Published in final edited form as:

Cardiovasc Pathol. 2017 ; 31: 26–33. doi:10.1016/j.carpath.2017.07.004.

Reduced Activin Receptor Like-Kinase 1 Activity Promotes Cardiac Fibrosis in Heart Failure

Kevin J. Morine, MD^{a,*}, Xiaoying Qiao, PhD^{a,*}, Vikram Paruchuri, MD^a, Mark J. Aronovitz^a, Emily E. Mackey^a, Lyanne Buiten^a, Jonathan Levine^a, Keshan Ughreja^a, Prerna Nepali^a, Robert M. Blanton, MD^a, S. Paul Oh, PhD^b, Richard H. Karas, MD, PhD^a, and Navin K. Kapur, MD^a

^aMolecular Cardiology Research Institute and Division of Cardiology, Department of Medicine, Tufts Medical Center, 800 Washington Street, Boston, Massachusetts, 02111, USA

^bDepartment of Physiology and Functional Genomics, University of Florida College of Medicine, 1600 SW Archer Road, Gainesville, FL 32610, USA

Abstract

Introduction—Activin receptor-like kinase 1 (ALK1) mediates signaling via the transforming growth factor beta-1 (TGFβ1), a pro-fibrogenic cytokine. No studies have defined a role for ALK1 in heart failure.

Hypothesis—We tested the hypothesis that reduced ALK1 expression promotes maladaptive cardiac remodeling in heart failure.

Methods and Results—In patients with advanced heart failure referred for left ventricular (LV) assist device implantation, LV Alk1 mRNA and protein levels were lower than control LV obtained from patients without heart failure. To investigate the role of ALK1 in heart failure, *Alk1* haploinsufficient (*Alk1*^{+/-}) and wild-type (WT) mice were studied 2 weeks after severe transverse aortic constriction (TAC). LV and lung weights were higher in *Alk1*^{+/-} mice after TAC. Cardiomyocyte area and LV mRNA levels of brain natriuretic peptide and β-myosin heavy chain were increased similarly in *Alk1*^{+/-} and WT mice after TAC. *Alk1* mice exhibited reduced Smad 1 phosphorylation and signaling compared to WT mice after TAC. Compared to WT, LV fibrosis and Type 1 Collagen mRNA and protein levels were higher in *Alk1*^{+/-} mice. LV fractional shortening was lower in *Alk1*^{+/-} mice after TAC.

Conclusions—Reduced expression of ALK1 promotes cardiac fibrosis and impaired LV function in a murine model of heart failure. Further studies examining the role of ALK1 and ALK1 inhibitors on cardiac remodeling are required.

Correspondence to: Navin K. Kapur, MD, Tufts Medical Center, 800 Washington Street, Box # 80, Boston, MA 02111, Telephone: 617-636-9371, Fax: 617-636-1444, Nkapur@tuftsmedicalcenter.org.

*These authors contributed equally to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

There are no relevant financial relationships to disclose.

Conflicts of interest: none

Keywords

Activin receptor like kinase 1; Heart failure; Cardiac fibrosis; Transforming growth factor beta

1. Introduction

Heart failure (HF) afflicts nearly 25 million people worldwide and is associated with a substantial burden of morbidity and mortality [1]. Cardiac fibrosis exaggerates myocardial stiffness, disorganizes contraction due to myocyte separation, disrupts electro-tonic connectivity, and worsens tissue hypoxia in HF. One of the most potent pro-fibrogenic cytokine systems governing cardiac fibrosis is the transforming growth factor beta (TGF- β) superfamily, which includes TGF- β 1 and 22 distinct bone morphogenetic proteins (BMPs) [2, 3]. TGF- β ligands signal via a heteromeric receptor complex consisting of type II and type I receptors, which phosphorylate downstream effectors such as SMADs. Smad 1 or Smad 2/3, respectively. TGF- β 1 signals mainly through TGF- β type II receptor (TGF β R2) and TGF- β type I receptor (activin receptor-like kinase 5; ALK5) and SMAD2/3, which promote type I collagen production and fibrosis in HF [2]. Previously we have shown that the TGF- β coreceptor endoglin is required for TGF β 1/ALK5 signaling in fibroblasts and reduced endoglin levels limit cardiac fibrosis via attenuation of SMAD2/3 signaling [4]. TGF- β 1 signals also through TGF β R2 and ALK1 via SMAD1/5/8. However, the role of ALK1 in cardiac remodeling remains poorly understood.

ALK1 is a cell surface serine-threonine kinase that serves an established role in maintaining vascular homeostasis [5, 6]. Loss of function mutations in human *ALK1* result in hereditary hemorrhagic telangiectasia type 2 (HHT2), characterized by the presence of arteriovenous malformations in the brain, lungs, visceral organs, and skins [7]. In mice, homozygous deletion of *Alk1* results in embryonic lethality due to severe defects in cardiovascular development [6, 8]. Global inducible knockout of *Alk1* in adult mice results in lethality associated with the formation of arteriovenous malformations in the gastrointestinal tract and subsequent development of high output HF [8, 9]. For this reason, several studies have employed the *Alk1* heterozygous mouse model to study the post-natal effects of ALK1 deficiency [6, 10, 11].

Recently, several reports have implicated a role for ALK1 as a negative regulator of fibrosis in vascular tissue, cartilage, the liver and the kidney [9, 16–19]. From these observations, we hypothesized that reduced ALK1 activity promotes cardiac fibrosis in HF by limiting SMAD1 activation. To explore this hypothesis, we used the well-established model of pressure overload-induced HF in *Alk1*^{+/-} mice.

2. Methods

2.1 Mouse Model of Pressure Overload Induced Heart Failure

Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science). Animal protocols were approved by the Tufts Medical Center Institutional Animal Care and Use Committee. Adult, male, 12–14 week old

wild-type ($Alk1^{+/+}$) and $Alk1^{+/-}$ mice underwent transverse aortic constriction (TAC) for 2 weeks using a 27G needle to generate left ventricular (LV) pressure overload [4, 12]. Sham-operated mice served as controls (n=6/group). At the conclusion of the studies, terminal hemodynamics were recorded using biventricular conductance catheters as previously described [12]. LV tissue was rinsed with sterile saline and stored for subsequent biochemical and histological analysis. $Alk1^{+/-}$ mice were obtained from The Jackson Laboratory. Genotyping of $Alk1^{+/-}$ and $Alk1^{+/+}$ mice was performed according to the PCR protocol recommended by the The Jackson Laboratory using REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich). The primers used for genotyping were wildtype $Alk1$ (TCT GAC TGT AAG TAG TCT GGC TCA G), mutant $Alk1$ (GGG TGG GAT TAG ATA AAT GCC TGC TCT), and a common primer (CCT GTT CAG ATG CCT TCA GGA TGA G).

2.2 Human samples

Viable LV free wall tissue was obtained from human subjects with end-stage HF (n=8) referred for left ventricular assist device implantation (LVAD). Non-failing LV tissue obtained from the National Disease Research Interchange served as controls (n=8). All tissue was rinsed with sterile saline, immediately frozen in liquid nitrogen and stored at -80°C until processed further. All tissue harvesting was performed in concordance with the National Institutes of Health and Tufts University Institutional Review Board guidelines.

2.3 Pressure-Volume Loop Analysis

Terminal hemodynamic evaluation was performed in all animals. Mice were anesthetized with 2.0% isoflurane administered via a non-invasive nose cone. Body temperature was monitored by a rectal thermistor probe and maintained at 37.5°C with heating pads and a cycling heat lamp. In the supine position, the right common carotid artery was surgically isolated. A silk tie was placed at the distal end of the vessel and an overhand loop with 7-0 nylon was placed at the proximal end of the vessel. A Millar PVR-1045 conductance catheter (Millar Instruments) was introduced after calibration using the cuvette method with freshly heparinized warm blood as previously described [13]. For LV cannulation, a micro vascular clip was placed proximal to the overhand loop then a transverse arteriotomy was performed with iris scissors and the conductance catheter was advanced to the clip. The proximal nylon was then tightened around the vessel and the catheter. After removal of the surgical clip, the catheter was advanced past the aortic valve into the LV. Pressure-volume loop acquisition and analysis was performed using IOX software (EMKA). After data acquisition, the mouse was euthanized by direct injection of 0.3 ml 1 N KCL into the left ventricle. The heart was removed and processed for further analysis.

2.4 Histologic Evaluation of Cardiac Hypertrophy, Fibrosis and Myocardial Capillary Density

Horizontal short-axis cut hearts were fixed in 10% formalin. Collagen abundance in the left ventricle was quantified by picrosirius red staining as a percentage of the total LV area. Cardiomyocyte cross-sectional area was quantified by identifying centrally nucleated and round cardiomyocytes on hematoxylin and eosin stained sections. Individual myocytes were traced after image acquisition and measured using ImageJ software (available online at

<http://imagej.nih.gov/ij/>). CD31+ positive cells were measured per high powered field by blinded observers.

2.5 Real time Quantitative Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the LV with Trizol (Life Technologies) and converted to cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR was performed in triplicate using 40 cycles at 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds with an ABI Prism 7900 Sequence Detection System. Primers for 18S ribosomal RNA, Endoglin, TGF- β 1, beta-myosin heavy chain (β -MHC), sarcoplasmic endoplasmic reticulum ATPase (SERCA), Type I collagen, and calcineurin were described previously [4, 12]. Primer sequences for *Alk1* were 5'-TGA CTT TCT GCA GAG GCA GA and 3'-CGA CTC AAA GCA GTC TGT GC, and for connective tissue growth factor (CTGF) were 5'-AGC CTC AAA CTC CAA ACA CC and 3'-CAA CAG GGA TTT GAC CAC.

2.6 Immunoblotting

Total protein was extracted from LV tissue using RIPA buffer containing protein inhibitor cocktail (cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail, Roche). Lysates were centrifuged at 12,000 rpm for 10 minutes and the supernatant was recovered. Protein concentration was measured using the BCA method. 25 μ g of total protein was loaded onto 4–12% Bis-Tris pre-cast gradient mini gels and blotted onto nitrocellulose membranes (Bio-Rad). Western blot analysis was performed with antibodies directed at ALK1 (Abcam #ab108207), GAPDH (Millipore #MAB374), phosphorylated SMAD1 (Cell Signaling #9516S), phosphorylated SMAD3 (Cell Signaling #9520), total SMAD1 (Cell Signaling #6944S) and total SMAD3 (Cell Signaling #9523). Following overnight incubation with the primary antibody diluted in 5% fat free milk, the membranes were incubated with the appropriate secondary antibodies for 2 hours. Bands were detected by chemiluminescence using an ECL kit (Pierce). Densitometry was performed using Image J software (NIH).

2.7 Echocardiography

Transthoracic echocardiography was performed under light sedation with isoflurane administered via nose cone with core body temperature maintained at $37.0 \pm 0.2^\circ\text{C}$. A HDI5000 Machine (ATL Inc.) and a 10 MHz linear array transducer were used to record short axis M-mode tracings. Left ventricular end-diastolic and end-systolic diameters were measured and averaged across three cardiac cycles. Fractional shortening was calculated using the equation $FS = (\text{End diastolic diameter} - \text{End systolic diameter}) / \text{End diastolic diameter}$.

2.7 Statistics

All statistical analyses were performed using Graph Pad Prism v6 (Graph Pad Software, Inc.). Comparison between two experimental groups was performed with the unpaired student's T test and for three groups or more with a one-way ANOVA with Tukey's multiple comparisons test. Survival was analyzed using a Mantel-Cox logrank test. α values less than 0.05 were accepted as statistically significant.

3. Results

3.1 ALK1 expression in human heart failure

To begin exploring a role for ALK1 in HF, ALK1 levels were measured in LV samples from patients with advanced HF referred for LVAD surgery (Table 1). ALK1 protein and mRNA levels were decreased compared to non-HF controls (Figure 1A-B). TGF- β 1 and endoglin mRNA expression was increased in patients with HF (Figure 1C), while expression of Alk5 was similar (Figure 1D-E). Expression of Serpine 3, also known as plasminogen activator inhibitor 1 (PAI-I), which has been associated with SMAD3 activation, was increased in HF patients (Figure 1F). mRNA levels of Inhibitor of differentiation 1 and 2, transcriptional targets of SMAD1 activation, were reduced in HF patients (Figure 1G-H).

3.2 The effect of Alk1 haploinsufficiency in pressure overload induced heart failure

Next, to explore the functional effect of reduced ALK1 expression in HF, we compared *Alk1*^{+/-} and WT mice subjected to TAC. Compared to WT, baseline LV Alk1 mRNA levels were 50% lower in *Alk1*^{+/-} mice (Figure 2A), as expected. Survival to 14 days was 75% (6/8) for WT mice and 56% (9/16) for *Alk1*^{+/-} mice ($p=0.36$). The pressure gradients across the constriction were similar between WT mice and *Alk1*^{+/-} mice (53 ± 9 vs. 52 ± 6 mmHg, $p=0.74$). There were no significant differences in tissue weights, hemodynamic or echocardiographic parameters between sham-operated WT and *Alk1*^{+/-} mice (Table 2). After two weeks of TAC, LV and lung weights were unchanged in WT mice compared to sham operated controls (Table 2). In contrast, *Alk1*^{+/-} mice exhibited increased LV and lung weights after TAC. LV end-systolic pressures were increased in both WT mice and *Alk1*^{+/-} mice following TAC, however end-systolic pressures were relatively lower in *Alk1*^{+/-} mice. In *Alk1*^{+/-} mice, the increase in LV end diastolic pressure was greater than WT mice after TAC. Compared to WT mice, dP/dT max and dP/dT min were decreased in *Alk1*^{+/-} mice. Following two weeks of TAC, *Alk1*^{+/-} mice demonstrated a greater decline in fractional shortening and exaggerated LV chamber dilatation compared to WT mice. These findings are consistent with impaired cardiac function in response to TAC in mice with reduced ALK-1 expression compared to controls.

3.3 Reduced ALK1 expression increases fibrosis

To examine the mechanisms underlying impaired cardiac function in *Alk1*^{+/-} mice following TAC, we first analyzed changes in cardiac fibrosis. LV mRNA levels of TGF- β 1 and endoglin were similarly increased after TAC in WT and *Alk1*^{+/-} mice (Figure 2B,D). LV mRNA expression of Alk5 was unchanged after TAC in WT and *Alk1*^{+/-} mice (Figure 2C). LV expression of PAI-I mRNA (Figure 2E), Type I collagen mRNA (Figure 2F) and protein (Figure 2G) was higher in *Alk1*^{+/-} mice compared to WT mice after TAC. We observed increased fibrosis in *Alk1*^{+/-} mice compared to WT controls following TAC (Figure 2H-I). Cardiomyocyte cross-sectional area was similarly increased in *Alk1*^{+/-} and not WT mice (Figure 3A-B). mRNA levels of β -MHC, SERCA, and calcineurin were similar in WT and *Alk1*^{+/-} mice after TAC (Figure 3C-E). Next, we found a similar reduction in myocardial capillary density in WT and *Alk1*^{+/-} mice (Figure 3F-G). These findings support that *Alk1*^{+/-} mice exhibit increased fibrosis with a similar cardiomyocyte hypertrophy and myocardial capillary density compared to WT mice.

3.4 Reduced ALK1 expression attenuates SMAD1 signaling

Next we measured phosphorylation of SMAD1 and 3 as downstream targets of ALK1 activity. While SMAD3 phosphorylation levels were similar in WT and *Alk1*^{+/-} mice after TAC (Figure 4A), SMAD1 phosphorylation was attenuated in *Alk1*^{+/-} mice (Figure 4B). mRNA levels of inhibitors of differentiation1 and 2 were increased in WT mice and suppressed in *Alk1*^{+/-} mice after TAC (Figure 4C-D). These data suggest that reduced Alk1 expression attenuates SMAD1 phosphorylation and signaling in pressure overload induced HF.

4. Discussion

We have identified a novel role for ALK1 in cardiac remodeling. We specifically show for the first time that ALK1 levels are reduced in patients with advanced HF and in a murine model of pressure overload induced HF that reduced ALK1 expression is associated with impaired cardiac function and worsened cardiac fibrosis. We further identified that compared to WT controls, reduced ALK1 expression does not affect SMAD3 activity, but is associated with decreased SMAD1 activity after TAC. These findings indicate that loss of ALK1 expression may play an important role in the pathophysiology of HF and that ALK1 may suppress cardiac fibrosis by maintaining SMAD1 activity in HF (Figure 5). These findings have important implications given that cardiac fibrosis is a major component of maladaptive remodeling and no prior studies have identified a functional role for ALK1 in HF.

Few studies have explored a role for the ALK family of receptors in pathological cardiac remodeling. Our finding that ALK1 expression and downstream signaling is reduced in a patient cohort with predominately non-ischemic cardiomyopathy suggests further investigation of ALK1 mediated signaling in a larger patient cohort is warranted. A recent study identified that ALK2 is expressed in cardiomyocytes and regulates calcineurin activation [14]. These investigators further showed that cardiomyocyte specific deletion of *Alk2* attenuated angiotensin II induced hypertrophy and fibrosis in mice. No phenotypic affect was observed with cardiomyocyte specific deletion of *Alk1*. Recently we demonstrated that global inducible knockout of *Alk1* expression in adult mice led to the development of gastrointestinal arteriovenous malformations and high output HF without structural or signaling changes consistent with pathological cardiac remodeling [8, 9]. We have now studied *Alk1* haploinsufficient mice and observed no change in cardiac structure or function at baseline compared to WT mice; however cardiac function worsened and cardiac fibrosis increased in *Alk1*^{+/-} mice after TAC. Consistent with prior observations of cardiomyocyte specific deletion of *Alk1*, we did not observe any change in cardiomyocyte cross sectional area, fetal gene marker or calcineurin expression compared to WT mice following TAC [14]. These findings may suggest that ALK1 mediated signaling in non-myocyte cell populations may be primarily responsible for any negative effects on cardiac remodeling. Our observations are supported by recent reports of increased renal fibrosis after unilateral ureteral obstruction and increased extracellular matrix production and fibroblast proliferation in cultured fibroblasts isolated from *Alk1*^{+/-}, not WT mice. Collectively, these findings suggest that ALK1 may serve as an important negative regulator of fibrosis and further that non-myocyte expression of ALK1 may be important for cardiac remodeling.

The mechanism by which ALK1 regulates cardiac fibrosis in HF remains poorly understood. Compared to WT mice, we observed reduced levels of phosphorylated SMAD1 and decreased expression of downstream SMAD1 effectors including inhibitor of differentiation 1 and 2. Furthermore, levels of phosphorylated SMAD3 were similarly increased in both WT and *Alk1*^{+/-} mice after TAC. These findings suggest that despite similar levels of TGFβ1 mRNA expression, loss of ALK1/SMAD1 activity may promote pro-fibrotic signaling via SMAD3 in the LV after TAC. Our observations are supported by recent reports showing that loss of ALK1 is associated with increased extracellular matrix production in scleroderma fibroblasts and hepatic stellate cells and that ALK1 antagonizes ALK5/SMAD2/3 activity in endothelial cells. Furthermore, prior reports have shown that treatment with recombinant BMP-7 promotes SMAD1 activity and attenuates renal fibrosis. Future studies are required to determine whether promoting ALK1/SMAD1 activity attenuates cardiac fibrosis in HF.

Our findings may have important clinical implications for ALK1 inhibitors under investigation as a therapeutic approach to limit angiogenesis in cancer [15]. *In vitro* and *in vivo* studies of ALK1 inhibition have consistently shown a profound effect on endothelial homeostasis and attenuation of neoangiogenesis in solid tumor animal models and thus generated interest in targeting ALK1 signaling as a new therapeutic approach for malignancies characterized by neoangiogenesis [16]. An anti-human ALK1 antibody (PF-3446962) and a ligand trap (ALK1-Fc; Dalantercept) designed to disrupt ligand-receptor interaction have been developed and are currently under clinical investigation [15, 17]. Phase I/II studies of PF-3446962 have shown an increased incidence of epistaxis and telangiectasia consistent with the biological effect of ALK1 disruption in humans and animal models. With the exception of one study of patients with malignant pleural mesothelioma noting high grade hypertension as a side effect, the available data from small clinical studies suggest the overall cardiovascular side effect profile of PF-3446962 is not adverse [18–22]. Phase I studies of Dalantercept did identify edema as a treatment related side effect in 54% of patients which the authors speculated was due to effects on the lymphatic system [23, 24]. LV pressure overload states, most commonly systemic hypertension and aortic stenosis, are prevalent in the general population. Our data suggest that in the context of LV pressure overload that ALK1 deficiency is associated with impaired systolic function. While predicting the effect of anti-ALK1 therapeutics on the cardiovascular system is difficult given the complexity of TGF-β signaling, diligent monitoring for cardiovascular complications is warranted in patients at risk for developing HF or with existing heart disease.

Our study has several limitations. *Alk1* mRNA levels were unchanged following two weeks of TAC compared to Sham operated controls in contradistinction to reduced *Alk1* expression seen in advanced human HF. The signaling observed in end stage human HF may differ from the TAC model in mice due to differences in the mechanism of injury (pressure overload vs. non-pressure overload) and the duration of the cardiomyopathy (weeks vs. years). Study of WT and *Alk1*^{+/-} mice following longer courses of TAC may more closely recapitulate signaling changes observed in end stage human HF and define the long term effect of reduced ALK1 expression on cardiac remodeling. We used a mouse model with reduced total body expression of ALK1 and were therefore unable to determine the effects of

reduced ALK1 activity in selected non-cardiomyocyte populations. Furthermore, extra-cardiac effects of reduced ALK1 signaling may have modified the response to pressure overload induced HF. Future studies employing cell-specific or a cardiac restricted ALK1 deletion model may help define the role of ALK1 signaling in HF.

5. Conclusions

Cardiac fibrosis mediated by TGF- β signaling is an emerging therapeutic target in HF. We now introduce that reduced ALK1 activity is a potentially important component of cardiac remodeling in HF and is associated with impaired cardiac function, increased cardiac fibrosis, and decreased SMAD1 signaling in pressure overload induced HF. Further investigation of the impact of ALK1 signaling on cardiac remodeling and ALK1 signaling in non-myocyte cell populations may identify novel therapeutic approaches to limit morbidity and mortality associated with this devastating disease.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (1R01HL133215-01) to N.K. and a Heart Failure Society of America Research Fellowship to K.M.

References

1. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al. Heart disease and stroke statistics—2015 update: a report from the American Heart Association. *Circulation*. 2015; 131:e29–322. [PubMed: 25520374]
2. Leask A. Getting to the heart of the matter: new insights into cardiac fibrosis. *Circulation research*. 2015; 116:1269–76. [PubMed: 25814687]
3. Massague J. TGFbeta signalling in context. *Nature reviews Molecular cell biology*. 2012; 13:616–30. [PubMed: 22992590]
4. Kapur NK, Wilson S, Yunis AA, Qiao X, Mackey E, Paruchuri V, et al. Reduced endoglin activity limits cardiac fibrosis and improves survival in heart failure. *Circulation*. 2012; 125:2728–38. [PubMed: 22592898]
5. Lamouille S, Mallet C, Feige JJ, Bailly S. Activin receptor-like kinase 1 is implicated in the maturation phase of angiogenesis. *Blood*. 2002; 100:4495–501. [PubMed: 12453878]
6. Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, et al. Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:2626–31. [PubMed: 10716993]
7. Johnson DW, Berg JN, Baldwin MA, Gallione CJ, Marondel I, Yoon SJ, et al. Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. *Nature genetics*. 1996; 13:189–95. [PubMed: 8640225]
8. Park SO, Wankhede M, Lee YJ, Choi EJ, Fliess N, Choe SW, et al. Real-time imaging of de novo arteriovenous malformation in a mouse model of hereditary hemorrhagic telangiectasia. *The Journal of clinical investigation*. 2009; 119:3487–96. [PubMed: 19805914]
9. Morine KJ, Qiao X, Paruchuri V, Aronovitz MJ, Mackey EE, Buiten L, et al. Conditional knockout of activin like kinase-1 (ALK-1) leads to heart failure without maladaptive remodeling. *Heart and vessels*. 2016 (In press).
10. Tual-Chalot S, Oh SP, Arthur HM. Mouse models of hereditary hemorrhagic telangiectasia: recent advances and future challenges. *Frontiers in genetics*. 2015; 6:25. [PubMed: 25741358]
11. Srinivasan S, Hanes MA, Dickens T, Porteous ME, Oh SP, Hale LP, et al. A mouse model for hereditary hemorrhagic telangiectasia (HHT) type 2. *Hum Mol Genet*. 2003; 12:473–82. [PubMed: 12588795]

12. Kapur NK, Paruchuri V, Aronovitz MJ, Qiao X, Mackey EE, Daly GH, et al. Biventricular remodeling in murine models of right ventricular pressure overload. *PloS one*. 2013; 8:e70802. [PubMed: 23936252]
13. Rockman HA, Ono S, Ross RS, Jones LR, Karimi M, Bhargava V, et al. Molecular and physiological alterations in murine ventricular dysfunction. *Proceedings of the National Academy of Sciences of the United States of America*. 1994; 91:2694–8. [PubMed: 8146176]
14. Shahid M, Spagnoli E, Ernande L, Thoonen R, Kolodziej SA, Leyton PA, et al. BMP type I receptor ALK2 is required for angiotensin II-induced cardiac hypertrophy. *American journal of physiology Heart and circulatory physiology*. 2016; 310:H984–94. [PubMed: 26873969]
15. Cunha SI, Pietras K. ALK1 as an emerging target for antiangiogenic therapy of cancer. *Blood*. 2011; 117:6999–7006. [PubMed: 21467543]
16. Hawinkels LJ, de Vinuesa AG, Paauwe M, Kruithof-de Julio M, Wiercinska E, Pardali E, et al. Activin Receptor-like Kinase 1 Ligand Trap Reduces Microvascular Density and Improves Chemotherapy Efficiency to Various Solid Tumors. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2016; 22:96–106. [PubMed: 26373572]
17. van Meeteren LA, Thorikay M, Bergqvist S, Pardali E, Stampino CG, Hu-Lowe D, et al. Anti-human activin receptor-like kinase 1 (ALK1) antibody attenuates bone morphogenetic protein 9 (BMP9)-induced ALK1 signaling and interferes with endothelial cell sprouting. *The Journal of biological chemistry*. 2012; 287:18551–61. [PubMed: 22493445]
18. Doi T, Lee KH, Kim TM, Ohtsu A, Kim TY, Ikeda M, et al. A phase I study of the human anti-activin receptor-like kinase 1 antibody PF-03446962 in Asian patients with advanced solid tumors. *Cancer medicine*. 2016; 5:1454–63. [PubMed: 27075560]
19. Goff LW, Cohen RB, Berlin JD, de Braud FG, Lyshchik A, Noberasco C, et al. A Phase I Study of the Anti-Activin Receptor-Like Kinase 1 (ALK-1) Monoclonal Antibody PF-03446962 in Patients with Advanced Solid Tumors. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2016; 22:2146–54. [PubMed: 26655846]
20. Necchi A, Giannatempo P, Mariani L, Fare E, Raggi D, Pennati M, et al. PF-03446962, a fully-human monoclonal antibody against transforming growth-factor beta (TGFbeta) receptor ALK1, in pre-treated patients with urothelial cancer: an open label, single-group, phase 2 trial. *Investigational new drugs*. 2014; 32:555–60. [PubMed: 24566706]
21. Simonelli M, Zucali P, Santoro A, Thomas MB, de Braud FG, Borghaei H, et al. Phase I study of PF-03446962, a fully human monoclonal antibody against activin receptor-like kinase-1, in patients with hepatocellular carcinoma. *Annals of oncology: official journal of the European Society for Medical Oncology*. 2016; 27:1782–7. [PubMed: 27329247]
22. Wheatley-Price P, Chu Q, Bonomi M, Seely J, Gupta A, Goss G, et al. A Phase II Study of PF-03446962 in Patients with Advanced Malignant Pleural Mesothelioma. *CCTG Trial IND*. 207. *Journal of thoracic oncology: official publication of the International Association for the Study of Lung Cancer*. 2016
23. Bendell JC, Gordon MS, Hurwitz HI, Jones SF, Mendelson DS, Blobe GC, et al. Safety, pharmacokinetics, pharmacodynamics, and antitumor activity of dalantercept, an activin receptor-like kinase-1 ligand trap, in patients with advanced cancer. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2014; 20:480–9. [PubMed: 24173543]
24. Niessen K, Zhang G, Ridgway JB, Chen H, Yan M. ALK1 signaling regulates early postnatal lymphatic vessel development. *Blood*. 2010; 115:1654–61. [PubMed: 19903896]

Highlights

- -ALK1 expression is decreased advanced human heart failure
- -Reduced ALK1 expression increases fibrosis after trans aortic constriction
- -Reduced ALK1 expression attenuates SMAD1 signaling

Figure 1. Reduced ALK1 Expression is Associated with Advanced Heart Failure

A and B, LV ALK1 protein and mRNA levels in patients with advanced heart failure (HF) compared to patients without heart failure (non-HF) (n=8/group). C-H, LV mRNA levels of TGF-β1, Alk5, Endoglin, PAI-I, Id1 and Id2. p<0.05: *, vs. non-HF.

Figure 2. Increased Fibrosis in *Alk1*^{+/-} Mice After Severe Pressure Overload Induced Heart Failure

A, LV *Alk1* mRNA levels in WT and *Alk1*^{+/-} mice after two weeks of TAC or sham-operated controls (n=6/group). B to F, LV mRNA levels of TGF-β1, *Alk5*, Endoglin, PAI-I and type I collagen. G, Quantification and representative Western blot of type I collagen protein expression normalized to GAPDH expression. H and I, Representative histological staining for collagen and quantification of LV fibrotic area. p<0.05: *, vs. WT Sham; †, vs. *Alk1*^{+/-} Sham; ††, vs. WT TAC.

Figure 3. Reduced ALK1 Expression Does Not Affect Cardiac Hypertrophy or Myocardial Capillary Density

A and B, Representative histological staining and quantification of LV cardiomyocyte cross sectional area in WT and *Alk1*^{+/-} mice after two weeks of TAC (n=6/group). C to E, LV mRNA levels of beta-myosin heavy chain (MHC), sarcoplasmic endoplasmic reticulum ATPase (SERCA) and calcineurin. F and G, Representative immunostaining and quantification of myocardial capillary density. p<0.05: *, vs. WT Sham; †, vs. *Alk1*^{+/-} Sham; ‡, vs. WT TAC.

Figure 4. Smad 1 Phosphorylation and Activity is Attenuated in *Alk1*^{+/-} Mice After Severe Pressure Overload Induced Heart Failure

A, Representative Western blot of phosphorylated SMAD3 (pSmad3), total SMAD3 and GAPDH. Quantification of pSMAD3 to total SMAD3 is shown in inset. B, Representative Western blot of phosphorylated SMAD1 (pSmad1), total SMAD1 and GAPDH. For pSMAD1 levels, the antibody used detects pSMAD1/5/8. The topmost band at 60 kD representing pSmad 1 was used for quantification of pSMAD1 levels. Quantification of pSMAD1 to total SMAD1 is shown in inset. C and D, LV mRNA levels of Id1 and Id2. p<0.05: *, vs. WT Sham; †, vs. *Alk1*^{+/-} Sham; ‡, vs. WT TAC.

Figure 5. Reduced ALK1 Activity Attenuates Smad 1 Signaling and Promotes Cardiac Fibrosis in Heart Failure

Left, With intact ALK1 activity, in response to LV pressure overload SMAD1 and SMAD3 signaling is activated.

Right, With reduced ALK1 activity, SMAD1 signaling is attenuated and associated with an increase in type I collagen production and cardiac fibrosis. Abbreviations: Activin like kinase 5 (Alk5); Transforming growth factor beta receptor II (TβRII); Endoglin (Eng)

Table 1

Clinical characteristics of patients with advanced heart failure

Patient	Age	Gender	Cardiomyopathy
1	48	F	Non-ischemic
2	58	M	Non-ischemic
3	62	F	Ischemic
4	44	F	Non-ischemic
5	52	M	Non-ischemic
6	64	M	Ischemic
7	60	M	Non-ischemic
8	55	F	Non-ischemic

Table 2Characterization of Left Ventricular Pressure Overload Induced by TAC in WT and *Alk1*^{+/-} Mice

	WT Sham	WT TAC	<i>Alk1</i> ^{+/-} Sham	<i>Alk1</i> ^{+/-} TAC
Mass				
Total body weight (g)	27.9 ± 1	27.2 ± 1.7	29.9 ± 0.9	27.6 ± 3.7
LV/TL (mg/mm)	5.8 ± 0.7	6.9 ± 0.6	5.7 ± 0.4	8.7 ± 1.1 ^{†‡}
Lung mass/TL (mg/mm)	9.2 ± 0.7	9.6 ± 0.7	8 ± 0.8	18.2 ± 8.6 ^{†‡}
Hemodynamic data				
Peak systolic pressure (mmHg)	97 ± 3	148 ± 16 [*]	102 ± 4	127 ± 11 ^{†‡}
End diastolic pressure (mmHg)	5 ± 2	16 ± 7 [*]	3 ± 1	22 ± 2 ^{†‡}
dP/dT max (mmHg/sec)	8450 ± 806	7577 ± 1163	9207 ± 462	4975 ± 1249 ^{†‡}
dP/dT min (mmHg/sec)	-7412 ± 956	-6879 ± 1113	-7835 ± 635	-4739 ± 1135 ^{†‡}
Heart rate (bpm)	531 ± 45	527 ± 34	495 ± 26	544 ± 36
Echocardiographic data				
LVEDD (mm)	2.4 ± 0.4	2.1 ± 0.6	3 ± 0.5	3.5 ± 0.1 [‡]
LVESD (mm)	0.8 ± 0.5	1.1 ± 0.5	0.9 ± 0.1	2.4 ± 0.1 ^{†‡}
FS (%)	71 ± 9	48 ± 12 [*]	71 ± 5	30 ± 9 ^{†‡}

Abbreviations: Left ventricle (LV); Tibia length (TL); Fractional shortening (FS). p<0.05:

* , vs. WT Sham;

† , vs. *Alk1*^{+/-} Sham;

‡ , vs. WT TAC.