (18). To test for any interaction between gonadal hormones in Uty protection against PH, we would first perform *Uty* knockdown experiments in gonad-intact male mice to identify whether loss of *Uty* still aggravates PH development in the presence of gonadal hormones. If the effect of *Uty* knockdown is enhanced or eliminated within the gonadal-intact environment, a comparison of the lung transcriptome of *Uty* knockdown mice in the presence and absence of gonads would help elucidate pathways downstream of Uty that are affected by a hormonal interaction. Based on these differences, cell-specific *in vitro* experiments could be performed with and without testosterone to further identify downstream Uty pathways that are testosterone dependent. The downstream hormone

interaction studies would focus on testosterone since it is the main gonadal hormone found in males, and our research identified a PH protective effect specific to males.

While *Uty* downregulation within the hypoxic lung resulted in 523 differently regulated genes, the exact mechanism as to how Uty imparts large transcriptomic changes is not known. Our studies investigating Uty as a histone demethylase in the context of PH echoed the literature which concludes that the Uty protein has lost its demethylase activity. Still, research suggests that Uty does play a role in epigenetic modification potentially through indirect mechanisms by associating with the BRG1 methyltransferase complex which alters the methylation state of the histone 3 lysine residue 4 (9). Further experiments looking at the methylation state of the H3K4 residue in the presence and absence of Uty could explore this hypothesis. Since there is no specific antibody for Uty, however, it is difficult to determine the direct chromatin or protein interactions of Uty. Ultimately, the generation of a flag-tagged Uty knock-in mouse or macrophage cell line would allow for the chromatin immunoprecipitation or co-immunoprecipitation experiments necessary to further parse apart the cellular mechanism of UTY.

Our data found that *UTY* is downregulated in lungs of mice exposed to chronic hypoxia versus normoxia and in male PAH patient lungs versus healthy lungs. This suggests that *UTY* expression is associated with PAH, and our further investigation found that *Uty* downregulation is also causal in PAH development. However, we do not know if hypoxia alone is sufficient to trigger the Uty downregulation found in hypoxic mice and PAH patients with poor gas exchange. Future studies aimed to investigate this could expose male wildtype lung macrophages, and other Uty expressing lung cell types, to hypoxic insult and measure the effect on Uty expression. If Uty is downregulated in cells as a

result of hypoxic insult alone, experiments could determine if loss of Uty expression is a hypoxia-inducible factor-alpha (HIF-1α) regulated process. Experiments could challenge cells with HIF-1α stabilizers and inhibitors and then measure if Uty expression is downregulated or unchanged, respectively. If Uty downregulation is HIF-1α dependent, it would explain why Uty is downregulated in hypoxic experimental conditions and in hypoxic male PAH patient lung tissue.

This work uncovered a sex-specific pathogenic immune response in PAH related to Uty expression. Males who express Uty in their lungs are protected from PAH development and exhibit lower Cxcl9 and Cxcl10 proinflammatory chemokines levels. Alternatively, males or females with little or no Uty expression exhibit an upregulation of these inflammatory factors and worse PAH. Since we found Uty expression in macrophages inversely regulates Cxcl9 and Cxcl10 expression which drives Uty-associated PAH protection or development, we hypothesize that bone marrow transplant experiments with bone marrow reconstitution from a wildtype male mouse to a male total Uty knockout mouse would be sufficient to extend Uty protection. Conversely, bone marrow reconstitution from a male Uty knockout mouse to a male wildtype mouse should eliminate Y chromosome protective effects in these animals.

To our knowledge, the *in situ* hybridization images using *Uty/UTY*-specific mRNA probes are the first to demonstrate Uty localization in male mouse and human lung tissue. Based on these images and our co-labeling with macrophage marker Cd68, we know that lung macrophages express Uty. These images also show Uty positive nuclei that lack Cd68 expression which indicates that Uty is also expressed in other lung cell types. Single-cell sequencing of male mouse lungs in combination with duplex *in situ* hybridization for Uty

and various cell-specific markers could be performed to identify other lung cell types that express Uty in the lung. Once identified, *in vitro* gain and loss of function studies could be performed to determine the cell-specific functions of Uty in relation to PAH development.

This dissertation presents a novel therapeutic strategy for the treatment of PAH through blocking the activity of the CXCL9 and CXCL10 chemokines via administration of a small molecule inhibitor of their shared receptor, CXCR3. We show that two-week treatment is sufficient to rescue PH development in intact female rats with monocrotaline-induced PH. Prior to moving this FDA-approved therapy to clinical trials for PAH, it is important to test the efficacy of this treatment in intact males and in a second animal model of PH. Since *UTY* is downregulated in PAH male lungs, it is reasonable that a therapy targeting the activity of chemokines regulated by Uty would be a viable strategy in PAH males as well as females who lack Uty altogether. However, it remains to be seen if testosterone interacts with or blocks Uty-mediated effects. Testing the efficacy of AMG487 therapy in intact males and comparing the protective effect to that of treated females would shed light on whether this treatment is applicable for both male and female PAH patients.

Furthermore, considering the animal models of PH that we use preclinically fail to perfectly recapitulate PAH in humans, it is important to also test our novel therapeutic strategy in an additional animal model of PH. This is particularly important since the monocrotaline model of PH is an inflammatory model and our treatment works through blocking an inflammatory process. Future studies are needed to test the efficacy of CXCR3 inhibition therapy in model where the mechanism of action is not rooted in inflammation, such as the Sugen/hypoxia model in rats which promotes PH development through vascular endothelial cell death and chronic hypoxia (19). Finally, the most

important test would be a clinical trial that examines the efficacy of AMG487 inhibition of CXCR3 in humans. Since we show increased Cxcl9 and Cxcl10 expression in the lungs increases endothelial dysfunction and production of the vasoconstrictive peptide ET-2, we believe that adding AMG487 treatment in combination with an endothelin receptor antagonist, which are already prescribed to PAH patients, has the potential to produce an additive beneficial effect in PAH patients.

We found that loss of *Uty* expression in male mice is associated with an increase in lung ET-2 expression. In humans, this increased ET-2 expression is exclusive to female PAH patients and could contribute to known clinical sex differences in response to treatment with endothelin receptor antagonists. In addition to vasoconstrictive properties, studies have shown that ET-2 can influence angiogenesis and cellular proliferation which are both pathways that are altered in PAH (13, 16, 20, 21). Future studies will examine whether ET-2 plays a causal role in PAH development via reducing the angiogenic response to hypoxia and increasing vascular smooth muscle cell proliferation. In addition, the structure of ET-2 peptide is strikingly similar to that of CXC proinflammatory chemokines (15). Future experiments should also test macrophage migration in response to ET-2. If these experiments show ET-2 contributes in a causal way to PAH development, this will identify a novel female-specific PAH disease process.

As mentioned throughout this text, females are up to 4x more likely to be diagnosed with PAH compared to males. Our studies show that males are protected from PAH development because of their expression of the Y chromosome gene Uty; however, Uty expression does not explain why males, once diagnosed, exhibit a more severe form of the disease. We found that *UTY* is downregulated in PAH males which indicates that the

protective effects of Uty may be abrogated in these males. We hypothesize that loss of *UTY* expression in males who are predisposed to PAH development by another factor associated with PAH may be a trigger for severe PAH development—similar to the “two-hit” phenomenon in PAH where those predisposed to PAH development require a “second hit” from a secondary PAH-associated factor to develop the disease (22). In this way, it would be interesting to examine if *Uty* downregulation would provoke PH development in mice with a heterozygous mutation in the *Bmpr2* gene. *BMPR2* is the most studied hereditary mutation associated with PAH development in patients, however, the penetrance of the mutation is low (~20%) (23). It has been shown that in order to develop PH, these mice must have an additional PAH-associated phenotype, such as inflammation. Since we found loss of *Uty* expression in male mice is associated with an upregulation of proinflammatory factors, we hypothesize that downregulating *Uty* expression will act as a “second hit” in *Bmpr2* mutant mice and trigger PAH.

Finally, all of the findings within this dissertation were a result of studying the effects of the Y chromosome gene *Uty* within the lung; however, PAH patients ultimately succumb to right ventricular failure. The ability of the right ventricle to compensate for the increased pulmonary pressures in PAH is the greatest indicator of disease severity, prognosis, and survival (24). There are known sex-specific differences in right ventricular function in PAH patients that are linked to sex hormones. It remails to be seen, however, if sex chromosomes influence right ventricular compensation to pressure overload. Studies examining the effects of *Uty* expression in response to pressure overload in a PH-independent model, such as pulmonary artery banding, would elucidate if Uty is also protective in mediating right ventricular failure.

Final remarks from the author

PAH is a rare but terminal pulmonary vascular disease that predominantly affects women. The current therapies for this devastating disease are largely inadequate, and research into novel therapeutic strategies is paramount to increase patient survival. It has been a privilege to study this disease, provide creative insight into its pathogenesis, and extend some hope to affected patients. It is my greatest hope that the preclinical therapies reported in this work are able to be tested in clinical trials and that this work continues beyond the pages of this dissertation. Finally, I hope that this dissertation serves as a call-to-action that encourages researchers to segregate data by sex in a well-powered and meaningful way, and as a how-to guide for translating a sex-specific phenotype from bench to bedside.

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