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ORIGINAL ARTICLE

Y-Chromosome Gene, *Uty*, Protects Against Pulmonary Hypertension by Reducing Proinflammatory Chemokines

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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 four times more prevalent in females. Understanding the molecular basis behind sex differences could help uncover novel therapies.

Objectives: We previously discovered that the Y chromosome is protective against hypoxia-induced experimental pulmonary hypertension (PH), which may contribute to sex differences in PAH. Here, we identify the gene responsible for Y-chromosome protection, investigate key downstream autosomal genes, and demonstrate a novel preclinical therapy.

Methods: To test the effect of Y-chromosome genes on PH development, we knocked down each Y-chromosome gene expressed in the lung by means of intratracheal instillation of siRNA in gonadectomized male mice exposed to hypoxia and monitored changes in right ventricular and pulmonary artery hemodynamics. We compared the lung transcriptome of *Uty* knockdown mouse lungs to those of male and female PAH patient lungs to identify common downstream pathogenic chemokines and tested the effects of these chemokines on human

pulmonary artery endothelial cells. We further inhibited the activity of these chemokines in two preclinical pulmonary hypertension models to test the therapeutic efficacy.

Measurements and Main Results: Knockdown of the Y-chromosome gene *Uty* resulted in more severe PH measured by increased right ventricular pressure and decreased pulmonary artery acceleration time. RNA sequencing 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 females with PAH. Treatment of human pulmonary artery endothelial cells with CXCL9 and CXCL10 triggered apoptosis. Inhibition of *Cxcl9* and *Cxcl10* expression in male Uty knockout mice and CXCL9 and CXCL10 activity in female rats significantly reduced PH severity.

Conclusions: *Uty* is protective against PH. Reduction of *Uty* expression results in increased expression of proinflammatory chemokines Cxcl9 and Cxcl10, which trigger endothelial cell death and PH. Inhibition of CLXC9 and CXLC10 rescues PH development in multiple experimental models.

Keywords: CXCL9; CXCL10; sex differences; endothelial cells; pulmonary arterial hypertension

At a Glance Commentary

Scientific Knowledge on the Subject: Idiopathic pulmonary arterial hypertension (PAH) is a pulmonary vascular disease that displays a marked sex bias with females being up to four times more affected. Decades of research have been spent studying the root cause of this sexual dimorphism, with an emphasis on studying the role of sex hormones. Our lab was the first to investigate the potential role of sex chromosomes in the context of PAH. We found that the male-specific Y-chromosome is protective against experimental pulmonary hypertension (PH); however, the mechanism of this protection was not known.

What This Study Adds to the Field: This study, an in-depth continuation of our previous research, identifies that the Y-chromosome gene, *Uty*, confers protection against the development of PAH in males by suppressing a proinflammatory pathway in the lungs. When Uty is lowly expressed in males or absent in females, increased expression of chemokines Cxcl9 and Cxcl10 result in vascular endothelial cell dysfunction and more severe PH development. We also demonstrate that targeting this downstream inflammatory pathway is sufficient to reduce PH severity in two preclinical rat models.

Pulmonary arterial hypertension (PAH) is a pulmonary vascular 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 plays a key role in this vascular remodeling, as 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 four times 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. Estrogen signaling has been implicated in PAH pathogenesis and shown to reduce expression of bone morphogenic protein 2 (BMPR2), a gene associated with PAH due to autosomal dominant mutations (6, 7). However, animal studies have also demonstrated that circulating estrogen is protective against pulmonary hypertension (PH) severity, and estrogen treatment has been shown to prevent and rescue PH (8–10) through mediating RV compensation to pressure overload (11, 12). These powerful yet paradoxical studies indicate that other sex-biasing factors contribute to the sex differences observed in PAH.

Our group examined the influence of sex chromosomes in PH and demonstrated that, in the absence of circulating gonadal hormones, the male-specific Y chromosome (ChrY) is protective against experimental PH (13). Although this study may 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* (ubiquitously transcribed tetratricopeptide repeat containing, Y-linked) as the protective ChrY gene. We also identified two proinflammatory chemokines, CXCL9 and CXCL10, downstream of Uty that contribute to PH pathogenesis in a sex-specific manner and found that knockdown (KD) of these chemokines is sufficient to reduce PH severity in global Uty knockout (KO) mice. We found that inhibition of the shared CXCL9 and CXCL10 receptor significantly reduced PH development in two experimental models of PH in female rats, suggesting that targeting autosomal genes downstream of *Uty* is sufficient to extend ChrY protection to females with PH.

Methods

For detailed methods, see the online supplement.

Models of Experimental PH and Treatments

ChrY KD experiments. Male C57BL/6J mice underwent gonadectomy (GDX) and, 30 days later, received recurring intratracheal instillation of short interfering RNA (siRNA) targeting a ChrY gene of interest or a scrambled control. All siRNAs were formulated for *in vivo* use. Mice were housed in hypoxia (Hx, 10% oxygen) for 3 weeks.

Cxcl9 and Cxcl10 KD experiments. Male global Uty KO (Uty-KO) mice and wild-type (WT) controls underwent GDX. After 30 days, mice received recurring intratracheal instillation of nontargeting siRNA (WT and Uty-KO groups) or a mixture of siRNAs targeting *Cxcl9* and *Cxcl10* (Uty-KO Si-Cxcl group). All siRNAs were formulated for *in vivo* use. Mice were housed in Hx for 3 weeks.

Cxcr3 inhibition. Female gonad-intact rats received a subcutaneous injection of either monocrotaline (MCT) or Sugen 5416. In the MCT model, 2 weeks after MCT injection, animals were injected subcutaneously every 12 hours with AMG487 (1 mg/kg) or vehicle for 2 weeks. In the Sugen-Hx (SuHx) model, after the injection of Sugen 5416, animals were housed in Hx for 3 weeks and then returned to normoxia for 2 weeks, where they received a subcutaneous injection of AMG487 (1 mg/kg) or vehicle every 12 hours.

Echocardiography

Two-dimensional Doppler and B-mode echocardiography were used to quantify pulmonary artery and RV parameters.

Hemodynamics and Gross Anatomical Evaluation

RV systolic pressure (RVSP) was measured by means of direct, open-chested catherization. The heart was dissected and weighed to calculate the Fulton index, a ratio of RV weight to left ventricle (LV) plus intraventricular septum (IVS) weight: RV/(LV + IVS).

Tissue Preparation, Staining, and Imaging

Tissues were formalin fixed, embedded in optical cutting temperature compound (Tissue-Tek), and stained according to product specifications. Formalin-fixed,

paraffin-embedded human lung sections were obtained from the Pulmonary Hypertension Breakthrough Initiative repository.

Real-Time qPCR

Quantitative reverse transcription PCR (RT-qPCR) was performed using iTaq Universal SYBR and reverse transcribed using the Omniscript RT Kit (Qiagen).

Bioinformatic Analysis

Total lung RNA was sequenced by the University of California, Los Angeles, Technology Center for Genomics & Bioinformatics. Differently expressed genes (DEGs) with a false discovery rate <0.1 were considered statistically significant. Public microarray data were used to define a list of inflammatory genes expressed higher in female versus male PAH patient lungs (17).

Cell Studies

Bone marrow was extracted from male Uty-KO or WT mice and incubated with colony-stimulating factor (20 ng/ml). Bone marrow–derived macrophages (BMDMs) were treated with interferon gamma (10 ng/ml) for 4 hours, and Cxcl9 and Cxcl10 expression was measured by means of RT-qPCR.

Healthy human male pulmonary artery ECs (PAECs) were spiked with CXCL9 (2 μM), CXCL10 (5 μM), or vehicle for 24 hours and stained for cleaved caspase-3.

Statistics

Mann–Whitney tests were used for comparisons of two groups. Two-way ANOVAs were used for comparisons of four groups. A significance level less than 5% ($P < 0.05$) was deemed significant. Values are expressed as mean ± SEM.

Results

Lung-Specific KD of Uty, but Not Other ChrY Genes Expressed in the Lung, Eliminates ChrY Protection against Hx-Induced PH

Our previously published work demonstrated that, in the absence of gonadal hormones, 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* (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 compared with healthy patient lungs (13).
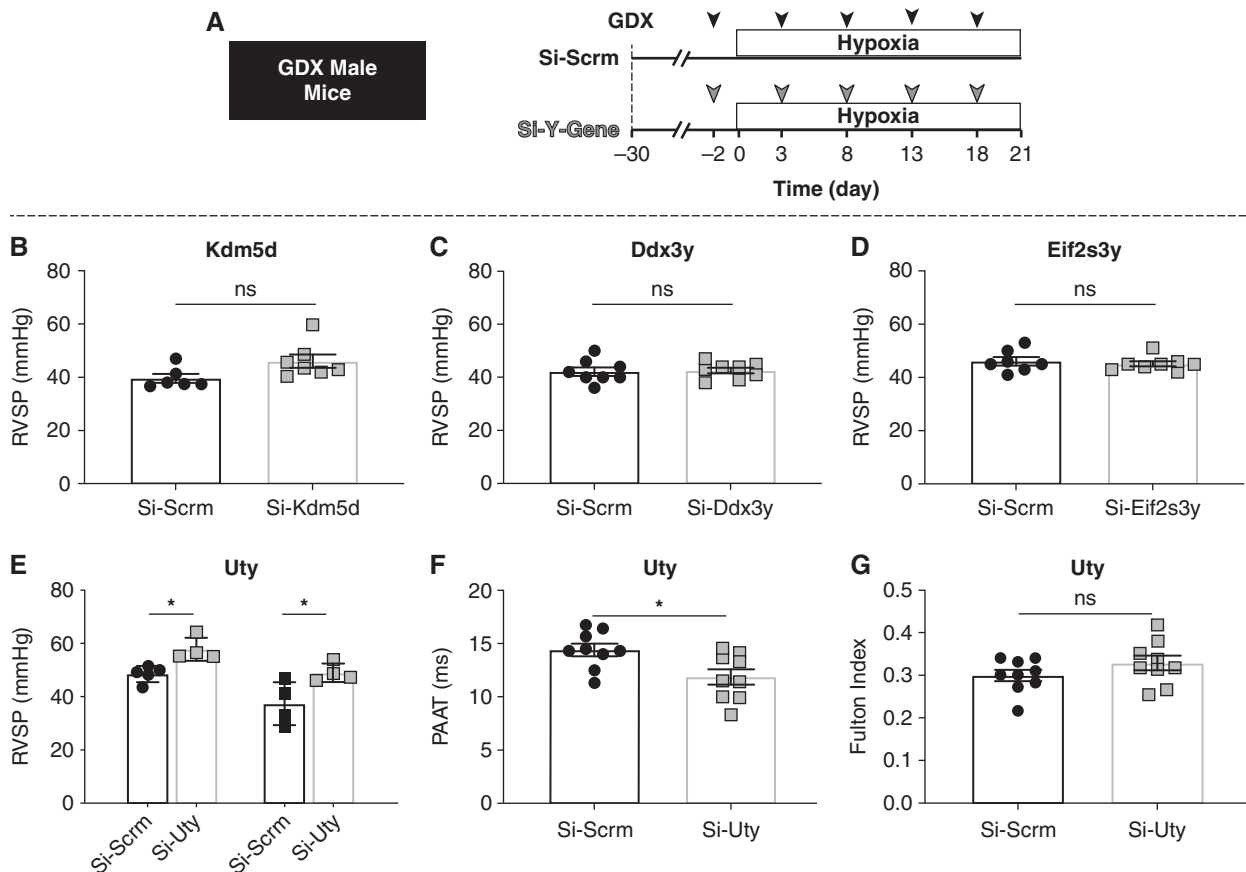

Figure 1. Lung-specific knockdown of *Uty*, but not other Y-chromosome (ChrY) genes expressed in the lung, eliminates ChrY protection against hypoxia (Hx)-induced pulmonary hypertension. (*A*) Experimental protocol: 30 days after gonadectomy (GDX), male mice receive scramble siRNA or siRNA targeting ChrY genes every 5 days and are placed in Hx for 3 weeks. (*B–E*) Right ventricular systolic pressure (RVSP) measured by direct RV catheterization for (*B*) Kdm5d, (*C*) Ddx3y, (*D*) Eif2s3y, and (*E*) Uty. (*F*) Pulmonary artery acceleration time (PAAT) measured by echocardiography. (*G*) Fulton index (RV/LV + IVS). *$P < 0.05$. IVS = intraventricular septum; LV = left ventricle; ns = not significant.

To determine which ChrY gene is responsible for PH protection, we individually knocked down each ChrY candidate genes in the lungs of GDX Hx mice and measured the effect on PH development (Figure 1A). We found no significant change in the RVSP, Fulton index, or pulmonary artery acceleration time (PAAT) of mice with KD of *Kdm5d* (Figure 1B; Figures E1A, E1D, and E1G in the online supplement), *Ddx3Y* (Figure 1C; Figures E1B, E1E, and E1H), or *Eif2s3y* (Figure 1D; Figures E1C, E1F, and E1I). However, KD of *Uty* resulted in significantly elevated RVSP compared with the Si-Scrm control group in two separate experiments (Figure 1E), which was concomitant with significantly lower PAAT and ratio of PAAT to RV ejection time (RVET) (Figure 1F; Figure E2A), although there was no

significant change in RV hypertrophy (Figure 1G). As experimental validation, we confirmed that WT mice subjected to Hx exhibited significantly elevated RVSP compared with normoxic mice and that Si-Scrm treatment alone had no significant effect on RV pressure (Figure E2B).

We found that *Uty* expression in Si-Uty–treated lungs was reduced by >50% compared with Si-Scrm lungs (Figure E3A). We further quantified cell-specific *Uty* knockdown and found that >50% KD was achieved in CD68$^+$ macrophages and vascular ECs and smooth muscle cells, indicating that the siRNA successfully reduced *Uty* transcripts in the cell types most relevant to PH and our study (Figures E3B–E3D).

We examined *Uty* expression in the total lung tissue of three different rodent

models of PH and found that *Uty* is downregulated as a result of Hx-induced PH in male mice and both the MCT and SuHx PH models in male rats, which is consistent with the downregulation of *UTY* found in human male PAH lungs (13) (Figure E4). We tested whether downregulation of Uty is a result of a pathogenic process in PH and PAH or a byproduct of hypoxia-inducible factor (Hif) upregulation, as Hif is upregulated in Hx and known to be associated with PH development (18). We found no change in *Uty* expression in response to *Hif1a* upregulation in alveolar macrophages (Figures E5A–E5D), indicating that Uty is not directly regulated by *Hif1a*. We also tested whether *Hif1a* is regulated by Uty expression and found no change in *Hif1a* expression as a result of global Uty-KO
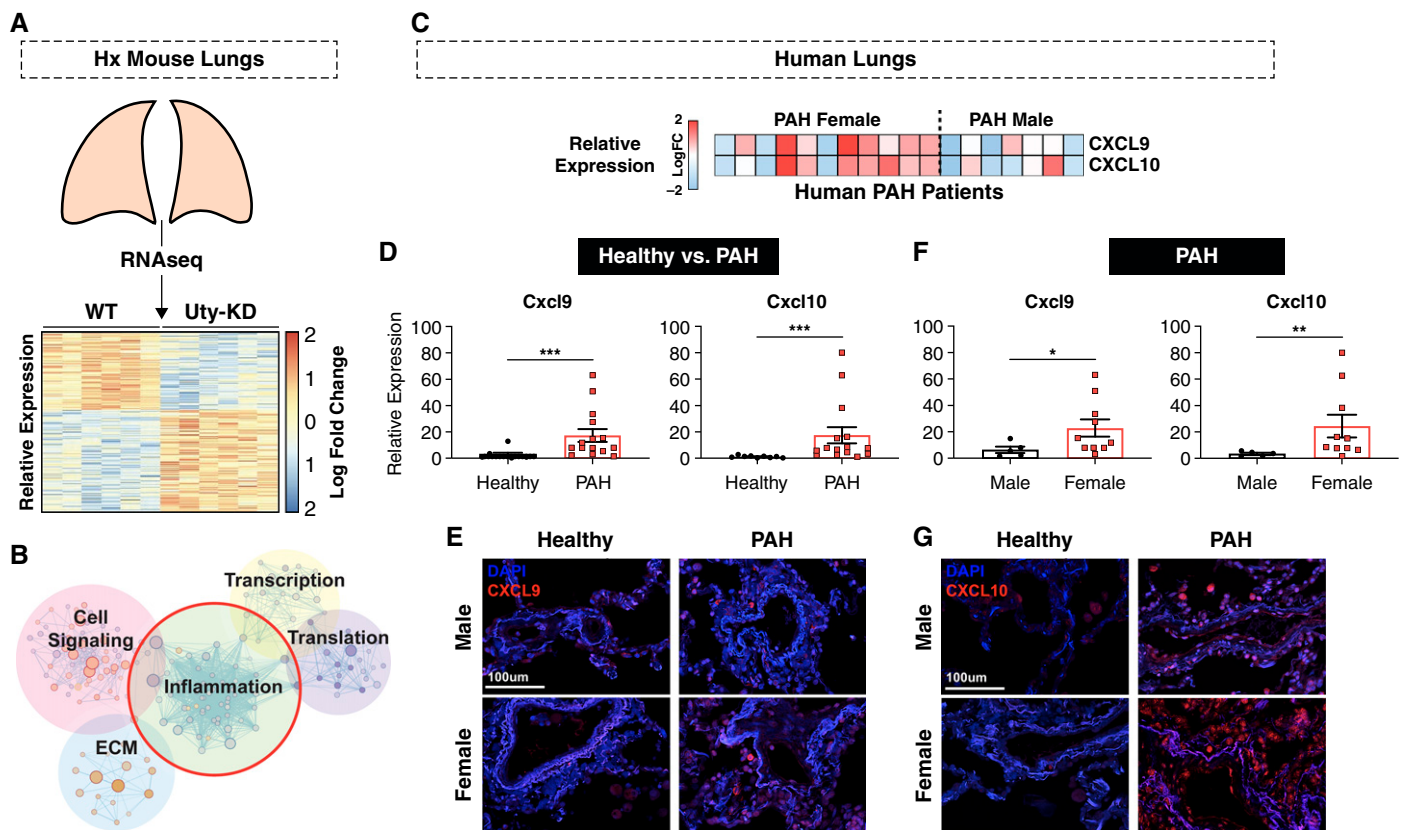

Figure 2. Proinflammatory chemokines CXCL9 and CXCL10 are autosomal downstream deterrents of *Uty* protection and are upregulated in PAH patient lungs in a sex-specific manner. (*A*) Comparison of RNA-sequencing (RNAseq) data from Si-Scrm and Si-Uty Hx mouse lungs revealed 523 differently expressed genes (DEGs) as represented by vertical columns on a heatmap depicting log fold change in expression. (*B*) Pathways enriched with DEGs include inflammation, cell signaling, extracellular matrix (ECM), transcription, and translation. (*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 (versus PAH males) and Si-Uty mice (versus Si-Scrm) as depicted in a heatmap representing individual patients' relative gene expression. (*D*) Relative expression of CXCL9 and CXCL10 in human healthy and PAH lungs as measured by real-time quantitative PCR (qPCR). (*E–G*) Representative immunofluorescence staining of CXCL9 (*E*) and CXCL10 (*G*) in male and female lung tissue from healthy and diseased (PAH) patients. (*F*) Relative expression of CXCL9 and CXCL10 in human male and female PAH lungs as measured by real-time qPCR. Scale bars, 100 μm. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Hx = hypoxia; KD = knockdown; WT = wild-type.

(Figures E5E–E5G). Our data suggest that Uty is downregulated as a result of the PH disease process but not because of an interaction with Hif1a alone.

In summary, these data indicate that *Uty* confers protection against PH. Reduction of *Uty*, but not the other ChrY genes expressed in the lung, is sufficient to exacerbate PH in GDX male mice, and PH and PAH pathology further downregulates Uty in a Hif1a-independent manner.

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 RNA sequencing on Hx Si-Scrm and Si-Uty mouse lung tissue and identified 523 DEGs (Figure 2A). Pathway enrichment analysis highlighted five main pathways,

with inflammation particularly enriched (Figure 2B). Because inflammation plays a major role in PAH pathogenesis and severity (2), we focused on inflammatory DEGs. We cross-referenced our inflammatory DEG set with an online microarray of PAH patient lung tissue and searched for genes that were elevated in both Si-Uty, compared with Si-Scrm, and female PAH samples, compared with male samples (17). This analysis yielded two top genes of interest: proinflammatory
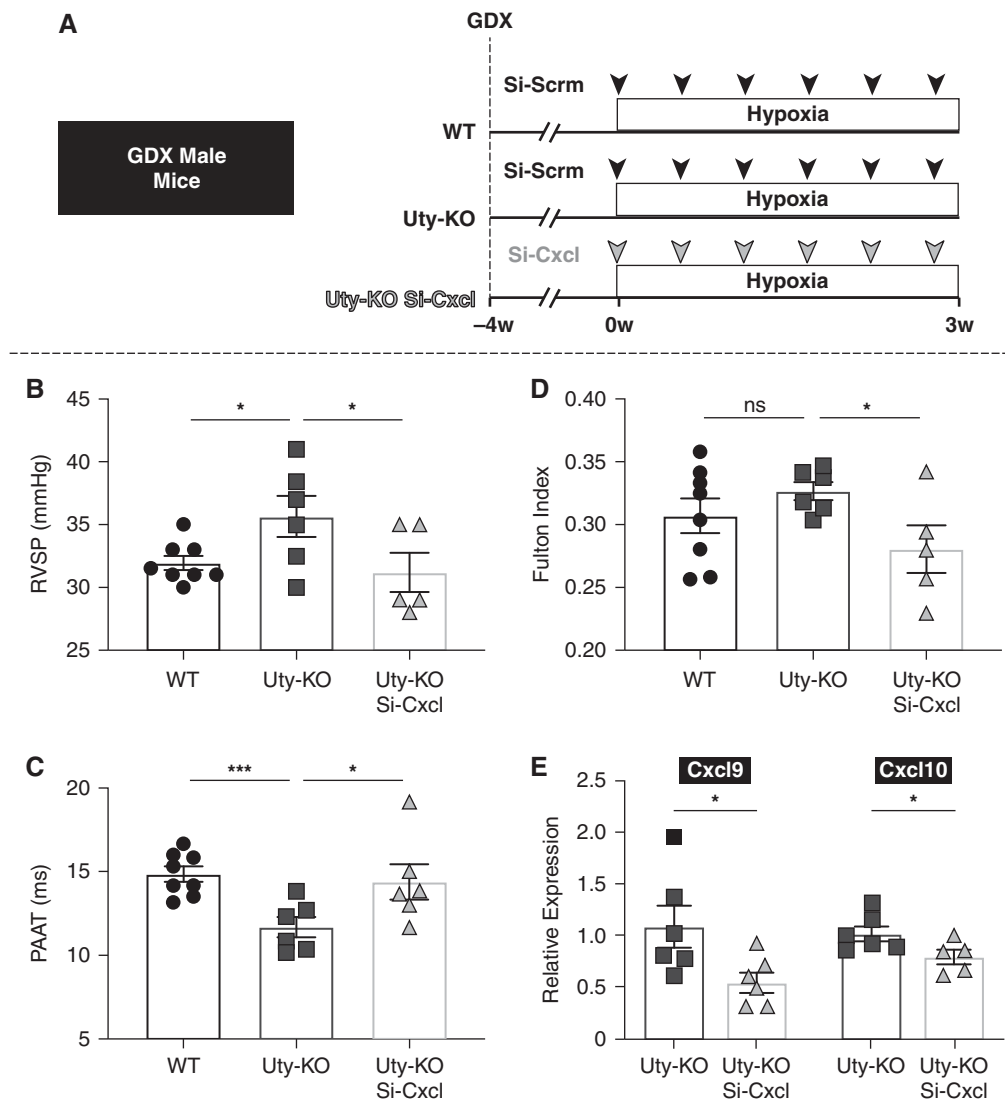

Figure 3. Global Uty-KO promotes more severe PH, which is prevented by KD of Cxcl9 and Cxcl10. (*A*) Experimental protocol: 30 days after GDX, age-matched global Uty-KO or WT male mice receive scramble siRNA or siRNA targeting Cxcl9 and Cxcl10 genes every 3–5 days and are placed in Hx for 3 weeks. (*B*) RVSP measured by direct RV catheterization. (*C*) PAAT measured by echocardiography. (*D*) Fulton index (RV/LV + IVS). (*E*) Quantification of *Cxcl9 and Cxcl10* transcripts in whole lung tissue by means of real-time qPCR (normalized to Si-Scrm). *$P < 0.05$ and ***$P < 0.001$. GDX = gonadectomy; HX = hypoxia; IVS = intraventricular septum; KD = knockdown; KO = knockout; LV = left ventricle; ns = not significant; PAAT = pulmonary artery acceleration time; PH = pulmonary hypertension; RV = right ventricle; RVSP = right ventricular systolic pressure; WT = wild type.
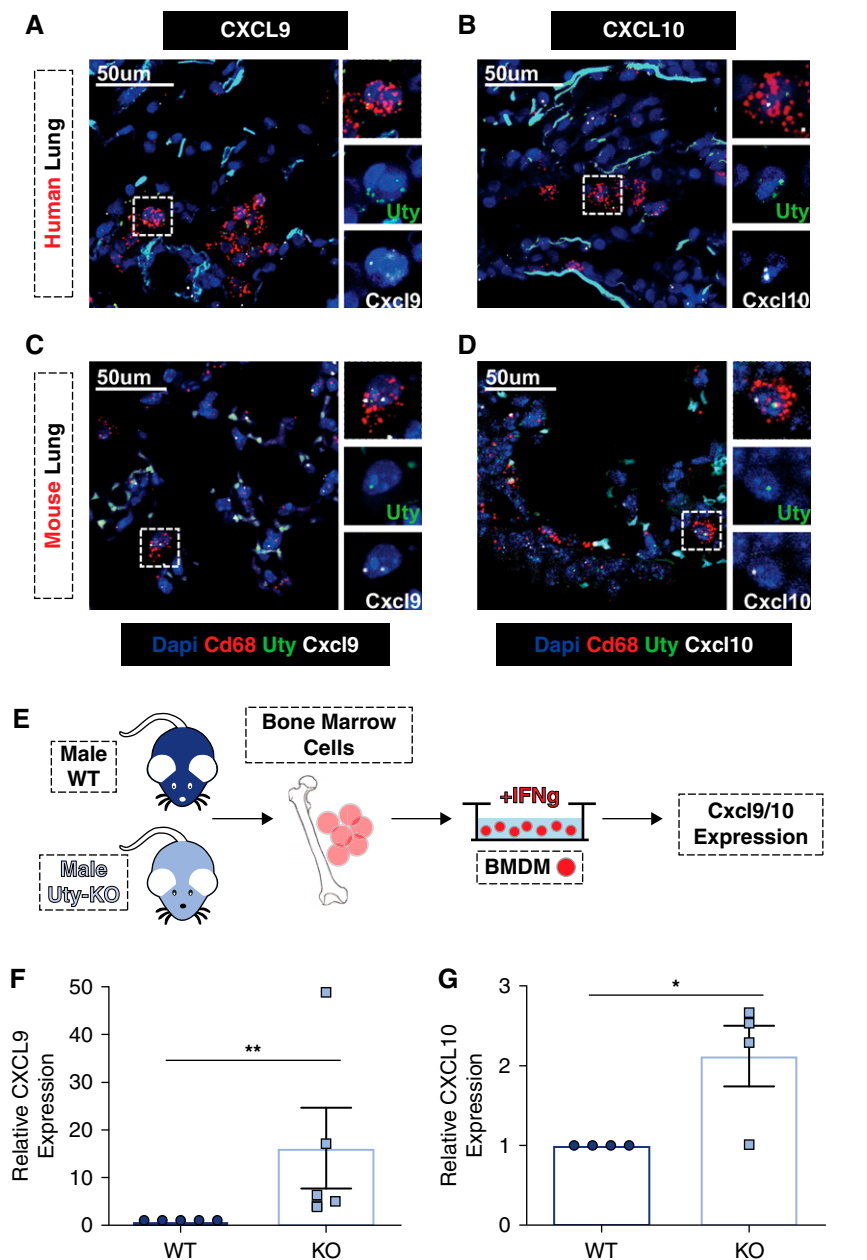
Figure 4. Uty, Cxcl9, and Cxcl10 are colocalized in macrophages, and Uty expression is inversely related to Cxcl9 and Cxcl10 expression. (*A–D*) Representative images depicting colocalization of *in situ* probes labeling *Cd68/CD68* (red), *Uty/UTY* (green), and *Cxcl9/CXCL9* (white) in mouse (*A*) and human lung sections (*C*). Representative images depicting colocalization of *in situ* probes labeling *Cd68/CD68* (red), *Uty/UTY* (green), and *Cxcl10/CXCL10* (white) in mouse (*B*) and human lung sections (*D*). (*E*) Schematic of bone marrow–derived macrophage (BMDM) *in vitro* experiments. (*F* and *G*) Relative expression of *Cxcl9* (*F*) and *Cxcl10* (*G*), as measured by real-time qPCR in BMDMs isolated from WT and Uty-KO mice. *$P < 0.05$ and **$P < 0.01$. Scale bars, 50 μm. KO = knockout; WT = wild type.

chemokines *CXCL9* and *CXCL10* (Figure 2C), both of which we validated to be upregulated in male and female PAH lungs compared with healthy lungs, with a more robust upregulation in female PAH patients compared with male patients (Figures 2D and 2F). These data are represented by immunofluorescence images of human healthy and PAH lungs (Figures 2E and 2G).

Taken together, we found that downregulation of *Uty* is concomitant with upregulation of *Cxcl9* and *Cxcl10* in Uty-KD mouse lungs (Figures E6A and E6B) and that *CXCL9* and *CXCL10* expression levels are upregulated in human PAH lungs in a sex-specific manner.

Global Uty-KO Promotes More Severe PH that Is Prevented by KD of Cxcl9 and Cxcl10

Because lung-specific Uty-KD resulted in increased PH severity and elevated *Cxcl9* and *Cxcl10* expression, we tested *1*) whether global Uty-KO mice also developed more severe PH and *2*) whether knocking down *Cxcl9* and *Cxcl10* in Uty-KO mice would be sufficient to reduce PH severity (Figure 3A). We found that GDX UTY-KO male mice exposed to chronic Hx exhibited elevated RV pressures and a reduced PAAT and PAAT/RVET ratio compared with GDX WT males (Figures 3B and 3C; Figure E7). More severe PH was also observed in gonad-intact Hx Uty-KO animals compared with intact Hx WT males, although there was no significant interaction between gonadal hormones and PH severity (Figures E8A–E8E). Combined KD of *Cxcl9* and *Cxcl10* in the lungs of Hx GDX Uty-KO mice resulted in a significant reduction in RV pressures and RV hypertrophy, coupled with a significant increase in PAAT and PAAT/RVET ratio compared with that in Uty-KO controls (Figures 3B–3D; Figure E7). We validated that *Cxcl9* and *Cxcl10* expressions were significantly reduced in the lungs of the Uty-KO mice that received targeting siRNA compared with Uty-KO mice that received nontargeting Si-Scrm (Figure 3E).

Overall, Uty-KO male mice exhibited worse PH severity compared with WT mice when exposed to Hx. Lung-specific KD of *Cxcl9* and *Cxcl10* is sufficient to attenuate PH development in GDX Uty-KO mice.

Uty, Cxcl9, and Cxcl10 Are Colocalized in Macrophages, and Uty Expression Is Inversely Related to Cxcl9 and Cxcl10 Expression

Given the increasing importance of macrophages in PH pathobiology (19–22) and macrophage expression of both CXCL9 and CXCL10 (23), we tested for Uty expression in lung macrophages. Stained tissue sections from mouse and human lungs confirmed Uty expression within Cd68^{+} macrophages in male tissue but not in female tissue (Figures E9A and E9B). Although we found Uty inversely related to
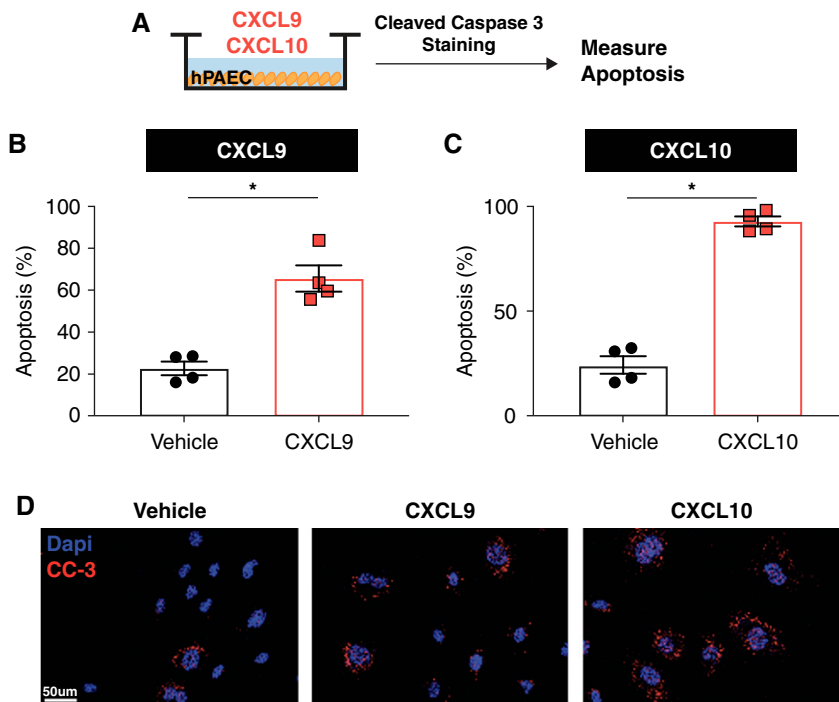
Figure 5. CXCL9 and CXCL10 recombinant protein triggers pulmonary artery endothelial cell (PAEC) dysfunction. (*A*) Schematic of human PAEC (hPAEC) experiments. (*B* and *C*) Quantification of percent apoptosis measured in PAECs treated with CXCL9 (*B*) or CXCL0 (*C*), as measured by cleaved caspase-3 immunofluorescence. (*D*) Representative images of cleaved caspase-3 (CC-3; red) immunofluorescence in vehicle, CXCL9-treated, and CXCL10-treated cells. *$P < 0.05$. Scale bar, 50 μm.

chemokines Cxcl9 and Cxcl10, we examined lung tissue for colocalization within macrophages and found Uty transcript colocalized with Cxcl9 (Figures 4A and 4C) and Cxcl10 (Figures 4B and 4D).

To identify whether Uty expression in macrophages directly influences Cxcl9 and Cxcl10 production, we measured *Cxcl9* and *Cxcl10* expression in BMDMs from male WT and Uty-KO mice (Figure 4E). BMDMs from Uty-KO mice expressed significantly more *Cxcl9* and *Cxcl10* compared with WT mice (Figures 4F and 4G).

UTY is a member of the Jumonji family of histone demethylases, but its role as an H3K27 demethylase is highly contested in literature (24–27). We examined whether the effects of UTY in the lung were mediated through demethylation and found that UTY expression in whole lung and isolated lung macrophages did not influence levels of H3K27 trimethylated protein as measured by Western blot (Figure E10).

In summary, we found that Uty, which colocalizes with Cxcl9 and Cxcl10 in lung macrophages, regulates *Cxcl9* and *Cxcl10*

expression but has no apparent effect on H3K27 demethylation.

CXCL9 and CXCL10 Expression Triggers Pulmonary Artery EC 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 PAECs. PAECs express the shared CXCL9 and CXCL10 receptor, CXCR3; and 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 (28). We incubated human PAECs with recombinant human CXCL9 and CXCL10 protein and measured the effect on EC dysfunction (Figure 5A). Treatment of PAECs with either CXCL9 or CXCL10 resulted in increased PAEC apoptosis, as measured by quantification of cleaved caspase-3 staining (Figures 5B–5D).

These data demonstrate that proinflammatory chemokines Cxcl9 and

Cxcl10, which are upregulated in the absence of Uty, induce PAEC apoptosis *in vitro*.

Blocking the Activity of CXCL9 and CXCL10 in Females Is Sufficient to Rescue PH in Two Preclinical Rat Models

Our data suggest that Uty confers PH protection through downregulating Cxcl9 and Cxcl10 expression (Figure 3) and that CXCL9 and CXCL10 promote PAEC dysfunction (Figure 5). Because we found these proinflammatory chemokines elevated in human PAH patient lungs and particularly high in female PAH patients (Figure 2), we tested whether we could extend the mechanism of Uty protection to females with PH by blocking the activity of CXCL9 and CXCL10 using a small-molecular inhibitor (AMG487) targeting CXCR3 (Figures 6A and 6B).

To better examine for protective effects, we used two preclinical models of PH in rats, the MCT and SuHx models, which are more severe than Hx in mice. We tested our preclinical treatment strategy on gonad-intact females to determine whether targeting CXCL9 and CXCL10 would be a viable treatment strategy for PAH. We found that AMG487 sufficiently rescued PH development in both the MCT and SuHx models, as rats treated with AMG487 2 or 3 weeks after PH stimulus exhibited significantly lower RV pressures concomitant with increased PAAT, PAAT/RVET ratios, and RV fractional area change (Figures 6C–6F; Figures E11A–E11D). In addition, RV hypertrophy was significantly reduced in AMG487-treated rats in the MCT model, and there was a trend toward reduced RV hypertrophy in the SuHx model (Figures 6G–6H). We validated that the RV pressures were elevated in both preclinical models compared with that in healthy control rats (Figure E12).

Because CXCL9 and CXCL10 induce PAEC apoptosis *in vitro* (Figure 5), we aimed to delineate whether this cellular mechanism contributes to the PH protection conferred by AMG487 treatment in rats. We found reduced EC apoptosis in the lungs of treated MCT and SuHx rats, which indicates that blocking the actions of CXCL9 and CXCL10 reduced pulmonary vascular EC dysfunction (Figures 6I–6L).

Taken together, our findings demonstrate that inhibiting the shared CXCL9 and CXCL10 receptor in gonad-intact female rats is a viable preclinical
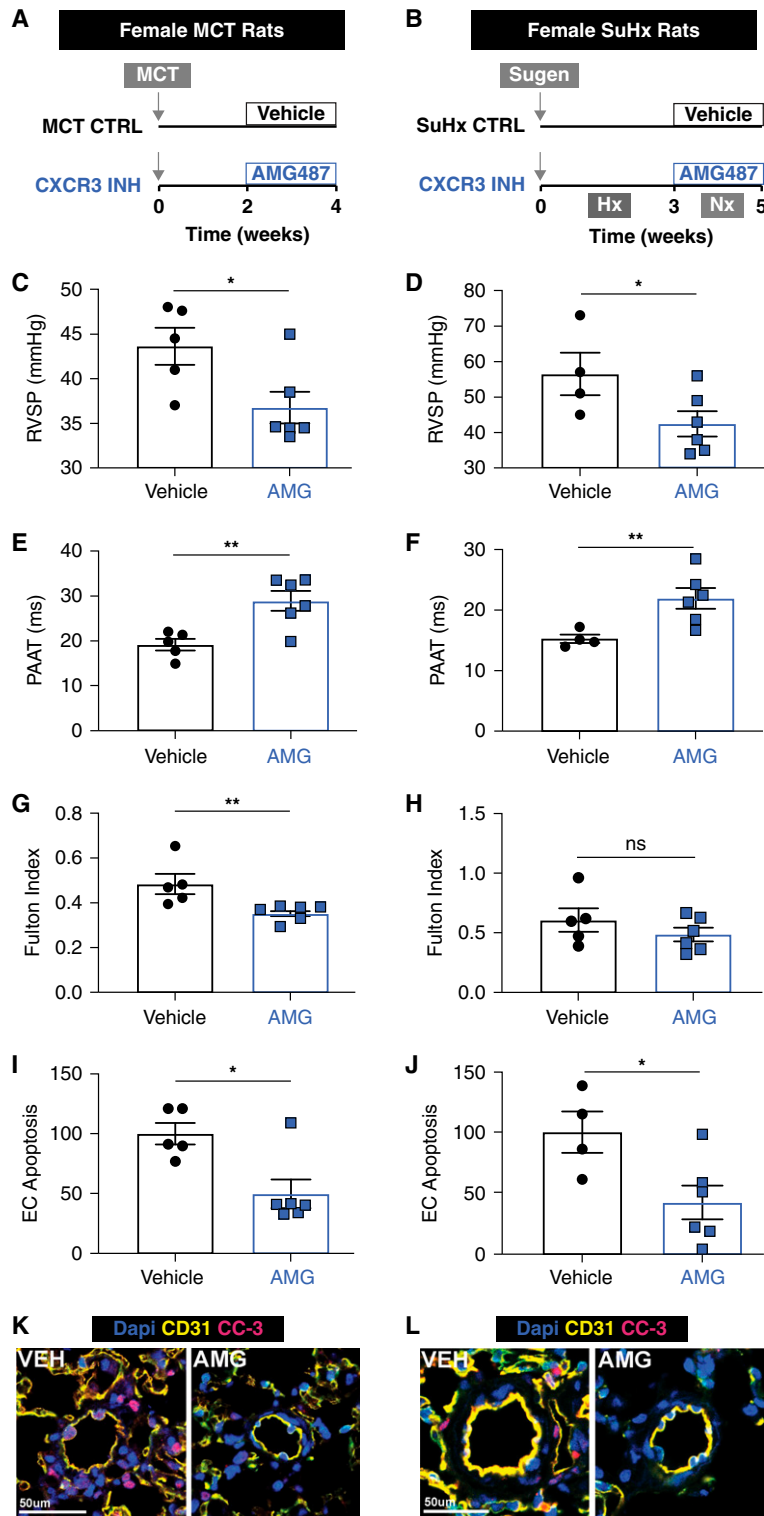
Figure 6. Blocking the activity of CXCL9 and CXCL10 is sufficient to rescue PH in two preclinical rat models. Schematic of *in vivo* experiments in female rats with (*A*) monocrotaline (MCT)-induced PH and (*B*) Sugen 5416-Hx (SuHx)–induced PH. All MCT model measurements are listed in the left column (*A*, *C*, *E*, *G*, *I*, and *K*), and SuHx measurements are listed in the right column (*B*, *D*, *F*, *H*, *J*, and *L*). (*C* and *D*) RVSP, (*E* and *F*) PAAT, and (*G* and *H*) Fulton index (RV/LV + IVS) measured in AMG487 (AMG)-treated PH rats compared with vehicle-treated PH controls. (*I* and *J*) Quantification and (*K* and *L*) representative images of apoptotic EC cells in CD31 (yellow) and cleaved caspase-3 (CC-3; pink) labeled sections from vehicle (VEH) and AMG-treated lungs. *$P < 0.05$ and **$P < 0.01$. Scale bars, 50 μm. CTRL = control; EC = endothelial control; INH = inhibition; IVS = intraventricular septum; LV = left ventricle; ns = not significant; PAAT = pulmonary artery acceleration time; RV = right ventricle; RVSP = right ventricular systolic pressure.
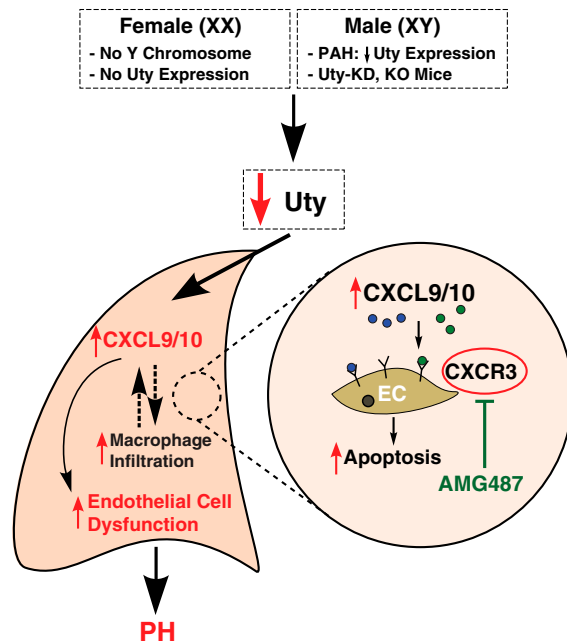
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Figure 7. Proposed mechanism of Uty/Cxcl9/Cxcl10 axis in PH pathogenesis. Uty absence (in females) or reduced expression (in males with PAH or Uty-KD or in KO mice) results in an upregulation of proinflammatory chemokines Cxcl9 and Cxcl10 in the lung. 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. EC = endothelial control; KD = knockdown; KO = knockout; PAH = pulmonary arterial hypertension; PH = pulmonary hypertension.

treatment strategy, as it reduced PH severity and lung vascular EC dysfunction in two experimental models of PH.

Discussion

We previously demonstrated that ChrY is protective against experimental PH (13), and this study is the first in the field to investigate the protective role of each ChrY gene expressed in the lung. We found that lung-specific KD of *Uty*—but not ChrY genes *Eif2s3y*, *Ddx3y*, or *Kdm5d*—in GDX male mice exposed to Hx increased PH severity, thereby eliminating ChrY protection (Figure 1). In agreement with our Uty-KD studies, our data also show global Uty-KO mice develop more severe PH than WT controls (Figure 3). We found that loss of *Uty* expression in PH lungs and cultured macrophages resulted in an upregulation of proinflammatory chemokines *Cxcl9* and *Cxcl10* (Figure 4; Figure E6). Interestingly, *CXCL9* and *CXCL10* are upregulated in PAH patient lungs with a more robust upregulation in female patients compared with males (Figure 2). We found that knocking down *Cxcl9* and *Cxcl10* was

sufficient to prevent more severe PH in Uty-KO mice, indicating that Cxcl9 and Cxcl10 are downstream of Uty protection (Figure 3). Stimulating human PAECs with exogenous CXCL9 or CXCL10 was sufficient to trigger PAEC dysfunction, a hallmark of PAH pathogenesis (Figure 5). Furthermore, we found that blocking CXCL9 and CXCL10 activity is a novel PH therapeutic strategy, as it reduced PH severity and EC apoptosis in two preclinical models (Figure 6). Figure 7 summarizes our findings.

We believe that our study is the first to directly explain ChrY protection against PH. A recent study from Yan and colleagues noted a connection between the ChrY testis-determining gene *Sry* and the PAH-related gene *Bmpr2* in cultured dermal fibroblasts (29). Unlike Uty, Sry expression is not detected in mouse or human lung tissue. Additionally, our previous study demonstrating ChrY protection utilized the well-published four core genotypes mouse model, which produces gonadal male and female mice with both XX and XY chromosomes (30). XY females, which were protected against PH compared with XX females, lack the testis-determining *Sry* gene (13). This indicates that Sry, a gene

absent from lung tissue and the XY female mouse model, is not responsible for ChrY protection against PH.

Our study found that Uty regulates the expression of the proinflammatory chemokines CXCL9 and CXCL10. Dysregulation of these small proteins and their receptor (23), which are known to promote chemotaxis and immune cell differentiation, is associated with EC apoptosis (31–33) and a myriad of systemic inflammatory diseases (34–38). Before this study, CXCL9 and CXCL10 were already associated with PAH severity, and plasma levels of CXCL10 were found to be upregulated in PAH patients compared with healthy controls (36, 37). However, sex differences in *CXCL9* and *CXCL10* expression in PAH patients and the mechanism by which they promote PAH development were not known. Our study identifies a sexually dimorphic *CXCL9* and *CXCL10* upregulation in human PAH lung tissues (Figure 2), mechanistically links these chemokines to Uty expression and PH pathogenesis through PAEC dysfunction, and shows that targeting their activity is a novel PH therapy.

UTY is a member of the Jumonji family of H3K27 histone demethylases; however, it has undergone biochemical changes, and the demethylase activity of UTY is contested (24, 26, 27). Although Walport and colleagues demonstrated some residual demethylase activity in the human UTY enzyme (25), our study is in agreement with Lan and colleagues, Hong and colleagues, and Shpargel and colleagues, who found no UTY enzymatic activity (24, 26, 27). Regardless, Uty expression has a widespread effect on autosomal gene expression (14), and dysregulation of Uty is associated with disease (15, 39). We found Uty expression is downregulated in male PAH patient lung tissue (13) as well as in the lung tissue of three different experimental models of PH (Figure E4). Reduced *UTY* expression, specifically in macrophages, is associated with increased atherosclerosis risk in men (15, 40, 41), and macrophage-specific Uty-KD impairs immune activation (41). We confirmed colocalization of Uty with Cxcl9 and Cxcl10 in lung macrophages using RNAscope *in situ* hybridization and found that macrophages derived from Uty-KO BM have higher expression of *Cxcl9* and *Cxcl10*, indicating that Uty expression in macrophages regulates Cxcl9 and Cxcl10 production (Figure 4). Although Hif1a signaling is associated with Hx and PH

development through chemokine signaling (18), we did not see evidence of a Hif1a/Uty interaction (Figure E5). RNAscope *in situ* probes were used for imaging after we determined that UTY antibodies likely detected ChrX paralog UTX, as they revealed a signal in females. We believe that our RNAscope images are the first to depict specific Uty expression in mouse and human lung tissue (Figure E9B).

Our study provides 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 (Figure 7). Although these results help explain why females who lack *UTY* have a higher incidence of PAH, they do not explain why PAH males have worse prognoses and clinical outcomes (42). We hypothesize that reduced *UTY* expression in some males, potentially because of a paternal haplotype that is known to influence *UTY* expression (43),

could prime them for PAH development and elevated Cxcl9 and Cxcl10 expression compared with males with higher *UTY* expression. Additionally, there are known sex differences in the immune system (44, 45), and further reduction of *UTY* expression in male lungs as a result of PAH pathology may trigger a harmful immune cascade that contributes to more severe PAH. There is also a known relationship between RV remodeling and PAH prognosis, which is linked to sex hormones (46, 47). In particular, testosterone was shown to contribute to more severe RV remodeling in PH, which could account for male PAH patients having worse prognoses (48). Although Uty expression may initially protect males from developing PAH compared with females, increased testosterone in males may lead to worse outcomes. Our studies did not find a significant interaction of gonadal hormones and PH severity in global Uty-KO mice (Figure E8E);

however, more investigation into the interaction between Uty and sex-biasing factors could provide additional insight into the complex and paradoxical sex differences in PAH. ∎

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