Smooth muscle cell-specific Tgfbr1 deficiency promotes aortic aneurysm formation by stimulating multiple signaling events

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Transforming growth factor (TGF)-β signaling disorder has emerged as a common molecular signature for aortic aneurysm development. The timing of postnatal maturation plays a key role in dictating the biological outcome of TGF-β signaling disorders in the aortic wall. In this study, we investigated the impact of deficiency of TGFβ receptors on the structural homeostasis of mature aortas. We used an inducible Cre-loxP system driven by a Myh11 promoter to delete Tgfbr1, Tgfbr2, or both in smooth muscle cells (SMCs) of adult mice. TGFBR1 deficiency resulted in rapid and severe aneurysmal degeneration, with 100% penetrance of ascending thoracic aortas, whereas TGFBR2 deletion only caused mild aortic pathology with low (26%) lesion prevalence. Removal of TGFBR2 attenuated the aortic pathology caused by TGFBR1 deletion and correlated with a reduction of early ERK phosphorylation. In addition, the production of angiotensin (Ang)-converting enzyme was upregulated in TGFBR1 deficient aortas at the early stage of aneurysmal degeneration. Inhibition of ERK phosphorylation or blockade of AngII type I receptor AT1R prevented aneurysmal degeneration of TGFBR1 deficient aortas. In conclusion, loss of SMC-Tgfbr1 triggers multiple deleterious pathways, including abnormal TGFBR2, ERK, and AngII/AT1R signals that disrupt aortic wall homeostasis to cause aortic aneurysm formation.

Recent clinical investigations have linked transforming growth factor (TGF)-β signaling disorders to the development of aortic aneurysms. When mutated, genes encoding TGFβ signaling components, such as the ligand, receptors, and intracellular mediators, can cause the formation of aortic aneurysms, leading to Loeys-Dietz syndrome (LDS)1-2. Familial thoracic aortic aneurysm and dissection (TAAD) can also be caused by mutations of TGFβ receptors (e.g. TGFBR13 and TGFBR24). In LDS patients, aortic tissue, which harbors defective TGFβ signaling components, paradoxically, but consistently, shows enhanced TGFβ activity, as evidenced by increased SMAD2 phosphorylation and the expression of CTGF2,5,6. These observations have led to a theory that TGFβ hyperactivity accounts for the aortic pathology7. Accordingly, loss-of-function mutation of the inhibitory TGFβ signaling component SKI predisposes patients to aortic aneurysm formation8. This notion is further supported by findings that mutations of genes not specific to TGFβ signaling transduction (e.g. ACTA2 and MYH11) also augment TGFβ signaling in the aortic wall, causing familial thoracic aortic aneurysm and dissection (TAAD)9. Further evidence for the causative role of TGFβ3 comes from studies of Marfan syndrome (MFS), in which affected organs contains abnormally high levels of active TGFβ10, and serological removal of TGFβ effectively prevents aortic dilation and elastic fiber fragmentation11.

However, evidence conflicting with the theory of TGFβ hyperactivity exists in the literature. For example, longitudinal studies of mouse models of LDS show that enhanced TGFβ signaling, canonical or non-canonical, is not evident until aortic pathology has advanced a relatively late stage12. It is also noteworthy that direct evidence supporting TGFβ hyperactivity as the mechanism underlying genetic TGFβ defects remains lacking, whereas
haploinsufficiency of TGFβ2<sup>13</sup>, TGFβ3<sup>2</sup>, and SMAD3<sup>14</sup> causes aortic aneurysm formation. In addition, a series of studies from Gomez et al. suggests that the expression of SMAD2 is upregulated in medial smooth muscle cells (SMCs) due to epigenetic modification. The elevated production of total SMAD contributes to the phenomenon of "TGFβ paradox" in a mechanism independent of the local concentration of TGFβ proteins<sup>15-18</sup>. These lines of evidence have raised a critical question about the exact role of TGFβ in the development of aortic pathology that is linked to genetic TGFβ signaling defects.

TGFβ is a potent growth factor that participates in vascular development via regulating cell differentiation and matrix metabolism<sup>19</sup>. Germline deletion of TGFβ signaling components such as receptors and ligands is thus embryonically lethal due to vascular defects of the yolk sac<sup>20</sup>. Further studies for the function of TGFβ in individual cell-groups demonstrate that deficiency of SMC-specific TGFβ induces persistent truncus arteriosus<sup>21,22</sup>. While the importance of TGFβ in vascular development is widely recognized, its physiological role in established vasculature, particularly the aortic wall, is less well understood. Patients suffering from genetic TGFβ disorders are usually born with a normal aorta with aneurysms developing in the postnatal life. Although the underlying mechanisms could be multifold, it is possible that the genetic mutations induce the aortic pathology through interrupting the physiological function of TGFβ. This hypothesis is consistent with the finding that a basal level of TGFβ protects aortas from chemical-induced aneurysm formation<sup>23</sup>. We have recently reported that the ablation of SMC-specific TGFβR1 causes aortic aneurysms and dissections<sup>24</sup>. Since TGFβR1 can form a receptor complex with multiple type II TGFβ receptor members<sup>25</sup>, it was unclear whether the phenotype resulting from the loss of SMC TGFβR1 is driven by the TGFβRII signal. Therefore, this current study evaluated the role of TGFβR2 in the phenotypic expression of the aortas deficient in TGFβR1. While our study was being completed, two alternate studies reported that the deletion of SMC-specific Tgfbr2 induces aortic aneurysmal degeneration with complete penetrance in immature but not adult mice<sup>26,27</sup>. The current study, however, using a series of genetic experiments in mice, investigated the functional relationship between TGFβ receptors and aorta homeostasis. Results from our study show that SMC-Tgfb1 deletion promoted aortic aneurysm formation in a manner partially dependent on Tgfbr2 in adult mice. The aneurysmal degeneration could be rescued by inhibition of phosphorylation of extra-cellular signal-regulated kinase (ERK) or blockade of angiotensin type I receptor (AT1R), indicating that multiple pathways deleterious to the aortic wall are activated following SMC-Tgfb1 deletion.

Results

SMC-specific deletion of the TGFβR3 receptor members. We used an inducible Cre-loxP system driven by a Myh11 promoter<sup>28</sup> to delete SMC-specific Tgfb1<sup>Tgfbr1<sup>Δ/Δ</sup></sup>, Tgfbr2<sup>Tgfbr2<sup>Δ/Δ</sup></sup>, or both Tgfbr1 and Tgfbr2<sup>Tgfbr1<sup>Δ/Δ</sup>, Tgfbr2<sup>Δ/Δ</sup></sup>. To confirm efficient gene-deletion in aortas, we monitored the recombination-events with a Gt(ROSA)26osr (R26R) reporter strain (n = 3–6 for each genotype). Shown in Supplementary Figure 1A are representative images of x-gal staining of the ascending aortic segments (ATAs) collected on the following day of the last dose of tamoxifen injection. Tamoxifen induced robust and similar degree of recombination-events in SMCs of Tgfbr1<sup>Δ/Δ</sup>, Tgfbr2<sup>Δ/Δ</sup>, and Tgfbr1<sup>Δ/Δ</sup>, Tgfbr2<sup>Δ/Δ</sup> ATAs, as evidenced by positive x-gal staining of vast majority of cells throughout the entire aortic layer. Genotyping assays on explanted SMCs confirmed the null allele for Tgfbr1 and Tgfbr2 (Supplementary Figure 1B). We further validated the deletion of these receptors with functional assays. In contrast to Tgfb1<sup>Δ/Δ</sup> controls, Tgfbr1<sup>Δ/Δ</sup> and Tgfbr2<sup>Δ/Δ</sup> SMCs showed no pSMAD2 expression or upregulation of TGFβ-responsive genes (e.g., Ctgf, col1a2, and Eln) after TGFβ stimulation (Supplementary Figure 2A, B). Activation of ERK by TGFβ was also abolished in these SMCs (Supplementary Figure 2C). Therefore, the system induced efficient deletion of the TGFβ receptors in vivo.

Deletion of SMC-specific Tgfbr1 in adult mice rapidly causes aneurysmal degeneration of the thoracic aorta.

With the validated Cre-loxP system, we induced Tgfb1<sup>Δ/Δ</sup> by tamoxifen injection in male mice at 9–13 weeks of age (n = 34) and injected age- and sex-matched Tgfb1<sup>+/+</sup> mice with tamoxifen (n = 17) as a control. In Tgfbr1<sup>Δ/Δ</sup> animals, aortic rupture was noted as early as 10 days after the first dose of tamoxifen (Fig. 1A). By day 28 (d28), 29% (10/34) of Tgfbr1<sup>Δ/Δ</sup> animals died from an aortic rupture located in ATA (2), proximal descending (DTA, 2), or suprarenal (SRA, 6) segments, but all Tgfbr1<sup>Δ/Δ</sup> controls survived (Fig. 1B). Surviving Tgfbr1<sup>Δ/Δ</sup> animals experienced progressive aortic enlargement. As determined by ultrasound scanning of a subset of age-matched (11 weeks) Tgfbr1<sup>Δ/Δ</sup> (n = 9) and Tgfbr1<sup>+/+</sup> (n = 5) mice on d28, Tgfbr1<sup>Δ/Δ</sup> mice exhibited a 23% and 78% increase in diameter in the ATA and suprarenal (SRA) aortic segments, respectively, whereas Tgfbr1<sup>Δ/Δ</sup> controls did not present significant changes (Fig. 1C, D). When gross examination was performed on d28, all Tgfbr1<sup>Δ/Δ</sup> aortas displayed one or more evident pathologies, which were manifested by fusiform dilation, intramural hematoma, intimal/medial tear, and contained rupture (Fig. 1E, Supplementary Figure 3). The prevalence of aortic pathologies varied depending on the aortic region. Of the 24 surviving Tgfbr1<sup>Δ/Δ</sup> animals, aortic lesions were noted in 100% of ATA segments, 33% of DTA segments, and 39% of SRA segments, but not in any infrarenal (AA) segments (Fig. 1E).

Next, we intensively characterized the progression of aortic pathologies over time. Because of the complete penetrance in ATA segments, we focused our evaluation on this location throughout the study. To assess the early histological changes, Tgfbr1<sup>Δ/Δ</sup> ATAs were harvested at d2 (n = 6), d5 (n = 7), and d10 (n = 12) and Tgfbr1<sup>+/+</sup> ATAs collected at d10 (n = 7) served as controls. Elastic fiber fragmentation was detected as early as 10 days after the first dose of tamoxifen in Tgfbr1<sup>Δ/Δ</sup> ATAs (Supplementary Figure 4A). Medial thickness of these ATAs did not change significantly and remained similar to that of the controls at this time point (Supplementary Figure 4B). Intimal/medial tears were caught randomly in cross-sections of d10 ATAs. To evaluate the distribution of this pathology in the entire ATA segments, we injected Evans Blue to animals prior to tissue collection to highlight areas with intimal/medial tears and examined the specimens with en face microscopy using the same protocol as previously described<sup>24</sup>. Specimens (n = 3 per genotype) were collected on d13, the time point around which Tgfbr1<sup>Δ/Δ</sup> aortas frequently rupture (Fig. 1B). As shown in Supplementary Figure 4C, scattered and randomly distributed blue
spots were observed on the luminal surface of Tgfbr1iko ATAs. Scanning electron microscopy (SEM) and histological evaluations confirmed that these focal lesions were intimal/medial tears that often extended deep into the media (Supplementary Figure 4C). Pathologies at a more advanced stage (d28) were characterized by randomly distributed areas of medial thinning or complete depletion (16/24, 67%), intimal-medial tears (17/24, 71%), intramural hematoma (12/24, 50%), and contained rupture (3/24, 13%). Intense adventitial fibrosis was evident in all Tgfbr1iko ATA segments (Fig. 2A,B). The pathological features displayed by the SRA segments were essentially the same as those presented by the ATA specimens (Supplementary Figure 5). False lumen formation, which was rarely detected in the ATA region, was occasionally noted for SRA specimens (Supplementary Figure 5E), indicating that animals with acute SRA dissections had a higher chance to survive than those with acute ATA dissections.

Deletion of SMC-specific Tgfbr2 is less deleterious to the aortic wall than deletion of Tgfbr1. TGFβ is thought to signal through Tgfbr2 and then Tgfbr1 receptor subunits 28, suggesting that Tgfbr2iko would lead to the same aortic phenotype as Tgfbr1iko. To test this hypothesis, we induced Tgfbr2iko in male mice (n = 18) at 9 to 13 weeks of age and evaluated aortic phenotypes at d28. Surprisingly, all Tgfbr2iko mice survived for at least 28 days. On gross examination, only one Tgfbr2iko mouse exhibited a fusiform aneurysm in the SRA segment, whereas all other mice displayed aortic morphology that was grossly undistinguishable from that of Tgfbr1iko controls (Fig. 3A). Aortic rupture was not observed in any of the Tgfbr2iko mice but occurred in 26% of Tgfbr1iko mice. The incidence of gross aortic pathology was 6% in Tgfbr2iko animals but 100% in Tgfbr1iko animals (Fig. 3B). When we performed ultrasound scanning for a subset of age-matched Tgfbr2iko animals (n = 9), Tgfbr2iko aortas showed only a modest increase in diameter over 28 days that was significantly less than that observed for Tgfbr1iko aortas (Fig. 3C). Pathologies detected in Tgfbr2iko ATA segments at the histological level were limited to elastic fiber breaks (7/18, 39%) and shallow intimal/medial tears (3/18, 17%).

![Figure 1](https://www.nature.com/scientificreports/)

**Figure 1.** Tgfbr1iko rapidly results in aortic rupture and aneurysmal degeneration. (A) A gross specimen of a Tgfbr1iko aorta showing a hematoma (arrow) resulting from rupture of the ascending aorta (ATA) on day (d) 13. (B) Kaplan Meier survival analysis of Tgfbr1iko (n = 34) and Tgfbr1f/f (n = 17) animals. (C,D) Ultrasound imaging of Tgfbr1iko (n = 5) and Tgfbr1iko (n = 9) aortas at sites of ATA (C) and suprarenal (SRA) (D) segments. Significance of genotype- and time-dependent differences was evaluated using two-way repeated measurement ANOVA. *P = 0.003, *P = 0.027. (E) Incidence of grossly evident pathologies at various anatomic locations of surviving Tgfbr1iko mice (n = 24). DTA: descending aorta; AA: infrarenal aorta. Note that nearly 40% of the aortas had pathologies at multiple locations.
scoring system to quantify these pathologies (See details in Materials and Methods and Supplementary Figure 6A–C) and found that Tgfbr2iko resulted in a significantly lower degree of aneurysmal degeneration than Tgfbr1iko (Fig. 3D). These in vivo observations suggest that the functions of Tgfbr2 and Tgfbr1 receptors in SMCs do not completely overlap, with Tgfbr1 being more critical than Tgfbr2 to the maintenance of aortic wall homeostasis in adult animals. An alternative explanation, however, is that the distinct phenotypes result from abnormal Tgfbr2 signal triggered by Tgfbr1 deficiency.

Deleterious effect of SMC-specific Tgfbr1 deficiency relies largely on Tgfbr2. The distinct effects of Tgfbr1 and Tgfbr2 deletion on aortic wall homeostasis suggest different underlying mechanisms. If the abnormal Tgfbr2 signaling accounts for the aortic disease, then removing Tgfbr2 should prevent aneurysmal degeneration of Tgfbr1iko aortas. We therefore generated the Tgfbr1iko.Tgfbr2iko strain to test this hypothesis. Male Tgfbr1iko.Tgfbr2iko mice (n = 17) at 9–11 weeks of age were treated with tamoxifen and followed up for 28 days. Removal of Tgfbr2 fully rescued aortic rupture induced by Tgfbr1iko (Fig. 4A). Compared with Tgfbr1iko, Tgfbr1iko.Tgfbr2iko significantly attenuated aortic dilation at ATA (23% vs. 8%) and SRA (78% vs. 8%) segments (Fig. 4B). On gross examinations of the Tgfbr1iko.Tgfbr2iko aortas, small intramural hematoma and tears were noted, but contained ruptures were not detected. The incidence of aortic pathology in the Tgfbr1iko.Tgfbr2iko group was 47%, which was significantly lower than that (100%) in the Tgfbr1iko group (Fig. 4C). In contrast to the severe aneurysmal degeneration of Tgfbr1iko ATAs (Figs 1 and 2 and Supplementary Figures 3 and 4), Tgfbr1iko.Tgfbr2iko ATA segments displayed isolated intimal/medial tears (5/17, 29%) and small areas of intramural hematoma (3/17, 18%), with medial thinning/depletion being less frequently detected (1/17, 6%). Adventitial fibrosis was observed only in the ATAs with evident gross pathology. Overall, Tgfbr1iko.Tgfbr2iko aortas displayed significantly less wall degeneration than Tgfbr1iko aortas (Fig. 4D). Therefore, abrogation of the Tgfbr2 signal ameliorated the aortic pathology induced by Tgfbr1iko. These in vivo observations indicate that SMC-specific Tgfbr2, in the absence of Tgfbr1, is deleterious to aortic wall homeostasis.
Additional deletion of SMC-Tgfbr2 inhibits activation of the ERK pathway in Tgfbr1<sup>iks</sup> aortas prior to aneurysmal degeneration. Activation of the ERK pathway has been identified as a major molecular event for aortic aneurysm development in various animal models<sup>7,12,29</sup>. To explore the role of the ERK pathway in Tgfbr1<sup>iks</sup> aortas, we evaluated the production and phosphorylation of pERK1/2 at various time points with western blotting assays. Compared with Tgfbr1<sup>f/f</sup> controls, Tgfbr1<sup>iks</sup> aortas produced two times more pERK1/2 by the time of completion of tamoxifen injection (d5, n = 5 per genotype), and this early augmentation was blunted by Tgfbr2<sup>iks</sup>. However, at the time that aortic rupture frequently occurred (d13, n = 5 per genotype), the production of pERK1/2 in Tgfbr1<sup>iks</sup>. Tgfbr2<sup>iks</sup> aortas returned to baseline levels (Fig. 5A), and immunohistochemistry (IHC) assays revealed a trend of reduction of pERK1/2 in cells located in the media of Tgfbr1<sup>iks</sup>-ATA segments (Fig. 5B). These assays uncovered a genotype-specific correlation between the exaggeration of early ERK signaling and subsequent development of aortic pathology, suggesting a role for the ERK pathway in Tgfbr1<sup>iks</sup>-driven aortic disease.

An unsolved puzzle in the study of TGFβ signaling disorders is the “TGFβ paradox”, which is characterized by increased pSMAD2 abundance in cells with defective TGFβ signaling components<sup>7,30</sup>. We measured pSMAD2 in our models, and not surprisingly, ATA segments deficient in Tgfbr1, Tgfbr2, or both exhibited impaired rather than enhanced SMAD2 phosphorylation (Supplementary Figure 7A, n = 5 per genotype), and IHC assays revealed that the reduction in pSMAD2 production was attributed primarily to cells located in the tunica media of ATA segments (Supplementary Figure 7B). In the absence of Tgfbr1, Tgfbr2 may assemble a signaling complex with other type I receptors, such as Alk1, to propagate the TGFβ signal through SMAD1/5/8, and this promiscuous receptor-receptor interaction may be an important mechanism of tissue fibrosis<sup>28</sup>. Therefore, we evaluated the production of pSMAD1/5/8 in our model (n = 5 per genotype). As the disease progressed to d13, the level of pSMAD1/5/8 became significantly higher in Tgfbr1<sup>iks</sup> than in Tgfbr1<sup>f/f</sup> ATA segments. However, pSMAD1/5/8 was also augmented in Tgfbr2<sup>iks</sup> and Tgfbr1<sup>iks</sup>. Tgfbr2<sup>iks</sup> aortas, with no differences among genotypes (Supplementary Figure 8). Thus, SMAD-dependent pathways may not be critical to the development of Tgfbr1<sup>iks</sup>-driven aortic disease.

Figure 3. Tgfbr2<sup>iks</sup> only causes mild aortic pathology with low phenotypic penetrance in 28 days. (A) Gross image of aortic specimens with the indicated genotypes. Dark patches appearing in Tgfbr1<sup>iks</sup> aortas reflect intramural hematoma. (B) Surgical evaluation of aortic pathology (aneurysm, intimal/medial tears, intramural hematoma, contained or free rupture). Fisher’s exact test was performed for data analysis. (C) Changes in aortic diameter at the indicated locations in Tgfbr1<sup>iks</sup> (n = 9) and Tgfbr2<sup>iks</sup> (n = 9) mice. Differences between groups were evaluated using unpaired t-tests. (D) Left: Histological evaluation of aortic structural degeneration. Images show typical histology of Tgfbr2<sup>iks</sup> and Tgfbr1<sup>iks</sup>-ATAAs. Arrows indicate a deep intimal/medial tear. Right: Degeneration score for Tgfbr1<sup>iks</sup> (n = 24) and Tgfbr2<sup>iks</sup> (n = 18) ATA segments. Data were analyzed using the unpaired t-test. Scale bars: 100 μm.
Pharmaceutical inhibition of ERK phosphorylation prevents aneurysmal degeneration of Tgfbr1\(^{i ko}\) aortas. The tight genotype-specific correlation between the early upregulation of pERK1/2 and the subsequent development of aortic disease raises the question of whether this correlation reflects causation. A previous study indicated that RDEA-119, a small chemical compound, selectively inhibits ERK phosphorylation and attenuates aortic dilation in MFS mice\(^{31}\). Therefore, we treated Tgfbr1\(^{i ko}\) mice with RDEA-119 (\(n = 7\)) and compared them to the solvent-treated controls (\(n = 8\)). Consistent with that study, RDEA-119 significantly inhibited ERK phosphorylation in the aortic wall (Supplementary Figure 9) and attenuated dilation of Tgfbr1\(^{i ko}\) aortas (Fig. 6A). Although the mice were treated with the same dosage as previously reported\(^{31}\), we noticed that animals that were treated but not in the control group began to lose weight a week after RDEA-119 treatment. Thus, we had to stop the experiment prematurely after 2 weeks. Because the aortic pathology in Tgfbr1\(^{i ko}\) aortas at this early time point was dominated by scattered intimal/medial tears (Supplementary Figure 4), we decided to use Evans Blue staining to catch aortic tears that would have otherwise been missed during routine histological evaluations.

Aortic ruptures were not noted in either group. Extravasation of Evans Blue was not detected in any aortas of RDEA-119-treated mice but was present in all ATA segments and three of eight SRA segments in placebo-treated mice (Fig. 6B), indicating that treatment with RDEA-119 fully prevents intimal-medial tears at the early stage. Representative images showing areas of extravasation of Evans Blue (indicated by arrows) are shown in Fig. 6C.

Losartan rescues the Tgfbr1\(^{i ko}\) phenotype without interrupting ERK phosphorylation. A large body of evidence suggests an important role for the AngII/AT1R signaling pathway in the pathogenesis of thoracic and abdominal aortic aneurysms\(^{11,32}\). In addition, an interaction between TGF\(\beta\) and AngII/AT1R signaling pathways was recently demonstrated in MFS and LDS mice\(^{12,33}\), thus calling for further mechanistic elucidation\(^{32}\).

To this end, we evaluated the role of the AngII/AT1R axis in the aneurysmal degeneration of Tgfbr1\(^{i ko}\) aortas. Compared with Tgfbr1\(^{f/f}\) controls, Tgfbr1\(^{i ko}\) induced a three-fold increase of angiotensin-converting enzyme (ACE) in the tunica media of the aortic wall, and this elevation persisted at d13 (Fig. 7A). ACE production was further quantified with western blotting assays. Consistent with the observation of IHC assays, an upregulation of ACE production was significant at both d5 and d13 compared with d5 (Fig. 7B). Because the upregulation of ACE was located primarily in medial SMCs (Fig. 7A), 
the reduction was most likely a result of the occurrence of intimal/medial tears and intramural hematoma that altered the cellular and protein compositions of the aortic tissue at this time point (Supplementary Figure 4C). To determine the importance of the AngII/AT1R axis in Tgfbr1iko-driven aortic disease, we disrupted this axis in Tgfbr1iko mice with pharmaceutical intervention. The effects of the treatment were then evaluated over a 28-day period. Mice in the experimental group were treated with losartan (n = 10), a specific AT1R inhibitor, and mice in the control groups were treated with hydralazine (n = 12), propranolol (n = 10), or placebo (n = 9) to assess the effect of lowering hemodynamic stress to the aortic wall. As expected, losartan, hydralazine, and propranolol lowered systolic blood pressure by 22–28 points, with no statistically significant differences among treatments (Supplementary Figure 10). Ultrasound scanning of aortas at various time points showed that losartan significantly inhibited aortic dilation. This therapeutic effect was not a result of reduction in blood pressure, as animals treated with hydralazine or propranolol exhibited aortic dilation equivalent to the placebo controls (Fig. 8A). Additionally, losartan treatment normalized aortic structure, resulting in a morphology that was indistinguishable from that of Tgfbr1f/f controls when evaluated with gross examination (Fig. 8B). Evidence of medial thinning, intramural hematoma, intimal/medial tears, or contained rupture was not detected in losartan-treated aortas (Fig. 8C). In contrast, lowering blood pressure alone failed to normalize aortic histology (Fig. 8B,C). Interestingly, propranolol seemed to attenuate aneurysmal degeneration, as compared with placebo (Fig. 8C). This improvement may be attributable to the drug effect of propranolol on heart rate and blood pressure, both of which lead to a significant reduction in wall stress. Additionally, aortic rupture did not occur when blood pressure was controlled at a relatively low level. This is in comparison to placebo-treated group, in which 2 out of 9 mice died from aortic rupture, indicating an impaired mechanical strength of Tgfbr1iko aortas. One of the mechanisms proposed for the therapeutic effect of losartan on the development of aortic aneurysms is the inhibition of ERK phosphorylation31,34. As our results show that both RDEA-119 and losartan ameliorated aortic pathology in Tgfbr1iko animals, we evaluated whether the therapeutic effect of losartan was achieved via inhibiting ERK phosphorylation. Tgfbr1iko mice were treated with losartan or placebo and specimens were collected at d5 (n = 5 for each group) and d13 (n = 5 for each group) to examine ERK phosphorylation. Surprisingly, losartan-treated aortas produced similar amounts of pERK1/2 as placebo-treated aortas at various time points (Supplementary Figure 11), suggesting that ERK phosphorylation was not inhibited by losartan in Tgfbr1iko aortas.

Discussion

TGFβ is indispensable for vasculogenesis in embryos35. Studies focusing on specific cell lineages have also demonstrated the importance of SMC-specific TGFβ in vascular development particularly patterning of aortas21,22,26. In the postnatal life, however, TGFβ is frequently considered as a culprit for vascular diseases, such as hypertension,
atherosclerosis, and stenosis/restenosis. Despite the recognition of its role in governing tissue homeostasis, its importance in maintaining the structural integrity of aortic walls was not appreciated up until recent studies showing that conditional deletion of SMC-specific Tgfbr2 in mice at an age of 6 weeks results in aortic aneurysm formation and dissection. A subsequent study showed that neutralization of pan-specific TGFβ isoforms at the early postnatal stage (<P45) increases the incidence of aortic rupture in MFS mice, suggesting a role for TGFβ in aortic wall maturation. Consistent with these reports, the present study demonstrates that deletion of SMC-specific Tgfbr1, the physiologic partner of Tgfbr2, also caused the collapse of aortic structure, which was manifested by aortic rupture, intimal-medial tears, intramural hematoma, and medial thinning/depletion.

Although ligand-ligand and ligand-receptor interactions are highly promiscuous among TGFβ superfamily members, it is believed that Tgfbr1 forms receptor complexes primarily with Tgfbr2 to transduce the TGFβ signal in SMCs. Theoretically, disruptions in Tgfbr1 or Tgfbr2 would lead to the same phenotype. In agreement with this prediction, the phenotype of TGFBR1 mutations is generally indistinguishable from that of TGFBR2 mutations in patients and animal models, though various sites of mutation in each gene may account for its wide spectrum of clinical presentations. Our experiments, however, identified different phenotypes for Tgfbr1iko and Tgfbr2iko aortas, with Tgfbr1iko causing more severe aortic pathology than Tgfbr2iko in adult mice. One explanation for this observation is that Tgfbr1 signaling governs a wider spectrum of biological processes than Tgfbr2 signaling in SMCs, as Tgfbr1 may assemble receptor complexes with type II receptors in addition to Tgfbr2. However, this plausible speculation could not explain the finding that Tgfbr1iko-induced pathology was significantly ameliorated by additional Tgfbr2iko. An alternative explanation is that Tgfbr2 signaling remains activated in the absence of Tgfbr1 and that this Tgfbr2-dependent signal contributes to the aortic pathology observed in Tgfbr1iko mice. Although Tgfbr2 is capable of self-phosphorylation, it requires a type I receptor to trigger the intracellular signaling cascade. In cells lacking both Alk1 and Tgfbr1, TGFβ is unable to alter the profile of global gene expression. Together with our results, these findings raise the hypothesis that the availability of Tgfbr2 in the absence of Tgfbr1 triggers the promiscuous binding of Tgfbr2 to Alk1, which leads to an aberrant TGFβ signal in SMCs that subsequently causes aneurysmal degeneration of the aortic wall. Li et al. reported that the impact of Tgfbr2iko is age-dependent, with its deleterious effects being more severe in immature than in mature aortas.

![Figure 6. Inhibition of ERK phosphorylation effectively protects Tgfbr1iko aortas from aneurysmal degeneration.](image)
We confirmed this observation in our study (data not shown). However, our data also showed that, in contrast to Tgfbr2iko, Tgfbr1iko remains to be devastating to mature aortas. Although the underlying mechanisms have yet to be defined, the diminished age-dependent protective effect under conditions of Tgfbr1iko may be attributable to the abnormal Tgfbr2 signal.

Aortic aneurysms resulting from the mutation of genes that are directly or indirectly committed to TGFβ signaling display enhanced activation of both SMAD-dependent and -independent pathways, as evidenced by the nuclear accumulation of pSMAD2 and pERK31. Further mechanistic studies with MFS mice demonstrate that an inhibition of ERK activation suppressed aortic dilation and improved aortic wall structure31. The neutralization of pan TGFβ ligands inhibited ERK phosphorylation and attenuated aneurysmal degeneration of the aortic wall37. These findings indicate that the intensified ERK signaling acts as a driving force for the aortic phenotype of the MFS. Our data show that genotype-specific alterations in pERK1/2 production during the early stages correlated with the subsequent phenotypic severity. Furthermore, inhibition of ERK phosphorylation prevented aortic dilation and the occurrence of intimal/medial tears in Tgfbr1iko aortas. These results reaffirm the theory that excessive ERK activity is deleterious to aortic wall homeostasis. We also showed that the early elevation of ERK phosphorylation was limited to a narrow window prior to the occurrence of intimal/medial tears, indicating that an early ERK elevation sets the stage for subsequent aneurysmal degeneration to occur. This finding is consistent with the observations that the contribution of β-arrestin2 and miR29b to aortic dilation is limited to the early stage in MFS mice41,42. Pathological exaggeration of the ERK signal can lead to activation of several pathways including enhanced production of MMP2 and MMP943. Further studies to characterize the relationship between the temporal changes of ERK phosphorylation and activation of the downstream pro-aneurysmal molecular cascades will provide a more in-depth understanding of ERK’s role in regulating the phenotypic expression of Tgfbr1iko aortas.

Although changes in SMAD-dependent pathways, including pSMAD2 and pSMAD1/5/8, were observed in our study, a correlation between these changes and phenotypic expression was not detected, indicating a modest role for the canonical pathways in Tgfbr1iko-driven aortic disease. This finding is consistent with the report that an upregulation of pSMAD2 in the aortic tissue was not evident prior to severe structural degeneration in LDS mice42. In the established human aortic aneurysms, an increased abundance of nuclear pSMAD2 has been repeatedly documented41,42. Further investigations have unraveled the epigenetic upregulation of the total SMAD2 production as a major contributor to the enhanced pSMAD2 accumulation in SMGs45. It is intriguing that the SMC-biased pSMAD2 confers a protective effect against aortic dissection by suppressing proteolytic

Figure 7. Angiotensin-converting enzyme (ACE) is upregulated in Tgfbr1iko aortas prior to discernable aneurysmal degeneration. (A) Immunohistochemistry assays for ACE production in ATAs at the indicated time points (n = 5 per group). Specimens stained with isotype-matched IgGs served as a negative control. Note the intense staining of ACE in cells of the tunica media. (B) Western blotting assays for ACE production in ATAs at the indicated time points (n = 5 per group). Data were analyzed using one-way ANOVA. Scale bar: 100 μm.
activity\(^{15,16}\). This notion is further supported by experimental studies. For example, studies from us\(^{24}\), as well as other groups\(^{26,27}\), have demonstrated that a disruption of the basal levels of TGF\(\beta\) activity breaks the aortic wall homeostasis, leading to aortic aneurysm formations and dissections. Serological removal of pan TGF\(\beta\) isoforms exacerbates the incidence of aortic rupture in mice receiving chronic infusion of angiotensin (Ang) II\(^{23,44}\). In the current study, we observed a reduced SMAD2 phosphorylation across various genotypes. While the blunted SMAD2 signal is not a primary contributor to the differential phenotypic expression, it might have created a context that renders the aortic wall more susceptible to the complex dysregulation of TGF\(\beta\) signaling, such as the abnormal TGFBRII signal induced by Tgfbr1 iko. Therefore, our results underscore the importance of both basal TGF\(\beta\) activity and the control of ERK activation in maintaining the aortic wall homeostasis.

Despite the limited benefits of losartan in Marfan patients\(^{45}\), experimental and clinical studies have consistently demonstrated an important role for the AngII/AT1R axis in the development of aortic aneurysms\(^{12,33,46}\). We also show that losartan treatment fully rescued the Tgfbr1 iko aortic phenotype. Studies from other groups suggest that the therapeutic effects of losartan are achieved via inhibition of the ERK pathway in the aortic wall\(^{31,37}\). However, we found that losartan treatment had only a modest impact on the production of pERK in Tgfbr1 iko aortas, indicating that the deleterious effects of the AT1R signaling may not be processed via stimulating ERK-phosphorylation in this particular context. An alternative explanation for this observation is that the therapeutic effect of RDEA-119 in Tgfbr1 iko mice might be achieved through an off-target effect. Although we could not exclude this possibility, this compound is highly selective to MEK\(^{47}\), and its cardiovascular toxicity has been found to be modest\(^{48}\). This suggests that inhibition of ERK phosphorylation accounts for the treatment effect of RDEA-119 in our experiments. In addition to ERK, the AT1R signal can activate several other kinases such as PKC, JNK, Akt, and JAK, all known to play a critical role in promoting aneurysmal degeneration\(^{26,31,49,50}\).

Oxidative stress, another pro-aneurysmal mediator, can also be promoted by the AT1R signal via mechanisms independent of ERK activation. In SMCs, for instance, AT1R signal stimulates the production of reactive oxygen species (ROS) via ARF6-mediated Nox1 upregulation\(^{51}\). It appears that the therapeutic benefits of AT1R blockade can be achieved via a disruption of multiple pro-aneurysmal pathways, with the primary target varying among animal models. A recent study showed that hydralazine inhibits PKC\(\beta\)-mediated ERK phosphorylation and prevents aortic dilation of MFS mice\(^{52}\). We included hydralazine as a control to assess losartan’s effect of lowering activity.
blood pressure in this study and did not detect any protective effects for this drug, indicating a modest role for PKCβ in mediating aneurysmal degeneration of Tgfbr1−/− aortas.

The mechanisms responsible for the activation of the local AT1R during aortic aneurysm development remain unclear. Our data show that the production of ACE was upregulated in Tgfbr1−/− aortas, particularly in the medial layer, indicating AngII-mediated AT1R activation. This mechanism appears to be unique to the Tgfbr1−/−, as the Myh11 mutation33 and fibrillin4 deletion34 are both associated with increased ACE production in the aortic wall. In addition to its ligand-dependent function, AT1R may act as a mechanosensor and can be activated in the heart35 and myogenic arteries36 in a ligand-independent fashion. Future studies are warranted to determine the importance of this mechanism in aortic aneurysm formation37.

Our study has a few limitations. First, mouse models were created via a postnatal homozygous gene deletion rather than a germline heterozygous gene mutation, as observed in TAAD and LDS patients1. Therefore, these models cannot be considered as models of TAAD or LDS. However, the Tgfbr1−/− model did recapitulate several key pathologies observed in TAAD and LDS patients, including aortic rupture, intimal-medial tears and dissections, and progressive aortic dilation26. It offers a unique platform for investigating the timing of TGFβ function over the course of aortic wall maturation, thus improving our understanding of TGFβ signaling disorders in the context of aortic aneurysm formation. In addition, although we linked Tgfbr1−/− to the activation of ERK and AngII/AT1R signaling pathways in Tgfbr1−/− aortas, it remains unclear whether this activation occurs via cell-autonomous mechanisms or is secondary to intermediate responses, such as mechanosensing at the tissue level27. Studies from other groups have shown that the local renin-angiotensin system (RAS) can be activated as a result of an upregulation of AT1R34 and ACE34 in aneurysmal aortas. While our study focused on ACE production, it is possible that the expression of AT1R is also increased in Tgfbr1−/− aortas. Finally, the ligand and the receptor complex for the aberrant TGFβRII signaling still remain to be determined. Further studies to quantify TGFβRII levels will help to understand the mechanism by which the loss of TGFβRII triggers the aberrant TGFβRII signaling in Tgfbr1−/− aortas. Our examination of a few selected TGFβ responsive mediators involved in canonical and non-canonical TGFβ signaling pathways was unable to identify any measurable readouts in SMCs lacking TGFβRI or TGFβRII after treatment with TGFβ31 (Supplementary Figure 2). However, we would not interpret this observation as evidence that excludes TGFβRII activity in Tgfbr1−/− aortas, as this model did recapitulate several in vitro SMC model to recapitulate the in vivo regulation of TGFβ signaling components. For example, SMCs with TGFβ2 mutations produced higher levels of TGFβ2 in the aortic tissue, but synthesized significantly less TGFβ2 in culture when compared with wild type controls4. Studies using high throughput approaches and the selected collection of the medial layer of the aortic tissue are warranted to screen for responsible mediators in order to shed light on proximal signaling components such as the ligand and the type I receptor members for the aberrant TGFβRII signaling.

In summary, we have provided evidence suggesting that the TGFβRII signaling pathway in SMCs is critical to the maintenance of structural integrity and homeostasis of aortas at an adult age. Our results suggest a novel mechanism for TGFβ signaling disorders. Namely, Tgfbr1−/− triggered an abnormal Tgfbr2 signal that is deleterious to the aortic wall. In addition, ERK and AngII/AT1R signaling pathways were also activated in the aortic wall following Tgfbr1−/−. Activation of these pathways caused the rapid collapse of aortic homeostasis, thereby promoting aortic aneurysm formation and dissection (Supplementary Figure 12).

Materials and Methods

Mice. The Tgfbr1+/− and Tgfbr2+/− strains were kindly provided by Dr. Karlsson (Lund University) and Dr. Moses (Vanderbilt University), respectively. The Myh11-CreER22 strain30 was obtained from Dr. Weiser-Evans (University of Colorado) with the permission of Dr. Offermanns (Semmelweis University). The Gt(Rosa)26sor reporter strain, colloquially referred to as “R26R,” was purchased from Jackson Laboratory. These floxed mouse lines were crossed with Myh11-CreER22 mice to establish colonies of R26R.Myh11-CreER22, Tgfbr1+/−.Myh11-CreER22, Tgfbr2+/−.Myh11-CreER22, and Tgfbr1−/−.Myh11-CreER22 mice. We used male mice at 9–13 weeks of age. Each strain was back-crossed to C57BL/6 females for at least five generations before breeding with other strains. Our breeding results show that the Myh11-CreER22 allele could be inherited by both male and female littermates. Because the transgene was initially inserted into the Y chromosome, chromosome-translocation of the Myh11-CreER22 allele must have occurred in our colony. Male mice carrying the Tgfbr1−/− but not the Myh11-CreER22 allele were saved from a colony that produced female carriers of the transgene and used as wild-type controls.

Animal treatment. Tamoxifen was administered via intraperitoneal injection (2.5 mg/day) for 5 consecutive days. The day of the first tamoxifen injection was considered to be d0. RDEA-119 (Selleckchem, S1089) was dissolved in 1% 2-hydroxypropyl-beta cyclodextrin (Sigma, 332607) in phosphate-buffered saline and administered twice daily by oral gavage at a dose of 25 mg/kg body weight11. Dissolvent was given to mice in the control group with the same volume and dosing schedule. Losartan was administered through a customized diet (Harlan) containing 1 mg active drug per gram of food, which delivered an estimated dose of 3 mg daily to a mouse consuming 3 grams of food per day. Hydralazine and propranolol were delivered to mice through drinking water at concentrations of 250 mg/l31 and 500 mg/l13, respectively. Blood pressure of these animals was measured with a non-invasive tail-cuff method (CODA, Kent Scientific, Torrington, CT USA)34. All treatments were started 1 week prior to the first dose of tamoxifen, except for RDEA-119 treatment, which began only 3 days after the first tamoxifen dose.

Gross examination. When a hemothorax and/or hematoma were noted during necropsy, the mouse was diagnosed with an aortic rupture. Otherwise, animals were evaluated for intimal/medial tears on d13 and advanced aortic wall remodeling on d28. These time points were chosen based on our pilot observation which...
showed complete penetrance of intimal/medial tears by d13 and grossly evident aortic pathologies by d28. For mice undergoing scheduled examination, 0.2 ml Evans Blue (5% in saline) was injected via tail vein 30 minutes prior to opening the chest. Immediately after opening the chest, 2.0 ml saline was injected into the left ventricle and drained from the right atrial appendage to remove blood and Evans Blue from the circulation. The use of Evans Blue to examine the changes of endothelial permeability in experimental aneurysms was invented several decades ago62. Other tracers such as horseradish peroxidase have also been used for the same purpose in previous studies63. We chose the use of Evans Blue staining to visualize the aortic tears because of its technical simplicity and high sensitivity to detect areas with a damaged endothelial barrier. More importantly, we have previously used scanning electron microscopy (SEM) to verify that areas with Evans Blue extravasation co-localize with one or more intimal/medial tears in our model24. Gross examination was then performed under an operating microscope by noting the presence and location of pathologies, including aneurysm formation, intramural hematoma, and contained rupture (Supplementary Figure 3). In addition to grossly evident pathologies, aortas with one or more spots of Evans Blue extravasation were also considered to be diseased. The incidence of aortic pathology for a given group was calculated as the percentage of mice with one or more of these aortic pathologies.

**Tissue collection.** Each aorta was divided into four segments: ascending aorta (ATA); descending aorta (DTA), from the takeoff of the left subclavian artery to the diaphragm; suprarenal aorta (SRA), distal to the diaphragm and proximal to the renal arteries; and infrarenal aorta (AA). Aortic tissues were either snap-frozen in liquid nitrogen for mRNA and protein assays or perfusion-fixed with 10% neutral buffered formalin for histology. The aortic root was not collected and the distal end of the perfusion-fixed ATA segments were labeled with a suture to ensure the subsequent sectioning to begin at the proximal end. All assays in this study were performed with ATA specimens, due to the complete phenotypic penetrance of the disease in this segment.

**Histology and morphometry.** Masson’s and Movat’s staining were performed as described previously24. ATA segments showing no grossly evident lesions but one or more spots of Evans Blue extravasation were opened longitudinally and evaluated en face to document early aortic lesions. X-gal staining (Mirus, MIR 2600) was performed on frozen sections (5.0-μm thickness), as per the manufacturer’s instructions. The medial area and length of internal and external elastic lamina were measured with ZEN lite (Zeiss) on cross sections and used for the approximation of medial thickness.

**Scoring system for quantifying the severity of aortic pathology.** The severity of ATA pathology was quantified on a 5-point scale for intimal/medial tears, intramural hematoma, and medial thinning/depletion. Intramural hematoma was deemed when red blood cells and/or thrombi were found between elastic laminae. Medial thinning was considered when the thickness of the media was thinner than that of the surrounding area, and the absence of the tunica media was noted as “medial depletion.” Equal weight was assigned to each category, and an example is provided in Supplementary Figure 5. Two sections approximately 500 μm apart were scored for each specimen, and the average of the total scores was calculated to represent the degree of aneurysmal degeneration of that sample.

(I) Intimal/medial tears

- Multiple sites with one or more tears penetrating full wall thickness: 5
- Multiple sites with one or more tears penetrating >50% but <100% of wall thickness: 4
- Single tear penetrating >50% but <100% of wall thickness or multiple tears penetrating <50% of wall thickness: 3
- Single tear penetrating <50% of wall thickness: 2
- Elastic fiber breaks without tears: 1
- No tears or elastic fiber breaks: 0

(I) Intramural hematoma

- Diffused hematoma: 5
- Multiple hematomas involving multiple layers: 4
- Single hematoma involving multiple layers: 3
- Single hematoma restricted to a single layer but spreading >50% of the wall: 2
- Single hematoma restricted to a single layer and spreading <50% of the wall: 1
- No hematoma: 0

(I) Medial loss (thinning or depletion)

- Multiple sites of medial depletion: 5
- Loss >50% of media in >50% of the wall: 4
- Loss >50% of media in <50% of the wall: 3
- Loss <50% of media in >50% of the wall: 2
- Loss <50% of media in <50% of the wall: 1
- No medial loss: 0

**SEM.** SEM was used to examine and characterize early aortic pathology, as previously described24.
Ultrasound imaging. The development and progression of aortic pathology was monitored via ultrasound examination. A high-resolution Vevo 2100 Imaging System with a MS550D (25–55 MHz) linear array transducer was utilized (VisualSonics) to acquire images. The diameters of ATA and SRA segments were measured at the maximum point of systole, as previously described41.

Immunohistochemistry assays. Assays were performed on formalin-fixed, paraffin-embedded sections. Citrate buffer- and heat-mediated antigen retrieval was carried out for pSMAD2 and pSMAD1/5/8 staining. Primary antibodies were purchased from Abcam (smooth muscle myosin heavy chain: ab53129), Santa Cruz (ACE: H-170), and Cell Signaling (pERK1/2: 4370; pSMAD2: 3101; and pSMAD1/5/8: 9511). Isotype-matched rabbit IgGs (Novus: NPB2-24893) served as a negative control. Antigen-specific signals were either detected with Alexa Fluor conjugated secondary antibodies (Life Technologies) or amplified with the ABC kit (Vector) and visualized via the DAB detection kit (Vector).

Western blot. ATA segments were homogenized in 50 mM Tris-HCl buffer supplemented with phenylmethylsulfonyl fluoride (0.1 μM), leupeptin (10.0 nM), and a phosphatase inhibitor cocktail (Invitrogen). The concentration of the protein solution was determined with the BCA protein assay kit (Thermo Scientific, 23225). Total protein (5 μg) was separated with SDS-PAGE gels, and membranes were blotted with primary antibodies purchased from R&D (Tgfbr2, MAB532), Santa Cruz (Tgfbr1, SC398), and Cell Signaling (SMAD2, 5339; SMAD3, 9523; pSMAD3, 9520; ERK1/2, 4696, and others as detailed above). Immunoblots were developed with Lumigen substrate (#TMA-6) and imaged with x-ray films. The intensity of the band of interest was quantified with ImageJ software, and data were normalized to β-actin or total protein of the assayed molecules.

Smooth muscle cell (SMC) culture and TGF-β1 treatment. Primary SMCs were explanted from R26R;Myh11-CreER<sup>T2</sup>, Tgfbr1<sup>−/−</sup>, and Tgfbr2<sup>−/−</sup> aortas on the following day of the last dose of tamoxifen and cultured in DMEM/12 plus 10% fetal bovine serum, as described previously64. The outgrowth at passages 2–3 was examined with flow cytometry analysis to determine the percentage of cells producing α-smo. With our established protocol, we have been consistently separate murine aortic SMCs with purity greater than 95%. Cells at passages of 9–10 were stocked for experiments. During experiments, cells were seeded at a density of 105 cells/cm<sup>2</sup> and allowed to recover overnight. After a 24-hour serum deprivation, cells were treated with TGF-β1 (1.0 ng/ml, R&D, 240-B-002) for 1 or 24 hours and lysed with RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (Invitrogen). Protein concentrations were measured with the BCA protein assay kit.

Gene expression. The probe and primer sets were purchased from Applied Biosystems, and quantitative RT-PCR assays were performed following a previously described protocol64.

Statistics. SigmaPlot version 13.0 was used for statistical analyses. Student t-tests, Fisher’s exact tests, Chi-square tests, one-way ANOVA, two-way repeated measurement ANOVA, and Kaplan Meier survival analyses were performed as appropriate, with P < 0.05 considered to be statistically significant. Datasets were evaluated using normality and equivalence variance testing. For those failing this evaluation, logarithmic and exponential transformations were employed to meet these requirements.

Study approval. The Institutional Animal Care and Use Committee (IACUC) at the University of Florida approved this study and animal experiments were carried out in accordance with the approved guidelines.

References

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Author Contributions
P.Y., B.M.S. and K.D. conducted all animal experiments, P.Y. and B.M.S. performed the ultrasound scanning and measurements, C.F. and P.Y. completed histology, IHC assays, western blot, and qRT-PCR, S.A.B. and S.P.O. participated in project design and data discussion, P.Y., C.F. and Z.J. performed data collection and analyses and prepared the figures, and Z.J. wrote the manuscript.

Additional Information
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