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Y Chromosomal Protection against Pulmonary Arterial Hypertension is not Mediated by Ddx3y

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of the requirements for the degree Master of Science
in Physiological Science

by

Mariam Barseghyan

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ABSTRACT OF THE THESIS

Y Chromosomal Protection against Pulmonary Arterial Hypertension

is not Mediated by Ddx3y

by

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Master of Science in Physiological Science
University of California, Los Angeles, 2015

Professor Arthur P. Arnold, Chair

Pulmonary arterial hypertension (PAH) is an incurable vascular disease characterized by elevated pulmonary arterial pressure, vascular remodeling, and lesions leading to right heart failure. It is more prevalent in females than males. In the laboratory, we use a hypoxia model (10% O₂) to induce PAH in mice. Our previous data indicate that in the absence of sex hormones mice with a Y chromosome are protected against hypoxic insult as their right ventricular developed pressures (RVDPs), measured by direct catheterization, are lower than that of mice without a Y chromosome. Because four Y chromosomal genes are expressed consistently in lung and heart (Ddx3y, Kdm5d, Uty, Eif2s3y), we hypothesized that one or more of these genes is/are responsible for the protective effect of the Y chromosome. We tested the role of Ddx3y by inducing hypoxia in wild type XX mice with and without a Ddx3y transgene. Our results showed no significant difference in the severity of PAH between the two groups. This result allows us to test for the roles of other candidates.
The thesis of Mariam Barseghyan is approved.

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2015
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Introduction

Pulmonary arterial hypertension (PAH) is currently an incurable pulmonary vascular disease that is characterized by elevated pulmonary arterial pressure, pulmonary vascular remodeling and lesions which lead to right heart failure and ultimately death [1]. PAH can be primary with unknown pathophysiology, also referred to as idiopathic PAH, or secondary to such diseases as connective tissue disease, heart or lung diseases, or systemic conditions [2, 3]. According to the European Society of Cardiology (ESC) and the European Respiratory Society (ERS) guidelines, pulmonary hypertension is suspected when direct right heart catheterization shows an increase in resting mean pulmonary arterial pressure (≥25 mmHg). The exact processes that initiate the pathological changes attributed to PAH are still unknown, however, it is commonly accepted that PAH has a multi-factorial pathobiology that involves various biochemical pathways and cell types [4].

Clinical studies demonstrate that women are more likely to be diagnosed with idiopathic PAH than men [5]. This suggests that the physiology of males protects them from acquiring PAH. In our study we tried to understand the underlying molecular reasons for this protection. It was historically believed that what made men and women physiologically different from one another were sex hormones. However, recently this view has been progressively challenged. Sex differences between males and females are not solely attributed to the effects of gonadal hormones. Mouse studies in which sex chromosome complement is independent of gonadal sex indicate that sex chromosome complement has strong effects contributing to sex differences in phenotypes such as metabolism [6]. Sex chromosomes are inherently different between males and females from the time of zygote formation. This sex determination precedes the gonad formation (sex differentiation). Together these two factors act directly on a tissue to produce sex differences [7].
Previously we used novel mouse models to tease apart differences between males and females that could be attributed to either sex hormones or sex chromosome complement. All mice were gonadectomized (GDX) at 75 days of age to eliminate the acute effects of sex hormones. To induce pulmonary hypertension (PH) mice were exposed to hypoxia (10% oxygen) for three weeks. The first model we used was the four core genotypes (FCG) mouse model. The FCG model allows for comparison of mice with the following chromosomal and gonadal type: XXM, X XF, XY M, XY F (“F” is for female and “M” is for male, defined by the type of gonad). Our studies using FCG model show that in hypoxia XY mice (XYM, XYF) develop less severe PAH than XX mice (XXM, XXF), regardless of their gonadal sex. We additionally used the XY* mouse model to determine if the cause of the observed difference is the presence of a Y chromosome, or is it due to the number of X chromosomes. The XY* model allows for comparison of mice with varying number and type of sex chromosomes: XO, XX, XY, XXY. We determined that the right ventricular developed pressure (RVDP) of hypoxic mice with a Y chromosome (XY, XXY) was significantly lower than that of mice without a Y chromosome (XO, XX). Since RVDP directly correlates with PH severity, we concluded that the Y chromosome confers protection against development of PH in gonadectomized mice.

Four Y chromosomal genes are expressed consistently in lung and heart (Ddx3y, Kdm5d, Uty, Eif2s3y) and are thus candidate protective genes [8, 9]. Here, we investigated the effect of Ddx3y, a Y chromosomal gene that has been shown to act as a cellular growth suppressor, on PAH [10]. Pulmonary arterial smooth muscle thickening (due to cellular proliferation) and endothelial remodeling are characteristic in PAH [11]. We used Ddx3y transgenic mice to study this disease and found no significant difference between the wild type and transgenic groups. Thus, we conclude that Ddx3y does not confer protection against PH. In our future investigations we will concentrate on the remaining three Y chromosomal genes (Kdm5d, Uty, Eif2s3y) hypothesized to protect from PH. Results of this work could contribute to description of
pathways involved in Y chromosomal protection from PAH and implementation of novel therapeutic methods of treatment.

**Materials and Methods**

**Ethics Statement**

All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the appropriate committee. All experiments in this study were conducted with UCLA IACUC approval.

**Mouse Models**

The Y-BAC (Bacterial Artificial Chromosome) mouse model was used in this study. Y-BAC mice are XX mice on the MF1 background that express a Y-gene of interest on their autosome (\textit{Ddx3y} specifically for this project). The \textit{Ddx3y} transgenic mice were obtained from Paul Burgoyne. Each BAC clone is thought to be integrated into one autosome either as a single copy or in multiple concatenated copies at a single locus. In addition to \textit{Ddx3y} transgenic mice, two other mouse models were used in our study: “Four core genotypes” (FCG) model and XY* model.

In FCG mice, the testis-determining \textit{Sry} gene is deleted from the Y chromosome and inserted as a transgene onto Chromosome 3 \cite{12}. Thus, development of testes is no longer controlled by Y chromosome and the effects of the sex chromosome complement on traits can be studied independent of the gonadal type. The FCG model allows for comparison of mice with the following chromosomal and gonadal type: XXM, X XF, XYM, XYF (“F” is for female and “M” is for male, defined by gonadal type). These mice are generated by a cross between XX gonadal females and XY\textsuperscript{−} (\textit{Sry}+) gonadal males (the \textit{Sry} testis-determining gene is deleted from the Y chromosome of these mice and inserted onto an autosome). Since the Y\textsuperscript{−} chromosome and the
Sry transgene segregate independently, gonadal sex of these mice is not determined by the sex chromosome complement (Figure 1) [13, 14].

The XY* mice have an aberrant Y chromosome that recombines abnormally with the X chromosome. In this model XX gonadal females were mated with XY* gonadal males. Mice genetically similar to XX, XY, XXY, XO, and XO+PAR (an extra pseudoautosomal region) are produced in this cross. The XO+PAR mice were grouped with XO mice in our final analysis due to their genetic similarity (Figure 1).

**Gonadectomy (GDX)**

All mice were GDX at 75 days after birth to bring gonadal hormone levels to zero and to reveal differences due to sex chromosomes when comparing XX and XY mice and long-lasting effects of gonadal hormones in groups differing in gonadal type. Mice were given a subcutaneous injection of carprofen under isoflurane anesthesia and gonads were surgically removed [15].

**Genotyping and Karyotyping**

The FCG mice were genotyped as described in Itoh et al. [12]. For the genotyping of Ddx3y transgenic mice, PCR was performed using the primers for: Ddx3y (osm5: TGTTAAGACGCAGCAAGCTGA, osm8.m1: TCTGAAGACAGCTACAGATAC), myogenin (Om1a: TTACGTCCATCGTGGACAGCAT, Om1b: TGGGCTGGGTGTTAGTCTTAT). The myogenin primers are for the internal control. The XY* mice were genotyped by either karyotyping or FISH (Leica Biosystems, Kreatech KI-30505).

**Experimental Design**

**Hypoxia Experiments**

Two hypoxic mouse groups were studied: MF1 XY*(n=4-5/genotype) mouse group and MF1 XX female mice with and without Ddx3y transgene (n=10/group). Mice were housed in an airtight hypoxic chamber (10% oxygen) for three weeks beginning one month after GDX.
Normoxia Experiments

One normoxic mouse group was studied: C57BL6/J FCG (n=5/genotype). Mice were housed at normoxic conditions in an animal vivarium for three weeks beginning one month after GDX.

Cardiac and Pulmonary Hemodynamics

At the end of hypoxia and normoxia all mice were anesthetized using intraperitoneal injection of ketamine/xylazine (80mg/10mg/1kg). Mice were then connected to a lung ventilator and surgery was started to open their thoracic cavity to expose the heart. Through direct cardiac catheterization, right ventricular developed pressure (RVDP) was measured. RVDP was then calculated from RV peak systolic pressure – RV peak diastolic pressure.

Gross Histological Evaluation

The right ventricular (RV) wall, the left ventricular (LV) wall, and the interventricular septum (IVS) were dissected, weighed, and the weight ratio of RV/(LV+IVS) was calculated as an index of RV hypertrophy.

Imaging

The right lung was fixed (4% paraformaldehyde (PFA)), sectioned and stained with standard hematoxylin and eosin, and Masson trichrome stain.

Statistical Analysis

For the FCG mouse model, we used 2-way ANOVAs to measure effects of sex chromosome complement (XX vs XY) and gonadal sex (male vs female). For the XY* mouse model, we ran 2-way ANOVAs with factors of number of X chromosome and gonadal sex (presence or absence of Y chromosome). We used 1-way ANOVAs to analyze data for Ddx3y mice. A 2-sided probability value of <0.05 was considered statistically significant. Means and standard errors of the mean (SEMs) are reported.
Results

GDX XY mice develop less severe PH compared to XX mice in hypoxia.

Previously, to study sex differences in pulmonary arterial hypertension we used GDX C57BL6/J FCG hypoxia mouse model. Our results showed that XY mice develop less severe PH compared to XX mice, regardless of gonadal type (XXF: 43.3±5.7mmHg; XXM: 46.5±4.5mmHg; XYF: 33.2±2.4mmHg; XYM: 40.4±2.3mmHg; p<0.05 indicates sex chromosomal effect; Figure 2). Here, we conducted a normoxia study on GDX C57BL6/J GDX FCG mice to check that the baseline pressure values were normal. As expected, RVDPs of normoxic mice were at the normal level (XXF: 25.5±1.6mmHg; XXM: 25.8±0.6mmHg; XYF: 23.8±0.7mmHg; XYM: 24.2±0.8mmHg). Additionally, there was no significant difference observed between XX and XY normoxic mice (p=0.1 indicates no sex chromosomal effect; p=0.8 indicates no gonadal effect; Figure 2).

GDX XY mice develop less severe pulmonary vascular remodeling compared to XX mice in hypoxia.

The representative Masson trichrome staining of fixed lung sections of FCG hypoxic mice discussed above show thicker pulmonary arterioles and more pulmonary fibrosis in XX mice compared to XY mice regardless of gonadal type (Figure 3). Thicker pulmonary arterioles and more pulmonary fibrosis is typical in PAH patients.

GDX C57BL6/J mice with Y chromosome develop less severe PH compared to mice without a Y chromosome in hypoxia.

To determine why XY mice respond better to hypoxic insult, we previously used GDX C57BL6/J XY* hypoxia mouse model. Our results indicate that this is due to the presence of Y chromosome: mice with Y chromosome develop less severe PH compared to mice without Y chromosome (XXY: 32.0±2.1; XY: 33.5±0.6; XX: 41.6±3.8; XO: 38.7±2.4, p=0.01 indicates Y chromosomal effect; Figure 4).
GDX MF1 mice with Y chromosome develop less severe PH compared to mice without a Y chromosome in hypoxia.

Since Ddx3y transgenic mice were available only an outbred MF1 background, we wished to confirm that the Y chromosome effect, found previously in C57BL6/J mice, was also found in the MF1 strain so that we could then vary dose of Ddx3y in MF1 mice to test if Ddx3y accounted for the protective effect of the Y chromosome. Therefore, we repeated the GDX XY* hypoxia experiment on MF1. Because MF1 is an outbred mouse strain, we accounted for litter effects by dividing individual RVDP by the mean RVDP of the litter. Litters were chosen to have representative mice from each four groups, as much as possible. With this experiment we replicated our previous findings and have shown that even on an outbred mouse strain, hypoxic mice with Y chromosome develop less severe PH compared to mice without a Y chromosome (XXY: 1.0±0.06; XY: 0.9±0.03; XX: 1.1±0.1; XO: 1.1±0.1; p=0.03 indicates Y chromosomal effect; Figure 5).

Ddx3y transgenic mice have as severe PH as wild type mice in hypoxia model.

Ddx3y was one of our candidate genes that are hypothesized to confer protection against PH. In this experiment litter effect was also taken into an account because mice were outbred MF1 strain. Individual RVDP was divided by the mean RVDP of the litter. Litters were chosen to have representative mice from each of the two groups. The hypoxia experiment on MF1 Ddx3y and wild type XX mice showed no significant difference between the two groups (Ddx3y: 1.0±0.04; WT: 1.0±0.05; p=0.6; Figure 6).

Discussion

Previous studies indicate that Y chromosome confers protection against PAH in an inbred mouse strain (C57BL6/J). Here, we confirmed this result on the MF1 strain, which is an outbred strain. Experiments on outbred strains may be more relevant to the human condition because
outbred mice are more genetically diverse. We have also established that XX female mice carrying a transgenic copy of \textit{Ddx3y}, a Y chromosomal gene that has been shown to act as a cellular growth suppressor, show no protection against PAH. These data therefore offer no support for the hypothesis that the protective effect of the Y chromosome is the result of expression of \textit{Ddx3y}.

According to REVEAL Registry, women are four times more likely to be diagnosed with idiopathic PAH than men [16]. Nonetheless, animal models of PH indicate that females are protected against development of PH likely due to the protective effect of estradiol [17]. This phenomenon is often referred to as the “estrogen paradox” of pulmonary hypertension [18]. According to Umar et al. estrogen rescues preexisting severe PH in rats by restoring lung and RV structure and function which is maintained even after removal of estrogen [18]. They further describe that the rescue is associated with stimulation of cardiopulmonary neoangiogenesis, suppression of inflammation, fibrosis, and RV hypertrophy all of which are likely mediated through estrogen receptor-\(\beta\) [17]. Even though in idiopathic PAH men are less frequently affected by the condition, they have worse outcome as compared to women [19, 20]. One study showed that male patients respond worse to PAH because they have proportionally worse RV function despite similar afterload. The authors hypothesize that adaptive remodeling of the RV in response to increased afterload in PAH is more effective in females [21].

Regardless of clear sex differences observed in idiopathic PAH, the role of sex chromosomes in the development of this disease has not been elucidated. In our studies we aimed to understand why women are more likely to be diagnosed with PAH while keeping in mind that sex differences are not only due to hormonal effects, but that they are also due to sex chromosomes. We have specifically shown that mice carrying a Y chromosome are less likely to be affected by PH, regardless of their gonadal type. We took this finding further in an attempt to describe specific gene or groups of genes on Y chromosome that confer protection against
PAH. The first gene out of the four hypothesized Y chromosomal genes (Ddx3y, Kdm5d, Uty, Eif2s3y) to protect from PH that we studied was Ddx3y. Based on the results of the study where we added Ddx3y transgene to XX female mice, we conclude that the Y chromosomal protection against PAH is not due to this gene.

In our upcoming studies we will test the effects of the remaining three genes (Kdm5d, Uty, Eif2s3y) on PH. For this we will use both transgenic XX mice with each of the three genes introduced one at a time and an in vivo siRNA silencing method to silence these genes, one at a time, in XY mice. Localized lung in vivo gene silencing is performed with the help of nebulizer to introduce siRNA directly to the lung and thus to silence genes of interest specifically in the lung. This method has been successfully used by many investigators [22, 23]. Lung-specific knock down would avoid possible negative effects on spermatogenesis as would be the case with global knock out of Y chromosomal genes. This will also allow us to define the role of Y chromosomal genes in the lung.

It is possible that the protection conferred by Y chromosome is due to the influence of not a single gene, but of a work in tandem of multiple genes. If our experiments indicate that the transgene achieves only partial protection relative to the protection conferred by the Y chromosome, then we will assess combined effects of more than one protective Y chromosomal gene. This could be done by testing the effect of Y-BAC chromosomal transgenes in pairs and in threes. The BAC transgenes are compatible with each other as is evidenced by mice we bred with multiple Y-BAC transgenes. Alternatively, pairs or threes of Y chromosomal genes could be knocked down using siRNA gene silencing technology. Once we identify specific gene or genes responsible for protecting against PH we will move to the next stage – description of molecular pathways involved in the protection.

Since PAH is more common in women than men, for future clinical implementation, it is also important to investigate if there is an interaction between the identified gene or genes that
protect from PH and estrogen. This could be done by using intact XX female mice with a transgene or transgenes of interest or by implanting a subcutaneous pellet of estrogen in GDX XX transgenic mice and comparing the severity of PAH in these mice with those without estrogen. This study will help us to better understand sex chromosomal and sex hormonal interaction and will bring us closer to developing therapeutics that could be administered clinically to the female population suffering from PAH.
Figure 1: FCG and XY* mouse models
Figure 2: GDX XY mice develop less severe PH compared to XX mice in hypoxia. RVDP is calculated from RV peak systolic pressure – RV peak diastolic pressure in normoxia or hypoxia. C57BL6/J background. *p<0.05, n=5 mice/group.
Figure 3: GDX XY mice develop less severe pulmonary vascular remodeling compared to XX mice in hypoxia. Representative Masson trichrome staining of lung sections of GDX FCG mice kept in hypoxia for 3 weeks showing thicker pulmonary arterioles and more pulmonary fibrosis in XX mice compared to XY mice regardless of gonadal type. C57BL6/J background.
Figure 4: GDX C57BL6/J mice with Y chromosome develop less severe PH compared to mice without a Y chromosome in hypoxia. RVDP is calculated from RV peak systolic pressure – RV peak diastolic pressure. *p=0.01, n=5 mice/group.
Figure 5: GDX MF1 mice with Y chromosome develop less severe PH compared to mice without a Y chromosome in hypoxia. RVDP is calculated from RV peak systolic pressure – RV peak diastolic pressure. Y-axis represents individual RVDP divided by litter mean RVDP to take into account litter effect. *p<0.05, n=4 or 5 mice/group.
Figure 6: *Ddx3y* transgenic mice have as severe PH as wild type mice in hypoxia model. RVDP is calculated from RV peak systolic pressure – RV peak diastolic pressure. Y-axis represents individual RVDP divided by litter mean RVDP to take into account litter effect. MF1 background.
References

