Dominant Frequency Increase Rate Predicts Transition from Paroxysmal to Long-Term Persistent Atrial Fibrillation

Running title: Martins et al.; Transition from Paroxysmal to Persistent AF

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Abstract

Background—Little is known about the mechanisms underlying the transition from paroxysmal to persistent atrial fibrillation (AF). In an ovine model of long-standing persistent AF (LS-PAF) we tested the hypothesis that the rate of electrical and/or structural remodeling, assessed by dominant frequency (DF) changes, determines the time at which AF becomes persistent.

Methods and Results—Self-sustained AF was induced by atrial tachypacing. Seven sheep were sacrificed 11.5±2.3 days after the transition to persistent AF and without reversal to sinus rhythm (SR); 7 sheep were sacrificed after 341.3±16.7 days of LS-PAF. Seven sham-operated animals were in SR for 1 year. DF was monitored continuously in each group. RT-PCR, western blotting, patch-clamping and histological analyses were used to determine changes in functional ion channel expression and structural remodeling. Atrial dilatation, mitral valve regurgitation, myocyte hypertrophy, and atrial fibrosis occurred progressively and became statistically significant after the transition to persistent AF, with no evidence for left ventricular dysfunction. DF increased progressively during the paroxysmal-to-persistent AF transition and stabilized when AF became persistent. Importantly, the rate of DF increase (dDF/dt) correlated strongly with the time to persistent AF. Significant action potential duration (APD) abbreviation, secondary to functional ion channel protein expression changes (CaV1.2, NaV1.5 and Kv4.2 decrease; Kir2.3 increase), was already present at the transition and persisted for one-year follow up.

Conclusions—In the sheep model of LS-PAF, the rate of DF increase predicts the time at which AF stabilizes and becomes persistent, reflecting changes in APD and densities of sodium, L-type calcium and inward rectifier currents.

Key words: atrial fibrillation, remodeling, fibrosis, ion channel, dominant frequency, Electrical Remodeling, Action potential duration, atrial dilatation, myocyte hypertrophy
Introduction

Atrial fibrillation (AF) is the most common arrhythmia in clinical practice. The natural history of AF usually starts with paroxysmal episodes. Some patients suffer paroxysmal AF indefinitely, mainly under anti-arrhythmic therapy, but a large proportion progress to persistent AF. In these patients, progression from paroxysmal to persistent and permanent AF is likely to reflect progressive electrophysiological and/or structural remodeling in both atria, making the arrhythmia more stable and long-lasting.\(^1\) However, despite more than 100 years of research, fundamental mechanisms governing transition from paroxysmal to persistent and permanent forms are poorly understood, and prevention and treatment remain suboptimal.

Electrical remodeling, reflected by shortening of atrial refractoriness, is known to develop within the first few days of AF.\(^2\) While some ion channel changes associated with electrical remodeling have been described in animal models and humans,\(^3-5\) it is unclear yet how these changes integrate to stabilize AF. Structural remodeling and fibrosis might also contribute to intra-atrial conduction disturbances and increase susceptibility for AF, yet their role in progression from paroxysmal to persistent AF remains to be elucidated. Recently, we developed a clinically relevant ovine model of intermittent right atrial (RA) tachypacing and demonstrated that after the first AF episode, dominant frequency (DF) of both the RA and left atrium (LA) increased gradually during a 2-week period, after which DF remained stable during follow-up.\(^6\) Here we have extended our study to investigate systematically whether gradual DF increase can predict when AF becomes persistent. We have modified the model such that pacing stops temporarily when AF is initiated during paroxysmal episodes and permanently once AF is sustained without reverting to sinus rhythm (SR). We compared three groups: Sham-operated, Transition (>7 days of self-sustained AF without reversal to SR) and self-sustained Long-
Standing Persistent AF (LS-PAF, > 1 year of AF without reversal to SR). We hypothesized that:
1) DF increase rate in paroxysmal AF predicts timing of transition from paroxysmal to persistent AF; and 2) electrophysiological remodeling occurs early in transition, whereas structural remodeling in the form of interstitial fibrosis appears more gradually and is belatedly manifest once self-sustained persistent AF has stabilized.

Methods

An expanded methods section is available in the Online Supplement.

Pacemaker implantation

Procedures were approved by the University of Michigan Committee on Use and Care of Animals and complied with National Institutes of Health guidelines. Twenty-one 6-8 month-old sheep (40 kg) had a pacemaker implanted subcutaneously, with an atrial lead inserted into the RA appendage. In a subset of thirteen sheep (7 LS-PAF, 1 transition and 5 in SR), an implantable loop recorder (ILR) was placed subcutaneously on the left side of the sternum (Figure S1A).

Pacing protocol

After 10 days recovery, sheep were assigned to either Sham-operated or atrial tachypacing groups. Sham-operated animals (N=7) had pacemakers programmed in sensing-only mode (OAO). Automatic mode switch was enabled in atrial-tachypaced animals (Figure S1B). The pacemaker was programmed to induce AF by burst tachypacing (30-sec pacing, 20 Hz, twice diastolic threshold) followed by 10-sec sensing. Pacemakers resumed pacing only if AF stopped and SR was detected. The Holter capabilities of the device were used to record intra-cardiac electrograms (EGMs) to accurately confirm the occurrence of AF, generate histograms, and follow the evolution of AF from the first episode of paroxysmal AF to the eventual establishment
of persistent AF. Persistent AF was defined as episodes lasting >7 days without reversal to SR. A subset of tachypaced animals was sacrificed after more than 7 days of self-sustained AF (Transition group, N=7). The remaining animals were sacrificed after one year of self-sustained AF (LS-PAF group, N=7). Pacemakers and ILRs were interrogated weekly.

**Electrogram acquisition and processing**

Persistence of SR was verified in sham-operated animals and pacemaker memories were checked to detect spontaneous AF episodes. Three recordings were obtained in tachypaced sheep during follow up: 1) RA-lead EGM; 2) ECG Standard lead I; and 3) ILR single lead recording (LA far-field signal).

**Serum measurements**

Procollagen III N-Terminal Propeptide (PIIINP) levels were measured by enzyme linked immunosorbant assay.

**Echocardiography**

LA and RA dimensions, severity of mitral regurgitation, left ventricular ejection fraction (LVEF), end-systolic and end-diastolic diameters were evaluated using echocardiography (Figures S2 and S3).^7^

**Heart removal and cell dissociation**

At termination of follow-up, hearts were removed and atria dissected (Figure S4) for myocyte isolation. patch-clamp recordings, western blotting, real-time PCR and histology (see Online Supplement).

**Computer Simulations**

We modified the Grandi-Pandit^8^ human atrial cell model to simulate action potential and propagation in 2D.
Statistical analyses

Normally distributed data are expressed as mean ± SEM. Normality of distributions was assessed using the Shapiro-Wilk test. A mixed regression model was applied to multiple group analyses and repeated measured data. Action potential durations (APD) and ionic current densities were compared using a two tailed unpaired Student’s-t tests. RT-PCR and Westerns blot data were analyzed using two-way ANOVA. A p<0.05 was considered statistically significant.

Results

Sheep model of persistent AF

Of 21 implanted sheep one sham-operated animal was excluded and sacrificed prematurely due to severe symptomatic systemic infection. No atrial arrhythmias occurred in any sham-operated animals during follow-up. Also, no tachypaced animals developed signs of heart failure or stroke. Figure 1 summarizes the time-course of AF development. The representative 3D plot (Figure 1A) relates percentage of AF episodes in a given day (Y-axis) to duration of episodes (X-axis) and weeks of follow-up (Z-axis). The first AF episode occurred after a median time of 5.5 days after initiation of pacing (mean, 15.0±5.9 days; range, 0-62 days, Figure 1B). AF episodes were then paroxysmal (<7 days duration), reaching self-sustained persistent AF (>7 days without reversal to SR) after a median of 43.5 days (mean 73.2±23.0 days; range, 19 to 346 days). Once in persistent, there was no further tachypacing as AF was detected uninterruptedly. Sheep in Transition and LS-PAF were sacrificed 11.5±2.3 days and 341.3±16.7 days, respectively, after occurrence of self-sustained persistent AF (i.e. after the last occurrence of SR).

Persistent AF leads to atrial dilatation
Echocardiographic findings (Table S1, Figures S2 and S3) revealed that LVEF was unchanged whereas RA and LA areas increased significantly in LS-PAF, compared with sham-operated and Transition groups (p<0.05, Figure S3). At last follow-up, LS-PAF animals showed significant mitral valve regurgitation (Figure S3B), yet LV end-diastolic volume, LV end-systolic volume or wall thickness were unchanged (data not shown), ruling out tachycardia-induced cardiomyopathy associated with AF. Although, compared to sham-operated animals, the dry weight of isolated atria in the transition group tended to be larger, only the atrial tissues from the LS-PAF group demonstrated a significant increase weight (Table S2).

**Persistent AF leads to atrial myocyte hypertrophy**

Mean LA and RA myocyte length and width, respectively, were similar for sham-operated animals (Figure S5). At transition, LA myocyte length and width increased significantly (p<0.001 and p<0.01, respectively); RA myocyte length did not change significantly (p=0.25) and a trend for wider cells was observed (p=0.08). At transition, LA cells were longer than RA cells (p<0.001), and after one year of AF, no further differences were observed for LA myocyte lengths or widths compared to transition. However, RA myocytes that initially did not exhibit significant changes at transition, showed a trend for longer cells and were significantly wider (p<0.001). In LS-PAF, LA myocytes were longer (p=0.002) and thinner (p=0.001) compared to RA.

**AF leads to atrial myofibroblast activation and fibrosis in the absence of heart failure**

AF-induced changes in the extracellular matrix were analyzed using histology, serum markers and molecular biology. There was a trend towards increased patchy fibrosis in RA, LA and PLA regions during AF progression, interstitial fibrosis increased in both LA (from 5.5±1.2 to 10.7±1.5%, p<0.05) and PLA (from 4.1±0.6 to 14.6±1.4%, p<0.001), particularly in LS-PAF
(Figure 2A-B, Table S3). These data correlated with measurements of PIIINP, a serum marker for collagen synthesis, which increased progressively, reaching maximal levels in LS-PAF which was increased significantly from Sham-operated animals at a similar time point (p=0.001 vs. sham, Figure S6). As expected from PIIINP serum levels, tissue protein levels of collagen III, analyzed by western blot, increased significantly in both atria during LS-PAF (Figures 2C and D). A significant increase in atrial α-smooth muscle actin (α-SMA), a marker of myofibroblast activation,9 was seen in both atria in Transition, but tended to decrease toward control levels in LS-PAF.

Electrophysiological remodeling is reflected by DF changes

During weekly interrogations, we investigated AF occurrence and recorded ongoing episodes. The DF of the first episode recorded from the RA lead was relatively slow at 7.5±0.1 Hz (range 6.5–8.25 Hz). Simultaneous DFs from the surface ECG and ILR after QRST subtraction were 7.7±0.2 Hz (range 6.5 to 9.25 Hz) and 9.0±0.1 Hz (range 8.9–9.4 Hz), respectively. Thus, as previously demonstrated,6 at the outset there was a significant DF difference between RA and LA (p<0.001, Figure 3). Thereafter, DF increased progressively in both atria. At both transition and LS-PAF, DFs recorded on the RA, surface ECG and LA were higher than during the first episode (p<0.001). However, in the 7 LS-PAF sheep, the last DFs recorded after 1 year of AF were not significantly different from prior corresponding values at transition. Thus, the major increase in DF occurred during paroxysmal AF and not during self-sustained LS-PAF.

Additionally, while a significant LA-to-RA frequency gradient was present during the first episode, this gradient diminished at transition (p=0.06) and LS-persistent time points (p=0.1), likely reflecting remodeling of refractory periods in both atria. In any given animal, once respective maximum DF values were achieved, they remained relatively stable even after one
year follow up; there was no significant difference between maximum DF at transition and at ~350 days.

The rate of DF increase predicts the onset of persistent AF

We analyzed several parameters to determine whether or not the time in paroxysmal AF and transition to self-sustained persistent AF could be predicted. We first surmised that a critical DF should be reached before self-sustained persistent AF developed, but the data did not support this hypothesis (Figure S7). Not only did maximal DF vary among animals, but the rate of DF increase during transition was also highly variable, ranging 0.003 to 0.15 Hz/day in the RA and 0.001 to 0.12 Hz/day in the LA. However, sheep that developed self-sustained persistent AF early, also had a steep slope of DF increase with time (dDF/dt), regardless of DF during the first episode, whereas those with a delayed onset of persistent AF had a shallower DF slope (Figure 4A). Thus we hypothesized that dDF/dt could predict when AF became persistent in each animal. Indeed, a strong nonlinear relationship was found between time to persistent AF onset and dDF/dt regardless of whether DF was determined in the RA, LA or surface ECG ($R^2 = 0.87, 0.92$ and 0.71, respectively, Figure 4B). The faster the DF increase, the quicker the animal developed self-sustained persistent AF. Furthermore, non-invasive measurement of dDF/dt (surface ECG lead I) correlated strongly with RA and LA dDF/dt (Figure S8).

Cellular and ionic mechanisms of electrical remodeling

We conducted patch-clamp experiments to determine whether the gradual DF increase during transition reflected development of remodeling at the cellular level. Action potential duration at 90 percent repolarization (APD$_{90}$) was significantly reduced in both RA and LA at transition and LS-PAF groups (Figure 5). Sheep from both groups tended to have more hyperpolarized resting membrane potentials than sham (p=NS, data not shown) for RA (-69.8±2.8 mV, -60.2±3.4 mV
and -57.6±4.6 mV, respectively) and LA myocytes (-72.1±4.1 mV, -66.6±3.6 mV and -63.5±2.3 mV, respectively). Action potential (AP) upstroke velocity (dV/dt_max) also tended to be lower in myocytes from AF animals, while AP amplitudes did not change significantly (data not shown). Myocytes from animals in AF also showed a loss of rate-adaptation of APD (Figure 5B).

Shortest pacing cycle length before AP alternans or failure to capture was significantly longer in sham than transition and LS-PAF groups (data not shown), as a consequence of APD and ERP shortening in both RA (345.7±37.5 ms, 165.7±62.6 ms and 203.3±26.5 ms, respectively, p<0.05 vs. sham) and LA (358.3±31.2 ms, 218.1±27.5 ms and 249.4±17.7 ms, respectively, p<0.05 vs. sham).

Next, we conducted Western blot analyses in the three groups on animals to test whether remodeling was related to altered intracellular calcium dysfunction. As illustrated in Figures S9 and S10 of the Online Supplement, while the Na⁺-Ca²⁺ exchanger was increased in the LA appendage, both total RyR2 and phosphorylated RyR2 proteins were decreased in the AF group, but the ratio of phosphorylated RyR2 to total RyR2 phosphorylation was unaffected.

Accordingly, the transition from paroxysmal to persistent AF did not seem to depend on Ca²⁺ leak or delayed afterdepolarizations.¹⁰

We then focused on possible alterations in sarcolemmal ion channels that might contribute to AF-induced changes in APD and refractoriness. Peak inward sodium current (I_{Na}) was significantly reduced at the transition time-point by about 50% in LA myocytes compared to sham (Figure 6A) and about 30% in RA myocytes. For LS-PAF, peak I_{Na} was decreased in both LA and RA myocytes (p<0.001 vs. sham). Similarly, peak L-type calcium current (I_{CaL}) was reduced in LA and RA at transition and LS-PAF (p<0.05, Figure 6B). Changes in I_{Na} and I_{CaL} resulted from concomitant decreases in expression of Naᵥ1.5 and Caᵥ1.2 proteins and SCN5A and
CACNA1C mRNA levels (Figure 6D-G; see Table S4 for primers used in RT-PCR).

In contrast to $I_{Na}$ and $I_{Ca,L}$, the density of the inward rectifier potassium current ($I_{K1}$) increased 2- to 3-fold at negative membrane voltages during the transition in both atria, and continued to increase for LS-PAF ($p<0.05$ vs. sham, Figure 7A). Since sheep atria predominantly express Kir2.3 channels, we measured Kir2.3 expression which was increased in LS-PAF animals (Figure 7B). There was no Kir2.3 increase in transition despite the larger current density compared to sham. The transient outward K$^+$ current ($I_{to}$) decreased by about 85% by transition (Figure S11) and remained low in LS-PAF ($p<0.001$, Figure S11). For LS-PAF animals, $I_{to}$ reduction could be explained by decreased Kv4.2 expression. However, reduced protein was not evidenced in the LA in transition animals, suggesting other mechanisms contributed to $I_{to}$ decrease. Lastly, Kv11.1 protein expression remained unchanged (Figure S11C-D).

Can ionic current changes explain DF increase?

To address the question of whether differential changes in ion currents demonstrated above could explain DF increase during transition from paroxysmal to persistent AF we generated APs for control, paroxysmal, and transition AF conditions using the Grandi-Pandit human atrial AP model (Figure 8A, Table S5). The ionic changes for the transition AF were based on our experimental patch clamp recordings. We did not have ionic current recordings for paroxysmal AF. Therefore, to represent paroxysmal AF, we retained the ionic changes made in transition AF, but reduced the magnitude of $I_{Ca,L}$ by only 30% (Table S5), such that the simulated APD$_{90}$ was shortened significantly by 17% in paroxysmal AF, compared to 51% in transition AF (Table S6).

We used a 2D sheet model of reentry to investigate whether AP differences between paroxysmal and transition AF simulations would explain the progressive DF increase
demonstrated in vivo. Sustained functional reentry (rotor) dynamics showed differential properties. The rotor in paroxysmal AF (Figure 8B, left) was short lived, and exhibited low rotation frequency (5.0 Hz) and considerable meandering (Figure 8C, left), eventually self-terminating upon collision with boundary edges. In contrast, in the transition AF model, the rotor was stable and persisted throughout the length of the simulation (Figure 8B, right) with significantly less rotor meander (Figure 8C, right) and higher DF (7.67 Hz) compared to the transition case. When reduction in INa density was not incorporated, the DF increased only slightly to 8.67 Hz, but the rotor was unstable and eventually stopped.

We also further investigated the roles of individual ionic changes in a subset of simulations. Rotors were simulated in 2D sheets, when individual ionic currents were changed, compared to controls. As discussed in detail in the Online Supplement (Figures S12-S15), the simulation results confirmed that changes in IK1 and ICaL are key determinants of rotor acceleration in paroxysmal and transition AF.

Fast versus slow transition

To search for determinants of the rate of AF progression, we separated slow and fast progressing animals sacrificed at transition depending on the median time to progression (<45 days: 4 animals; >45 days: 3 animals). As discussed in detail in the Online Supplement (Figures S16 and S17), the major factor contributing to the larger dDF/dt in the fast transition animals was greater APD shortening secondary to ICaL reduction. On the other hand, the slow transition animals seemed to require an additional IK1 increase and greater structural remodeling.

Discussion

The most important results of this study are: 1) Intermittent rapid tachypacing results in a
progressive increase in DF during paroxysmal AF; 2) When DF stabilizes (dDF/dt>0), AF becomes persistent; 3) DF increase correlates strongly with time to persistent AF; 4) APD abbreviation, secondary to ion channel gene expression changes (Na\textsubscript{v}1.5 Ca\textsubscript{v}1.2, and Kv4.2 decrease; Kir2.3 increase), is already present or occurs rapidly during transition and explains the DF increase; 5) In the absence of LV dysfunction, there is a progressive increase in atrial dilatation, mitral valve regurgitation, myocyte hypertrophy, and atrial fibrosis, which became significant after DF had stabilized. Altogether, these results demonstrate that the rate of DF increase during transition predicts the time at which AF stabilizes and becomes persistent, reflecting changes in APD and densities of $I_{\text{Cal}}$, $I_{Kl}$, $I_{Na}$ and $I_{lo}$. Thus, this is the first detailed characterization of the electrophysiological and structural remodeling involved in the transition from paroxysmal to persistent AF and self-sustained LS-PAF.

**In-vivo changes in DF during AF**

Various animal models and clinical studies have demonstrated the presence of a spatial distribution of DF during AF. Left-to-right frequency gradients were found in isolated sheep hearts, supporting the hypothesis that AF results from rapidly successive wavefronts emanating from fast sources localized in the LA.\textsuperscript{13} Similar gradients were confirmed in humans with AF,\textsuperscript{14,15} but were shown to be present mainly during paroxysmal AF, but not always in persistent or permanent AF since longer periods in AF lead to a more complex remodeling making both atria suitable for harboring reentrant sources.\textsuperscript{14-16} Patients with persistent AF usually demonstrate higher DFs compared to paroxysmal AF.\textsuperscript{15} We recently demonstrated in the ovine model that intermittent tachypacing results in AF with a progressive increase in DF over a 2-week period after the first detected AF episode.\textsuperscript{6} There we used an algorithm of 30-seconds pacing followed by a 10-second blanking period, over a 22-week follow-up, whether or not AF was detected.
Thus, continuous pacing likely induced a DF that was similar in all animals studied at that time.⁶ To increase the clinical relevance of our AF model, here we modified our pacing algorithm by transforming the 10-second blanking period into a real sensing period, consequently avoiding unnecessary pacing and generating a model of self-sustaining AF. Therefore, in this more realistic model of lone AF, there was a progressive increase in DF that was different for each animal, ranging from 4–50 weeks. The first AF episode was slow followed by a progressive and significant increase in DF during paroxysmal AF, reaching maximal value at the transition to persistent AF. Most important, DF did not increase further even after 1 year of self-sustained persistent AF.

Progressive DF increase during transition is a consequence of electrical remodeling. Sustained AF shortens APD and effective refractory period, decreasing wavelength and facilitating acceleration and stabilization of sustained reentry. The main determinants of APD shortening are the decrease in $I_{CaL}$ and increase $I_{K1}$.¹⁷,¹⁸ In a canine model of constant rapid atrial pacing, appreciable APD shortening occurred after 1 day of pacing, and was near-maximal after 7 days.¹⁹ At the electrophysiological level, reduced $I_{CaL}$ and $I_{so}$ were observed, without significant change in $I_{K1}$.¹⁹ However, human remodeling as a consequence of AF has been shown to be more complex, involving many changes in potassium currents like decreased of $I_{to}$⁵ and $I_{Kur}$⁵ and increased $I_{Ks}$⁵, $I_{Kf}$ and the constitutively-active $I_{KACH}$.⁴ The contribution of these changes to APD shortening has been analyzed in computer modeling studies suggesting that $I_{K1}$ increase is a predominant mechanism of APD shortening.²⁰

Our results support previous numerical predictions. $I_{Na}$ and $I_{CaL}$ decreases secondary to reductions in $NaV1.5$ and $CaV1.5$ expression, and $I_{K1}$ density increases secondary to increased Kir2.3 expression were the major ionic changes observed at transition (remodeling during
paroxysmal AF); they evolved parallel with DF increase, and changed negligibly throughout the one-year follow-up in self-sustained persistent AF. Upregulation of $I_{K1}$ is known to enhance cardiac excitability through cell hyperpolarization and increased sodium channel availability, and to increase frequency and stability of rotors driving AF and VF. Our computer simulations reaffirmed these results since rotors were found to be more stable, less meandering and persistent for transition compared to paroxysmal time-points (7.7 vs. 5.0 Hz, respectively). Similarly, $I_{Na}$ reduction, despite reducing excitability slightly, also contributed to rotor stabilization, as shown by the fact that rotors generated in the absence of $I_{Na}$ reduction exhibited considerable meandering leading to their eventual annihilation. Most important, however, our numerical results predict that downregulation of $I_{CaL}$ and upregulation of $I_{K1}$ to be the most important contributors to the increased DF and rotor stabilization, particularly during transition AF.

**Structural remodeling and the link to electrical remodeling**

We observed significant structural remodeling in this model of lone, persistent AF. Myocyte hypertrophy and atrial dilatation increased progressively among sham-operated sheep and sheep at transition or LS-PAF, which suggests there might be a connection between atrial enlargement and DF increase. Additionally, PIIINP, a serum marker of interstitial fibrosis, increased gradually from the first episode through transition into the last follow-up. Collagen protein levels and interstitial fibrosis also tended to increase during transition and became frankly manifest once self-sustained LS-PAF developed. Notably, a significant increase in atrial $\alpha$-SMA protein, a marker of myofibroblast activation, was seen in both atria in transition, but this protein tended to decrease toward control levels in LS-PAF animals. These changes might reflect processes similar to those described for myocardial infarction: a myofibroblast proliferative phase gives way to a maturation phase in which the cellularity of tissue decreases and local extracellular
matrix is cross-linked forming a collagen-based fibrotic scar. Therefore, the increase in α-SMA protein may reflect increased atrial myofibroblast activation and proliferation induced by atrial tachypacing induced upregulation of transient receptor potential canonical-3 (TRPC3) channels, which may have contributed not only to development of fibrosis, but also to electrical remodeling through release of profibrotic cytokines. One such cytokine is platelet-derived growth factor (PDGF), which, as recently demonstrated, can reduce atrial myocyte APD and $I_{CaL}$ in ways that resemble the effects of persistent AF as shown in this study. Finally, we found no change or downregulation of most calcium-handling proteins and no change in the phosphorylated RyR2/total RyR2 ratio, which makes it unlikely that, in this animal model of lone AF, the rate of change of AF-induced differential ion channel expression depends on sustained AF-induced intracellular Ca$^{2+}$-handling remodeling or increased SR Ca$^{2+}$ leak. However, it is still possible that Ca$^{2+}$ loading produced by the high AF frequency activated the Ca$^{2+}$-dependent calmodulin-calcineurin-NFAT system to cause transcriptional downregulation of $I_{CaL}$.25

Predicting transition from paroxysmal to persistent AF

Mid- to long-term follow-up studies in patients have shown that the rate of progression from paroxysmal to persistent or permanent AF ranges between 14.6% and 35.3% during 1 to 12 years. A 30-year follow-up study reported a cumulative probability risk of progression of 29% (95% CI, 16 to 42%) with most transitions occurring within 15 years after diagnosis. Many independent clinical, echocardiographic and electrocardiographic predictors of transition have been described including age, hypertension, previous ischemic attack or stroke, chronic obstructive pulmonary disease, presence of cardiomyopathy/heart failure, atrial enlargement, valvular diseases, filtered P-wave duration ≥150 ms, or P-wave dispersion on
ECG. A report using pacemaker memory interrogation found 24% risk of transition at a mean follow-up of 5 months. Interestingly, slope of change in AT/AF burden as a function of time was examined. Patients developing sustained AF showed increase of AF burden over time, compared with patients remaining in paroxysmal AF who demonstrated no progressive change in AF duration (14 sec/day vs. 0 sec/day, p<0.001). Similarly, in our study, we observe a very different rate of increase of dDF/dt and episode durations between animals quickly developing persistent AF and those remaining in paroxysmal AF for a long period before transition. Both LA and RA dDF/dt predicted the transition to persistent AF. This is not surprising since DF increased in both atria until AF became persistent (Figure 3). Thus ionic remodeling occurred in both atria, though somewhat more intensely in the LA than RA, particularly for I_{CaL} (Figure 6B) and I_{K1} (Figure 7), both of which contributed to the increased DF and rotor stabilization (Figures S12 and S13). Finally, the possible contribution of cytokines released from ion-channel mediated activation and differentiation of fibroblasts into hypersecretory myofibroblasts as a common link to structural and electrical remodeling in the fibrillating atria is likely to be important and deserve further exploration.

Limitations

We used a pacing induced self-sustained AF model in which continued periods of SR were not allowed since pacing was resumed as soon as AF terminated (5-10 seconds of SR between spontaneous termination of AF, detection of SR and initiation of the following pacing run). Thus, it is unknown whether the time from paroxysmal to persistent AF would be predictable in other animal models or humans. In addition, while this model enabled us to investigate the consequences of sustained AF in the absence of other co-morbidities, we are well aware that the majority of human AF cases associate with cardiovascular disease. Nevertheless, our results
show that the time to AF persistence is predicted by the rate of change of DF and explained primarily by downregulation of $I_{Cal}$ and upregulation of $I_{K1}$. The results also suggest that the wide range of dDF/dt values and time to persistent AF might be explained by temporal differences in the remodeling of these two channels in animals exhibiting slow-versus-fast progression to LS-PAF, which requires further study. Other currents may be important, including $I_{K1}$\textsuperscript{18} and $I_{KACH}$, whose constitutively active isoform is upregulated.\textsuperscript{4,31} Activation of $I_{KACH}$ with adenosine accelerates DF in patients with paroxysmal AF\textsuperscript{16}. Therefore studies should be performed to analyze the time-course of $I_{KACH}$ changes during AF progression. In our study $I_{lo}$ decrease and $I_{K1}$ increase in transition did not correlate with protein expression, suggesting other genes encoding ion channel alpha- or beta-subunits might be important. Alternatively, open-channel probability in the case of $I_{K1}$\textsuperscript{31} might help explain the changes we have observed.

Similarly, while our analysis of Ca\textsuperscript{2+}-handling proteins suggested that the transition from paroxysmal to persistent AF did not depend on Ca\textsuperscript{2+} leak or DADs, more extensive studies involving intracellular Ca\textsuperscript{2+} measurements will be required for definite proof.\textsuperscript{25} Lastly, we decided not to model LS-PAF since no major electrophysiological changes occurred during the one-year follow-up. However, additional structural remodeling after the transition (e.g., atrial dilatation, fibrosis, myocyte hypertrophy) are likely to affect DF, but were not incorporated in the simulations.

**Clinical Implications**

Our model of intermittent atrial tachypacing resembles human AF in the absence of comorbidities. Similar to humans, AF in sheep follows a very heterogeneous temporal progression to persistent AF, ranging from 4–50 weeks. Therefore, analyzing dDF/dt by surface ECG in
human AF could help stratify paroxysmal AF patients depending on risk of progression and be of great help in guiding physicians to individualize therapy. Patients exhibiting steep dDF/dt increases during follow-up could be rapidly referred to an EP lab for ablation before persistent AF develops, which would reduce ablation time and number of procedures. Conversely, patients with low dDF/dt could be considered at low risk for progression and be treated longer with anti-arrhythmic drugs before being referred to an EP lab.

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Conflict of Interest Disclosures: Dr. Jalife is on the Scientific Advisory Board of avertAF. Dr. Berenfeld is the Scientific Officer of Rhythm Solutions, Inc.

References:


Figure Legends:

**Figure 1.** Time-course of AF development. A: representative 3D plot of percentage of AF episodes in a given week (Y-axis) vs episode duration (X-axis) and weeks of follow-up after initiation of pacing (Z-axis). The first paroxysmal episode occurred 3 weeks after initiation of pacing. Duration of episodes progressively increased until persistent AF developed (week 12). B: summary of temporal measurements. AF: Atrial fibrillation; LS-PAF: Long-Standing Persistent AF.
**Figure 2.** AF-induced changes in extracellular matrix. A: Mean±SEM values for patchy fibrosis (left) and interstitial fibrosis (right) in right atrium (RA), left atrium (LA) and posterior left atrium (PLA) of sham-operated (N=6), transition (N=7) and LS-PAF (N=7). Twenty pictures per slide were randomly selected and analyzed; *p<0.05; **p<0.001 vs. sham. B: Representative picrosirius red staining of PLA of sham-operated, transition and LS-PAF. C and D: Western blots of α-smooth muscle actin (SMA) and Collagen III (Col III) in LA and RA tissue homogenates relative to GAPDH. N=6 for each group. *p<0.05, **p<0.01 vs. sham. N = number of animals.

**Figure 3.** Dominant frequency increases in RA and LA (A) and surface ECG (B) during progression of AF. N=14 for RA, N=8 for LA. #p<0.001 for RA vs. LA, **p<0.001 vs. sham. N = number of animals.

**Figure 4.** Rate of increase in DF during paroxysmal AF predicts transition to persistent AF. A: Representative graphs for three animals. Left, sheep with the highest dDF/dt (0.14 Hz/day, time to transition 19 days); middle, intermediate dDF/dt (0.03 Hz/day, time to transition 46 days); right, lowest dDF/dt (0.003 Hz/day, time to transition 346 days); left and right from transition group, middle from LS-PAF group. B: log-log plots of time from first episode to onset of self-sustained persistent AF versus dDF/dt for the RA (intracardiac electrode), LA (loop recorder) and ECG (surface Lead 1). Each point represents an animal. dDF/dt correlated with time to develop self-sustained persistent AF. N=14 for RA and ECG, N=8 for LA.

**Figure 5.** APD and frequency dependence in myocytes from sham, transition, and persistent AF.
A: Action potential duration (APD$_{90}$ at 1Hz) is reduced in both atria at transition from paroxysmal to persistent AF. For RA: $N=$3/n=13 (sham), $N=$3/n=13 (transition), $N=$3/n=14 (LS-PAF); for LA: $N=$3/n=18 (sham), $N=$3/n=14 (transition), $N=$3/n=18 (LS-PAF). $^*$p<0.05. Right: Representative LA APs are superimposed. B: Cycle length (CL) dependence of APD$_{90}$. For RA: $N=$3/n=13 (sham), $N=$3/n=13 (transition), $N=$3/n=14 (LS-PAF); for LA: $N=$3/n=18 (sham), $N=$3/n=14 (transition), $N=$3/n=18 (LS-PAF). $^*$p<0.05 Transition and LS-PAF vs. sham 1000 ms CL. #p<0.05 Sham at 300ms vs. sham at v1000ms CL. $N=$ number of animals; n= number of cells.

**Figure 6.** Sustained AF reduces functional expression of Na$^+$ and L-type Ca$^{2+}$ channels. A: Current-voltage relationships for $I_{Na}$ in myocytes from LA (left) and RA (right). For LA: $N=$3/n=12 (sham), $N=$4/n=21 (transition), $N=$5/n=21 (LS-PAF); for RA: $N=$3/n=10 (sham), $N=$4/n=18 (transition), $N=$5/n=18 (LS-PAF). $^*$p<0.05 vs. sham, # p<0.05 vs. transition. B: Current-voltage relationships for $I_{CaL}$ in myocytes from LA (left) and RA (right). For the LA: $N=$3/n=13 (sham), $N=$4/n=17 (transition), $N=$4/n=11 (LS-PAF); for the RA: $N=$3/n=12 (sham), $N=$4/n=16 (transition), $N=$4/n=14 (LS-PAF). $^*$p<0.05 vs. sham. C: Representative traces for $I_{Na}$ (upper) and $I_{CaL}$ (lower) in myocytes from LA of sham-operated and LS-PAF animal. D-E: Western blot analysis of Na$_V$1.5 and Ca$_V$1.2 protein expression in LA tissue homogenates (D) and RA tissue homogenates (E). Top, Representative blots; bottom, Quantification of protein expression relative to GAPDH. $N=$6. F-G: Real time RT-PCR analysis of SNC5A and CACNA1C gene expression in tissue homogenates from LA (F) and RA (G); quantification of gene expression relative to GAPDH. $N=$6. $^{**}$p< 0.01 vs. sham. $N=$ number of animals; n= number of cells.
**Figure 7.** Sustained AF increases functional expression of Kir2.3. A: Current-voltage relationships for $I_{K1}$ in myocytes from LA (top) and RA (bottom). For LA: $N=3/n=7$ (sham), $N=5/n=10$ (transition), $N=2/n=4$ (LS-PAF); for RA: $N=3/n=6$ (sham), $N=3/n=10$ (transition), $N=3/n=9$ (LS-PAF). *p<0.05 vs. sham. B: Western blots for Kir2.3 in LA tissue homogenates. Top: representative blots of 2 different groups; bottom: quantification of protein expression relative to GAPDH. $N=6$. *p<0.05 vs. sham. $N=$ number of animals; $n=$ number of cells.

**Figure 8.** Simulations predict consequences of ion channel remodeling on rotor frequency. A: Action potential traces for sham, paroxysmal and transition AF predicted by experimentally derived ion channel changes (Figures 6-7). APD$_{90}$ was abbreviated in both paroxysmal and transition AF compared to sham. Resting membrane potential was hyperpolarized -2 mV. B: Rotor in paroxysmal (left) had lower frequency than transition AF. C: Rotors in paroxysmal AF meandered considerably and eventually self-terminated upon collision with boundary. In transition AF, the rotor was stable, had higher frequency and persisted throughout the simulation.
Figure 2
Figure 3
Figure 4
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Figure 6
Figure 7
Figure 8
Dominant Frequency Increase Rate Predicts Transition from Paroxysmal to Long-Term Persistent Atrial Fibrillation

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Dominant Frequency Increase Rate Predicts Transition from Paroxysmal to Long-Term Persistent Atrial Fibrillation

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Supplemental Methods:

Pacemaker implantation: All procedures were approved by the University of Michigan Committee on Use and Care of Animals and complied with National Institutes of Health guidelines. Twenty-one 6-8 month-old sheep (~40 Kg) were used for implantation.

Anesthesia was achieved using propofol IV for the induction (4-6 mg/kg) and isoflurane gas at 5-10 ml/kg for maintenance. An endocardial 6 to 8 Fr, bipolar, active fixation and steroid-eluting lead was inserted into the right atrial appendage through the left external jugular vein. Once properly placed, the proximal end was screwed to the atrial port of a sterile dual chamber pacemaker (St. Jude Medical, Inc, St Paul, MN). The ventricular port was occluded using specific plugs. The pacemaker canister was then inserted in a subcutaneous pouch at the base of the neck. In a subset of thirteen sheep (8 paced animals and 5 ones in SR), an implanted loop recorder (ILR, Reveal® XT, Medtronic, Inc. Minneapolis, MN. USA) was placed subcutaneously on the left side of the sternum in close proximity to the LA (Supplemental Figure S1, Panel A).

Pacing protocol: After 10 days of recovery, sheep were assigned to either the Sham-operated group or to one of the atrial tachypacing groups. The sham operated animals had the device programmed in a sensing (OAO) mode only. Pacing voltage output was programmed at least twice the diastolic threshold for 0.4 ms duration to ensure appropriate atrial capture. The automatic mode switch (AMS) mode was enabled in the atrial tachypacing animals in order to avoid unnecessary pacing and allow AF to self-sustain once initiated (Supplemental Figure S1, Panel B). The AMS algorithm reliably generated tachypacing-induced self-sustained AF because the pacemaker resumed pacing only if AF stopped and sinus rhythm was detected. The pacemaker was programmed to induce AF by burst tachypacing; i.e., 30-sec pacing at 20 Hz at twice diastolic threshold followed by 10 sec sensing. The pacemaker resumed pacing only if AF stopped and SR was detected. In addition, devices had the capability of storing information on the history of AF, including the number and duration of AF episodes and the precise moment of each episode’s occurrence. The Holter capabilities of the device were used to record intracardiac electrograms (EGMs) to accurately confirm the occurrence of AF, generate histograms and follow the evolution of AF. This was an attempt to reproduce the actual evolution of human AF, from the initiation of premature atrial beats, to paroxysmal and eventually persistent AF. Persistent AF was then defined as episodes lasting more than 7 days without reversal to sinus rhythm and necessity for resumption of pacing. Thus, in addition to the Sham-operated group (N=7) a subset of animals assigned to the fast atrial pacing group was sacrificed after 7 days of self-sustained AF (Transition group, N=7). The rest of the animals was sacrificed after one year of self-sustained AF (LS-PAF group, N=7). The ILR was programmed to identify AF episodes lasting >6 sec based on RR irregularity during the 10 sec sensing. Both pacemaker and ILR were interrogated weekly during the study period.

Electrogram acquisition and processing: After group assignment, a weekly interrogation was performed. Persistence of sinus rhythm was verified in sham-operated animals and pacemaker memories were checked to detect if spontaneous episodes of AT/AF occurred. Three recordings were obtained in the tachypaced sheep during the follow-up: 1) RA lead tip EGM with a case reference; 2) Standard Lead I of the resting ECG exported at a 512 Hz sampling rate; and 3) ILR single lead recording (representing a LA far-field signal) exported as a vector PDF file. The EGM waveforms encoded in the PDF files were magnified and then digitized in a custom made Matlab program (MathWorks, Natick MA). The digitized signal was then superimposed on the original EGM image for visual inspection. If a miss-match was found, the cause was determined
and adjustments made to correct them and ensure quality data. Recordings obtained by the ILR, whose canister is external to the LA, contain a mixture of atrial and ventricular activity. To analyze the atrial activity, the ventricular activity (dubbed QRST) was subtracted from the original recordings. A principal component analysis based AF estimation (PCA) was used for QRST removal. After QRST removal, a biased-free bidirectional Butterworth band-pass filter (4-35 Hz) was applied to each trace, as previously described. The fast Fourier transform (FFT) was then used as previously described to extract the dominant frequency (DF) from 5 sec-long signals from the ILR and RAA electrograms. Finally, DF values from RAA and ILR electrograms were compared to identify in-vivo differences between the two regions in the left and right atria.

**Serum measurements:** Serum was obtained from all animals at baseline, after the initiation of paroxysmal AF (i.e. as soon as the first episode was detected), at the transition from paroxysmal to persistent AF and after 1 year of self-sustained LS-PAF maintenance. All samples were obtained from a peripheral vein, the serum extracted, and stored at -20°C. PIIINP (Biotang, Waltham MA) levels were measured by enzyme linked immunosorbant assay according the manufacturers’ specifications. The sensitivity (lower detection limit) for the assay was 12.5 ng/ml. All samples were run in duplicate and measured at 450nm.

**Echocardiography:** Echocardiograms were obtained in awaked sheep in the sternal recumbency position using a Vivid Q echocardiograph (GE Healthcare, Horten, Norway) at baseline, at the time of transition from paroxysmal to persistent AF and at the last follow-up for the LS-PAF group. LA and RA dimensions and areas, severity of mitral regurgitation, left ventricular ejection fraction (LVEF), end-systolic and end-diastolic diameters, and septal and posterior wall thickness were evaluated using standard criteria of the American Society of Echocardiography.9

**Heart removal and cell isolation:** After the end of the follow-up, hearts were quickly removed via thoracotomy and placed in a cold cardioplegic solution. LA and RA walls were removed, weighted and cut in three different portions. The posterior portion was used for molecular biology, middle portion was used for histology analysis and the anterior portion was used for cell dissociation. The posterior left atrium (PLA) was sectioned longitudinally and stored for subsequent histology and molecular biology analysis.

Cell isolation was performed as previously described. Left and right atrial samples for dissociation were transferred into a stock solution containing (in mM): NaCl (120), KCl (5.4), MgSO4 (5), Pyruvate (5), Glucose (20), Taurine (20), HEPES (20) and nitrilotriacetic acid (5). Tissue was chopped into chunks of about 1 mm3 with scissors. Chunks were stirred for 12 min in the above-mentioned solution at 37°C oxygenated with 100% O2. Every 3 min, the tissue was transferred to a fresh solution by filtering solution through gauze. Chunks were then transferred to the calcium free protease digestion solution containing (in mM): NaCl (120), KCl (5.4), MgSO4 (5), Pyruvate (5), Glucose (20), Taurine (20), HEPES (20) and protease type XXIV (Sigma) for 45 min. After the end of the protease digestion, chunks were transferred to the collagenase digestion solution containing (in mM): NaCl (120), KCl (5.4), MgSO4 (5), Pyruvate (5), Glucose (20), Taurine (20), HEPES (20), CaCl2 (0.05) and collagenase type I (Worthington) for 2 digestion time points. At 15 and 30 minutes, the filtrate containing myocytes was decanted and centrifuged for 2 min at 500 rpm. Supernatant was aspirated and pellets resuspended in KB solution containing (in mM): L-Glutamic Acid (50), KOH (70), KCl (30), L-Aspartic Acid-K (10), KH2PO4 (10), MgSO4·7H2O (2), Glucose (20), Taurine (20), Creatine (5), EGTA (0.5) and HEPES (10). Myocytes were centrifuged a second time to aspirate supernatant, and resuspended in KB before use.
Cell dimensions (length and width) were measured in the \(I_{Ca}\text{L}\) extracellular solution before the recordings of the currents at 40x from images recorded using a 40x oil-immersion objective lens (N.A. 1.30) attached to a Nikon Eclipse Ti inverted microscope.

**Western Blotting:** Sheep LA and RA tissue samples were washed with protease inhibitors (Roche, protease inhibitor tablet) containing PBS and flash frozen in liquid nitrogen. Frozen tissue (50–100mg) was homogenized in 1ml of lysis buffer containing (in mM): Tris•HCl (25), NaCl (150), EDTA (1), NaF (4), Sodium ortho-vanadate (2), Triton X-100 1% and protease inhibitor. The homogenate was centrifuged at 10000 rpm for 5 minutes; the supernatant was used for western blotting. The tissue lysates (20 μg) were then subjected to one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. The blots were incubated overnight in cold room with one of the following antibodies, mouse monoclonal α-SMA (1:2000); rabbit GAPDH antibody (1:5000) both from Sigma-Aldrich, St. Louis, MO; rabbit Cav1.2 antibody (1:500); rabbit Na\(_{v}\)1.5 antibody (1:500); rabbit KV11.1 antibody (1:1000); rabbit Kv4.2 antibody (1:250) all from Alomone Labs, PO Jerusalem, IL; rabbit Col III antibody (1:1000) from Abcam Cambridge, MA; mouse monoclonal Kir2.3 (1:250) from NeuroMab, Davis, CA. The protein bands were visualized using enhanced chemiluminescence (Thermo Scientific, Rockford, IL).

**Real-time RT-PCR:** Sheep left and right atrial samples were washed in RNase/DNase free ice cold PBS. Clean samples were preserved in RNA stabilizing agent (Ambion, Austin, TX) and stored at -80⁰C till further use. RNA was isolated from the myocardial tissue using RNAeasy kit from Qiagen (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Isolated RNA from these samples was treated with DNase for 15 min at room temperature (Qiagen, Valencia, CA). 2 μg of DNA-free total poly A tail RNA (mRNA) was first subjected to synthesis of cDNA using Oligo dT primers using SuperScript III First-Strand Synthesis System from Invitrogen (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA from 20 ng of total RNA was then subjected to RT-PCR using sybergreen real time PCR master mix (Qiagen, Valencia, CA). For real time PCR sheep specific primers were designed using predicted sequences (Table S4). No-template controls and no-RT controls were run during each experiment to detect any RNA and/or genomic DNA contamination.

**Patch-clamp recordings:** Ion currents and action potentials were recorded in the whole-cell patch-clamp configuration using a MultiClamp 700B amplifier and Digidata 1440A digitizer (Molecular Devices, Sunnyvale, CA). Patch pipettes had resistances of 2 - 6 MΩ when filled with intracellular pipette solution and placed in extracellular solution. After formation of a GΩ seal, the patch membrane was ruptured and cell capacitance (Cm) was determined by integration of capacitive transients elicited by 10-mV hyperpolarizing and depolarizing steps (10 ms duration) from a holding potential of -80 mV. Data acquisition and analysis was performed using pCLAMP software (ver.10.3; Molecular Devices, Sunnyvale, CA). Current amplitudes were divided by Cm and expressed as current densities (pA/pF) to normalize for variable cell sizes. Action potentials and \(I_{K1}\) were adjusted for liquid junction potential.
L-type calcium current ($I_{CaL}$) was recorded with pipette solution containing (in mM): CsCl (120), TEA-Cl (20), MgCl$_2$ (1), Mg-ATP (5.2), HEPES (10), EGTA (10), adjusted to pH 7.2 with CsOH; and extracellular solution containing (in mmol/L): TEA-Cl (148), Na$_2$HPO$_4$ (0.4), MgCl$_2$ (1), glucose (5.5), CsCl (5.4), CaCl$_2$ (1.8), HEPES (15), adjusted to pH 7.4 with CsOH. Activation of $I_{CaL}$ was elicited by 300-ms voltage steps from a holding potential of -50 mV. Amplitude of $I_{CaL}$ was measured as the difference between peak inward current and current at the end of the voltage step.

Sodium currents ($I_{Na}$) were recorded at room temperature (20-22 °C) with pipette resistances <2.8 MΩ when filled with pipette filling solution containing (in mM): NaCl (5), CsF (135), EGTA (10), MgATP (5), Heps (5), pH 7.2. The extracellular bathing solution contained (in mM): NaCl (5), MgCl$_2$ (1), CaCl$_2$ (1.8), CdCl$_2$ (0.1), glucose (11), CsCl (132.5) and Heps (20); pH was maintained at 7.4 with CsOH at room temperature. Appropriate whole-cell capacitance and series resistance compensation (≥60%) was applied along with leak subtraction. To assess the $I_{Na}$ density, cells were held at -160 mV and stepped to various test potentials from -100 to 30 mV in 5 mV increments, with 200 ms duration pulses and 2800 ms interpulse intervals. Voltage-dependent activation of $I_{Na}$ was assessed by generating conductance voltage relationships (m-infinity curves) and fitting the data with a standard Boltzman function (Origin 8.1, Northampton, MA, USA). Voltage-dependence of inactivation was assessed by holding the cells at -160 mV followed by a 300 ms test pulse from -140 to -40 mV in 5 mV increments; interpulse interval was 2700 ms. Recovery from inactivation was studied by holding cells at -160 mV and applying two 20 ms test pulses (S1, S2) to -45 mV, separated by increasing increments of 1 ms to a maximum S1-S2 interval of 50 ms. The S1-S1 interval was kept constant at 2000 ms.

The conventional whole-cell recording technique was employed to record the transient outward K+ current ($I_{to}$). Electrophysiological recordings were conducted at room temperature. The bath solution contained (in mM): NaCl (136), KCl (4), CaCl$_2$ (1.8), MgCl$_2$ (2), HEPES (10), tetrodotoxin (0.03), nifedipine (0.005), pH adjusted at 7.4 with NaOH. Recording pipettes contained (mM): KCl (135), MgCl$_2$ (1), EGTA (10), HEPES (10), glucose (5), pH adjusted at 7.2 with KOH. Borosilicate glass electrodes were pulled with a Brown–Flaming puller (model P-97), yielding appropriate tip resistances when filled with pipette solution < 3 MΩ. Appropriate whole-cell capacitance and series resistance compensation (≥70%) was applied. Leakage compensation was not used. Ito was record using a step protocol with a holding potential of -70 mV and stepping from -40 to +60 mV in 10 mV increments of 5 s at each potential every 20 s. Ito was measured as the difference between the peak current and the current at the end of the 5 s pulse.

Inward rectifier current ($I_{Ki}$) was recorded with pipette solution containing (in mM): KCl (148), MgCl$_2$ (1), EGTA (5), Creatine (2), ATP (5), Phosphocreatine (5); adjusted to pH 7.2 with KOH and extracellular solution containing (in mM): NaCl (148), Na$_2$HPO$_4$ (0.4), MgCl$_2$ (1), Glucose (5.5), KCl (5.4), CaCl$_2$ (1), HEPES (15), pH adjusted at 7.4 with NaOH. Activation of $I_{Ki}$ was elicited by a step protocol utilizing 400-msec steps ranging from -120 to +20 mV in 10 mV increments with a holding potential of -50mV and with 2 seconds between successive steps. 5 µM nifedipine was added to block $I_{CaL}$ channels and the Ca2+-sensitive $I_{Cl}$-BaCl$_2$ (1mM) was used to isolate $I_{Ki}$ from other background currents.

Action potentials were elicited using square wave pulses (30 – 50 pA amplitude, 10 – 30 ms duration) generated by a DS8000 digital stimulator (World Precision Instruments, Sarasota, FL) and recorded at 37°C with pipette solution containing (in mM): MgCl$_2$ (1), EGTA (1), KCl (150), HEPES (5), phosphocreatine (5), K$_2$ATP (4.46), b-hydroxybutyric acid (2), adjusted to pH
7.2 with KOH; and extracellular solution containing (in mM): NaCl (148), NaH₂PO₄ (0.4), MgCl₂ (1), glucose (5.5), KCl (5.4), CaCl₂ (1), HEPES (15), EGTA (1), pH adjusted at 7.2 with NaOH.

APD rate adaptation was analyzed by steady state stimulation at progressive shorter cycle lengths (CL) starting at 1000 ms, decreasing the CL slowly by 100 ms steps down to 300 ms and then by 20 ms steps after 300 ms cycle length. Action potentials at 1000, 500 and 300 ms CL were plotted in the rate adaptation curve.

**Histology**: Tissue samples were sectioned longitudinally to the atrial wall plane at 4 µm, fixed in 10% buffered formalin, embedded in paraffin, and stained with picrosirius red. Patchy and interstitial fibrosis was quantified in both atria and in the PLA at 10x and 20x magnifications, respectively, using the BioQuant software (Bioquant Image Analysis Corporation, Nashville, TN). A minimum of 20 randomly selected pictures per slide were analyzed by a blinded investigator, carefully excluding endocardial, epicardial and peri-vascular regions.

**Computer Simulations**: We implemented modified versions of the Grandi-Pandit⁴ model of the normal human atrial cell to simulate the cardiac action potential and its robust propagation in 2D cardiac tissue. The formulation for the fast sodium current in the original model was replaced with that of a mammalian ventricular myocyte model⁵ to achieve propagation in 2D cardiac tissue. In addition, the maximum conductance value for the inward rectifier potassium current, Iₖᵢ, was increased by 30% to achieve tissue excitability and smooth propagation. The conduction velocity in the tissue was adjusted to 0.58 m/s.⁶,⁷

Atria in SR (equivalent to the Sham group in our study) and at the transition stage from paroxysmal to persistent AF (equivalent to the Transition group) were simulated by modifying the magnitudes of Iₙₐ, Iₖᵳ, Iᵣᵣ, and Iₖᵢ as observed in the experiments (listed in Table S5). Paroxysmal AF was simulated by incorporating all ionic changes similar to that in transition AF, except for Iₖᵳ, whose density was reduced by 30% only, such that the APD₉₀ has values approximately half way in between SR and transition AF. See supplemental tables S5 and S6). The steady-state cardiac action potentials were obtained by pacing the models for 50 seconds at 1 Hz. In all cases, reentry in 2D sheets (6 cm²) was initiated using the S1-S2 cross-field protocol.

**Statistical analyses**: Normally distributed data are expressed as mean ± SEM. Normality of distributions was assessed using the Shapiro-Wilk test. A mixed regression model was applied to multiple group analyses and repeated measured data. Action potential durations (APD) and ionic current densities were compared using a two tailed unpaired Student’s-t tests. RT-PCR and Westerns blot data were analyzed using two-way ANOVA. A p<0.05 was considered statistically significant.

**Supplemental Results**:  

**AF remodeling and Ca²⁺-handling proteins.** We conducted Western blot analyses in five different calcium handling proteins (ryanodine receptor type two-, RyR2; SR Ca²⁺-ATPase, SERCA, phospholamban, PLN; Na⁺-Ca²⁺ exchanger, NCX and Ca²⁺/calmodulin-dependent protein kinase-II, CaMKII) in the left and right atria appendages in each of the three groups of sheep (i.e., sham, transition and persistent AF). As illustrated in Figures S9 and S10, RyR2, SERCA, PLN and CaMKII were either down-regulated or unchanged, while NCX was increased only in the LA. In addition, RyR2 phosphorylation was not affected and thus, the ratio of
pRyR2/total RyR2 was unchanged. Altogether, these results suggest that in this model one should not expect an increase in the spontaneous release of Ca\(^{2+}\) from the SR or that DADs and triggered activity would be involved in transition from paroxysmal to more stable forms of the arrhythmia.

**Roles of individual ionic changes.** Rotors were simulated in 2D sheets, when individual ionic currents were changed, compared to controls. When \(I_{K1}\) alone was increased by 100% compared to controls (Figure S12), the action potential shortened by 23%, the resting membrane potential hyperpolarized by -2 mV (Figure S12A) and the rotors with DF of 4.7 Hz were seen (Figure S12B). When \(I_{CaL}\) alone was decreased by 30% compared to controls (as simulated in paroxysmal AF), APD\(_{90}\) was reduced by 37%, and resulted in a meandering rotor with a frequency of 4.7 Hz (Figure S13A). When \(I_{CaL}\) was decreased by 65%, as observed in persistent AF, APD was greatly reduced (~64%), which provided sufficient substrate for a very stable rotor at 8.0 Hz (Figure S13B). Decreasing \(I_{Na}\) density by 75% resulted in an unstable, meandering rotor with DF = 3.38 Hz (Figure S14), whereas decreasing \(I_{Na}\) density alone by 50% resulted in an unstable rotor with DF of 3.8 Hz (Fig. S15). These simulation results thus indicate that changes in \(I_{K1}\) and \(I_{CaL}\) are key determinants of rotor acceleration in paroxysmal and transition AF.

**Fast versus slow transition.** To search for determinants of the rate of AF progression, we separated slow and fast progressing animals sacrificed at transition depending on the median time to progression (<45 days: 4 animals; >45 days: 3 animals). As expected, dDF/dt was significantly larger for the animals progressing quickly to persistent AF (p=0.005 for LA), which also showed significantly shorter APDs in the LA than the slow transition animals, with a trend for shorter RA APDs in the former than the latter (Figure S16B). A trend for smaller \(I_{CaL}\) current densities suggested a mechanism for the APDs in the fast transition sheep (Figure S16C). Conversely, \(I_{K1}\) appeared to be relatively larger in slow transition animals (Figure S16D) which had significantly larger atrial dimensions (124% vs. 45% increase in LA atrial area; p=0.014, Figure S17). However, the degree of fibrosis, the cell dimensions and atrial weights were similar in both groups. Accordingly the major factor contributing to the larger dDF/dt in the fast transition animals was greater APD shortening secondary to \(I_{CaL}\) reduction. On the other hand, the slow transition animals seemed to require an additional thrust to achieve AF persistence, which was brought about by additional \(I_{K1}\) increase and greater structural remodeling (Figure S17C and D).

**References**


Supplemental Tables:

Table S1: Echocardiographic findings. (LVEF = left ventricular ejection fraction; LA = left atria; RA = right atria; *p<0.05 vs sham; †p<0.05 vs Transition)

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Transition AF</th>
<th>LS-PAF</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LVEF (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>73.7±2.4</td>
<td>75.3±1.7</td>
<td>73.7±2.2</td>
<td>0.82</td>
</tr>
<tr>
<td>Last follow-up</td>
<td>75.5±1.4</td>
<td>75.6±1.0</td>
<td>72.7±1.7</td>
<td>0.30</td>
</tr>
<tr>
<td><strong>LA area (cm²)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.6±0.3</td>
<td>7.2±0.4</td>
<td>7.7±0.2</td>
<td>0.56</td>
</tr>
<tr>
<td>Last follow-up</td>
<td>10.0±0.8</td>
<td>12.8±1.1</td>
<td>20.9±2.1*</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>RA area (cm²)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5.2±0.4</td>
<td>5.5±0.2</td>
<td>5.9±0.3</td>
<td>0.30</td>
</tr>
<tr>
<td>Last follow-up</td>
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<td>8.6±1.0</td>
<td>14.3±1.0*</td>
<td>0.006</td>
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<tr>
<td><strong>Mitral regurgitation, /4</strong></td>
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</tr>
<tr>
<td>Baseline</td>
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<td>0.19</td>
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<tr>
<td>Last follow-up</td>
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<td>0.8±0.3</td>
<td>1.2±0.2*</td>
<td>0.03</td>
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Table S2. Regional dry weights in the atria of sham, transition and LA-PAF animals.

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<tr>
<th>Tissue Region</th>
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<th>Transition</th>
<th>LA=PAF</th>
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<tbody>
<tr>
<td>LA</td>
<td>6.5 ± 0.6 g</td>
<td>10.6 ± 1.3 g (NS)</td>
<td>15.4 ± 2.1 g (p&lt;0.02)</td>
</tr>
<tr>
<td>RA</td>
<td>7.4 ± 0.8 g</td>
<td>9.9 ± 1.2 g (NS)</td>
<td>17.3 ± 2.8 g (p&lt;0.04)</td>
</tr>
<tr>
<td>PLA</td>
<td>9.8 ± 1.7 g</td>
<td>13.9 ± 1.3 g (NS)</td>
<td>20.1 ± 2.9 g (p&lt;0.04)</td>
</tr>
</tbody>
</table>
Table S3: Structural remodeling associated with AF. (RA = right atria; LA = left atria; PLA = posterior left atria; *p<0.001 vs sham; †p=0.005 vs sham; ‡p<0.001 vs sham and Transition AF; §p<0.05 vs sham).

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Transition AF</th>
<th>LS-PAF</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td><strong>LA cell size</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length, µm</td>
<td>153.3 ± 4.1</td>
<td>188 ± 4.0*</td>
<td>186.1 ± 4.1*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Width, µm</td>
<td>16.2 ± 0.4</td>
<td>18.2 ± 0.4†</td>
<td>19.0 ± 0.4*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>RA cell size</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length, µm</td>
<td>155.4 ± 3.7</td>
<td>161.9 ± 4.2</td>
<td>168.8 ± 3.8</td>
<td>0.07</td>
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<tr>
<td>Width, µm</td>
<td>17.0 ± 0.9</td>
<td>18.3 ± 0.4</td>
<td>21.4 ± 0.5§</td>
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<tr>
<td><strong>Patchy Fibrosis, %</strong></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>RA</td>
<td>5.4±0.4</td>
<td>5.4±0.4</td>
<td>6.3±0.6</td>
<td>0.30</td>
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<tr>
<td>LA</td>
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<td>5.8±0.8</td>
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<tr>
<td>PLA</td>
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<td>6.6±0.8</td>
<td>9.3±1.2</td>
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<tr>
<td><strong>Interstitial Fibrosis, %</strong></td>
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<tr>
<td>RA</td>
<td>5.1±0.9</td>
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<tr>
<td>LA</td>
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<td>7.0±0.6</td>
<td>10.7±1.5§</td>
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<tr>
<td>PLA</td>
<td>4.1±0.6</td>
<td>7.9±0.7</td>
<td>14.6±1.4‡</td>
<td>&lt;0.001</td>
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Table S4: Primers used for RT-PCR.

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<th>Reverse Primer (5'-3')</th>
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<tr>
<td>CACNA1C</td>
<td>Cav1.2</td>
<td>XM_004007606.1</td>
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<td>GAGGTAAGCGTTGGGTGAAG</td>
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<td>TGAGGTAGAGGTCCAGGAT</td>
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<td>Kv4.2</td>
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<td></td>
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<td>CGGGGATCTTGTACTCCTC</td>
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<td></td>
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<td>GCCTGGTTGTCAGGTCTAT</td>
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Table S5: Modifications in currents of GP atrial model to implement paroxysmal and transition AF.

<table>
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<tr>
<th>Current</th>
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<th>Transition AF</th>
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<tr>
<td>(I_{Na})</td>
<td>-50%</td>
<td>-50%</td>
</tr>
<tr>
<td>(I_{CaL})</td>
<td>-30%</td>
<td>-65%</td>
</tr>
<tr>
<td>(I_{K1})</td>
<td>+100%</td>
<td>+100%</td>
</tr>
<tr>
<td>(I_{to})</td>
<td>-75%</td>
<td>-75%</td>
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Table S6: Action potential durations (APDs) obtained in the three models: sham, paroxysmal and transition AF.

<table>
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<tr>
<th>APD</th>
<th>Sham (ms)</th>
<th>Paroxysmal AF (ms)</th>
<th>Transition AF (ms)</th>
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<tr>
<td>APD_{30}</td>
<td>5.5</td>
<td>15.0</td>
<td>8.5</td>
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<tr>
<td>APD_{50}</td>
<td>42.0</td>
<td>64.5</td>
<td>31.0</td>
</tr>
<tr>
<td>APD_{90}</td>
<td>203.0</td>
<td>168.5</td>
<td>99.5</td>
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Supplemental Figures:

**Figure S1: Experimental and pacing protocols.** A: Fluoroscopy image showing the RA lead screwed to the right atrial (RA) appendage (arrowhead) and the implantable loop recorder (ILR, black arrow) fixed subcutaneously in close proximity to the left atrium (LA). B: Top: A 30-second burst of tachypacing (20 Hz) during sinus rhythm (SR) induces a short-lasting episode of AF; bottom: intracardiac electrogram recorded from the RA showing AF termination (left), detection of SR by the automatic mode switching (AMS) algorithm and automatic resumption of pacing.
Figure S2: Echocardiographic evidence of sustained AF-induced atrial dilatation. Parasternal long-axis view of the heart of a sham-operated (A) and a long-standing persistent AF animal (B). Note substantial atrial dilatation in B (scale bar on the left of each image in cm).
Figure S3: Quantification of echocardiographic findings. A: Left ventricular ejection fraction (LVEF) did not change over the time of the study. B and C: Both atria were significantly dilated in the LS-persistent AF animals. D: Mitral valve regurgitation, measured in arbitrary units (AU) of severity, where 1 is mild and 4 is severe, was significant in LS-persistent AF animals. *p<0.05 vs. sham, #p<0.05 vs. transition; N=6, N=7 and N=7 for the sham-operated, transition and LS-PAF groups. N = number of animals.
Figure S4. After the heart was explanted, the atria were removed and cut in the following three sections: RA wall (panel A), PLA (panel B) and LA wall (panel C). The posterior part of the PLA was used for histology (fibrosis analysis), whereas both RA and LA walls were cut in three pieces: anterior (A), medial (M), and posterior (P). PLA and posterior LA differ since they were separated from each other by the pulmonary veins (which can be seen in the PLA photography). Scale bar = 1 cm.
Figure S5: Sustained AF induces atrial myocyte hypertrophy. A: Average lengths and widths for cells isolated from RA (open symbols) and LA (filled symbols). N=3/n=60 (sham), N=4/n=70 (transition), and N=5/n=90 (LS-PAF). B: Representative phase contrast micrographs. For RA LS-PAF myocytes: ‡p=0.001 vs. sham for width. For LA Transition and LS-PAF myocytes: *p=0.01 and **p<0.001 vs. sham for width and length, respectively; #p<0.001 and ##p=0.002 vs. RA LS-PAF myocytes for width and length, respectively. N = number of animals; n = number of cells.
Figure S6: Sustained AF increases serum levels of Procollagen III N-Terminal Propeptide (PIIINP). *p< 0.001 vs. sham, #p=0.06 vs. baseline.
Figure S7: Relationship between DF and time to transition to persistent AF. No correlation was found between the DF at transition and the time from the first episode of AF to the transition to self-sustained persistent AF.
Figure S8: Correlations between dDF/dt measured from the signal obtained through the RA intracardiac lead, the ILR and the ECG (lead I). A correlation between the dDF/dt measured from LA and RA (left), ECG and LA (middle) and ECG and RA (right) was observed.
Figure S9: Calcium handling protein changes. Western blot analysis of SERCA (panel A), phospholamban (Panel B), Sodium-calcium exchanger (NCX, panel C) and CaMKII (panel D). Left: representative blots; right: quantification of protein expression relative to GAPDH. N=6 per group. N= number of animals.
Figure S10: Ryanodine receptor (RyR2) changes. Western blot analysis of RyR2 (top), and phosphorylated RyR2 (pRyR2, bottom). Left: representative blots; right: quantification of protein expression relative to GAPDH. N= 6 per group. N = number of animals.
Figure S11: Sustained AF reduces functional expression of the transient outward potassium channel (I_{to}) but not hERG. A and B: Current-voltage relationships for I_{to} in cells from the LA (A) and the RA (B). For the LA: N=2/n=6 (sham), N=3/n=5 (transition), N=6/n=10 (LS-PAF); for the RA: N=2/n=6 (sham), N=3/n=5 (transition), N=3/n=5 (LS-PAF). *p<0.05 vs. sham for the transition and LS-PAF groups. C and D: Western blot analysis of Kv4.2 and Kv11.1 protein expression in LA (C) and RA (D) tissue homogenates. Top, Representative blots; bottom, Quantification of protein expression relative to GAPDH. N=6. Note significant reductions of Kv4.2, but not Kv11.1 (hERG) *p<0.05 vs. sham, **p<0.01 vs. sham. N= number of animals.
**Effect of increase in $I_{K1}$ (100%) only**

**Figure S12**: Effects of increasing $I_{K1}$ alone in the Grandi-Pandit human atrial model. A: Increasing $I_{K1}$ by 100% alone as seen in myocytes from transition sheep hyperpolarized the resting membrane potential by -2 mV and significantly shortened the APD (23%) with respect to sham. B: $I_{K1}$ increase alone resulted in a meandering rotor at 4.7 Hz.
Figure S13: Effects of reducing $I_{\text{CaL}}$ alone in the Grandi-Pandit human atrial model. A: When $I_{\text{CaL}}$ was reduced by 30%, as simulated in paroxysmal AF, $\text{APD}_{50}$ and $\text{APD}_{90}$ were reduced (~37%), which resulted in a meandering rotor that eventually died out. B: When $I_{\text{CaL}}$ was reduced by 65%, as observed in transition AF, $\text{APD}_{50}$ and $\text{APD}_{90}$ were greatly reduced (~64%). This reduction in APD provided sufficient substrate for a very stable rotor with DF=8.0 Hz.
Figure S14: Effects of reducing $I_{to}$ alone in the Grandi-Pandit human atrial model. A. reducing $I_{to}$ by 75% resulted in only slight increases in APD$_{30}$ and APD$_{50}$. B: This condition yielded a meandering and unstable rotor whose DF was 3.38 Hz.
Figure S15: Effects of reducing $I_{Na}$ alone in the Grandi-Pandit human atrial model. A. reducing $I_{Na}$ by 50% negligibly changed APD$_{90}$. B: this condition resulted in an unstable rotor whose DF was 3.82 Hz.
Figure S16: Electrophysiological differences between fast and slow transition animals. A. dDF/dt was significantly higher in fast transition sheep (0.07±0.02 Hz/day; N=7) than slow transition sheep (0.02±0.007 Hz/day; N=7; **p=0.007; *p=0.036). B. Mean APD at 30-90% repolarization was shorter in fast than slow transition animals. C, I_{CaL} tended to be lower in fast than slow transition animals; top, LA bottom, RA. D, I_{K1} tended to be larger in slow transition animals. N=4, fast transition; N=3 slow transition sheep. N= number of animals.
Figure S17: Structural differences between fast and slow transition animals. LA area was significantly increased in both groups (**p=0.007; *p<0.05), although a more pronounced atrial dilatation was observed in slow transition animals (124% vs. 45% increase in LA atrial dilatation; p=0.014, panel A). Trends for higher degree of fibrosis (panel B), longer and wider cells (panel C) and heavier atria (panel D) were observed. N=4 fast transition; N=3 slow transition sheep. N = number of animals.