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# Atrial myofibroblast activation and connective tissue formation in a porcine model of atrial fibrillation and reduced left ventricular function

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ABSTRACT

*Aims:* Atrial fibrillation (AF) is associated with fibrosis that slows electrical conduction and causes perpetuation of the arrhythmia. The molecular characterization of AF pathophysiology may provide novel therapeutic options. This study was designed to elucidate profibrotic signaling and myofibroblast activation in a porcine model of atrial tachypacing-induced AF and reduced left ventricular function.
*Materials and methods:* Ten domestic pigs were randomized to sinus rhythm (SR) or AF groups. Prior to AF induction and on day 14 the animals underwent echocardiographic examinations. Profibrotic pathways were analyzed in right atrial tissue obtained from AF animals compared to SR controls using histology, immunofluorescence microscopy, Western blot, and real-time PCR.
*Key findings:* AF was associated with atrial dilation, increased atrial fibrosis, and enhanced expression of collagens I and V in right atrial tissue after 14 days follow-up. The fraction of α-smooth muscle actin (SMA)-producing activated myofibroblasts was elevated in AF, whereas the abundance of vimentin-expressing inactive fibroblasts was not affected. Profibrotic signaling involved upregulation of TGF-$\beta_1$, Smad2/3, and CTGF.
*Significance:* The transformation of atrial fibroblasts into myofibroblasts through activation of TGF-$\beta_1$ and CTGF emerged as potential cellular trigger of fibrogenesis. Prevention of fibroblast-to-myofibroblast switching may serve as target for remodeling-based antiarrhythmic AF therapy.



## 1. Introduction

Atrial fibrillation (AF) is associated with electrical and structural remodeling of the atria. Fibrosis and increased levels of extracellular matrix (ECM) proteins form a primary substrate for AF maintenance and perpetuation [1–3]. Fibrosis impairs electrical conduction velocity, allowing for the generation of re-entrant circuits in atrial tissue. Human and animal studies revealed a positive correlation between the amount of fibrotic fibers and AF burden [4]. The specific nature of the cells responsible for enhanced collagen production is still poorly understood. In the healthy heart, non-excitable fibroblasts comprise >50% of cardiac cells [5,6]. Under pathologic cardiovascular conditions, fibroblasts may differentiate into α-smooth-muscle-actin (α-SMA)-producing myofibroblasts that are not present in healthy myocardium [3,7]. These cells are capable of synthesizing excessive collagen fibers, cytokines, growth factors, and chemokines involved in reactive fibrosis development [8,9]. The auto- and paracrine activity of myofibroblasts contributes substantially to the stimulation and activation of profibrotic signaling cascades of other fibroblasts. In addition, direct coupling of myofibroblasts to cardiomyocytes affects electrical properties of cardiomyocytes and may lead to a more depolarized resting membrane potential, heterogeneity of conduction, depolarization of resting membrane potential, shortening of action potentials, and spontaneous cellular depolarizations [9,10].

TGF-β is a key regulator of profibrotic signaling and contributes substantially to the pathophysiology of maladaptive processes in heart disease [9]. TGF-β induces the differentiation of resident cardiac fibroblasts into myofibroblasts with para- and autocrine activity [11,12]. A key role of TGF-β for the development of AF-associated cardiac fibrosis has been suggested [13]. Profibrotic effects of TGF-β are mediated via Smad-proteins and altered expression of the connective tissue growth factor

*Abbreviations:* AF, atrial fibrillation; CTGF, connective tissue growth factor; DAPI, 4′,6-diamidino-2-phenylindole; ECM, extracellular matrix; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HDAC, histone deacetylase; HF, heart failure; LVEF, left ventricular ejection fraction; SMA, smooth muscle actin; Smad, Sma (from *Caenorhabditis elegans*) and MAD (mothers against decapentaplegic gene from *Drosophila*) homolog; SR, sinus rhythm; TGF, transforming growth factor.

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(CTGF) gene [9]. CTGF is a well characterized downstream target of TGF-β that promotes fibrotic response and ECM production [14,15].

In AF, fibroblast-to-myofibroblast switching and underlying signaling mechanisms remain incompletely understood. This study was designed to assess atrial myofibroblast differentiation, fibrosis, and profibrotic pathways using an established porcine model of AF [16–20].

## 2. Materials and methods

### 2.1. Atrial fibrillation animal model

AF-associated profibrotic remodeling was evaluated using a porcine model [16–20]. AF was induced by rapid atrial burst pacing in sex-matched domestic swine (<6 months of age; body weight 25 to 35 kg) as described earlier in detail [19]. Cardiac tissue was obtained from previously reported pigs [19] 14 days after the initiation of atrial burst pacing. Reduced left ventricular ejection fraction (LVEF) associated with AF and additional clinical characteristics of study animals have been described elsewhere [19]. This study has been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health (NIH publication No. 86-23, revised 1985) and with the EU Directive 2010/63/EU, and the current version of the German Law on the Protection of Animals was followed The study has been approved by the Regierungspräsidium Karlsruhe (Karlsruhe, Germany; approval number G-165/12).

### 2.2. Western blot analysis

After euthanization cardiac tissue was processed as described [16–21]. The right atrial appendage was dissected, rapidly frozen in liquid nitrogen and stored at −80 °C. Homogenized cardiac samples (Yellow line DI 18 basic homogenizer, IKA, Essex, UK) were subjected to cell lysis in radioimmunoprecipitation (RIPA) buffer containing 20 mM Tris-HCl, 0.5% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM $Na_3VO_4$, 1 mM NaF, and inhibitors of proteases (Complete) and phosphatases (PhosStop) (Roche Applied Science, Indianapolis, IN, USA). The bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL, USA) was used to determine protein concentrations. Equal amounts of protein were separated on 6–20% SDS polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and developed using primary antibodies directed against vimentin (ab8069; Abcam, Cambridge, UK), α-SMA (A2547; Sigma-Aldrich, St. Louis, MO, USA), CTGF (PA1-16634; Thermo Fisher Scientific, Waltham, MA, USA), Smad2/3 (sc-6200; Santa Cruz Biotechnology, Heidelberg, Germany), phospho-Smad2/3 (sc-11769; Santa Cruz Biotechnology), Smad4 (ab137861; Abcam), type I collagen (NB600-408, Novus Biologicals, Littleton, CO, USA), or type V collagen (ab112551; Abcam), and appropriate horseradish peroxidase (HRP)-conjugated mouse *anti*-goat (sc-2354; Santa Cruz Biotechnology) or donkey *anti*-rabbit (NA934V; GE Healthcare Life Sciences, Piscataway, NJ, USA) secondary antibodies. Signals were developed using the enhanced chemiluminescence assay (ECL Western Blotting Reagents, GE Healthcare, Buckinghamshire, UK) and quantified with ImageJ 1.41 software (National Institutes of Health, Bethesda, MD, USA). After removal of primary and secondary antibodies, the membranes were reprobed with *anti*-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (G8140-01; US Biological, Swampscott, MA, USA) and corresponding goat *anti*-mouse secondary antibodies (sc-2005; Santa Cruz Biotechnology). Protein content was normalized to GAPDH for quantification of optical density.

### 2.3. Quantitative real time PCR

Quantitative real time PCR (RT-qPCR) was performed using the StepOnePlus (Applied Biosystems, Foster City, CA, USA) PCR system as reported [22–25]. Total RNA was isolated from porcine right atrial appendage using TRIzol-Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. DNA synthesis was carried out by reverse transcription with the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, USA) using 3 μg of total RNA. Optical detection plates (96 wells; Applied Biosystems) were then loaded to a total volume of 10 μl per well, consisting of 0.5 μl cDNA, 5 μl TaqMan Fast Universal Master Mix (Applied Biosystems), and 6-carboxyfluorescein (FAM)-labeled TaqMan probes and primers (TaqMan Gene Expression Assays; Applied Biosystems) detecting porcine TGF-$\beta_1$ (Ss03382325_u1; Life Technologies, Darmstadt, Germany), Smad3 (Ss03382808_u1; Life Technologies), and CTGF (Ss03392397_m1; Life Technologies). Pre-designed primers and probes detecting porcine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Ss 03374854_g1; Life Technologies) were used for normalization. The validity of GAPDH as housekeeping gene for RT-qPCR analyses was previously indicated by a comparison between GAPDH, hypoxanthine phosphoribosyltransferase 1, and β-actin levels in pigs, confirming stable housekeeping gene expression throughout different cardiac regions and rhythm conditions [22].

### 2.4. Histological evaluation of fibrosis

Right atrium was dissected from the heart. Sections for microscopic analysis were fixed in 10% formalin, embedded in paraffin and cut to 7 μm thickness. Slices were deparaffinized, rehydrated, and stained with Picro-Sirius Red (AB150681, Abcam, Cambridge, UK). Stained collagen fibers were visualized with a Nikon Eclipse TE2000-E microscope (Nikon GmbH, Düsseldorf, Germany) and quantified with ImageJ 1.41 software (National Institutes of Health) in blinded fashion.

### 2.5. Immunofluorescence analysis

For immunofluorescence staining paraffinized sections were dewaxed and rehydrated. After washing with PBS, sections were fixed in ice-cold acetone, rinsed, and incubated in 1% BSA in PBS and 0.5% Triton X-100 (Merck Millipore, Darmstadt, Germany) (pH 6.0). Permeabilized cells were incubated over night with primary antibodies against troponin T (ab64623; Abcam) and either α-SMA (A2547; Sigma-Aldrich) or vimentin (ab8069; Abcam). Primary antibodies were removed, and the following secondary antibodies were applied: Texas Red-conjugated horse-*anti*-mouse antibody (TI-2000; VectorLabs, Burlingame, CA, USA); Alexa 555-conjugated donkey-*anti*-rabbit antibody (A-31572; Life Technologies, Darmstadt, Germany), and Alexa 488-conjugated donkey-*anti*-goat antibody (A-11055; Life Technologies). In addition, cells were stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) to visualize all nuclei. Sections were analyzed with a Nikon Eclipse TE2000-E microscope (Nikon, Düsseldorf, Germany) and ImageJ 1.41 software (National Institutes of Health, Bethesda, MD, USA) in blinded fashion. Myofibroblasts located in blood vessels were excluded from the analysis.

### 2.6. Echocardiography

Echocardiography was performed after 14-day follow up prior to euthanization (Philips Healthcare Sonos 5500, Hamburg, Germany) as reported [19]. Animals were sedated and anesthetized, and all examinations were done under equal conditions during SR. AF was electrically converted to SR prior to examination. Sizes of left atria and left ventricles were measured in M-mode. Left ventricular ejection fraction (LVEF) was calculated from M-mode measurements using the Teichholz formula ($V = [7/(2.4 + LVId)] \cdot [LVId]^3$).

### 2.7. Statistics

Experimental animal data are expressed as mean ± SEM. Statistical analyses were performed with GraphPad Prism 6.0 software (GraphPad