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The Y Chromosome Regulates BMPR2 Expression via SRY: A Possible Reason "Why" Fewer Males Develop Pulmonary Arterial Hypertension

To the Editor:

Reduced lung *BMPR2* (bone morphogenetic protein receptor type 2) expression and female predominance are two major features of most pulmonary arterial hypertension (PAH) subtypes (1). In addition, germline mutations in *BMPR2* are present in more than 75% of patients with heritable PAH, and about 20% of patients with idiopathic PAH (2). However, only 14% of males, compared with 42% of females, who harbor *BMPR2* mutations develop PAH (3).

There is a growing body of molecular and *in vivo* work supporting the concept that sex and *BMPR2* are intimately related to each other, to PAH pathogenesis, and perhaps to right ventricular adaptation. For example, estrogen receptor α binds to the *BMPR2* promoter in Cos-7 cells, leading to decreased expression and signaling of BMPR-II, whereas female human pulmonary artery smooth muscle cells exhibit estrogen-driven suppression of BMPR-II signaling (4, 5); however, estrogen signaling appears to support right ventricular response to stress (6). Although likely relevant, a focus only on sex hormones ignores an obvious difference between females and males: the sex chromosomes (XX vs. XY). Importantly, recently in the *Journal*, Umar and colleagues demonstrated a protective effect of the Y chromosome in murine hypoxia-induced pulmonary hypertension (7). We explored the hypothesis that the higher female incidence in PAH is driven in part by factors specific to the Y chromosome that enhance *BMPR2* expression, with a focus on the transcription factor *SRY* (sex-determining region Y).

To start, we analyzed *BMPR2* proximal regulator sequences using the transcription element search system and transcription factor database (http://www.gene-regulation.com). These analyses predicted *BMPR2* upstream regulatory regions had at least five SRY binding sites. This suggested to us that SRY may regulate *BMPR2* mRNA expression.

We next evaluated *SRY* expression in different cell types and found that *SRY* expression was low in multiple different male lung vascular cell lines but high in dermal fibroblast cell lines from multiple control patients and patients with PAH (data not shown). We then sought to determine whether *SRY* regulates native *BMPR2* expression. We used RNA interference to knockdown *SRY* expression in fibroblasts from a male patient with PAH. We found that reducing *SRY* expression resulted in decreased *BMPR2* mRNA and protein expression (Figure 1A). Although Smad 1/5/8 protein expression was not demonstratively reduced (data not shown), the breadth of canonical and noncanonical BMPR2 signaling was not assessed. We then used a *SRY* expression construct to overexpress *SRY* in a female HEK293 cell line (which does not express *SRY*). We found that *SRY* overexpression resulted in ∼20% increased *BMPR2* expression compared with control (Figure 1B).

We next investigated whether *BMPR2* is regulated by *SRY* in a dose-dependent manner. A *BMPR2* promoter expression construct pGL3-BMPR2-Luciferase (containing the predicted *SRY* binding sites) was used in a cotransfection assay with a varying amount of *SRY* expression construct. We found that an increased

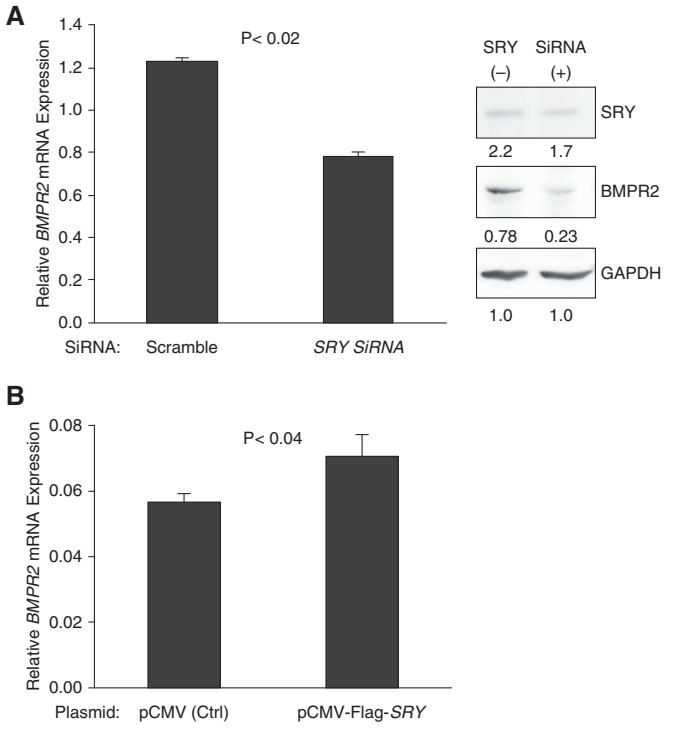

Figure 1. *SRY* (sex-determining region Y) positively regulates *BMPR2* (bone morphogenetic protein receptor type 2) expression in human cells. (*A*) *SRY* knockdown resulted in lower *BMPR2* mRNA and protein expression in male fibroblasts. The dermal fibroblasts, derived from a male patient with pulmonary arterial hypertension, were transfected with scrambled siRNA or *SRY* siRNA. *BMPR2* mRNA levels were quantified by real-time PCR, and protein levels by Western blot. (*B*) SRY overexpression resulted in increased BMPR2 mRNA expression in female HEK293 cells (which do not express native SRY). HEK293 cells were transfected with plasmids: control pCMV or pCMV-FLAG-SRY (which expressed SRY). BMPR2 mRNA levels were quantified by real-time PCR.

Funding was provided by NIH P01 HL 108800 (E.D.A.), NIH R01 HL134802 (E.D.A.), and NIH R01 HL 102020 (R.H.) awards.

Author Contribution: L.Y. conducted experiments, analyzed data, and wrote the manuscript; J.D.C. analyzed data; L.K.H. and B.N. conducted experiments; and L.Y., R.H., and E.D.A. conceived the study; designed the experiments; acquired, analyzed, and interpreted the data; and wrote and edited the manuscript.

Originally Published in Press as DOI: 10.1164/rccm.201802-0308LE on September 25, 2018

quantity of SRY resulted in an increased expression of luciferase, suggesting that SRY can positively regulate the *BMPR2* promoter in a dose-dependent manner (Figure 2A).

We then used a chromatin immunoprecipitation assay to determine whether SRY directly bound to human *BMPR2* promoter sequences, using the female cell line HEK293 overexpressing human influenza hemagglutinin–tagged SRY. We found that SRY bound to *BMPR2* promoter sequences (Figure 2B). Next, we repeated the chromatin immunoprecipitation assay in fibroblast cell lines from a female and a male to determine whether endogenous SRY can also bind to *BMPR2* promoter sequences. Although the female cells (lacking SRY) showed no binding, the SRY in the male fibroblasts bound to *BMPR2* promoter sequences (Figure 2C). Taken together, our chromatin immunoprecipitation data show that SRY binds to *BMPR2* promoter sequences.

Our data thus demonstrate that SRY binds to and positively regulates the *BMPR2* promoter. These findings add to our understanding of the contribution of sex to PAH and its relationship to *BMPR2*. Given the crucial role of reduced *BMPR2* in PAH, including data that reduced BMPR-II signaling (regardless of the presence of a *BMPR2* mutation) is detrimental for the pulmonary vasculature, factors that regulate *BMPR2* are likely to modify overall PAH risk and resilience. *SRY* appears to be one of these factors.

SRY is a member of the SOX (SRY-like box) family of transcription factors, and is known to both positively and negatively regulate gene expression (8). The *SRY* gene is localized on chromosome Yp11.2, and as such is expressed exclusively in males. It is a critical gene for male sex determination, as well as other components of development (9). However, SRY also appears to modulate several other pathways through *cis* and *trans* effects

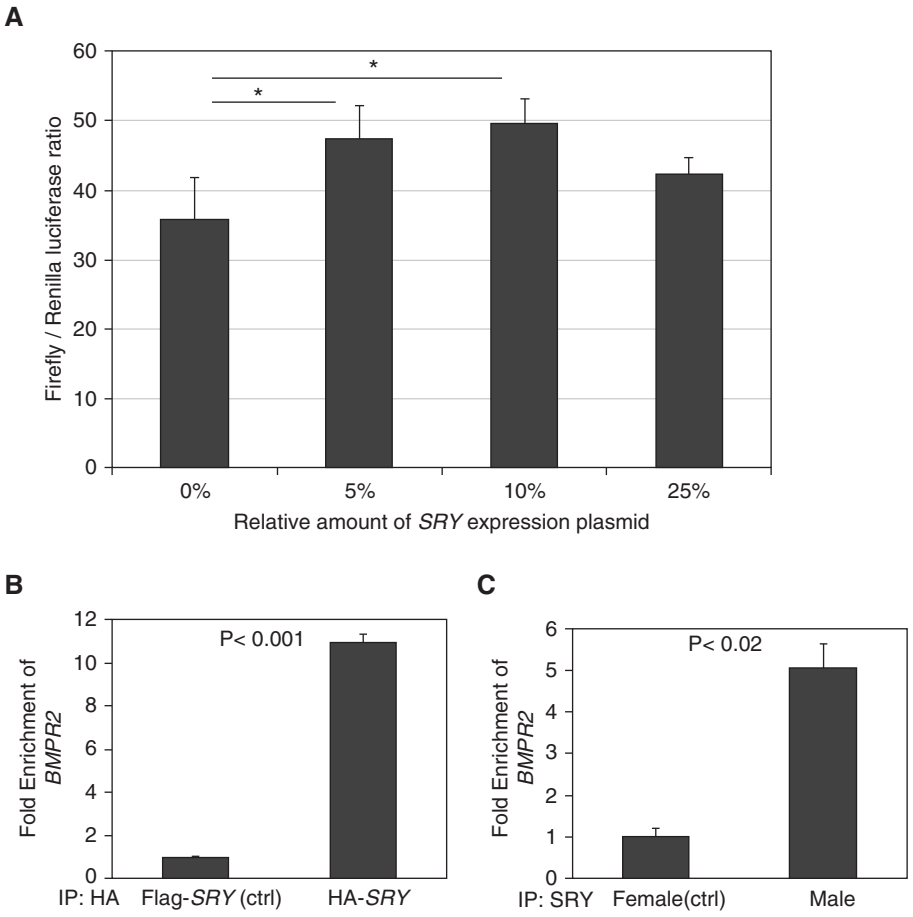

Figure 2. *SRY* (sex-determining region Y) directly binds to the *BMPR2* (bone morphogenetic protein receptor type 2) promoter. (*A*) *SRY* induction of a *BMPR2* promoter−driven luciferase. Dual luciferase assay was performed in human microvascular endothelial cells (HMECs). HMECs were transfected with a pGL3-luc-BMPR2 firefly vector, an internal control herpes simplex virus thymidine kinase promoter *Renilla* vector, and a variable amount of SRY expression plasmids. The transfected cells were lysed, and luminescence was measured on a luminometer. The relative luminescent ratios of luciferase firefly/*Renilla* were plotted to indicate *BMPR2* expression (*$P < 0.05$). (*B*) *SRY* binds to the *BMPR2* promoter in *HEK293* cells. Chromatin immunoprecipitation (ChIP) assay was performed in HEK293 cells (female) overexpressing a control plasmid (Flag-SRY) or plasmid HA-SRY. Anti-HA antibody was used to immunoprecipitate HA-tagged SRY. PCR was performed to detect the *BMPR2* promoter sequence, using the EpiTect ChIP quantitative PCR assay designed for human *BMPR2*. Results were calculated as fold enrichment of *BMPR2* and represent the mean ± SEM. (*C*) Endogenous *SRY* binds to the *BMPR2* promoter in cells of a male patient with pulmonary arterial hypertension (PAH). ChIP assay was performed in dermal fibroblasts derived from one female patient with PAH and one male patient with PAH. Anti-SRY antibody was used to immunoprecipitate endogenous SRY. PCR was performed to detect the *BMPR2* promoter sequence, using EpiTect ChIP quantitative PCR assay designed for human *BMPR2*. Results were calculated as fold enrichment of *BMPR2* and represent the mean ± SEM. ctrl = control; HA = human influenza hemagglutinin; IP = immunoprecipitation.

across the genome, including pathways highly relevant to PAH pathogenesis, such as WNT signaling (8, 10, 11).

One of the findings in our study was that the positive effect of SRY on *BMPR2* expression had an upper limit, above which adding more SRY did not result in a corresponding increase in *BMPR2* expression. This is not a surprising finding, as most transcription factors have a limited range of function. Furthermore, *BMPR2* expression is likely controlled by a coordinated action of multiple different transcriptional regulators, and thus one transcription factor would not be expected to modify its expression to an unlimited extent. For example, previous studies have identified other factors that regulate *BMPR2* expression, such as estrogen receptor α (4, 5). Thus, the integration of multiple factors may explain why SRY has a limited, but important, capacity to increase *BMPR2* expression. Each factor may play a role in PAH susceptibility or resilience. It is important to acknowledge that in some assays, the effect sizes were modest; this may reflect the fact that multiple factors likely contribute to *BMPR2* expression, not simply SRY activity. Finally, much of this work was conducted using PAH fibroblasts because of the low expression of *SRY* in typical lung vascular cells. Although the amount of data suggesting that fibroblasts may contribute to PAH pathogenesis is growing, future work will determine whether SRY contributes to variations in lung vascular cell health and function.

In conclusion, SRY binds to and positively regulates *BMPR2* expression. This builds on recent novel work by Umar and colleagues, which demonstrated the relevance of the Y chromosome to pulmonary hypertension (7). Our findings advance the concept that protective factors on the Y chromosome contribute to pulmonary hypertension, with a focus on the reduced male incidence in PAH via sex-specific *BMPR2* regulation. ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

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Exposure to Humidifier Disinfectants Increases the Risk for Asthma in Children

To the Editor:

In South Korea, several types of chemical disinfectants that had been widely used in humidifiers since 1994 were found to be associated with lung injury and widespread lung fibrosis (1–4). After the humidifier disinfectants (HDs) were found to be the cause of the lung injury, they were withdrawn from the market in 2011, after which no new cases of lung injury have been reported (1). Although much research has examined the acute lung injury resulting from HD exposure in terms of outcomes such as mortality and HD-induced lung injury (HDLI), the long-term consequences of HD usage on health effects have not been reported. Patients with HDLI and people exposed to HDs have complained of asthma symptoms, but only one case of occupational asthma resulting from isothiazolinone exposure has been reported (5). A previous

This study was supported by the Korea Ministry of Environment as the Environmental Health Action Program (2016001360006), the Environmental Health Center for Hazardous Chemical Exposure funded by Ministry of Environment Republic of Korea (2018), and the research of Korea Centers for Disease Control and Prevention (2015-ER6601-00). This work was also supported by the Research Program funded by the Korea Centers for Disease Control and Prevention (2008-E33030-00, 2009-E33033-00, 2011-E33021-00, 2012-E33012-00, 2013-E51003-00, 2014-E51004-00, 2014-E51004-01, 2014-E51004-02, and 2017-E67002-00).

Author Contribution: J.Y. planned the project, acquired the data, helped interpret the data, and wrote the manuscript; S.-Y.L. contributed to the study conception, interpreted the results, and wrote the manuscript; S.-H.L. performed most of the animal experiments and data analysis and helped write the manuscript; E.M.K. was responsible for data transformation, database management, and statistical analysis; S.J., H.-J.C., E.L., and S.-IY. helped with the writing and review of the manuscript; S.-J.H. conceived and planned the project, acquired the data, analyzed the data, interpreted the results, and critically reviewed the manuscript.

Originally Published in Press as DOI: 10.1164/rccm.201805-0840LE on September 7, 2018