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Mas1 Receptor Activation is Necessary and Sufficient to Transduce ACE2 Effect in PAH, But Ang(1-7) Alone is Insufficient

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ABSTRACT

ACE2 has shown effectiveness in treating pulmonary hypertension in multiple animal models and has some promise in early human trials. The key barrier to translation is that enzymatically active ACE2 is difficult to manufacture and exhibits a short half-life in humans, making chronic administration challenging. Understanding the mechanism of effect is thus key to finding ways to bypass ACE2 while still reproducing therapeutic effects. In this study, we test the hypotheses that ACE2 produces its therapeutic effect through increased Mas1 signaling and that Ang(1-7) is sufficient as the Mas1 ligand. We found that the ACE2 effect is blocked in Mas1 knockout mice and that the Mas1 agonist AR234960 reproduces the ACE2 effect, indicating that Mas1 activation is necessary and sufficient for the ACE2 therapeutic effect. However, neither AlbudAb-stabilized Ang(1-7) nor Ang(1-7) stabilized through the use of protease inhibitors were capable of reproducing ACE2 effectiveness, indicating that Ang(1-7) alone does not activate Mas1 in this context. RNA-seq suggests that the key mechanisms downstream of Mas1 responsible for the therapeutic effect of ACE2 and AR234960 are the rescue of cytoskeletal and microtubule defects. Together, these findings indicate that direct activation of Mas1 will likely be effective in treating pulmonary arterial hypertension, but raise the question of the identity of the endogenous ligand(s).

1 | Introduction

Cytoskeletal defects are fundamental to pulmonary arterial hypertension (PAH) through multiple physiologic mechanisms. Stiffening of the pulmonary vasculature reduces vascular compliance, increases hydraulic impedance, and increases cardiac workload [1]. Related cytoskeletal defects increase vascular leak, which exposes smooth muscle cells and the obverse side of the endothelium to plasma proteins, increasing inflammatory reactions, as well as increasing circulating cell recruitment, driving endothelial dysfunction and smooth muscle proliferation [2, 3]. Intracellular transport depends on a functional cytoskeletal architecture, and some of the metabolic defects in PAH, such as insulin insensitivity, may be caused by failure to properly translocate the glucose transporter in response to signaling [4].

One potential cause of these defects is the gene most commonly responsible for heritable PAH, BMPR2 [5, 6]. Although BMPR2 is most commonly thought of as signaling through SMAD transcription factors, many disease-causing mutations are in the cytoplasmic tail domain, which is dispensable for SMAD signaling. The BMPR2 tail domain directly interacts with and

regulates several proteins that control cytoskeletal function in a fairly direct fashion, including SRC [7], LIMK1 [8], and TCTEX [9]. However, even without BMPR2 mutation, idiopathic PAH patients have cytoskeletal problems. We performed whole exome sequencing on 36 IPAH patients with no family history of disease and found that they had multiple rare genomic variants that converged on cytoskeletal function [10]. The IPAH patients did have a genetic cause, focused on cytoskeletal defects; their lack of family history was because the odds of getting multiple genomic variants in any nearby generation are vanishingly small. As a whole, then, alterations in cytoskeletal dynamics are at the core of both heritable and idiopathic PAH.

In 2012, we published that treating Bmpr2 mutant mice with recombinant ACE2 resolved their PAH while resolving their cytoskeletal defects [11]. It is still the most effective treatment we have found in this model; unlike other models, very little reduces PAH in $Bmpr2^{R899X}$ mutant mice. Moreover, both before and after this, ACE2 was found to treat PAH in a variety of other models, including in fibrotic mice [12], monocrotaline rats [13], pressure overload [14], hypoxic rats [15, 16], and pneumonectomy [17, 18].

In 2018, we published a small single-dose trial of recombinant human ACE2 in PAH patients [19]. It showed indications of molecular efficacy and, surprisingly, reduced pulmonary vascular resistance in some patients. However, enzymatically active ACE2 requires proper glycosylation to be produced in a functional form [20, 21] and thus requires expression in adherent mammalian cell lines, limiting production. Attempts have been made to make ACE2 in other cell types [22], but so far, enzymatic activity has not been demonstrated. In addition, the half-life of recombinant human ACE2 is short [23]. Taken together, this will mean that using ACE2 to treat chronic diseases like PAH will be extremely challenging.

To overcome this problem, it may be necessary to reproduce ACE2's therapeutic effect through reproducing ACE2's effect on downstream components through other means. This requires explicitly testing the assumed mechanism for ACE2 activity, which is as follows.

ACE2 is an enzyme that cleaves one amino acid off the 8-peptide AngII to form Ang(1-7), resulting in a decrease in signaling through angiotensin receptors AT1R and AT2R, and an increase in signaling through the receptor Mas1 with Ang(1-7) as the ligand (Figure 1). Although there may be some benefit to the reduced signaling through AT1R and AT2R [24], the bulk of the benefit is hypothesized to be through Mas1 activation.

The goal of this project was to explicitly test the above mechanism, using mouse models, with RNA-seq to understand molecular effects.

2 | Methods

2.1 | Sugen-Hypoxia in Mice

Most of the mouse models below use the Sugen-Hypoxia (SuHx) model. Sugen (Tocris) is delivered to mice suspended at 0.5 mg/100 μL in CMC solution. CMC solution is 0.5% carboxymethylcellulose sodium, 0.4% polysorbate, and 0.9% benzyl alcohol, dissolved in 0.9% saline. The Sugen suspension is remade weekly. Sugen suspension is injected at 20 mg/kg (= 100 μL of the above solution in a 25 g mouse). Sugen is injected IP on Day 1, and weekly thereafter.

Mice are placed in 10% normobaric hypoxia for 3 weeks, with O_2 and CO_2 levels continuously monitored.

2.2 | Ethical Approval

The Institutional Animal Care and Use Committee at Vanderbilt University Medical Center approved all animal studies through the following protocol numbers: M1600048 (ACE2, Ang(1-7)-Albudab), M2200005 (AR234960), and M2200003 (Alt00).

2.3 | Mas1 Knockout Mice

Mas1 knockout mice were used with permission from Dr. Michael Bader, who originally created them, as described here [25].

2.4 | Mouse Phenotyping

Right ventricular systolic pressure (RVSP) was directly measured via the insertion of a 1.4 F Mikro-tip catheter transducer (Millar Instruments Houston, Texas, the United States) into a surgically exposed right internal jugular vein as previously described [26].

Immunohistochemistry was performed as previously described [27], using an alpha-smooth muscle actin antibody (ab5694 Abcam Cambridge, Massachusetts, the United States).

2.5 | Compounds

AlbudAb-linked Ang(1-7) was received courtesy of Dr. David Hall at GSK and was used at 0.1 mg/kg injected subcutaneously

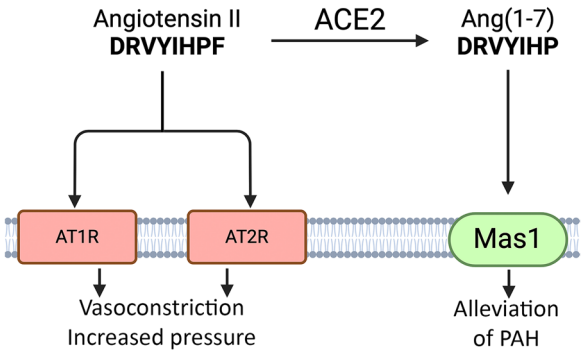

FIGURE 1 | Initial hypothesis for ACE2 therapeutic effect in pulmonary hypertension; ACE2 cleaves one amino acid from angiotensin II to produce Ang(1-7), which signals through the Mas1 receptor to produce beneficial effects. This study demonstrates that this is partially incorrect; ACE2 and Mas1 are required, but Ang(1-7) is not the ligand.

twice/week for the 3 weeks of SuHx. Control mice were given Albudab alone.

Alt00 is an aminopeptidase inhibitor, which prevents N-terminal proteolytic degradation of angiotensin metabolites, including Ang(1-7) [28]. This was used in osmotic pumps in combination with Lisinopril, which inhibits ACE, which can also degrade Ang(1-7). Alt00 was received courtesy of Alterras Therapeutics GmbH, Vienna, Austria.

Recombinant human ACE2 was delivered at 1.2 mg/kg/day through osmotic pumps at pH 7.5, 100 mM glycine, 150 mM NaCl, and 50 μM $ZnCl_2$ for the final 14 days of the SuHx treatment (it cannot be used longer because mice start to develop antibodies to the human ACE2).

AR234960 was purchased from MedChemExpress and was injected twice daily at 0.25 mg/injection. A stock solution of AR234960 was made in DMSO at 12.5 mg/mL. The working solution, which is only good for 24 h, was made by diluting the stock to 10% DMSO with 40% PEG400, 5% Polysorbate 80, and 45% saline.

2.6 | RAS Equilibrium Analysis

The equilibrium levels of angiotensin metabolites in murine heparinized plasma samples were quantified with liquid chromatography-tandem mass spectrometry (LC-MS/MS) performed at Attoquant Diagnostics (Vienna, Austria) using previously validated and described methods [28].

2.7 | RNA-Seq

We performed RNA-Seq on whole lung from 9 control mice (6 male, 3 female), 3 mice treated with ACE2 (male), 3 with AR234960 (male), and 6 with Alt00/Lisi (3 male, 3 female). Larger numbers of control mice were used because each group (ACE2, AR234960, and Alt00/Lisi) was sequenced with contemporaneous controls. Lung was flash-frozen in liquid nitrogen immediately after sacrifice. RNA was isolated from the whole lung using RNEasy kits (Qiagen) and delivered to Novogene (Sacramento, California, the United States) for paired-end 150 sequencing on an Illumina platform. A nominal read depth of 20 million RNA (40 million ends) per animal was used, with the actual falling between 20.2 and 29.1 million reads. These were uploaded to and analyzed on the Partek platform, using the STAR aligner to align to the mm39 reference mouse genome. An average of 94% of reads aligned to the genome (91.4%–95.6%). Counts were normalized to Counts Per Million, and the results and raw data were uploaded to GEO (GSE155124 and GSE272518). Group differences were assessed using DESeq. 2, a newer statistical method designed explicitly for RNA-seq data [29].

Gene ontology was determined using Partek's Gene Set Enrichment tool or using Webgestalt [30]. Gene ontology significance is corrected for multiple testing using the Benjamini and Hochberg False Discovery Rate.

2.8 | LIMK Westerns

Samples were broken up in our bead beater with Lysing matrix D tubes (3 × 15 s) in RIPA buffer with a 1:50 dilution of protease inhibitors and phosphatase inhibitor cocktails 1 and 2 (Sigma cat. # P8340, P-2850, and P-5726). Samples were then sonicated for 1 min at 20% power and then allowed to sit on ice for 20 min. Samples were then centrifuged for 15 min at 13k RPM at 4°C. The cleared supernatants were transferred to new 2 mL microfuge tubes. Protein quantification was determined by BCA. The samples were divided into three different tubes to avoid repeated freeze–thawing. The samples were denatured at 70°C for 10 min in (final concentration) 1× LDS BUFFER and 1× reducing reagent (NuPage). The protein amounts were 15 μg per lane on a NuPage 4-12 Page gel with MES running buffer. The ladder marker was PS2 plus BLUE from Thermo Fisher. Gel proteins were transferred to a PVDF membrane with a Bio-Rad TRANS BLOT TURBO SYSTEM. PVDF blot was blocked in BioGenex Power Block. Our antibody was purified with Mouse Anti-LIMK1 (cat # 6117484, BD Transduction Labs), used at a 1:1000 concentration in NFDM, and incubated overnight at 4°C. The next morning, the NFDM in 1× TTBS with antibody was removed, and the blot was washed 4 × 15 min in 1× TTBS; after this, the blot was incubated for 1 h in 1× TTBS with a 1:5000 dilution of Goat anti-Mouse secondary labeled with HRP. After 1 h this antibody was removed, and the blot was again washed as above. Then, the blot was immersed in 5 mL of Super Signal West Pico Plus Chemiluminescent substrate and rocked for 5 min before imaging in our iBRIGHT 1500 imager.

3 | Results

3.1 | The Mas1 Receptor is Necessary and Sufficient to Transduce ACE2 Therapeutic Effect on PAH

We tested the hypothesis that Mas1 is necessary and sufficient to transduce the ACE2 therapeutic effect using the Sugen-hypoxia model. Hypoxia alone produces RVSPs of 28–30 mmHg in C57Bl/6 J mice in our hands; adding weekly injections of the VEGF inhibitor Sugen increases this by about 5 mmHg and generally results in stronger remodeling. We found that ACE2 was effective in reducing RVSP but not in Mas1 knockout mice (Figure 2A). The ACE2 effect could be reproduced using the specific small molecule Mas1 agonist AR234960 [31].

The right ventricle generally muscularizes under load stress; results for RV muscularization were similar, with a large increase with SuHx compared to normoxic mice, which was alleviated with ACE2 and AR234960 (Figure 2B). ACE2 still appeared to reduce right heart muscularization even in Mas1 knockout mice, though, suggesting that Mas1 may be dispensable for ACE2 effect on the RV. Finally, increased pulmonary pressures result in increased muscularization; counts of small muscularized vessels tracked RVSP, with a mild increase in muscularized vessels, which was alleviated by

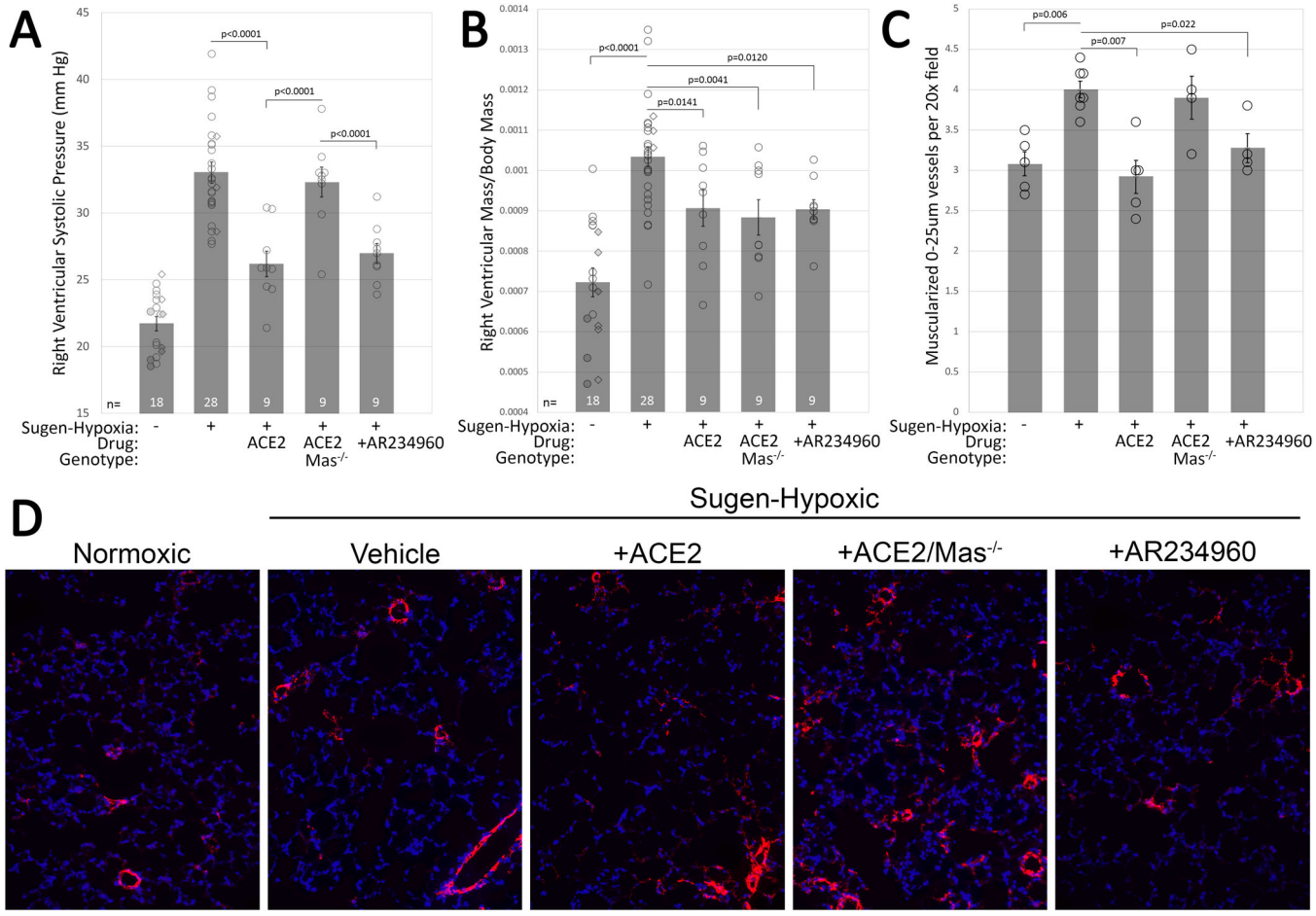

FIGURE 2 | Mas1 activation is necessary and sufficient to reproduce the ACE2 effect on the pulmonary vasculature (A) Right Ventricular Systolic Pressure. Each symbol indicates pressure from one animal. The diamonds in the first two columns are $Mas1^{-/-}$ mice; filled symbols in the normoxic group were treated with ACE2. Error bars are SEM. *p* values are Tukey HSD after ANOVA. (B) Right ventricular muscularization. Symbols, error bars, and statistics are as per (A). (C) Muscularized vessels. Each symbol is an average of vessel counts in ten 20× fields in a single mouse; each symbol is thus an average from an individual animal. Error bars are SEM. *p* values are nonparametric Wilcoxon values after ANOVA (overall ANOVA $p = 0.0003$). (D) Representative immunohistochemistry images for muscularized vessels. Blue is nuclear stain (DAPI); red is alpha-smooth muscle actin.

either ACE2 or AR234960, but not in Mas knockout mice (Figure 2C,D).

Taken together, these results indicate that Mas receptor is necessary and sufficient to reproduce ACE2 therapeutic effect in pulmonary vascular resistance.

3.2 | Ang(1-7) Cannot Reproduce ACE2 or AR234960 Effect on Pulmonary Hypertension, Suggesting It Is Not the Correct Mas1 Ligand in This Context

Here, we are testing the second part of the hypothesis in Figure 1 that the mechanism of Mas1 activation is the Ang(1-7) produced by ACE2. We once again used the SuHx model and two different methods of increasing Ang(1-7). Increasing Ang(1-7) in vivo is difficult because it has a very short half-life in vivo (30 min at most [32]). The first model uses an artificially stabilized Ang(1-7) provided by GlaxoSmithKline (GSK). Here, Ang(1-7) is fused to albumin-binding domain antibodies (AlbudAb), which substantially increases stability and half-life [33]. The second model uses a drug provided by Alterras Therapeutics (Alt00, an aminopeptidase inhibitor) in combination with Lisinopril to reduce Ang(1-7) degradation. This was an important alternate because of the possibility that the Albudab reduced Ang(1-7) binding affinity for Mas1.

To verify that the Alt00/Lisinopril combination was molecularly functional, we checked blood levels of angiotensin pathway components; we found that Alt00/Lisi was capable of reproducing the Ang(1-7) levels produced by ACE2 (Figure 3A). High-dose Alt00/Lisi could drive Ang(1-7) levels to eight times that produced by ACE2 (not shown).

We found that neither stabilized Ang(1-7) nor Alt00/Lisi reduced RVSP (Figure 3B), right ventricular mass (Figure 3C), or lung vessel muscularization (Figure 3D,E). In fact, high-dose Alt00/Lisi resulted in an increase in RVSP over SuHx alone (Figure 3B). The large number of mice included in these experiments is because this result was so counterintuitive that the entire set of experiments were repeated three times independently.

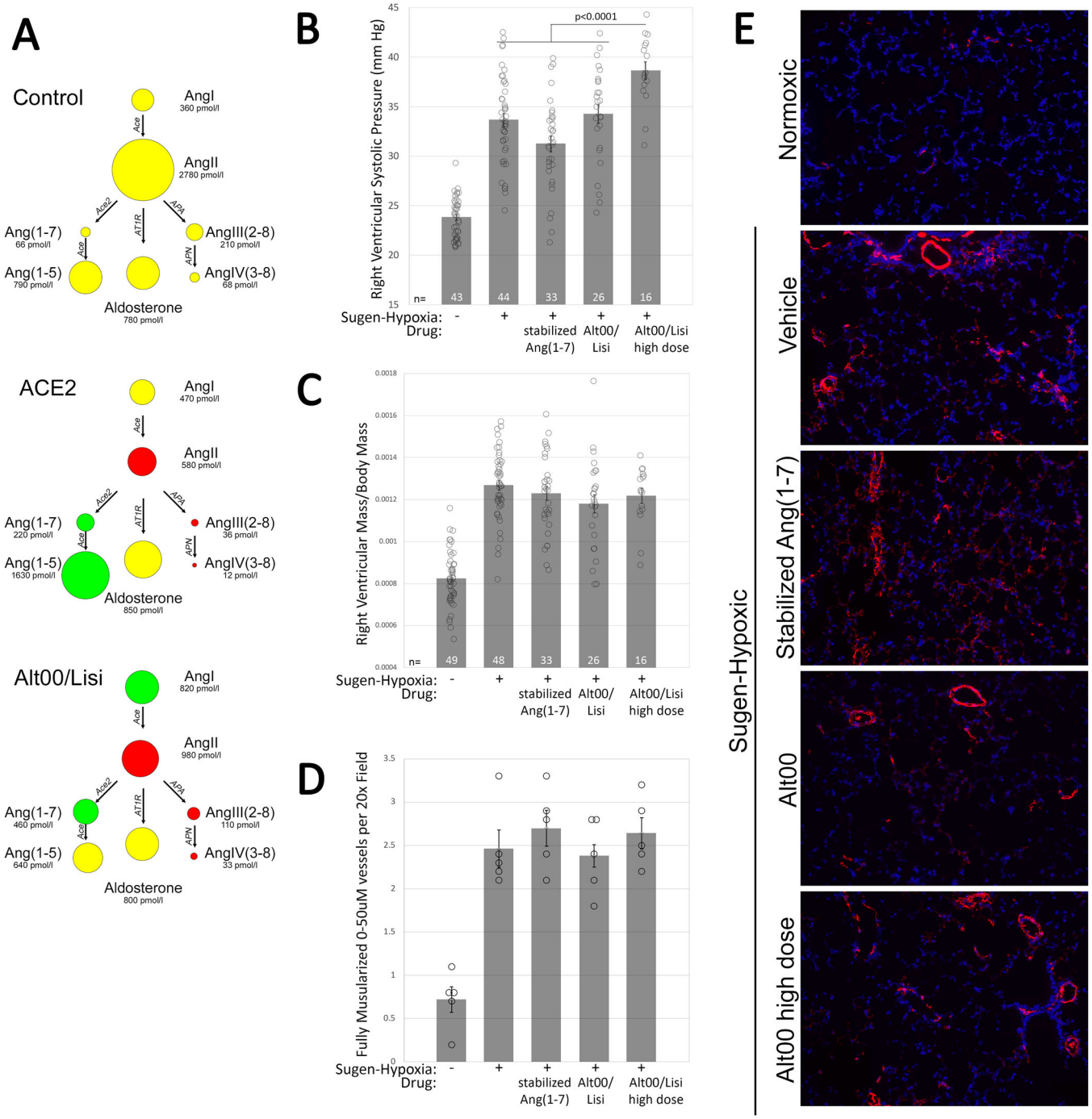

FIGURE 3 | Ang(1-7) does not reproduce the effects of ACE2 or Mas1 activation on the pulmonary vasculature. (A) Analysis of the effect of ACE2 and Alt00 + Lisinopril on Angiotensin system components by mass spectrometry, in vivo in serum. The area of circles is proportional to quantity; red indicates reduced from control, and green indicates increased and is an average of treated animals. (B) Right Ventricular Systolic Pressure. Each symbol indicates pressure from one animal. Error bars are SEM. p values are Tukey HSD after ANOVA. (C) Right ventricular muscularization. Symbols, error bars, and statistics are as per (B). (D) Muscularized vessels. Each symbol is an average of vessel counts in ten 20× fields in a single mouse; each symbol is thus an average from an individual animal. Error bars are SEM. p values are nonparametric Wilcoxon values after ANOVA (overall ANOVA $p = 0.0003$). (E) Representative immunohistochemistry images for muscularized vessels. Blue is the nuclear stain (DAPI); red is alpha-smooth muscle actin.

3.3 | RNA-Seq Suggests the Critical Commonality Between ACE2 and AR234960 Is in Rescue of Actin Cytoskeleton and Microtubule Network Function

We performed RNA-Seq on whole lungs from 9 control mice, 3 mice treated with ACE2, 3 with AR234960, and 6 with Alt00/Lisi. Using DESeq. 2 [29], we found that at a p value of < 0.05, a fold change of > 1.5, and a minimum of 8 reads per million in any group, ACE2 had 264 genes altered compared to vehicle (all with Sugen-Hypoxia), AR234960 had 230 genes altered, and Alt00/Lisi had 75 genes altered. Because of the overlap in these lists, these totaled 490 genes altered in any of the conditions. In

considering the overlap between these lists, however, we moved to a different set of criteria; a gene was considered to be altered by one of the other treatments if it moved in the same direction and was not statistically different than the treatment that had $p < 0.05$ significance. It is important to remember in interpreting this that none of these treatments are expected to directly modify gene transcription or any transcription factor; rather, differentially regulated genes are second-order effects downstream of the functions that are being modified by the drugs.

Using these criteria, we found significant overlap between treatment groups. Because they are the two treatments which ameliorated pulmonary hypertension, the arguably most important overlap is between ACE2 and AR234960 effects, at 49 genes (Figure 4A and Table S1a). 37 of these 49 genes fall into a narrow set of highly significantly ($p = 6\times10^{-12}$) overrepresented gene ontology groups focused on cell migration or movement of subcellular components (Table S2a). Examples of these genes are plotted in Figure 4B. Many of the genes are broadly responsible for regulating the actin cytoskeleton or microtubule networks; Ephrin B1 (Efnb1) is a regulator of adhesion and migration [34]; Fam107a regulates cytoskeletal organization in contexts such as focal adhesion, membrane ruffle, and stress fibers [35]; Limk1 is directly regulated by Bmpr2 [8] and regulates actin polymerization [36]; and microtubule-associated protein 6 (Map6) interacts with Tctex1 [37], another Bmpr2 interacting protein [9], in regulating movement of intracellular cargos via the microtubule network [38]. However, the overlap also includes some signaling molecules, including downregulation of Wnt3a (shown) and Wnt7a.

The almost complete overlap between ACE2 and Alt00/Lisi is to be expected. As shown in Figure 2A, the effect of Alt00/Lisi is to reproduce the Ang(1-7) levels created by ACE2; however, ACE2 has additional effects, such as reducing AngII levels, which would be expected to produce additional effects. Of the 122 overlapping genes (Table S1b), 90 fall into significantly overrepresented gene ontology groups, including tube development, proliferation, migration, and inflammation (Table S2b). Although these all seem relevant to pulmonary hypertension, the fact that pressures actually go up when we overdrive this (Figure 3B) indicates that, apparently, these are not being moved in a therapeutic direction by Ang(1-7) alone. Illustrative examples of these genes are plotted in Figure 4C. Bmper is an endothelial BMP inhibitor [39]. Calreticulin (Calr) is a calcium-binding chaperone protein that is mutated in some thrombocythemias [40]; the loss of which can drive myeloproliferative pulmonary hypertension in mice [41]. Ccl21b is involved in the recruitment of T-cells [42]. Matrix metalloproteinase (MMP) 19 is involved in the breakdown of extracellular matrix; in lungs, it is primarily expressed in fibroblasts and decreased in profibrotic [43]. S100A4, one of a family of small multifunctional calcium-binding proteins, is dysregulated in many lung diseases, including COPD, asthma, pulmonary fibrosis, and pulmonary hypertension [44].

We would expect whole lung treated with ACE2 to modulate targets outside of those with our other treatments since, in addition to altering Ang(1-7) and the Mas1 ligand, it regulates AngII levels and other components of the angiotensin system, affecting multiple additional downstream receptors. Considering the 127 genes that are significantly changed with ACE2 treatment (Table S1f), but neither Alt00/Lisi (Ang(1-7)) nor AR234960 (Mas1 agonist), 68 fall into overrepresented gene ontology groups at FDR of 0.05 or better. These are substantially overlapping groups once again related to adhesion and motility (Table S2c).

The final group is one that we have the least a priori mechanistic explanation for—genes altered by AR234960, but not by ACE2. There are 144 genes in this category (Table S1g), of which 130 fall into overrepresented gene ontology groups at FDR of 0.05 or better (Table S2d). The top three groups statistically were stimulus–response groups (oxygen-containing compounds, lipids, and organic cyclic compounds), followed by circulatory system development, extracellular matrix, adhesion, and motility groups. Examples of these presented in Figure 4D include angiopoietin-like 4 (Angptl4), a regulator of insulin sensitivity [45]; endothelin receptor b is one of the targets of existing PAH therapy [46]; the Glp1 receptor is a central metabolic regulator and the semaglutide target [47]; Phosphodiesterase 3a (Pde3a) is part of another pathway currently a PAH drug target [48]; and the eicosanoid receptor Ptgfr has been proposed as an idiopathic pulmonary fibrosis target [49]. The mechanism by which AR234960, a strong and fairly specific Mas1 agonist, has significant in vivo targets not found in ACE2 is unclear. Two potential explanations, outside the scope of this investigation, are that AR234960 has additional receptor targets besides Mas1 (it is less specific than thought) or that ACE2 inhibits some Mas1 targets through other means.

Because Limk1 is a direct Bmpr2 target and lies in the presumed therapeutic group (regulated by ACE2 and AR234960), we checked protein levels by western blot and found that it is inhibited by SuHx compared to normoxia, but protein levels are significantly increased by either ACE2 or AR234960 (Figure 4E).

4 | Discussion

This project had two important conclusions. First, the hypothesis that the ACE2 effect was driven by Mas1 activation was demonstrated to be almost entirely correct—Mas1 knockout resulted in the loss of the ACE2 effect, and Mas1 activation reproduced it (Figure 2). The exception to this is the possibility that Mas1 is dispensable for ACE2's therapeutic effect in the heart (Figure 2B). Since examining RV response was not the goal of this study, further investigation would be required to verify this. However, it is possible that this is the source of the epidemiologic finding that PAH patients on angiotensin receptor blockers fare better [24].

The second important conclusion—and the most surprising—is that Ang(1-7) is not the ligand for Mas1 in this context (Figure 3). Our initial trial used AlbudAb stabilized Ang(1-7). The lack of effect there made us concerned that the AlbudAb may have changed Ang(1-7) binding, and so we moved on to the Alt00/Lisinopril combination. By titrating the dose, we could reasonably reproduce the same Ang(1-7) levels produced by ACE2 (Figure 3A) or even higher levels. However, not only did

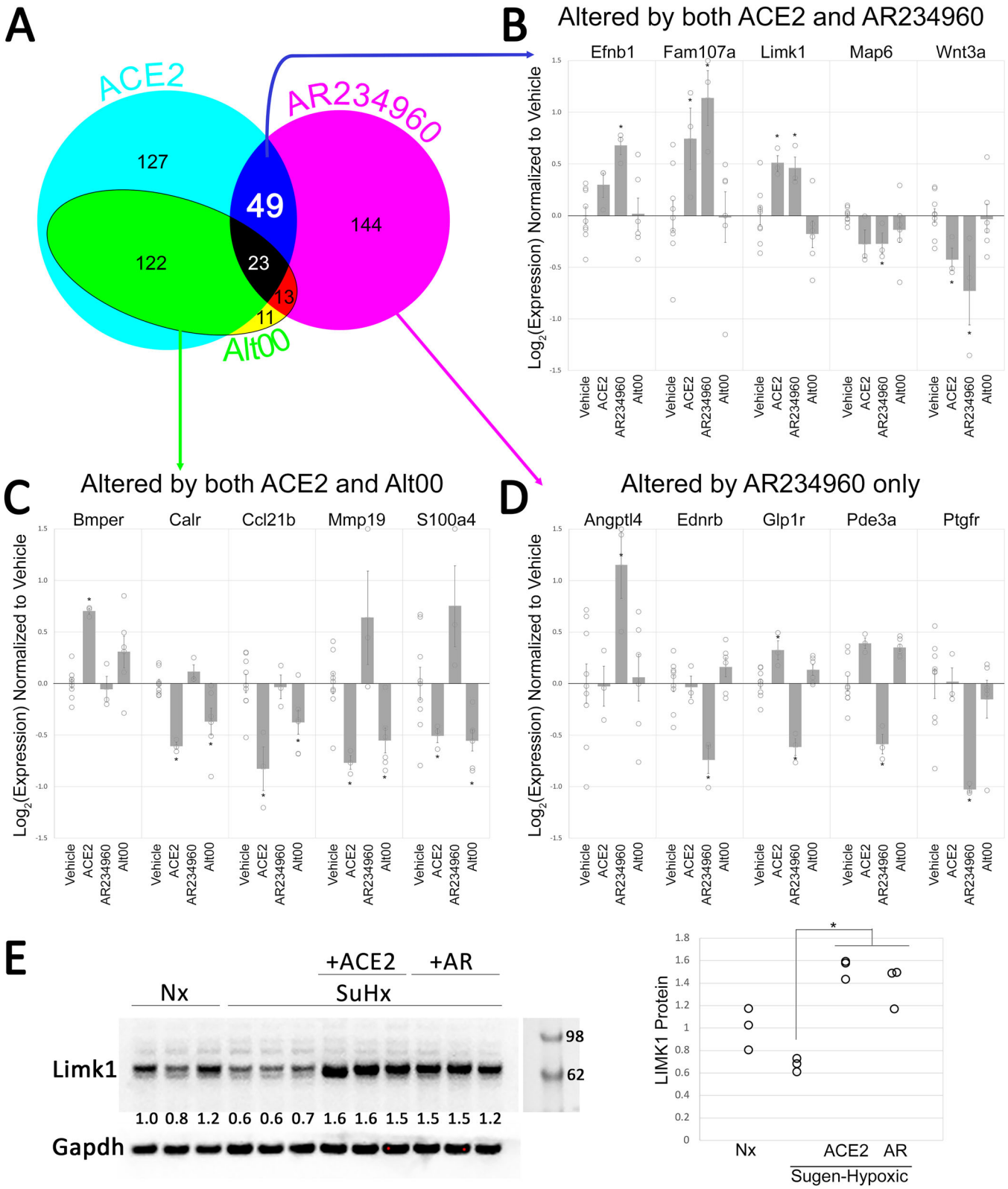

FIGURE 4 | RNASeq (A) Venn diagram indicating overlap between genes differentially regulated by ACE2 (blue circle), AR234960 (purple circle), and Alt00/Lisi (green ellipse). Numbers indicate the number of genes regulated in common when these overlap. Sizes are roughly proportional to numbers. (B) Examples of genes differentially regulated in common with ACE2 and AR234960. (C) Examples of genes differentially regulated in common with ACE2 and Alt00/Lisi. (D) Examples of genes differentially regulated only by AR234960. For B–D, error bars are SEM, circles are values from individual animals, bar heights are averages, and $*p < 0.05$ by Wilcoxon nonparametric test compared to SuHx with vehicle only. (E) Western blot for LIMK1. Numbers are densitometry, normalized to Gapdh, with densitometry plotted at right. $*p < 0.05$ by Wilcoxon nonparametric test.

this not work, higher levels of Ang(1-7) resulted in slightly (and statistically significantly) higher RVSP. This strongly indicates that Ang(1-7) is not the correct ligand for Mas1, at least in the context of the ACE2 effect on pulmonary hypertension.

Although the bulk of the literature indicates that Ang(1-7) is the ligand for Mas1, we are not the first group to notice that this might not be true. Groups that explicitly look at binding affinity have found that Ang(1-7) cannot actually initiate detectable signaling through Mas1 [31]. Other groups have suggested that the actual ligand for Mas1 may be Ang(1-5) [50, 51]. This fits well with both the literature and our results for the following reasons. Ang(1-7) rapidly breaks down into Ang(1-5). Studies that thought Ang(1-7) was the ligand did not check for degradation or actual binding—it could thus easily be true that Ang (1-5) was the actual ligand in those studies. Further, in our study, Alt00/Lisi reproduced most of the effects of ACE2 on the Angiotensin system but did not increase Ang(1-5) levels since it explicitly prevented the breakdown of Ang(1-7) (Figure 3A). One hypothesis, then, consistent with both much of the literature and our findings, is that the actual ligand for Mas1 in this context is Ang(1-5). Of course, there are alternate, more complicated hypotheses—for instance, we did not measure Ang (1-9), and there are a variety of studies that show ACE2 having additional cleavage targets or activities [52].

Our molecular studies (Figure 4) were largely confirmatory of our earlier studies and the broader literature. As before [11], we found that the key ACE2 targets, confirmed by overlap with AR234960, were the rescue of the actin cytoskeleton and microtubule network-related genes (Figure 4B and Tables S1a and S2a). These fundamental functions impacting cell–cell junctions, migration, proliferation, stiffness, and transport are directly regulated by the Bmpr2 cytoplasmic tail, with all of the above heavily implicated in PAH [53–56].

This study leaves several important outstanding questions of both practical and mechanistic importance. Is there a direct Mas1 agonist that can be brought to the clinic? If not, can one be made? If not, can we identify the endogenous Mas1 ligand and induce that? In addition, although we have strong evidence that the primary impact of Mas1 activation is the rescue of cytoskeletal and microtubule functions, we do not know in detail what we are rescuing here, given the large number of potential downstream functions. Knowing this will be important either for finding alternate points of intervention or for better understanding molecular endpoints during trials of a Mas1 agonist. These studies are a solid and necessary step along the way of bringing this axis of therapy to patients, but they leave open a number of other questions before trials are practical.

Author Contributions

Conceptualization: James West and Anna Hemnes. Data curation: James West. Formal analysis: James West. Funding acquisition: Anna Hemnes, Erica Carrier, and James West. Investigation: Megha Talati, Erica Carrier, Anandharajan Rathinasabapathy, Ying Cai, Tom Blackwell, Santhi Gladson, Christie Moore, Sheila Shay, Ethan Sevier, and Benjamin Vigl. Methodology: Ibragim Gaidarov and Megha Talati. Resources: Benjamin Vigl and Hongpeng Jia. Project administration: Santhi Gladson and Sheila Shay. Supervision: James West and Santhi Gladson. Visualization: James West. Writing – original draft preparation: James West. Writing – review and editing: Anna Hemnes, Erica Carrier, and James West.

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Disclosure

James West serves as the submission's guarantor. He takes responsibility for the integrity of the work as a whole, from inception to published article.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.