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Original Article

Transforming growth factor-β inhibition attenuates pulmonary arterial hypertension in rats

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Abstract: The role of transforming growth factor-β in the pathogenesis of pulmonary arterial hypertension is unclear. We examined the effects of T9429, an antibody against transforming growth factor-β receptors, on hemodynamic, histological and functional parameters in the rat model of monocrotaline-induced pulmonary hypertension. One week after monocrotaline injection (60 mg/kg) in 28 Wistar rats, T9429 (0.1mg/kg daily) was administered intraperitoneally in 19 rats (268±10g) via an osmotic mini-pump for 7 days. One week thereafter, right ventricular systolic pressure, pulmonary vascular remodeling and exercise tolerance were evaluated. Compared to the monocrotaline group (25.5±1.9mmHg), right ventricular systolic pressure was lower (p=0.0014) in the monocrotaline+antibody group (18.4±0.8mmHg). This was translated into attenuated right ventricular hypertrophy (p=0.0063) and longer (p=0.0155) exercise duration (2.08±0.29min versus 6.19±1.02min). Pulmonary arterial wall thickness (in vessels 50 -200μm) was comparable between the two groups, but the monocrotaline+antibody group displayed lower number (p<0.0001) of pre-capillary arterioles (<50μm, in 20 randomly selected fields) with a muscularized media (23.33±3.15 versus 6.64±0.75). Our results suggest that transforming growth factor-β receptor blockade improves vascular remodeling and attenuates pulmonary hypertension, a finding with potential therapeutic implications.

Keywords: Pulmonary arterial hypertension, transforming growth factor-β, pulmonary vascular remodeling, exercise tolerance

Introduction

Pulmonary arterial hypertension (PAH) is an uncommon but serious disease, associated with high morbidity and mortality [1]. Despite considerable advances recently, several aspects of the pathophysiology of the disease remain obscure.

Endothelial dysfunction in the pulmonary vascular tree initiates complex molecular processes, eventually affecting all components of the pulmonary arterial wall [2]. Pulmonary vascular remodeling consists of adventitial, medial and intimal thickening, secondary to fibroblast, smooth muscle and endothelial cell proliferation. Bone morphogenetic proteins are involved in this process [3], but the role of the transforming growth factor-beta (TGF-β) branch of this super-family remains incompletely understood.

TGF-β is a multifunctional cytokine, involved in the regulation of proliferation, differentiation, migration and survival of various cell types [4]. Since TGF-β is known to induce differentiation and migration of fibroblasts and smooth muscle cells in the media of systemic [5] and pulmonary arteries [6], altered cellular responses to TGF-β may contribute to the pathophysiology of PAH. In fact, this notion emerged as early as in 1990 [7, 8] and subsequent studies in patients

[9-14] have described mutations in all TGF-β receptor sub-types, confirming the initial observations. For example, mutations in type I TGF-β receptor have been observed in PAH associated with hereditary hemorrhagic telangiectasia [9, 10] and mutations in type II TGF-β receptor have been reported in endothelial cells within plexiform lesions, obtained from patients with idiopathic PAH [11]. Lastly, abnormal TGF-β signaling, resulting in exaggerated growth response [12], has been reported in endothelial and smooth muscle cells in pulmonary arteries from patients with idiopathic PAH [13].

Despite the recognized involvement of TGF-β in the pathogenesis of PAH, previous studies have provided an unclear picture of its precise pathophysiologic role. A recent report indicated that the expression of TGF-β receptors and its downstream signaling were decreased in smooth muscle cells, located in the lungs and pulmonary vessels of rats with monocrotaline-induced PAH [15]. In contrast, using the same animal model, another study [16] reported increased expression of TGF-β isoforms in the lungs. Furthermore, increased TGF-β levels were found in the lungs of sheep with PAH induced by air embolism [7] and in the pulmonary arterial tree of patients with idiopathic PAH [8].

In view of the contradictory results of previous reports [7-16], the aim of the present study was to further examine the pathophysiologic role of TGF-β, by inhibiting its downstream signaling. For this purpose, rats with monocrotaline-induced PAH were treated with a TGF-β antibody; we hypothesized that TGF-β inhibition may attenuate monocrotaline-induced PAH, if commenced at an early stage of the disease. Moreover, we examined whether this possible beneficial effect could be translated into improved functional parameters.

Materials and methods

Experimental animal population

The animal study population consisted of 40 Wistar rats (261±5g). The experimental procedures conform to National Research Council Guide for the care and use of laboratory animals and the study protocol was approved by the appropriate state authority. The experimental animals were randomly assigned into three groups in a 1:1:2 allocation ratio, namely to control group (n=10), monocrotaline group (n=10) and monocrotaline plus anti-TGF-β group (n=20). This unbalanced randomization design minimizes the number of experiments in the control groups, without compromising the statistical power [17, 18].

Induction of PAH and TGF-β inhibition

PAH was induced by monocrotaline (Crotaline, Sigma-Aldrich Ltd., Athens, Greece), administered as a single subcutaneous injection of 60 mg/kg [19]. For TGF-β inhibition, we used a TGF-β (pan)antibody (T9429, Sigma-Aldrich Ltd., Athens, Greece), which non-selectively blocks all TGF-β receptor sub-types [20]. Treatment was initiated one week after monocrotaline injection and was administered at a daily dose of 0.1mg/kg for a total of 7 days. This dosage was selected based on prior work (reviewed in [21]) and was confirmed in a series of preliminary experiments (n=10), in which a marked reduction in right ventricular (RV) pressure and an attenuation of pulmonary vascular remodeling was noted (unpublished observations). Due to the un-blinded nature of these experiments, they were not included in the final experimental animal population. The total dosage of 0.7mg/kg was administered continuously via the intraperitoneal route, using osmotic mini-pumps (ALZET, Cupertino, CA, USA).

Study end-points

Exercise capacity and RV pressure were evaluated two weeks after monocrotaline injection, followed by histological assessment.

Exercise capacity

Exercise capacity was assessed with the use of the modified forced swimming test [22]. In brief, the animals were forced to swim in a cylinder beaker (height:50cm; diameter:30cm) filled with water (25°C) to a height of 25cm. The actual swimming time (measured with a stopwatch) represents the total time, i.e., from immersion until near-drowning, from which floating time was subtracted.

RV pressure measurements

Following intubation of the trachea, the animals were mechanically ventilated (model 7025, Ugo Basile, Comerio, Italy) and anesthesia was

maintained with isoflurane. Open chest measurements were performed as previously described [23], after slight modification in our laboratory [24]. Pressure recordings were performed using the Fukuda Denshi/Datascope (Model IB5006) system.

Histology

Histology was performed by an author (C.G.) blinded to treatment assignment. The animals were sacrificed with potassium chloride; the heart and lungs were resected *en block* and fixed in neutral-buffered formalin (10%).

RV hypertrophy

A central transversal section of the heart was embedded in paraffin, cut in 2μm-thick sections and stained with hematoxylin-eosin. RV hypertrophy was expressed as:
right ventricular free wall thickness / (left ventricular free wall thickness + interventricular septal thickness) /2.

Vascular remodeling

Lungs were initially cut in 2mm-thick sections, embedded in paraffin and were finally cut in 2μm-thick sections. Lung parenchyma sections were stained with hematoxylin-eosin and immunohistochemically for α-smooth muscle actin (1:100, Dako, Glostrup, Denmark). Pulmonary vascular remodeling was examined as previously described [25, 26]: (a) In 20 (per animal) medium-sized pulmonary arteries (with an external diameter 50-200μm). Random circular vessel profiles were selected; the external diameter and the medial muscular layer thickness were measured and reported as: muscular wall thickness / external diameter. (b) In (normally non-muscular) pre-capillary pulmonary arterioles (with an external diameter less than 50μm) associated with alveolar sacs and ducts. Muscularisation was assessed as the number of vessels displaying over 75% of the circumference positive for α-smooth muscle actin in 20 randomly selected fields (using the x100 magnification).

Statistical analysis

All values are given as mean ± standard error of the mean. According to the Kolmogorov-Smirnov test for normality, differences in continuous variables between the three groups were assessed with one-way analysis of variance (followed by post-hoc Newman-Keuls test), in case of normal distribution; in the absence of normal distribution, the non-parametric Kruskal-Wallis analysis of variance was used (followed by median test). Significance was defined at an alpha level of 0.05.

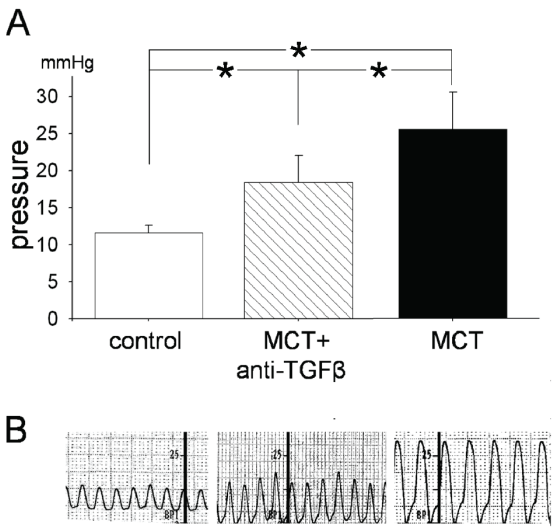

Figure 1. Right ventricular pressure. Right ventricular systolic pressure differed (asterisk denotes $p<0.05$) in the control, the monocrotaline (MCT) *plus* transforming growth factor-β antibody (anti-TGFβ) group and the monocrotaline groups (**A**). A representative example from each group is shown in panel (**B**).

Results

Animal study population

Of the 40 Wistar rats initially included in the study, 2 rats (1 from the monocrotaline group and 1 from the monocrotaline *plus* anti-TGF-β group) died during pressure measurements and were excluded. Thus, the final study population consisted of 38 rats that were assigned to the three groups as follows: control group (n=10; 253±10g), monocrotaline group (n=9; 256±3g) and monocrotaline *plus* anti-TGF-β group (n=19; 268±10g). Body weight was comparable in the three groups (H=0.79, p=0.67).

RV systolic pressure

There was a significant variance in RV systolic pressure between the three groups (F=31.5,

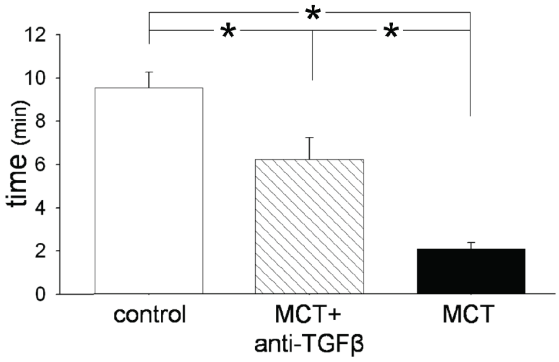

Figure 2. Exercise duration. Exercise duration in the three groups. Asterisk denotes statistically significant differences.

p<0.0001). This was due to *lower* (p=0.0014) RV pressure (18.4±0.8mmHg) in the monocrotaline *plus* anti-TGF-β group, when compared to the monocrotaline group (25.5±1.9mmHg). RV pressure in the monocrotaline *plus* anti-TGF-β group was *higher* (p=0.0009) than in controls (11.6±0.3mmHg). All values are depicted in **Figure 1A** and a representative example from each group is shown in **Figure 1B**.

Exercise tolerance

There was a significant variance in the exercise duration in the three groups (F=8.62, p=0.0010). This was due to *longer* (p=0.0155) exercise duration in the monocrotaline *plus* anti-TGF-β group (6.19±1.02min) than in the monocrotaline group (2.08±0.29min). However, exercise duration was *shorter* (p=0.0467) in the former group compared to controls (9.51±0.74min). All values are depicted in **Figure 2**.

RV hypertrophy

There was a significant variance in RV hypertrophy (F=12.3, p<0.0001) in the three groups. This was due to *lower* values (p=0.0063) in the monocrotaline *plus* anti-TGF-β group (0.33±0.02), compared to the monocrotaline group (0.45±0.02). However, RV hypertrophy was more pronounced (p=0.0171) in the monocrotaline *plus* anti-TGF-β group than in controls (0.22±0.01). All values are illustrated in **Figure 3**.

Pulmonary vascular remodeling

There was a significant variance (F=20.3, p<0.0001) in wall thickness (corrected for vessel size) in pulmonary vessels with an external diameter ranging from 50 to 200μm. This was due to *lower* values in the control group (0.202±0.005), compared to either the monocrotaline *plus* anti-TGF-β (0.270±0.009) or to the monocrotaline groups (0.292±0.010, p=0.0001 for both comparisons). However, no significant difference was found (p=0.12) between the latter two groups.

There was a highly significant variance (F=48.5, p<0.0001) in the number of pre-capillary arterioles (with external diameter less than 50μm) displaying a muscularized media. This variance was due to a *lower* number (p<0.0001) in the monocrotaline *plus* anti-TGF-β group (6.64±0.75), when compared to the monocro-

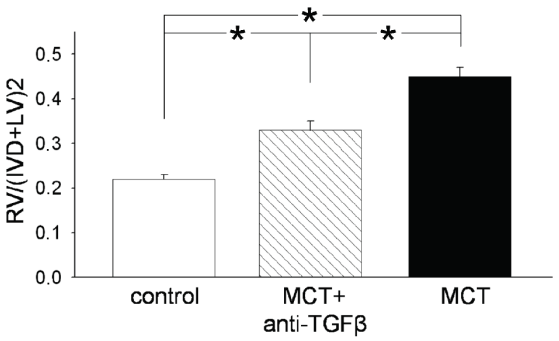

Figure 3. Right ventricular hypertrophy. Right ventricular hypertrophy in the three groups. Asterisk denotes statistically significant differences.

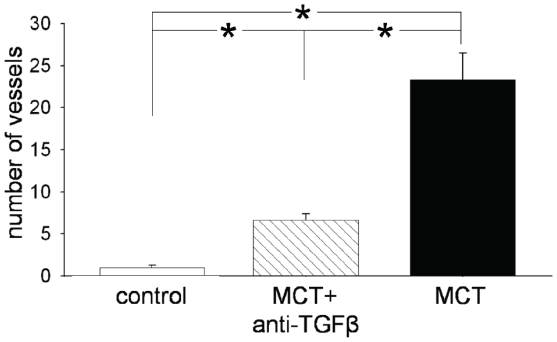

Figure 4. Muscularized pre-capillary arterioles. Number of muscularized pre-capillary arterioles vessels in the three groups. Asterisk denotes statistically significant differences.

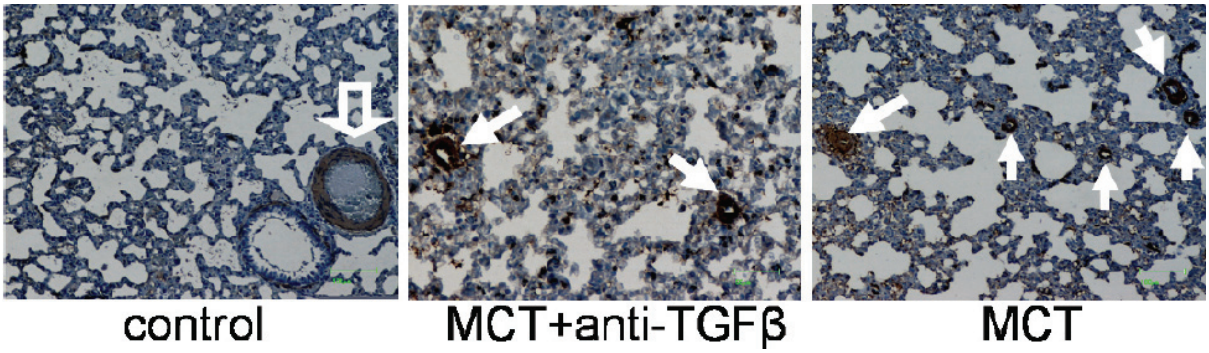

Figure 5. Muscularized pre-capillary arterioles. Examples of muscularized arterioles (filled arrows) in the 3 groups. Note the lower number in the treatment group and their absence in the control group. The open arrow indicates a positive control for α-smooth muscle actin staining in a pulmonary artery >100μm.

taline group (23.33±3.15). However, this number was *higher* (p=0.0145) in the monocrotaline *plus* anti-TGF-β group than in the control group (1.00±0.25). Values are depicted in **Figure 4** and representative examples are shown in **Figure 5**.

Discussion

The present study examined the effects of TGF-β inhibition in the rat model of monocrotaline-induced PAH. This model is widely used, because of its similarities with human familial or idiopathic PAH, with respect to alterations in molecular signaling and histological changes [27].

Main findings

We report attenuation of PAH development after continuous TGF-β-receptor (pan)antibody administration during an early stage of the disease. Such treatment ameliorated pulmonary vascular remodeling, resulting in markedly lower RV pressure, attenuated RV hypertrophy and improved exercise tolerance.

Possible beneficial mechanisms of TGF-β-inhibition in PAH development

TGF-β super-family plays a crucial role in vasculogenesis and cell growth, illustrated by the finding that TGF-β-knockout mice die *in utero* of multiple abnormalities [28]. During PAH development, TGF-β has been shown to participate in cellular and molecular processes [3]; stimulation of TGF-β receptors activates intracellular proteins Smad2 and Smad3 that, in conjunction with Smad4, enter the nucleus and regulate transcription of target genes, eliciting exaggerated growth responses in vascular endothelial and smooth muscle cells [27]. Moreover, the Smad-family signaling interacts with other growth promoting signaling circuits such as Ras, the mitogen-activated protein kinase kinase (MKK)4-Jun kinase and MKK3-p38 [29].

Despite the recognized involvement of TGF-β in the pathogenesis of PAH, its precise role *in vivo* is incompletely understood [27]. In the present study, we hypothesized that inhibition of TGF-β downstream signaling may ameliorate PAH development. Our findings implicate increased TGF-β activity in the pathogenesis of PAH, a notion concurrent with a previously reported [30] four-fold increase in TGF-β levels in monocrotaline-induced PAH in rats. The sites of increased TGF-β production are unknown, but possible candidates include pulmonary vascular smooth muscle cells and alveolar macrophages, the number of which increases sharply in PAH [31].

TGF-β and vascular remodeling

In addition to intimal lesions, pulmonary vascular remodeling consists of medial hypertrophy, which is considered characteristic of PAH. In the present study, we examined two important aspects of medial smooth muscle cell hypertrophy, namely, *first*, the appearance of a media in (normally non-muscular) pre-capillary pulmonary arterioles and, *second*, vessel wall thickness in medium-sized muscularized arteries (with an

external diameter 50-200μm).

A pathological feature common to all forms of PAH is the appearance of smooth muscle cells in small pulmonary arterioles. The underlying mechanisms are incompletely understood, but differentiation of pericytes into smooth muscle cells that subsequently proliferate is a likely candidate [32]. Here, we report a marked reduction in the number of arterioles displaying a muscularized wall after TGF-β receptor blockade. This reduction reinforces previously raised hypotheses, implicating a major role of TGF-β in this process [27, 32]. In contrast, we found no significant effect of TGF-β receptor blockade on medium-sized pulmonary arteries.

The explanation for this difference may be two-fold: *First*, heterogeneity in vascular smooth muscle cells between regions of the pulmonary vascular tree is well recognized, with significant variations in the responses to growth stimuli [33, 34]. *Second*, different mechanisms of medial hypertrophy may be operative: in contrast to differentiation of pericytes in pre-capillary arterioles, neo-intimal formation and wall thickening of more proximal muscular arteries has been attributed to migration and proliferation of smooth muscle cells [27, 32]. TGF-β has been shown to promote smooth muscle cell migration *in vitro* [6], but TGF-β-receptor sub-types may exert diverse or even opposing effects in this process [35]. Thus, the neutral effect on medial hypertrophy in our experiments may be attributed to the non-selective blockade of all TGF-β-receptor sub-types by the compound used in our study.

Comparison with previous studies

Our results are in line with two previous experimental studies [6, 36] that used the same rat model. Long *et al* [6] demonstrated increased TGF-β expression in remodeled pulmonary arteries; TGF-β receptor antagonism *in vivo* prevented PAH, RV hypertrophy and vascular remodeling [6]. Likewise, Zaiman *et al* [36] reported attenuation of PAH development after treatment with an orally active TGF-β receptor inhibitor. Taken together, our present findings and previous studies [6, 36], indicate that increased TGF-β signaling is involved in the pathogenesis of PAH and support future therapeutic strategies aiming at decreasing TGF-β activity.

The findings of the present study contrast those of Zakrzewicz *et al* [15], who reported a significant down-regulation of TGF-β receptor expression and some of their associated Smad proteins. In addition, both baseline and induced TGF-β signaling were also reduced in pulmonary arterial smooth muscle cells isolated from monocrotaline-treated rats, when compared with controls [15]. The explanation for these differing results is difficult and may be attributed to previously highlighted methodological issues [36]. However, the most likely explanation for the variation in TGF-β-receptor expression observed in previous studies [6, 15, 30, 36] is the time-point of such measurements. Specifically, in the study by Zakrzewicz *et al* [15] TGF-β-receptor expression was evaluated four weeks after monocrotaline injection, as opposed to one week by Tanaka *et al* [30] and three weeks by Long *et al* [6]. In fact, the data presented by Zakrzewicz *et al* [15] indicate *increased* TGF-β type II receptors two weeks after monocrotaline injection, which subsequently *decrease* by four weeks. Nonetheless, the course of TGF-β activity during PAH development is unknown and further studies examining this issue are required. Importantly, such knowledge would indicate the optimal time-frame for anti-TGF-β intervention.

Effects of timing of anti-TGF-β treatment initiation

Based on the available evidence [6, 15, 30, 36], we hypothesized that anti-TGF-β intervention would be most effective, if applied early during PAH development. Our hypothesis is clinically pertinent, given the increasing percentage of patients being diagnosed at early stages, due to high index of suspicion among physicians and to the refinement of diagnostic techniques [37].

Previous studies [6] characterizing the rat model of monocrotaline-induced PAH indicated that pulmonary artery pressure and RV hypertrophy are significantly abnormal on day 7 onwards after monocrotaline-injection. Thus, the time point chosen in our protocol for treatment initiation corresponds to an early phase of the disease. In our experiments, RV systolic pressure was lower by approximately 40% in anti-TGF-treated rats, compared to the PAH control group. In the two previously reported studies [6, 36], treatment with anti-TGF-β antibody was initiated later in the course of the disease, i.e., 3 weeks after monocrotaline injection: Long *et al* [6] reported a 20-25% reduction in RV sys-

tolic pressure and RV hypertrophy (22.3% and 23.5%, respectively) after two weeks of treatment. Using a similar compound, Zaiman *et al* [36] demonstrated a 15% reduction in mean pulmonary artery pressure and a 26% increase in cardiac output, as well as improved RV diastolic and systolic function indices. Taken together, the present and previous findings [6, 36] indicate that reversal of PAH with anti-TGF-β treatment is feasible.

Although the results of different studies should be compared cautiously, the reduction in RV systolic pressure observed in our experiments was more pronounced than in prior reports [6, 36]. The explanation for this apparent difference may be twofold: *first*, it may be due to the variety of compounds used; *second*, the markedly decreased RV systolic pressure after anti-TGF-β treatment in our experiments may reflect treatment initiation early in the course of the disease. These hypotheses merit further investigation in future studies.

Treatment effects on exercise capacity

To our knowledge, our study is the first to report on the effects of TGF-β inhibition on the exercise capacity. Such assessment is important not only from a physiological point of view, but also because exercise capacity is one of the major endpoints frequently used in clinical studies. Here, we report an impressive improvement, i.e., a threefold longer exercise time in rats treated with an anti-TGF-β antibody than in PAH controls. This finding indicates that the histological and hemodynamic effects of TGF-β inhibition can be translated into improved functional capacity.

Strengths and limitations of the study

We feel that our study contributes to the current understanding of the pathophysiology of PAH. The combined evaluation of hemodynamic, histological and functional parameters adds value to our work. Nonetheless, three limitations should be acknowledged. *First*, we did not measure TGF-β levels in the plasma or in the lungs of animals after monocrotaline injection as well as in control rats; nonetheless, previous studies [6, 7, 16, 36] have reported increased TGF-β expression in the lungs in a variety of experimental settings, as well as in patients [8]. *Second*, a more comprehensive evaluation of the effects of treatment initiation would require the comparison between early and late treatment administration. *Lastly*, we used a anti-TGF-β (pan)antibody, which non-selectively blocks TGF-β receptors; thus, we did not address the issue of selective inhibition of each receptor sub-type.

Clinical implications

In search of additional effective therapeutic strategies of PAH, there has been a recent focus on treatments aiming at reversing PAH. Our results provide proof-of-concept for approaches targeted at TGF-β signaling manipulation. Moreover, our study indicates that treatment of PAH may be more effective when applied at early stages of the disease, as recently demonstrated in a clinical study [38].

Conclusions

Our study confirms the pathophysiologic role of TGF-β early in the course of experimental PAH. In the *in vivo* monocrotaline-rat model, TGF-β inhibition attenuated pulmonary vascular remodeling and RV hypertrophy, lowered RV systolic pressure and improved exercise tolerance. Better understanding of the molecular events that lead to the development of PAH will ultimately benefit patients by providing tools for early intervention.

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