Constitutive Expression of a Dominant-Negative TGF-β Type II Receptor in the Posterior Left Atrium Leads to Beneficial Remodeling of Atrial Fibrillation Substrate

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Rationale: Fibrosis is an important structural contributor to formation of atrial fibrillation (AF) substrate in heart failure. Transforming growth factor-β (TGF-β) signaling is thought to be intricately involved in creation of atrial fibrosis.

Objective: We hypothesized that gene-based expression of dominant-negative type II TGF-β receptor (TGF-β-RII-DN) in the posterior left atrium in a canine heart failure model will sufficiently attenuate fibrosis-induced changes in atrial conduction and restitution to decrease AF. Because AF electrograms are thought to reflect AF substrate, we further hypothesized that TGF-β-RII-DN would lead to increased fractionation and decreased organization of AF electrograms.

Methods and Results: Twenty-one dogs underwent injection-electroporation in the posterior left atrium of plasmid expressing a dominant-negative TGF-β type II receptor (pUBc-TGFβ-DN-RII; n=9) or control vector (pUBc-LacZ; n=12), followed by 3 to 4 weeks of right ventricular tachypacing (240 bpm). Compared with controls, dogs treated with pUBc-TGFβ-DN-RII demonstrated an attenuated increase in conduction inhomogeneity, flattening of restitution slope and decreased duration of induced AF, with AF electrograms being more fractionated and less organized in pUBc-TGFβ-DN-RII versus pUBc-LacZ dogs. Tissue analysis revealed a significant decrease in replacement/interstitial fibrosis, p-SMAD2/3 and p-ERK1/2.

Conclusions: Targeted gene-based reduction of TGF-β signaling in the posterior left atrium—with resulting decrease in replacement fibrosis—led to beneficial remodeling of both conduction and restitution characteristics of the posterior left atrium, translating into a decrease in AF and increased complexity of AF electrograms. In addition to providing mechanistic insights, this data may have important diagnostic and therapeutic implications for AF. (Circ Res. 2016;119:69-82. DOI: 10.1161/CIRCRESAHA.115.307878.)

Key Words: atrial fibrillation • electroporation • fibrosis • gene therapy • heart failure

Atrial fibrillation (AF) is the most common heart rhythm disorder and is a major cause of stroke and heart failure (HF).1 Unfortunately, current therapies for AF, including drugs and ablation, are suboptimal in the setting of persistent AF and structural heart disease (eg, HF).2 Recent investigations have, therefore, attempted to better understand the pathophysiological mechanisms underlying AF, to develop more mechanism-guided approaches to treat AF.

The development of AF is heralded by electrophysiological and structural alterations, which serve to maintain, promote, and propagate AF. Of the structural changes that occur in AF, fibrosis is considered especially important in the generation of the substrate leading to AF, especially in the setting of HF, with fibrosis likely contributing to reentry by causing inhomogeneous conduction in the atrium.3–5 Although the underlying molecular mechanisms that lead to the development of fibrosis are complex, several recent studies suggest that the transforming growth factor-β1 (TGF-β1) signaling pathway may be an important contributor to the development of atrial fibrosis.6–9 TGF-β1 is an inflammatory, profibrotic cytokine that stimulates the production of extracellular matrix proteins such as collagen, fibronectin, and proteoglycans in a number of different organ systems, including the heart.10,11 TGF-β is also known to cause generation of reactive oxygen species, with NADPH oxidase 4-generated reactive oxygen species thought to at least partially mediate TGF-β effects in the atrium.12 Serum levels of TGF-β
have been shown to be increased in patients with AF undergoing defibrillation.10 Similarly, tissue expression of TGF-β is increased in the atria of patients with AF secondary to valvular heart disease.11 Moreover, transgenic overexpression of TGF-β in the mouse causes selective fibrosis of atrial, but not ventricular myocardium,12 resulting in in-homogenous atrial conduction and increased AF inducibility.11

We and others have recently reported the use of gene-based approaches to target key mechanisms underlying the development of AF substrate, with a resulting reduction in spontaneous and inducible AF.15–18 Because structural remodeling, specifically fibrosis, is a key to the development of AF substrate in the setting of structural heart disease, we now report a novel gene–based approach to decrease the development of atrial fibrosis in the setting of HF. We hypothesized that (1) targeted reduction of fibrosis in the atrium in a canine HF model of AF with (1) retrovirus/lentivirus expressing TGF-β-DN-RII or GFP and (2) plasmid expressing TGF-β-DN-RII or LacZ. Western blotting was performed for p-SMAD2/3 and e-RK1/2 to assess for signaling resulting from gene transfection. See Expanded Methods in the Online Data Supplement for full details.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AF</td>
<td>atrial fibrillation</td>
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<tr>
<td>APD</td>
<td>action potential duration</td>
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<td>CI</td>
<td>conduction inhomogeneity</td>
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<td>CV</td>
<td>conduction velocity</td>
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<td>ERP</td>
<td>effective refractory period</td>
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<td>HF</td>
<td>heart failure</td>
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<td>MAP</td>
<td>monophasic action potential</td>
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<td>LAA</td>
<td>left atrial appendage</td>
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<td>PLA</td>
<td>posterior left atrium</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
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<tr>
<td>TGF-β-DN-RII</td>
<td>dominant-negative TGF-β type II receptor</td>
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<td>VTP</td>
<td>ventricular tachypacing</td>
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Methods

Detailed Methods are provided in the Online Data Supplement. An overview of the Methods used is given below.

In Vitro Studies

Cloning of Plasmid Vectors

The TGF-β-DN-RII plasmid was cloned into a human polyubiquitin C (pUBc) backbone. The final product was pUBc/HA-V5-His/TGF-β-DN-RII. Chemically competent *Escherichia coli* were transformed with the plasmid. DNA was purified and resuspended in sterile saline for injection. The control plasmid used was pUBc-LacZ.

Studies in Fibroblasts and Myocytes

National Institutes of Health (NIH) 3T3 cells were transfected with pUBc-TGF(β)-DN-RII and pUBc-LacZ in the absence and presence of TGF-β. Western blotting was performed for p-SMAD2 and type I collagen to assess for gene transfection.

In addition, canine atrial fibroblasts as well as atrial myocytes were isolated and transfected in the absence and presence of TGF-β with (1) retrovirus/lentivirus expressing TGF-β-DN-RII or GFP and (2) plasmid expressing TGF-β-DN-RII or LacZ. Western blotting was performed for p-SMAD2/3 and e-RK1/2 to assess for signaling resulting from gene transfection. See Expanded Methods in the Online Data Supplement for full details.

In Vivo Studies in Intact Animals

Three groups of dogs were studied: (1) HF dogs (n=12) that underwent injection with LacZ expressing plasmid (pUBc-LacZ; ie, pUBc-LacZ group), (2) HF dogs injected with plasmid expressing TGF-β-DN-RII (pUBc-TGF(β)-DN-RII; n=9; ie, pUBc-TGF(β)-DN-RII group), and (3) sham controls (n=3; 2 of these animals received pUBc-LacZ and 1 received pUBc-TGF(β)-DN-RII). During an initial procedure, a left lateral thoracotomy was performed. The animals underwent epicardial electrophysiological mapping followed by gene injection and by epicardial implantation of a left ventricular pacemaker. After animals were allowed to recover for 3 to 5 days, the first 2 groups of animals (pUBc-LacZ group and pUBc-TGF(β)-DN-RII group) were subjected to ventricular tachypacing (VTP) at 240 bpm. The sham controls were not subjected to VTP. Clinical status assessment and pacing were verified daily. Three weeks after the initial procedure, animals underwent repeat open-chest electrophysiological mapping (terminal study).

Echocardiography

Comprehensive echocardiography was performed before the baseline study and then immediately before the terminal study. Echocardiographic data included left ventricular end–diastolic and systolic dimensions, ejection fraction, and left atrial volumes.

Open-Chest Electrophysiological Mapping

Effective Refractory Periods: Effective refractory periods (ERPs) were obtained using 2 rectangular, 21-electrode plaques epicardially positioned on the PLATRA and left atrial appendage (LAA). ERPs were obtained from 5 evenly distributed sites in both the PLA and the LAA.

AF Inducibility: AF was induced with burst pacing as previously described.19,20 AF was defined as episodes lasting >3 s. AF inducibility was defined as the percentage of burst pacing attempts that induced AF. Sustained AF was defined as AF >60 s.

Activation Mapping: High-density epicardial mapping was performed sequentially in the LAA and at 2 adjacent sites in the PLA (so as to encompass the entire PLA). At each site, 10-s recordings were made during sinus rhythm and pacing with cycle lengths of 400, 300, and 200 ms. See Expanded Methods in the Online Data Supplement for full details.

AF Electrogram Mapping: AF Electrograms were recorded to determine the following electrogram characteristics: (1) dominant
frequency, (2) organization index, (3) fractionation interval, and (4) Shannon entropy. Each of these electrogram characteristics has been described in detail in the Expanded Methods in the Online Data Supplement.

Monophasic Action Potential Recordings: Monophasic action potentials (MAPs) were recorded from the LAA and the PLA. A dynamic restitution pacing protocol was performed as previously described.22 Electrophysiological Data Analysis: Analysis of each of the above-mentioned electrophysiological parameters was performed as described in Expanded Methods (Online Data Supplement).

Gene Injection/Transfer

After the completion of the initial electrophysiological study, plasmid was injected subepicardially in the PLA to encompass the entire PLA, and electroporation was performed at each site of gene injection. See Expanded Methods in the Online Data Supplement for full details.

Tissue Analysis

After the completion of the terminal electrophysiological study, the heart was removed and the atria snap frozen as previously described by us.15,23 The explanted left atrium (PLA/pulmonary veins [PVs] and LAA) were subjected to the following analysis: real-time polymerase chain reaction, Western blot, immunofluorescence, Masson-Trichrome staining, immunohistochemical analysis for macrophages, and protein oxidation (carbonylation). See Expanded Methods in the Online Data Supplement for full details.

Statistical Methods

All data is reported as mean±SE. Comparison of conduction parameters (at different pacing cycle lengths) before and after gene injection in pUBc-TGFβ-DN-RII and pUBc-LacZ dogs was performed using 2-way repeated measures ANOVA; individual means were compared using the Holm–Sidák method. The change in conduction parameter(s) with chronic VTP was compared between the 2 gene groups using a linear model, ANOVA analysis with the study group, parameter(s) with chronic VTP was compared between the 2 gene groups. Comparison of ERPs before and after gene injection in pUBc-TGFβ-DN-RII and pUBc-LacZ dogs was performed using paired t tests. Comparisons of all other electrophysiology parameters (restitution parameters, AF duration, and AF electrograms) and tissue parameters (eg, fibrosis and gene expression) between pUBc-TGFβ-DN-RII and pUBc-LacZ dogs were performed by unpaired t tests (continuous variables) or by the χ² test (categorical variables). Comparisons of tissue characteristics between the PLA and LAA in the same animals were performed using paired t tests. In addition, for the occurrence of AF events (AF inducibility), we used hierarchical logistic regression where pacing attempt within each dog and within each study group was the unit of analysis.

Results

Efficacy of pUBc-TGFβ-DN-RII in Disrupting Canonical TGF-β Signaling in Fibroblasts

The schematic diagram in Figure 1 shows the canonical TGF-β signaling cascade and how the cytoplasmic (kinase)-deficient pUBc-TGFβ-DN-RII blocks this signaling cascade. Online Figure IA and B shows that both the pUBc-TGFβ-DN-RII and the pUBc-LacZ plasmids efficiently transfected NIH 3T3 fibroblasts. The fusion product of pUBc-TGFβ-DN-RII was 25 kDa and pUBc-LacZ was 100 kDa (corresponding to predicted sizes for each). In addition, TGF-β activated fibroblasts transfected with pUBc-TGFβ-DN-RII showed an additional band ≈45 kDa (Online Figure I)—in addition to a predicted 25-kDa band, a band in the 45-kDa range has been previously reported for TGF-β-DN-RII,24,25 likely representing a glycosylated form of the receptor. As previously reported with a similar TGF-β-DN-RII construct,25 pUBc-TGFβ-DN-RII significantly attenuated TGF-β-induced p-SMAD2 and collagen production in fibroblasts (Online Figure IC and D).

Similar attenuation of canonical TGF-β signaling was noted in isolated canine atrial fibroblasts. As shown in Online Figure IIA, pUBc-TGFβ-DN-RII attenuated TGF-β-induced p-SMAD2/3 production in canine atrial fibroblasts. In contrast, there was no discernible evidence of canonical TGF-β signaling in isolated canine atrial myocytes; as shown in Online Figure IIB, TGF-β stimulation did not significantly increase p-SMAD2/3 generation in the atrial myocytes.

Efficacy of pUBc-TGFβ-DN-RII in Disrupting Noncanonical TGF-β: pERK1/2 Signaling in Atrial Fibroblasts and Myocytes

A schematic diagram in Figure 1 shows the canonical TGF-β signaling cascade and how the cytoplasmic (kinase)-deficient pUBc-TGFβ-DN-RII blocks this signaling cascade. As previously reported with a similar TGF-β-DN-RII construct,25 pUBc-TGFβ-DN-RII significantly attenuated TGF-β-induced p-SMAD2 and collagen production in fibroblasts (Online Figure IC and D).

Echocardiography

Online Figure III shows that as previously demonstrated by us30 and by others in this HF model, there was a significant increase in left ventricular dimensions and decrease in left ventricular systolic function (ejection fraction) after 3 weeks of VTP. There was also an expected increase in left atrial size. There was no difference in ventricular dimensions, systolic function, or left atrial size between the pUBc-TGFβ-DN-RII and the pUBc-LacZ groups.

Effects of TGF-βdnRII on Conduction Inhomogeneity and Conduction Velocity

We determined the effects of pUBc-TGFβ-DN-RII gene transfer on atrial conduction using high-density epicardial plaques. Previous reports have repeatedly demonstrated that fibrosis contributes to the formation of AF substrate in HF primarily by increasing conduction inhomogeneity (CI, heterogeneity) in the atrium, as measured by the CI index.31 Changes in overall conduction velocity (CV) in the presence of fibrosis in this model have been shown to much less pronounced.5 Our primary electrophysiological end point of interest was therefore CI, with CV being a secondary end point. In PLA injected with pUBc-LacZ, the expected increase in CI with HF was significantly attenuated in pUBc-TGFβ-DN-RII–injected PLA (Figure 2A). Figure 2A, panel I provides a representative example of a lack of significant increase in CI seen in PLA injected with pUBc-TGFβ-DN-RII, compared with pUBc-LacZ–injected PLA, where there is a marked increase in CI after 3 weeks of VTP. Figure 2A, panels II shows in pUBc-LacZ PLA, the expected increase in CI that is known to occur in the HF atrium1 was seen at all cycle lengths. Figure 2A, panel III shows that the expected increase in CI with HF was significantly attenuated in pUBc-TGFβ-DN-RII–injected PLA (no significant increase in CI at any cycle length). Online Figure IV shows that the increase in CI with VTP was significantly lower in pUBc-TGFβ-DN-RII compared with pUBc-LacZ.
In the un.injected LAA, there was no significant increase in CI in either the pUBc-TGFβ-DN-RII or the pUBc-LacZ group (Online Figure V). Online Figure VIA shows that sham controls had no significant change in CI after gene injection.

CV was also assessed in both groups of animals. As shown in Figure 2B, HF led to a decrease in CV in pUBc-LacZ PLA (slower CV noted both during sinus rhythm as well as during pacing). However, in pUBc-TGFβ-DN-RII–injected PLA a significant decrease in CV was only noted at the fastest pacing cycle length, that is, 200 ms. In the LAA, there was no significant change in CV with HF (in either gene group, Online Figure VII). Online Figure VIA shows that sham controls had no significant change in CV after gene injection.

**Effects of TGF-β-DN-RII on Atrial Repolarization**

*Effective Refractory Periods*

ERPs lengthened significantly after 3 weeks of pacing when compared with baseline in both pUBc-TGFβ-DN-RII and pUBc-LacZ animals, with the magnitude of ERP increase being similar in both the groups (Figure 3A). Similar changes were noted in the LAA, with no difference between the pUBc-TGFβ-DN-RII and the pUBc-LacZ animals (Online Figure VIII).

*APD Restitution Characteristics*

An example from 1 animal from each group is shown in Figure 3B, panel I. The maximum slope of the of the APD restitution curve was steeper in pUBc-LacZ–injected PLA when compared with pUBc-TGFβ-DN-RII–injected PLA (3.10±0.78 versus 1.09±0.17; P<0.05; Figure 3B, panel II). There was no significant change in APD restitution slope in the LAA in either group (Online Figure IX).

There was no significant difference in alternans of MAPd90 and MAP amplitude between the pUBc-LacZ– and pUBc-TGFβ-DN-RII–injected groups in either the PLA (Figure 3C, panels II and II) or the LAA (Online Figure X).

As shown in Online Figure VIB, the PLA of sham control dogs had an APD restitution slope close to pUBc-TGFβ-DN-RII–injected PLA.

*Effects of TGF-β-DN-RII on AF Inducibility and AF Electrogram Characteristics*

**AF Inducibility**

The duration of induced AF (defined as AF >3 s) was markedly lower in pUBc-TGFβ-DN-RII–injected animals compared with pUBc-LacZ–injected animals (Figure 4A, panel I). AF inducibility (ie, percentage of burst pacing attempts that induced AF) was also significantly lower in pUBc-TGFβ-DN-RII–injected animals compared with pUBc-LacZ–injected animals (Figure 4A, panel II). The decrease in AF in pUBc-TGFβ-DN-RII continued to be significant (P=0.023) after hierarchical logistic regression was performed to determine the effect of variability between animals on AF inducibility. No AF was noted in the sham controls.

**AF Electrogram Characteristics**

In previously published data from our laboratory, we compared AF electrogram characteristics in dogs with HF and dogs without any structural heart disease.19 We discovered that AF in dogs with HF was significantly slower and more organized than AF in dogs without structural heart disease (in which AF was induced by vagal stimulation). AF electrograms in HF demonstrate the following changes in AF electrogram characteristics compared with vagal-induced AF in normal dogs: decrease in dominant frequency (a frequency domain measure of activation rate), increase in organization index (a frequency domain measure of temporal organization or regularity), increase in fractionation interval (the mean interval between deflections detected in the electrogram segment), and decrease in Shannon entropy (a statistical measure of complexity). Moreover, percent fibrosis in the HF atrium was negatively correlated with dominant frequency and positively correlated with fractionation interval.

Because pUBc-TGFβ-DN-RII–injected animals demonstrated a significant decrease in atrial fibrosis (see histological evaluation in next section), we compared AF electrogram characteristics between pUBc-LacZ– and pUBc-TGFβ-DN-RII–injected atria, to determine if there is an association between fibrosis and AF electrogram characteristics. Figure 4B shows an example of atrial electrograms in pUBc-LacZ– and pUBc-TGFβ-DN-RII–injected PLA. As shown in Figure 4C, AF electrograms from pUBc-TGFβ-DN-RII–injected PLA were significantly faster and less organized (higher dominant frequency, lower organization index, lower fractionation interval, and higher Shannon entropy) than AF electrograms recorded from pUBc-LacZ–injected PLA. In contrast, there was no difference in AF electrogram characteristics in the LAA between the pUBc-TGFβ-DN-RII and the pUBc-LacZ dogs (Online Figure XI). Taken together, these data indicate that atrial fibrosis contributes to both the frequency and organization characteristics of AF electrograms, with increased fibrosis leading to slowing and organization of AF electrograms.

**Fibrosis and Inflammation**

To assess differences in structural remodeling between groups, we systematically assessed atrial fibrosis in both the groups of animals. pUBc-TGFβ-DN-RII–injected PLAs were much less likely to harbor regions of dense, interstitial fibrosis when compared with pUBc-LacZ–injected PLAs (Figure 5). Figure 5A, panel I shows representative micrographs of dense, interstitial fibrosis in 2 pUBc-LacZ–injected PLAs. The sections clearly show clear evidence of myocyte dropout, indicating this is replacement fibrosis, because of the broad geographic areas of dense collagen, without embedded or intervening myocytes. In comparison, Figure 5A panel II shows a lack of myocyte dropout and a paucity of interstitial fibrosis in 2 pUBc-TGFβ-DN-RII–injected PLAs. The fibrosis seen...
Figure 2. Effect of plasmid expressing a dominant-negative TGF-β type II receptor (pUBc-TGFβ-DN-RII) on conduction inhomogeneity (CI) and conduction velocity (CV) in posterior left atrium (PLA). A, Panel I, Examples of isochronal maps showing the effect of gene injection on CI in the PLA. Top 2 panels show an example of significant increase in CI in a pUBc-LacZ–injected PLA. Bottom 2 panels show the lack of an increase in CI in a pUBc-TGFβ-DN-RII–injected PLA. Panel II, pUBc-LacZ–injected PLA shows significant increase in CI after 3 weeks of ventricular tachypacing. Panel III, In pUBc-TGFβ-DN-RII injected PLA, there is no significant increase in CI after 3 weeks of ventricular tachypacing. B, Panel I, pUBc-LacZ–injected PLA shows significant decrease in CV after 3 weeks of ventricular tachypacing. Panel II, In pUBc-TGFβ-DN-RII injected PLA, a significant decrease in CV is only noted with the fastest pacing cycle length, that is, 200 ms. TGF-β indicates transforming growth factor-β.
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here is seen to surround myocytes. No broad geographic areas of dense collagen are seen (and there is no myocyte drop out).

Figure 5A panel III shows that percent dense interstitial fibrosis was significantly lower in pUBc-TGF\(\beta\)DN-RII compared with pUBc-LacZ–injected PLAs. As expected, there was no significant difference in interstitial fibrosis in the LAA between the 2 groups of animals (Online Figure XII). As shown in Online Figure VIC, sham control dogs had no evidence of atrial fibrosis.

To assess whether the fibrosis noted in this model was inflammatory in nature, macrophage staining was performed in gene-injected PLA in both pUBc-TGF\(\beta\)DN-RII– and pUBc-LacZ–injected dogs. As shown in Figure 5B, there was no difference in macrophage staining between pUBc-TGF\(\beta\)DN-RII– and pUBc-LacZ–injected animals. Furthermore, we did not detect any evidence of apoptotic bodies in either group of animals.

The absence of macrophage infiltration, along with the nature of the fibrosis as described above (dense fibrosis with myocyte dropout) indicate that fibrosis seen in the HF model is a replacement in character, with the beneficial effects of pUBc-TGF\(\beta\)DN-RII being mediated by a reduction in this replacement fibrosis.

In Vivo Gene Expression

Polymerase Chain Reaction

Gene expression by polymerase chain reaction was noted in the PLA of both pUBc-TGF\(\beta\)DN-RII– and pUBc-LacZ–injected animals (plasmid copy number=2.17×10^8±7.3×10^7). Transcription product was either absent or was barely detectable in the adjoining, uninjected LAA.

Western Blotting

As shown in Figure 6A, pUBc-TGF\(\beta\)DN-RII–transfected PLA demonstrated His-tagged TGF-\(\beta\)-DN-RII protein close to...
its predicted size of ≈25 kDa. In addition, an additional band was noted at ≈45 kDa as previously described for this truncated receptor. Prolonged exposure was required—likely because of relatively low or inhomogeneous gene expression (see Discussion section in this article)—to discern bands at these expected sizes; as shown in Figure 6A, prolonged exposure revealed 2 specific, expected bands at ≈25 and 45 kDa (bands only seen in tissue injected with active gene and not in any control lane), respectively. Prolonged exposure unfortunately also led to the appearance of several nonspecific bands,
Figure 5. Effect of plasmid expressing a dominant-negative TGF-β type II receptor (pUBc-TGFβ-DN-RII) on fibrosis and inflammation. A, Panel I, Example of dense, interstitial fibrosis in posterior left atrium (PLA) from 2 dogs receiving pUBc-LacZ. Panel II, PLA from 2 dogs receiving pUBc-TGFβ-DN-RII shows very little interstitial fibrosis. Fibrosis is stained blue. III, % dense, interstitial fibrosis in pUBc-LacZ vs pUBc-TGFβ-DN-RII injected PLA. B, Macrophage staining in pUBc-LacZ vs pUBc-TGFβ-DN-RII injected PLA. Panel I, Examples of nuclear, macrophage, and merged staining in pUBc-LacZ and pUBc-TGFβ-DN-RII injected atria (top and bottom subpanels, respectively). Macrophage (MΦ) staining is green. Nuclei were detected with DAPI (4′,6-diamidino-2-phenylindole; blue). Panel II, Bar graph shows quantitation of percent macrophage positive nuclei in the experimental groups. TGF-β indicates transforming growth factor-β.
We also evaluated the expression and distribution of TGF\(\beta\)-DNRII using immunofluorescence (Figure 6B). Figure 6B panel I shows expression of His-tagged TGF\(\beta\)-DNRII protein, with no expression being seen in uninjected (control) PLA (Figure 6B, panel II). As Figure 6B, panel I demonstrates, although gene expression was noted in most randomly selected 20× panels, it was not entirely homogeneous in all of these panels.

**Effect of TGF\(\beta\)dnRII on Canonical and Noncanonical TGF-\(\beta\) Signaling and on Protein Oxidation**

Next, we assessed the signaling mechanisms downstream of TGF-\(\beta\) inhibition by a TGF\(\beta\)-DNRII approach. TGF-\(\beta\) knockdown by pUbC-TGF\(\beta\)-DNRII led to a decrease in p-SMAD2/3 in the injected PLA (compared with pUbC-LacZ–transfected PLA; Figure 7A). Sham controls, as expected, had significantly less p-SMAD2/3 compared with pUbC-LacZ–transfected PLA (Online Figure VID).

Noncanonical TGF-\(\beta\) signaling was also assessed by examining expression of phosphorylated TGF-\(\beta\) activated kinase 1, pERK1/2 and phospho-p38MAP kinase. pERK1/2 was markedly reduced in pUbC-TGF\(\beta\)-DNRII compared with pUbC-LacZ (Figure 7B). Phospho-p38MAP kinase trended to be lower in TGF\(\beta\)-DNRII–injected atria, whereas phosphorylated TGF-\(\beta\) activated kinase 1 was not changed (Online Figure XIV A and B).

Assessment of carbonylation revealed no attenuation of protein oxidation by pUbC-TGF\(\beta\)-DNRII compared to pUbC-LacZ (Online Figure XV).

**Discussion**

In this study, we demonstrate that gene-based targeting of profibrotic signaling in the atrium is not only feasible, but it helps limit adverse fibrotic remodeling in the HF atrium, with a resulting decrease in inducible AF. More specifically, we demonstrate that: (1) gene-based targeting of TGF-\(\beta\) signaling in the canine atrium is feasible, with a plasmid expressing dominant-negative type II TGF-\(\beta\) receptor (TGF-\(\beta\)-RII-DN) under the control of a long-acting UbC promoter; (2) inhibition of TGF-\(\beta\) signaling in the PLA by TGF-\(\beta\)-RII-DN resulted in a significant decrease in HF-induced atrial fibrosis, leading to a significant decrease in the conduction heterogeneity that characterizes fibrosis-induced AF substrate; (3) TGF-\(\beta\)-RII-DN expression results in flattening of the restitution slope in the PLA; and (4) the improvement in conduction and restitution characteristics translates into a significant decrease in the duration of inducible AF. In addition, our results demonstrate that a decrease in atrial fibrosis by TGF-\(\beta\) inhibition is accompanied by a significant change in the complexity and organizational characteristics of induced AF.

**TGF-\(\beta\) Signaling Is a Therapeutic Target to Reduce Fibrosis in the HF Atrium**

Of the morphological changes that occur in the fibrillating atrium—fibrosis, hypertrophy, necrotic and apoptotic cell loss, and dilation—fibrosis is considered especially important in the creation of AF substrate, both in the absence and presence of HF. Patients with AF display increased atrial fibrous tissue content, along with increased expression of collagens I and III. Attral extracellular matrix remodeling in AF patients with HF is characterized by increased collagen type I fraction, upregulation of MMP-2 (matrix metalloproteinase-2) protein, and downregulation of the tissue inhibitor of metalloproteinase. Expression of the active form of MMP-9 and monocyte chemoattractant protein-1, an inflammatory mediator, is increased in patients with AF. The regional distribution
the close relationship between TGF-β-regulating SMAD2/3 activation. Other studies also indicate a remodeling in chronic disease states. Cucoranu et al re-
sion and knockout models. TGF-β has been implicated in the development of diabetic nephropa-
ty, ulcerative colitis, hepatic, pulmonary and skin fibrosis. TGF-β seems to also be a major signaling pathway under-
ing induction of cardiac fibrosis, as evidenced by overexpres-
sion elicited marked atrial fibrosis, altered conduc-
tion characteristics, and increased AF inducibility. Recently, Lamirault et al, using microarray analysis, illustrated that gene expression of TGF-β is upregulated in patients with AF secondary to valvular heart disease in right atrial preparations.

In view of the above, TGF-β signaling in the atrium seems to be a viable therapeutic target in AF. Lee et al attempted to target TGF-β signaling in AF by using pirfenidone—a nonspecific blocker of TGF-β, tumor necrosis factor-α, and multiple other cytokines—and showed that this approach could prevent the development of fibrosis (and resulting AF) in a canine model of HF. We hypothesized that a more targeted strategy that selectively inhibits canonical TGF-β signaling in the atrium—and more specifically in the region of the PVs and PLA—would be sufficient to attenuate the formation of fibrotic substrate in AF.

Previous attempts at selectively targeting TGF-β signaling have used small molecules or monoclonal antibodies that are specifically targeted to the TGF-β–signaling pathway, for example, in the setting of metastatic cancer and renal fibrosis. There have also been attempts at targeting TGF-β signaling more selectively in a single organ by using a gene-based approach. A gene-based approach using a soluble, dominant-negative TGF-β type II receptor—as a competitive inhibitor of TGF-β—when used in the postmyocardial mouse ventricle, mitigated cardiac remodeling by affecting cardiac fibrosis, and infarct tissue dynamics (apoptosis inhibition and infarct contraction). In this study, we used a related dominant-negative gene-therapy approach to decrease TGF-β signaling in the HF atrium. To our knowledge, this is the first report of a targeted gene-therapy approach to decrease atrial fibrosis (with a view toward modifying AF substrate). This is also the first attempt, to our knowledge, of using such a gene-based approach to target cardiac fibrosis in a clinically relevant large animal model of AF. TGF-β-RII-DN not only decreased atrial fibrosis in this study but also seemed to do so by affecting both canonical and noncanonical TGF-β signaling. Indeed, TGF-β-RII-DN had a greater effect on noncanonical pERK1/2 than canonical p-SMAD2/3 in gene-transfected atria. Because TGF-β-RII-DN

Figure 7. Effect of plasmid expressing a dominant-negative TGF-β type II receptor (pUBc-TGF-β-DN-RII) on downstream signaling. A, Western blot showing p-SMAD2/3 in pUBc-TGF-β-DN-RII vs pUBc-LacZ–injected posterior left atrium (PLA). B, Western blot showing pERK1/2 in pUBc-LacZ–injected PLA vs PLA from sham control dogs. TGF-β indicates transforming growth factor-β.
was found to attenuate pERK1/2 not only in atrial fibroblasts but also in atrial myocytes, it is possible that at least some of the beneficial effects of TGF-β-DN-RII on atrial fibrosis may be mediated by paracrine cross-talk between myocytes and fibroblasts (as described previously by several investigators), with TGF-β having been shown to participate in bidirectional regulatory signaling between fibroblasts and cardiomyocytes.

Electrophysiological Mechanisms Underlying Attenuation of AF Substrate by TGF-β-RII-DN Expression in the PLA

In addition to causing structural remodeling (fibrosis) in the atrium, HF is also known to promote alterations in atrial ionic currents, for example, decrease in atrial $I_{\text{Na}}$, $I_{\text{Cal}}$, and $I_{\text{Ks}}$ and increase in $I_{\text{NCX}}$ (i.e., electrophysiological remodeling). The contribution of TGF-β signaling to formation of atrial fibrosis has been discussed earlier. In addition, it seems that TGF-β signaling also be contributing to electrophysiological remodeling in the atrium. TGF-β1 released by myofibroblasts has been shown to differentially regulate transcription and function of ion channels involved in cardiac activation and repolarization, for example, $I_{\text{Na}}$, $I_{\text{CaL}}$, and $I_{\text{Ks}}$, in both atrial and ventricular myocytes. Although individual ion channels were not examined in this study, we did examine for evidence of electric remodeling by assessing the restitution characteristics of the left atrium. Indeed, inhibition of TGF-β signaling in the atrium, in addition to attenuating fibrosis, also flattened the restitution slope of the PLA. A steepening of the APD restitution slope with atrial APD alternans has been shown to correlate with increased vulnerability to AF. The slope of the restitution curve thought to affect propensity for wavebreak and substrate for reentry, including in the PVs. A decrease in restitution slope in the PVs and PLA may, therefore, attenuate arrhythmogenic substrate in the atrium. Because TGF-β signaling can affect $I_{\text{Na}}$, it is possible some of the salutary effects of TGF-β-RII-DN on CI are also mediated at least partially by the ion-channel effects of TGF-β signaling (in addition to the improvement in conduction resulting from a decrease in fibrosis).

Limitations of Current Therapies for AF and Need for New Mechanism-Guided Therapies for AF

Because a majority of AF triggers and drivers originate in the PVs and PLA, catheter ablation to electrically isolate the PVs/PLA has recently emerged as a viable therapy for AF. Nonetheless, high-ablation success rates have only been achieved in selected patients. Even with extensive, linear ablation in the atrium—which can lead to a decrease in atrial contractility and an increase in the incidence of complications—ablation success rates in the setting of structural heart disease do not seem to exceed 50% to 60%. The limited efficacy of current treatment options has led to a major research effort to better understand the mechanisms underlying this arrhythmia. A better understanding of the molecular mechanisms underlying electrophysiological remodeling in the atrium has also led to recent efforts to selectively target some of these mechanisms by using a biological, that is, gene-based approach. We have previously shown that vagal-induced AF in normal dogs can be successfully prevented by atrial injection of plasmid expressing C-terminal Go- and Go+. Amit et al showed that refractory period shortening in AF can be prevented in a rapid atrial pacing model of AF by overexpression of dominant-negative mutants of the $I_{\text{Na}}$ channel, with a resulting decrease in fibrosis. As conduction changes also contribute to the creation of AF substrate, Igarashi et al showed that gene transfer of connexin 40 and 43 led to improved conduction and reduced AF relative to controls in the same rapid atrial pacing model of AF. More recently, Trappe et al have shown that knockdown of caspase 3 by atrial Ad-siRNA-Cas3 gene transfer suppresses or delays the onset of persistent AF by reduction in apoptosis and prevention of intra-atrial conduction delay (in the above-mentioned porcine model of AF).

Our approach differs from the ones mentioned above in that it is the first, to our knowledge, that has specifically targeted fibrosis as AF substrate. Fibrosis is not only a major contributor to AF substrate in the setting of structural heart disease but is also thought to contribute to the maintenance of AF in patients with long-standing AF in the absence of any overt valvular heart disease or HF. Even more importantly, it is thought to be a major determinant of the failure of ablative approaches to AF, with increasing fibrosis correlating directly with the decreasing success of AF ablation. As a result, any therapeutic approach that can prevent or attenuate the progression of fibrotic remodeling would likely have benefit in reducing the ability of the atria to sustain AF. Our approach in targeting TGF-β signaling in the atrium—performed in a model of AF where fibrosis is thought to be the major substrate underlying AF—successfully attenuated the formation of fibrosis in the HF atrium, with resulting attenuation of the adverse electrophysiological remodeling—specifically the increase in CI—that is characteristic of the HF atrium and a concomitant decrease in the duration of induced AF. A gene-based approach that targets a key molecular signaling pathway underlying fibrosis may have significant therapeutic potential in patients with AF. Nonetheless, to conclusively demonstrate its therapeutic potential, this targeted gene-based approach would have to be systematically compared with systemically administered small-molecule inhibitors of TGF-β signaling. Furthermore, the benefits of such an approach would be weighed against the possible risks associated with the invasive delivery approaches required for targeted gene delivery in the atrium.

Effect of Fibrosis on AF Electrogram Characteristics: Insights Gained From Inhibition of TGF-β Signaling in the PLA

Several investigators have suggested that the characteristics of electric signals (electrograms) recorded from the atria reflect the pathophysiological substrate underlying AF. The need for a better understanding of the mechanisms underlying AF electrogram formation is heightened by the emerging data that mapping AF may improve ablation outcomes. The ability to use such electrograms as a marker of key AF mechanisms may translate into an increase in the specificity and success of current ablation procedures, as well as provide suitable targets for novel biological therapies.

To determine if fibrosis contributes to AF electrogram characteristics, we compared in this study AF electrogram characteristics in dogs that received active TGF-β-DN RII (with a resulting decrease in fibrosis) with dogs that received empty
vector. We discovered that TGF-β-DN RII dogs (that had mild–moderate interstitial fibrosis compared with the more severe fibrosis seen in dogs receiving empty vector) had significantly more fractionated and disorganized AF electrograms than dogs that received empty vector. These results are consistent with our recent findings, where we discovered that in the setting of HF, AF electrograms over regions of dense fibrosis were more organized and less fractionated compared with AF electrograms overlying lesser degrees of fibrosis. Although the finding that AF electrograms are slower and more organized in HF may seem contradictory to some clinical studies that indicate that complex-fractional atrial electrograms % are higher in patients with persistent than in patients with paroxysmal AF, the precise structural substrate underlying AF electrograms has not characterized in these previous studies. Indeed, the observation that f-wave frequency of AF on surface ECGs decreases with increasing age further supports the notion that increasing atrial fibrosis—a phenomenon well described in the aging heart—may also be contributing to slowing and organization of AF in the HF atrium. It is therefore entirely possible that dense replacement fibrosis as seen in advanced HF may lead to a coalescing and organization of activation wavefronts in the atrium, with an increased organization of AF electrograms. In comparison, a lesser degree of fibrosis—as noted in the current atrium, with an increased organization of AF electrograms. In to a coalescing and organization of activation wavefronts in the of AF in the HF atrium. It is therefore entirely possible that of this targeted gene-based approach would ideally have to be set up microscopic conduction barriers (and resulting anisotropy) that may be conducive to the creation of disorganized electrograms. Regardless of the precise mechanisms underlying the differences in AF electrogram characteristics in the presence of dense versus milder degrees of fibrosis, the findings of this study may have translational significance, in that they may allow for potential use of AF electrograms to detect fibrosis burden in the atrium, as well as to potentially hone in on regions of dense fibrosis in the atrium. Future studies are therefore necessary to understand the precise contribution of fibrosis to AF inducibility, duration, and organization.

**Study Limitations**

The study was performed in a model that is known to have easily inducible AF— in large part because of atrial fibrosis—but does not harbor spontaneous AF. It is therefore not known if TGF-β inhibition would have inhibited spontaneous AF. Further testing of this targeted gene-based approach would ideally have to be performed in the setting of spontaneous AF. Future studies will also need to systematically examine how TGF-β contributes to not just structural but also electrophysiological remodeling in AF.

In our study, gene expression in the region of injection was not entirely homogenous. Although in-homogenous gene expression may theoretically enhance heterogeneity of conduction and repolarization and therefore increase potential for proarrhythmia, we did not find this to be the case in our study. Future studies need to more systematically examine whether homogenous gene expression is indeed required for a beneficial therapeutic effect.

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**Disclosures**

Dr Arora has an ownership interest in Rhythm Therapeutics, Inc.

**References**


Novelty and Significance

What Is Known?

- Clinical and experimental evidence has shown that structural remodeling, specifically atrial fibrosis, creates a substrate that serves to maintain, promote, and propagate atrial fibrillation (AF).
- Transforming growth factor-β1 (TGF-β1) signaling is central to the genesis of atrial fibrosis.
- Current pharmacological and ablative approaches to AF have limited efficacy and the potential to cause adverse effects; consequently, there has been an emphasis to develop more mechanism-based therapies for AF that target the specific molecular mechanisms underpinning the genesis of AF.

What New Information Does This Article Contribute?

- TGF-β1 inhibition in the failing left atrium can be successfully achieved with a dominant-negative transgene.
- Targeted downregulation of TGF-β1 in the posterior left atrium with a nonviral gene-based approach results in decreased atrial fibrosis and inducible AF.
- Our results highlight the contribution of TGF-β1 signaling to the genesis of AF and demonstrate the feasibility of a targeted, nonviral gene-based approach for prevention of structural substrate for AF.

AF is the most common sustained cardiac arrhythmia and accounts for significant morbidity and mortality. Present therapeutic approaches to AF have major limitations, including limited efficacy and potentially serious adverse effects. Clinical and experimental studies have demonstrated that structural remodeling (fibrosis) is an important feature of AF; the resulting alteration in atrial tissue composition and function serves to maintain, promote, and propagate AF. Although the precise mechanisms that lead to formation of atrial fibrosis are complex; recent work suggests that the TGF-β1 signaling pathway may be an important contributor to the development of fibrosis. TGF-β1 is an inflammatory, profibrotic cytokine that is a potent stimulator of collagen-producing cardiac fibroblasts. Using a novel nonviral gene-based approach, we demonstrate that targeted inhibition of TGF-β1 signaling can be successfully achieved in the intact atrium, with a resulting decrease in fibrosis. This decrease in atrial fibrosis attenuates the conduction heterogeneity that is known to occur in heart failure, thereby leading to a decrease in inducible AF. In addition to providing mechanistic insights into the pathophysiology of AF, this study demonstrates that gene-based approaches targeting key AF mechanisms (eg, profibrotic signaling pathways) may have significant therapeutic potential in AF.
Constitutive Expression of a Dominant-Negative TGF-β Type II Receptor in the Posterior Left Atrium Leads to Beneficial Remodeling of Atrial Fibrillation Substrate

Aaron Kunamalla, Jason Ng, Vamsi Parini, Shin Yoo, Kate A. McGee, Todd T. Tomson, David Gordon, Edward B. Thorp, Jon Lomasney, Qiang Zhang, Sanjiv Shah, Suzanne Browne, Bradley P. Knight, Rod Passman, Jeffrey J. Goldberger, Gary Aistrup and Rishi Arora

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Animal Studies

Purpose-bred hound dogs (weight range: 25-35 kg; age range: 1-3 years) used in this study were maintained in accordance to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) as approved by the Animal Care and Use Committee of Northwestern University. Before undergoing the procedures listed below, all animals were sedated with diazepam 0.22 mg/kg IM and were induced with propofol (3-7 mg/kg IV). After animals were unresponsive, they were intubated and positive-pressure ventilated, and anesthesia was maintained with isoflurane 0.5-1.5 %. The surgical field was scrubbed with chlorhexidine and isopropyl alcohol, sprayed with betadine solution, and draped with sterile towels and sheets. The chest was then opened via a left lateral thoracotomy.

Twenty-one hounds were randomized into 3 groups: 1) HF dogs (n=12) that underwent injection with LacZ expressing plasmid (pUBc-LacZ) (i.e. ‘pUBc-LacZ’ group) 2) HF dogs injected with plasmid expressing TGFβ-DNRII (pUBc-TGFβ-DN-RII) (n=9) (i.e. ‘pUBc-TGFβ-DN-RII group’) and 3) sham controls (n=3; two of these animals received pUBc-LacZ and one received pUBc-TGFβ-DN-RII). During an initial procedure, a left lateral thoracotomy was performed and the pericardium was incised; animals underwent invasive electrophysiology study (EPS), gene injection, echocardiogram and epicardial implantation of a left ventricular pacemaker. After animals were allowed to recover for 3-5 days, ventricular tachypacing was initiated at 240 bpm. Clinical status assessment and pacing was verified daily. Three weeks after the initial procedure, animals underwent terminal echocardiogram, EPS and cardiac extraction for histology and molecular studies.

Cloning of plasmid vectors

pUB6/V5-His/LacZ was purchased from Invitrogen. The dnTGFβ receptor II with HA tag was purchased as a CMV plasmid from Addgene. The CMV plasmid was cloned into the UB6 backbone. The final product was pUB6/Ha-V5-His/dnTGFβ receptor II. Chemically competent E. coli that is a derivative of DH5alpha (New England Biolabs, #C2987H), were transformed with the plasmid. DNA was purified with the EndoFree Plasmid Giga Kit (Qiagen, #12391) and resuspended in sterile saline for injection.

In-vitro studies in NIH3T3 fibroblasts

NIH 3T3 cells were transfected with the plasmid expressing TGFbetaDNRII and LacZ. Cells were plated at 250,000 cells/well into each well of a six well plate: 2 untransfected wells, 2 UBC- TGFbetaDNRII wells, and 2 UBC-LacZ wells. Cells were then transfected using Lipofectamine 2000 (Life Technologies, #11668) and left to express for 48 hours. After 48hrs, 10ng/ml of TGFβ1 was added to stimulate collagen production in half of the wells (1 untransfected, 1 TGFbetaDNRII, 1 LacZ). 24 hours later (72hrs after initial transfection), total protein was lysed with M-per (Mammalian Protein Extraction Reagent, Thermo Scientific, #78503) containing protease and phosphatase inhibitors for western blot analysis (same as below).

NIH 3T3 cells were also transfected (using the methods described above) with the TGFbetaDNRII plasmid at increasing concentrations (0 µg, 1.2 µg, 1.8 µg, and 2.5 µg). After 48 hours, total protein was lysed and used for western blot analysis.

Primary Antibodies: (1) Rb pAb to V5 tag (Abcam, Cambridge, MA, #ab15828); (2) HisProbe-HRP, 1:5000 (Thermo Scientific, Rockford, IL, # 15165); (3) anti-Type I Collagen (Southern Biotech, Birmingham, AL. Cat# 1310-01)

Secondary antibodies: (1) Anti-rabbit IgG (Cell Signaling, Danvers, MA, #70745S); (2) Anti-mouse IgG (Cell Signaling, Danvers, MA, #7076S)

All band densities were quantified using the Image J software and normalized to GAPDH or Cadherin.
**In-vitro studies in canine atrial fibroblasts and myocytes**

Coronary perfusion of explanted left atrium:
While the dog was still deeply anesthetized, the hearts was quickly removed and immersed in cold cardioplegia solution containing (mM) NaCl 128, KCl 15, HEPES 10, MgSO$_4$ 1.2, NaH$_2$PO$_4$ 0.6, CaCl$_2$ 1, glucose 10, and heparin (0.001 U/mL); pH 7.4. All solutions were equilibrated with 100% O$_2$. The aorta was cannulated, and the heart was perfused with cold cardioplegia solution until effluent was clear of blood and heart was cold (5-10 min). The ventricles were cut away, the left circumflex coronary artery was cannulated, and the LA was dissected free. The left atrium was slowly perfused with cold cardioplegia while leaks from arterial branches were ligated with suture to assure adequate perfusion. The LA was then perfused with Tyrode’s at 37°C for 5 min to remove cardioplegia solution and assess for viability—i.e., the reestablishment of beating. If viable, the LA was then perfused at ~12 mL/min with Ca$^{2+}$-free Tyrode’s solution for ~20 min, followed by ~40 min of perfusion with the same solution containing Liberase (Liberase TH Research Grade, Roche 05401151001) and 1% BSA; all at 37°C. Thereafter, the LA tissue was transferred to dish and cut into small pieces (~0.5 cm$^2$). These tissue pieces were then transferred to conical plastic tubes, and fresh enzyme solution (37°C) was added. The tissue pieces were triturated in the fresh enzyme solution for 5-15 min for 15 min. The triturated tissue suspension was then filtered through nylon mesh (800 μm).

Fibroblast isolation:
The enzyme solution supernatant was spun at 2000g for 5 min to isolate the fibroblasts. To gradually increase the calcium concentration the supernatant was discarded and exchanged with Tyrode’s containing 600uM calcium. The cells were then filtered through a 400 micron mesh filter and left to equilibrate for 5 min. The process was repeated with another spin at 2000g for 5 min and the fibroblast pellet was resuspended in 1200uM Ca and left for 5 min.

Myocyte isolation:
The filtered cell suspension was briefly centrifuged at ~500g, then enzyme solution poured off, and cell suspension resuspended in Tyrode’s solution containing 200μM Ca$^{2+}$ and 0.1% BSA. This resuspension was then and filtered through a nylon mesh (210 μM) was then and filtered through a 400 micron mesh filter and left to equilibrate for 5 min. The process was repeated with another spin at 2000g for 5 min and the fibroblast pellet was resuspended in 1200uM Ca and left for 5 min.

Generation of retrovirus or lentivirus expressing TGFβ-DN-RII
Panotropic GP2-293 retroviral or lentiviral packaging cells (Clontech, San Diego, CA) were seeded at a density of 2.5 × 10$^6$ cells in collagen-I-coated T-25 flasks (BIOCOAT; BD Biosciences, Mountain View, CA) 24 h before plasmid transfection in antibiotic-free DMEM-10, such that the cells were ~70–90% confluent at the time of transfection, at which point the cells were rinsed with PBS to remove residual serum. A mixture of 2 μg of retroviral plasmid (dominant-negative TGF-β type II receptor (TGFβ-DN-RII) or control green fluorescent protein (GFP)) and 2 μg of VSV-G envelope plasmid were cotransfected in DMEM with 10% FBS using LipofectAMINE-2000 (Invitrogen, Gaithersburg, MD) according to the manufacturer’s protocols with the following modifications. Cells were transfected for 24 h. After 24 h of total transfection time, the supernatant was aspirated, the cells were rinsed gently in PBS, and 3 ml of fresh DMEM with 10% FBS was added to each flask. After 24 h, virus-containing supernatant was collected and used to infect target cells.

Fibroblast culture and transfection:
After a final spin, the pellet was transported to a cell culture hood and resuspended in 20ml of DMEM (containing Pen/Strep and 10% FBS) and the fibroblasts were divided onto uncoated 100cm$^2$ dishes. The
fibroblasts were left to attach for 1hr and then the media was replaced to remove dead cells and unattached myocytes. After about 4 days the fibroblasts were confluent and split into a 6 well plate at 120,000 cells/well. The next day retrovirus or plasmid containing our active construct (TGFβ-DN-RII) and our control construct (GFP in retrovirus, LacZ in plasmid) were added. Two wells were transfected with the TGFβ-DN-RII retrovirus, and two wells were transfected with the GFP retrovirus or LacZ plasmid. They were left for 72hrs to express. TGFβ1 treatment was added at 10ng/ml for the last 24hrs to one well in each group.

Myocyte culture and transfection:
After isolating the cardiomyocytes and raising the Ca++ to 1.8mM in 1X Tyrodes Solution, the supernatant was removed and replaced with M199 media (supplemented with ITS which consists of Insulin, Transferrin, and Selenite, Pen/Strep, 10%FBS). Cells were plated at low density on a 6-well culture plate coated with a 1:3 dilution of ECM gel (Sigma, E1270) and maintained at 37°C. After two hours, dead and unattached myocytes were removed and fresh media was added. The next day a lentivirus containing our active construct and our control construct were added to the 6-well plate. Two wells were transfected with the TGFβ-DN-RII lentivirus, and two wells were transfected with the GFP expressing lentivirus and they were left for 72hrs to express. TGFβ1 treatment was added at 10ng/ml for the last 24hrs to one well of each group.

Echocardiography
To measure cardiac function, comprehensive echocardiography was performed pre-operatively (in animals sedated with Innovar) and after three weeks of left ventricular tachypacing (prior to sacrifice). All animals were imaged by a single, experienced operator using a commercially available ultrasound system with harmonic imaging (Vivid 7, GE Healthcare, Waukesha, WI). Short-axis, long-axis, and apical views of the heart were obtained. Echocardiographic data collected for analysis included: left ventricular end-diastolic dimension, left ventricular end-systolic dimension, biplane ejection fraction (performed using the Simpson’s method of discs) and left atrial end-systolic and end-diastolic volumes (performed using the area-length method). All measurements were made offline using an EchoPAC workstation (GE Healthcare, Waukesha, WI).

Open chest electrophysiological mapping
**Effective Refractory Periods (ERPs).** ERPs were obtained using two rectangular plaques epicardially positioned on the posterior left atria (PLA) and left atrial appendage (LAA). Each plaque had 21 electrodes each (7x3 electrodes, inter-electrode distance = 5 mm). A Bloom stimulator interfaced with a GE Prucka Cardiolab system (GE Healthcare, Waukesha, WI) were used to perform programmed stimulation. ERPs were measured by delivering an 8 pulse drive train at 400 ms coupled to a premature stimulus. The coupling interval of the premature stimulus was decreased in 10 ms increments, and the ERP was defined as the longest coupling interval that fails to conduct. ERPs were obtained from five distributed sites in the both the PLA and LAA.

**Atrial fibrillation (AF) inducibility.** AF was induced with burst pacing at maximum output using cycle lengths of 180 ms to 100 ms with 10 ms decrements for 10 seconds for each cycle length. An AF episode of was defined as an episode lasting more than 3 seconds. AF inducibility was defined as the percentage of burst pacing attempts that induced AF.

**Activation Mapping.** High density activation mapping was performed using a UNEMAP mapping system (Univ. of Auckland, Auckland, New Zealand). A triangular plaque containing 130 electrodes (inter-electrode distance of 2.5 mm) was used to record 117 bipolar EGMs at a 1 kHz sampling rate. Mapping was performed sequentially in the LAA and in two adjacent sites in the PLA. At each site, 10-second recordings were made during sinus rhythm and pacing with cycle lengths of 400 ms, 300 ms, and 200 ms. Pacing was performed in LAA when recording from the PLA and vice versa.

**AF Electrogram Mapping.** The UNEMAP systems was also used to record electograms during AF. AF was induced by burst pacing if necessary. At least four successive recording were made in the LAA and
two adjacent sites in the PLA. AF EGMs were recorded in order to determine the following EGM characteristics: 1) Dominant Frequency (DF), 2) Organization Index (OI), 3) Fractionation Interval (FI) and 4) Shannon’s Entropy (ShEn). Briefly, DF is a frequency domain measure of activation rate. OI is a frequency domain measure of temporal organization or regularity. FI is the mean interval between deflections detected in the EGM segment. ShEn is a statistical measure of complexity.

**Monophasic Action Potential Recordings.** A hand-held monophasic action potential (MAP) probe (Model 200, EP Technologies, Sunnyvale, CA) was used to sequentially record epicardial MAPs from the LAA and the PLA. During LAA recording pacing was performed in the PLA and vice versa. A dynamic restitution pacing protocol was performed. The pacing protocol consisted of 50 paced beats at each of the following cycle lengths in milliseconds: 400, 350, 300, 280, 260, 250, 240 with continued 10 ms decrementing until 2-to-1 atrial capture or AF was achieved. The MAP signals were recorded using the Prucka Cardiolab System (GE, Waukesha, WI) at a sampling rate of 977 Hz and stored for offline analysis of restitution and alternans.

**Data Analysis**

**Conduction Inhomogeneity.** MATLAB (Mathworks, Natick, MA) was used for all offline signal analysis in this study. Conduction inhomogeneity analysis was performed using the high-density UNEMAP recordings obtained during sinus rhythm and pacing. The bipolar electrograms were high-pass filtered at 30Hz, rectified, and then low-pass filtered at 20 Hz. The times of the filtering peaks were considered the activation time for that activation. Conduction inhomogeneity was calculated as described by Lammers et al. Total activation time measuring the amount of time to activate the entire area of the plaque was also measured.

**AF Electrogram Analysis.** The high-density UNEMAP recordings obtained during AF were analyzed for dominant frequency (DF), organization index (OI), fractionation intervals (FIs), and Shannon entropy (ShEn) as described by Koduri et al. Each of these measures has been explained in detail below. DF is a frequency domain measure of activation rate. OI is a frequency domain measure of temporal organization or regularity. FI is the mean interval between deflections detected in the EGM segment. ShEn is a statistical measure of complexity.

**Dominant Frequency (DF).** DF is a frequency domain measure of activation rate. Following bandpass filtering with cutoff frequencies of 40 and 250 Hz and rectification, the power spectrum of the EGM segment was computed using the fast Fourier transform. The frequency with the highest power in the power spectrum was considered the DF.

**Organization Index (OI).** OI is a frequency domain measure of temporal organization or regularity. It has been shown that AF episodes with recordings with high OI are more easily terminated with burst pacing and defibrillation. OI was calculated as the area under 1-Hz windows of the DF peak and the next three harmonic peaks divided by the total area of the spectrum from 3 Hz up to the fifth harmonic peak.

**Fractionation Interval (FI).** FI is the mean interval between deflections detected in the EGM segment. Deflections were detected if they meet the following conditions: 1) the peak-to-peak amplitude was greater than a user determined noise level, 2) the positive peak was within 10 ms of the negative peak, and 3) the deflection was not within 50 ms of another deflection. The noise level was determined by selecting the amplitude level that would avoid detection of noise-related deflections in the iso-electric portions of the signal. FIs ≤120 ms have been considered CFAE. The 120 ms criterion was used to calculate the % CFAE in each region for both low density and high density mapping. FI is dependent on both the AF cycle length and the fractionation of the EGM.

**Shannon’s Entropy (ShEn).** ShEn is a statistical measure of complexity. The 4000 or 3908 (depending on the 1kHz or 977 Hz sample rate) amplitude values of each EGM segment were binned into one of 29 bins with width of 0.125 standard deviations. ShEn was then calculated as:

\[
ShEn = -\sum_{i=1}^{29} p_i \log_{10} p_i
\]

where \( p_i \) is the probability of occurrence of amplitude values in the ith bin.
In this equation, \( p_i \) is the probability of an amplitude value occurring in bin \( i \).

The above measures were assessed for each pixel/electrode on each plaque. There was a small number of electrodes (<10%) where signal (EGM) quality was inadequate (e.g. due to noise, poor contact) for assessment of the above measures. These pixels are shown as grey in figures 6 and 7.

**MAP Restitution.** The MAPs for each beat were characterized by the following measures: (1) time of activation, the maximum positive slope of the phase 0 depolarization, (2) peak amplitude, and (3) duration of the MAP at 90% repolarization (MAPd90). To obtain dynamic restitution curves for each dog and site (PLA and LAA), the MAPd90 of the last beat of each pacing cycle length was plotted against the diastolic interval of the preceding beat. The diastolic interval was calculated as the time between the 90% repolarization time of the second-to-last beat to the activation time of the last beat. Fourth degree polynomial fitting was used to capture the potentially biphasic shape of the cardiac action potential restitution curve. From the polynomial function, the maximum slope within the range of its measured DIs was determined.

**MAP Alternans.** After excluding the first and last beats of each 50 beat sequence of MAPs, the remaining 48 beats were analyzed to detect the presence of alternans (period doubling) in two MAP features: MAP amplitude and MAPd90. To obtain the magnitude spectra (the square root of the power spectra) for these measures the following steps were performed: (1) the 48 beat sequence of each measure was linearly detrended; (2) a Hanning window was applied to the sequence; and (3) a 100-point fast Fourier transform. The alternans magnitude was defined as the magnitude of the spectra at the 0.5 cycles per beat. The alternans magnitude for MAP amplitude was normalized to the mean peak MAP amplitude for the 48 beats.

**Gene injection/transfer**

After the completion of the initial EPS, 10 mg of plasmid DNA (diluted in 5 ml sterile saline) was injected sub-epicardially in the PLA with a 27-gauge needle; 5-6 injections (approximately 1 ml each) were used to encompass the entire PLA. Immediately after the injection, 8 electrical pulses (amplitude, 200 V; duration, 10 ms; intervals, 1 sec) were delivered to the myocardium through electrodes spaced 1 cm apart (Genetrodes, BTX) using a square-wave electroporation generator (ECM 830, BTX, Harvard Apparatus).

**Tissue analysis**

**Real-time PCR**

Frozen tissue samples were frozen crushed and homogenized. Total RNA was isolated using Trizol Reagent (Life Technologies, 15596-026). Contaminating DNA was removed using DNA-free DNA Removal Kit (Life Technologies, AM1906). cDNA was synthesized from .5 μg of total RNA with TaqMan reverse transcription reagents SuperScript VILO (Life Technologies, #11755050) and mixed with TaqMan Fast Advanced Master Mix (Life Technologies, #4444965). Quantitative real-time PCR (qRT-PCR) was carried out using Applied Biosystems® 7500 Fast Real-Time PCR System (Life Technologies). Relative mRNA levels were calculated by the (type of software) after normalization of each experimental sample to GAPDH levels.

**Western Blot Analysis**

The atrial tissue was snap frozen in liquid nitrogen, homogenized, and separated into membrane and cytosolic fractions according to the procedures of the Mem-Per Plus Membrane Protein Extraction Kit (Thermo-Scientific, #89842) or T-Per Tissue Protein Extraction Reagent (Thermo-Scientific, 78510) for total protein. Halt Protease & Phosphatase Inhibitor Cocktail (Thermo-Scientific, #78446) were added to all buffers. Protein concentrations were determined using Pierce BCA Protein Assay Kit (Thermo-Scientific, #23227). Proteins were fractionated by SDS-PAGE, transferred to polyvinyl difluoride.
(PVDF) membrane, blocked with 5% BSA, and blotted 1:5000 with HisProbe-HRP Conjugate (Thermo-Scientific, 15165), blotted 1:4000 with anti-NADPH oxidase 4 (Abcam, #ab109225), and blotted 1:1500 with anti-p-ERK ½, and blotted 1:1000 anti-pSMAD2 (Cell Signaling, #3108S), and blotted 1:5000 anti-GAPDH (Cell Signaling, #5174S) for all total protein and cytosolic, and blotted 1:10000 anti-pan cadherin (Abcam, #ab6528) for all membrane fractions. All band densities were quantified using the Image J software and normalized GAPDH/Cadherin.

Primary Antibodies: (1) HisProbe-HRP Conjugate 1:5000 (Thermo-Scientific, #15165); (2) anti-NADPH oxidase 4 1:4000 (Abcam, #ab109225); (3) anti-pERK1/2 (p44/42) 1:2000 (Cell Signaling, Danvers, MA, #9101S); (4) anti ERK1/2 (p44/42) 1:2000 (Cell Signaling, #4695S); (5) anti-pSMAD2 1:1000 (Cell Signaling, Danvers, MA, #3108S) (6) anti pSMAD2/3 1:1000 (Cell Signaling, Danvers, MA #8828); (7) anti SMAD2/3 1:5000 (Cell Signaling, Danvers, MA #8685); (8) anti pTAK 1:1000 (Cell Signaling, Danvers, MA #4531), rabbit (1:1000); (9) anti TAK 1:1000 (Cell Signaling #4505); (10) anti phospho-p38 MAP kinase 1:1000 (Cell Signaling #9211S); (11) anti p38 MAP kinase 1:1000 (Cell Signaling #9212S); (12) anti-GAPDH 1:5000 (Cell Signaling, Danvers, MA, #5174S) for all total protein and cytosolic; (13) anti-pan Cadherin 1:10,000 (Abcam, #ab6528) for all membrane fractions.

Secondary antibodies: (1) Anti-rabbit IgG (Cell Signaling, Danvers, MA, #7074S); (2) Anti-mouse IgG (Cell Signaling, Danvers, MA, #7076S)

All band densities were quantified using the Image J software and normalized to GAPDH or Cadherin.

**Immunofluorescence**

Dog atrial tissue was excised after in-vivo electrophysiological study and PLA, and LAA regions were dissected. The preparations were frozen in optimal cutting temperature compound (OCT) (Leica Instruments GmbH, Germany) at ~50°C in 2-methyl butane cooled by dry ice, and stored at -80°C.

Sections taken from -80 freezer were fixed with 4% PFA, and washed three times in PBS. The sections were then permeabilized by incubating them in PBS containing 0.1 Triton X-100 (BDH) for 10 min. After washing three times in PBS, the sections were blocked in 10% normal goat serum (NGS; Sigma) in PBS for 1 hr. The sections were incubated with primary antibodies diluted with PBS containing 1% Bovine Serum Albumin (BSA; Sigma) and 10% NDS in a humid container at 4°C overnight. The sections were then washed three times in PBS, and incubated with secondary antibodies diluted with same solution as primary antibodies in a humid box at room temperature in the dark for 1 hour. After sections were washed three times in PBS, they were then mounted with Dapi containing mounting media (Vector Labs) and sealed with nail polish. Labeling was visualized using an epi-fluorescent microscope (Axiovision observer, Zeiss) or laser scanning confocal microscope (LSM510 Meta, Zeiss). Acquired images were analyzed by LSM examiner, axiovision, Zen2011, or image J.

Primary antibodies: (1) mouse monoclonal anti-cadherin (used at 1:200, Abcam); (2) rabbit polyclonal anti-V5 (used at 1:200, Abcam).

Secondary antibodies: (1) goat anti-rabbit IgG conjugated to Alexa 488 (used at 1:2000, Invitrogen); (2) goat anti-mouse IgG conjugated to Alexa 568 (used at 1:2000, Invitrogen)

**Immunohistochemical analysis of canine myocardium for macrophages.**

Experimental canine myocardial tissue was embedded in freezing medium and sectioned and fixed for immunohistochemistry. To detect macrophages in canine tissue, antibody PM-2K (PMID reference 12689859) was overlaid after blocking solution. Control was isotype immunoglobulin. Signal was detected with FITC (green) secondary antibody. Nuclei were imaged with DAPI stain. To quantify macrophage density in experimental groups, percent macrophage positive nuclei were enumerated after blinded analysis.

**Masson's Trichrome stain:**
Paraffin embedded tissue sections were cut 4 µm apart. Paraffin was removed by placing the tissue section in histology grade xylene for two minutes and the process was repeated four times changing xylene solution after every two minutes. Finally, the xylene was washed away with ETOH for one minute in absolute ETOH, then again for one more minute with fresh absolute ETOH, followed by wash in 95% ETOH for 30 seconds, and subsequently in 70% ETOH for 45 seconds. ETOH was then washed with water for one minute. The frozen preparations were serially sectioned (at -25°C) at 10 µm thickness. Sections were mounted on Superfrost Plus slides (VWR) and stored at -80°C until use. The tissue section was then ready for staining. The section was treated with Bouin’s mordant at room temperature overnight. The following day the tissue section was rinsed in running water to remove excess yellow. The tissue section was stained in Weigert’s Iron Hematoxylin Solution for 5 minutes. Next, it was washed under running water for 5 min and briefly rinsed in distilled water. The section was then stained in Beibrich Scarlet-Acid fuchsin for 5 minutes, followed by a rinse in distilled water. Subsequently, the tissue section was stained in phosphomolybdic-phosphotungstic acid solution for 5 minutes. The issue section was then stained in Aniline Blue solution for 5 minutes. Immediately after staining, the tissue was dipped once in 1 % Glacial acetic acid for 2 minutes. The tissue section was then dehydrated through ethanol series, which include 70%, 90% and absolute ethanol (twice). Then, the tissue section was placed in xylene 5 minutes twice. A coverslip was finally placed on the tissue section for microscope examination.

Quantification of Fibrosis
The slides were digitized with the NanoZoomer 2.0-HT: C9600-13 scanner (Hamamatsu Photonics, Iwata City, Japan) at 20X magnification. In-house developed Matlab (Natick, MA) algorithm was used to quantify pixels with blue (fibrosis) and red (viable myocardium) colors of Masson’s Trichrome staining. Ratio of blue to red pixel density was represented as percentage of fibrosis. Evaluation consisted of quantifying percentage of dense interstitial fibrosis based on Trichrome staining with predetermined sensitivity and threshold on acquired TIFF images. Prominent fibrosis of interstitium with loss of surrounding myocardial fibers at 5X magnification was defined as dense interstitial fibrosis. Epicardial fibrosis, peri-vascular fibrosis and peri-neural connective tissue were not considered interstitial fibrosis.

Protein oxidation (Carbonylation)
The oxidation status of atrial tissue was determined by following the recommended protocol for the Oxiselect Protein Carbonyl Immunoblot Kit (Cell Biolabs, Inc. - Cat # STA-308). First, a standard SDS-PAGE electrophoresis gel was run and transferred to a PVDF membrane. The membrane was then immersed in a dinitrophenylhydrazine (DNPH) solution for the derivatization of the carbonyl group followed by incubations with the anti-DNP primary antibody (1:3000) and secondary antibody (1:5000) that were supplied with the kit. The western blot was developed on film using standard chemiluminescence techniques and the band densities were analyzed using JPEG software.
Supplemental Figures and Figure Legends
Online Figure VII

A. Conduction Velocity in Control (LacZ) LAA (uninjected tissue)

B. Conduction Velocity in TGFβdnRII LAA (uninjected tissue)

Pacing cycle length (PCL): NSR 400 ms 300 ms 200 ms
Online Figure IX

![Graph showing LAA max slope for Control (LacZ) and TGFβdnRll, with NS indicating no significant difference.](image)
Online Figure XI

A. Mean DF (Hz)

B. Mean OI

C. Mean ShEn

D. Mean Fl (ms)
Online Figure XII

Percent interstitial fibrosis
Control (LacZ) vs TGFβdnRII

Control (LacZ)

TGFβdnRII

Percent fibrosis

0 5 10 15 20 25

NS
Online Figure XIII

Control (LacZ)

1° 2°

100 kD His-tagged protein

140 kD Cadherin

*cytoplasmic fraction
**Figure legends**

**Online Figure I.** A. Expression of pUBc-LacZ in NIH-3T3 fibroblasts. B. Expression of pUBc-TGFβ-DN-R11 in NIH-3T3 fibroblasts. C. Effect of pUBc-TGFβ-DN-R1I on TGFβ induced collagen I. D. Effect of pUBc-TGFβ-DN-R1I on TGFβ induced p-SMAD2.

**Online Figure II.** A. Effect of TGFβ-DN-R1I on p-SMAD2/3 in canine atrial fibroblasts. B. Effect of TGFβ-DN-R1I on p-SMAD2/3 in canine atrial myocytes; C. Effect of TGFβ-DN-R1I on p-ERK1/2 in canine atrial fibroblasts. D. Effect of TGFβ-DN-R1I on p-ERK1/2 in canine atrial myocytes.

**Online Figure III.** Left atrial volume and left ventricular ejection fraction, left ventricular end diastolic volume and left ventricular systolic volume in and end systolic volumes pUBc-TGFβ-DN-R1I versus pUBc-LacZ treated atria.

**Online Figure IV.** Comparison of increase in Conduction Inhomogeneity index with HF in pUBc-TGFβ-DN-R1I versus pUBc-LacZ treated PLA.

**Online Figure V.** Conduction Inhomogeneity index in pUBc-TGFβ-DN-R1I versus pUBc-LacZ treated atria in the left atrial appendage (LAA).

**Online Figure VI.** Assessment of Conduction, Fibrosis, Restitution and pSMAD2/3 expression in sham control dogs. A. Panel I: Conduction inhomogeneity (CI) in the PLA of sham control dogs; Panel II: Conduction velocity (CV) in the PLA of sham control dogs; B. Restitution slope in sham control dogs; C. Fibrosis in sham control dogs; D. pSMAD2/3 expression in the PLA of LacZ injected HF dogs versus sham control dogs.

**Online Figure VII.** Conduction velocity in the LAA of pUBc-TGFβ-DN-R1I versus pUBc-LacZ injected animals.

**Online Figure VIII.** Effective refractory period (ERP) change in the LAA of pUBc-TGFβ-DN-R1I versus pUBc-LacZ injected animals LAA.

**Online Figure IX.** Restitution slope in the LAA in UBc-LacZ and pUBc-TGFβ-DN-R1I injected animals.

**Online Figure X.** Alternans for MAPd90 and MAP amplitude in the LAA in pUBc-LacZ and pUBc-TGFβ-DN-R1I injected animals.

**Online Figure XI.** AF frequency characteristics (DF, OI, ShEn, FI) in the LAA in UBc-LacZ and pUBc-TGFβ-DN-R1I injected animals.

**Online Figure XII.** Interstitial fibrosis in the LAA in UBc-LacZ and pUBc-TGFβ-DN-R1I injected animals.

**Online Figure XIII.** In pUBc-LacZ injected PLA, His-tagged fusion protein is noted at the expected size (100kD).

**Online Figure XIV:** A. Western blot showing phosho-p38 MAP kinase/p38 MAP kinase in pUBc-TGFβ-DN-R1I versus pUBc-LacZ injected PLA and B. Western blot showing pTAK1/TAK1 in pUBc-TGFβ-DN-R1I versus pUBc-LacZ injected PLA.
Online Figure XV: Carbonylation in the PLA of pUBc-TGFβ-DN-RII versus pUBc-LacZ injected dogs.

Supplemental References


