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Galectin-3 Regulates Atrial Fibrillation Remodeling and Predicts Catheter Ablation Outcomes

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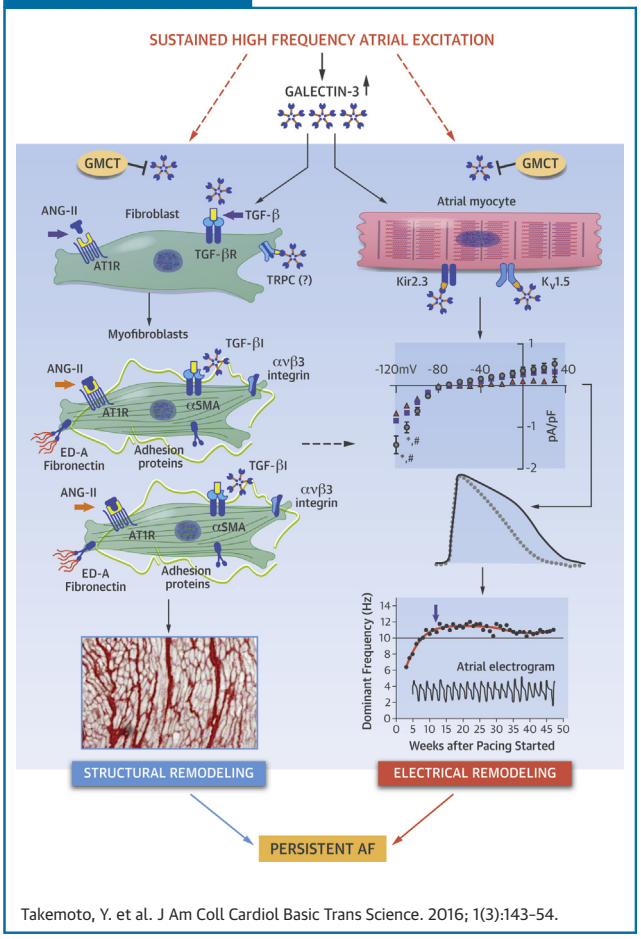
VISUAL ABSTRACT



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HIGHLIGHTS

- **Intracardiac serum galectin (Gal)-3 levels are shown to be greater in patients with persistent than paroxysmal atrial fibrillation (AF), and the Gal-3 level was an independent predictor of AF recurrences after a single ablation procedure.**
- **In a sheep model, the Gal-3 inhibitor GM-CT-01 (GMCT) reduced atrial fibroblast proliferation in vitro.**
- **GMCT mitigated atrial dilation, myocyte hypertrophy, fibrosis, and the expected increase in DF during transition to persistent AF.**
- **GMCT-treated sheep hearts had longer action potential durations, and fewer rotors and wavebreaks during AF than control.**
- **GMCT increased the number of spontaneous AF terminations and reduced overall AF burden.**

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ABBREVIATIONS AND ACRONYMS

AF = atrial fibrillation

APD_{90} = action potential duration at 90% repolarization

BNP = brain natriuretic peptide

CRP = C-reactive protein

CS = coronary sinus

DF = dominant frequency

Gal = galectin

GMCT = GM-CT-01

LA = left atrium

PLA = posterior left atrium

SMA = smooth muscle actin

TGF = transforming growth factor

SUMMARY

Atrial fibrillation (AF) usually starts as paroxysmal but can evolve relentlessly to the persistent and permanent forms. However, the mechanisms governing such a transition are unknown. The authors show that intracardiac serum levels of galectin (Gal)-3 are greater in patients with persistent than paroxysmal AF and that Gal-3 independently predicts atrial tachyarrhythmia recurrences after a single ablation procedure. Using a sheep model of persistent AF the authors further demonstrate that upstream therapy targeting Gal-3 diminishes both electrical remodeling and fibrosis by impairing transforming growth factor beta–mediated signaling and reducing myofibroblast activation. Accordingly, Gal-3 inhibition therapy increases the probability of AF termination and reduces the overall burden of AF. Therefore the authors postulate that Gal-3 inhibition is a potential new upstream therapy to prevent AF progression. (J Am Coll Cardiol Basic Trans Science 2016;1:143–54)

Atrial fibrosis is a hallmark of atrial fibrillation (AF)-induced structural remodeling and plays an important role in AF perpetuation (1). The β-galactoside binding lectin galectin (Gal)-3 is highly expressed in fibrotic tissues (2–4); it associates with cardiac fibrosis during heart failure (5) and increases risk for heart failure and mortality (6). Gal-3 contains a carbohydrate-binding domain linked by an amino-terminal nonlectin region that favors formation of Gal-3 pentamers (7). Gal-3 pentamers bind to transforming growth factor (TGF)-β receptors causing cell surface retention and internalization of the receptors, which is important for mediating duration and directionality of signaling through Smad and Akt proteins (8,9).

Recent studies demonstrated that serum Gal-3 is elevated in AF patients with preserved left ventricular function (10). Contrastingly, others suggested that elevation of Gal-3 was driven by cardiometabolic comorbidities and not heart rhythm (11). In the present study, we endeavored to determine whether Gal-3 mediates structural and electrical remodeling during AF progression. We conducted a blinded saline-controlled study in atrially tachypaced sheep with persistent AF (12) to investigate the effects of GM-CT-01 (GMCT), a galactomannan that inhibits Gal-3 by binding to its carbohydrate-binding domain (4). Our results suggest a role for Gal-3 in atrial remodeling of persistent AF in humans, and provide compelling experimental evidence that Gal-3 inhibition reduces AF burden by mitigating structural and electrical remodeling.

METHODS

An expanded methods section is available in the Supplemental Appendix.

CLINICAL STUDY PROTOCOL AND FOLLOW-UP. All clinical procedures were approved by the Institutional Review Board of the University of Michigan. An electrophysiologic study was performed under conscious sedation. Blood samples were collected from the coronary sinus (CS) and left atrium (LA) for measurement of Gal-3, C-reactive protein (CRP), and brain natriuretic peptide (BNP). Mean AF cycle length was measured on lead V_1 and correlated with Gal-3 levels in both CS and LA. Catheter navigation, mapping and ablation were performed with the guidance

Fibrillation" (Drs. Jalife, Berenfeld and Oral), and University of Michigan Health System - Peking University Health Science Center Joint Institute for Translational and Clinical Research (UMHS-PUHSC) project: Molecular Mechanisms of Fibrosis and the Progression from Paroxysmal to Persistent Atrial Fibrillation (Dr. Jalife). Dr. Noujaim was supported by NIH grant (R00HL105574). Dr. Takemoto has been supported by the JHRS fellowship program from Medtronic Japan as well as the Uehara Memorial Foundation, and by a postdoctoral fellowship from the American Heart Association (14POST18220000). Dr. Berenfeld serves as the Scientific Officer for and owns stock in Rhythm Solutions Inc.; he has served as a consultant for Acutus Medical; and the modified pacemakers used for tachypacing were a donation by St. Jude Medical to his lab at the University of Michigan. Drs. Berenfeld and Jalife have received research grant support Medtronic, Inc. Dr. Oral is the Chief Medical Officer for Rhythm Solutions Inc. Dr. Jalife has served as a consultant for Topera-Abbott Laboratories. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose. Drs. Takemoto, Ramirez, and Yokokawa contributed equally to this work. Drs. Oral and Jalife contributed equally to this work.

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of an electroanatomical mapping system (CARTO-3, Biosense Webster, Inc., South Diamond Bar, California). The ablation strategy for AF involved antral pulmonary vein isolation followed by ablation of complex fractionated atrial electrograms, when needed. Patients were seen in an outpatient clinic 3 months after the procedure and every 6 months thereafter. Any atrial arrhythmias (tachycardia, flutter, or fibrillation) lasting >30 s, 3 months after the ablation indicated recurrence.

PROTOCOL FOR BLINDED SALINE-CONTROLLED SHEEP TRIAL. All animal procedures were approved by the University of Michigan Committee on Use and Care of Animals and complied with National Institutes of Health Guidelines. Twenty-four male sheep (≈40 kg) underwent subcutaneous pacemaker implantation with an atrial lead attached to the right atrial appendage. After 1 month recovery, sheep were randomized to: 1) saline-treated AF (control); 2) GMCT-treated AF; or 3) sham-operated non-AF groups. Intermittent tachypacing was initiated in the AF animals with automatic mode switch enabled for tachypacing only during detected episodes of sinus rhythm. Device onboard memory was used to store number, duration and timing of AF episodes, as well as intracardiac electrograms for post-acquisition analysis. After 35 days of self-sustained AF without pacing, animals were euthanized and hearts were removed for either ex vivo optical mapping, or patch-clamping and molecular biology experiments. Investigators were blinded to treatment until the end of follow-up.

RESULTS

Fifty-five patients (Supplemental Table 1) who underwent de novo radiofrequency ablation to eliminate paroxysmal (n = 29) or persistent AF (n = 26) were investigated. Serum Gal-3, CRP, and BNP were measured in blood samples from the LA cavity and CS prior to ablation (**Figure 1A**). Gal-3 levels were greater in patients with persistent than paroxysmal AF, but CRP and BNP levels were not significantly different. Notably, mean AF cycle length in V_1 was inversely related to Gal-3 serum levels in both LA and CS (**Figure 1B**), and LA volume index correlated positively with Gal-3 serum levels in both LA and CS (**Figure 1C**).

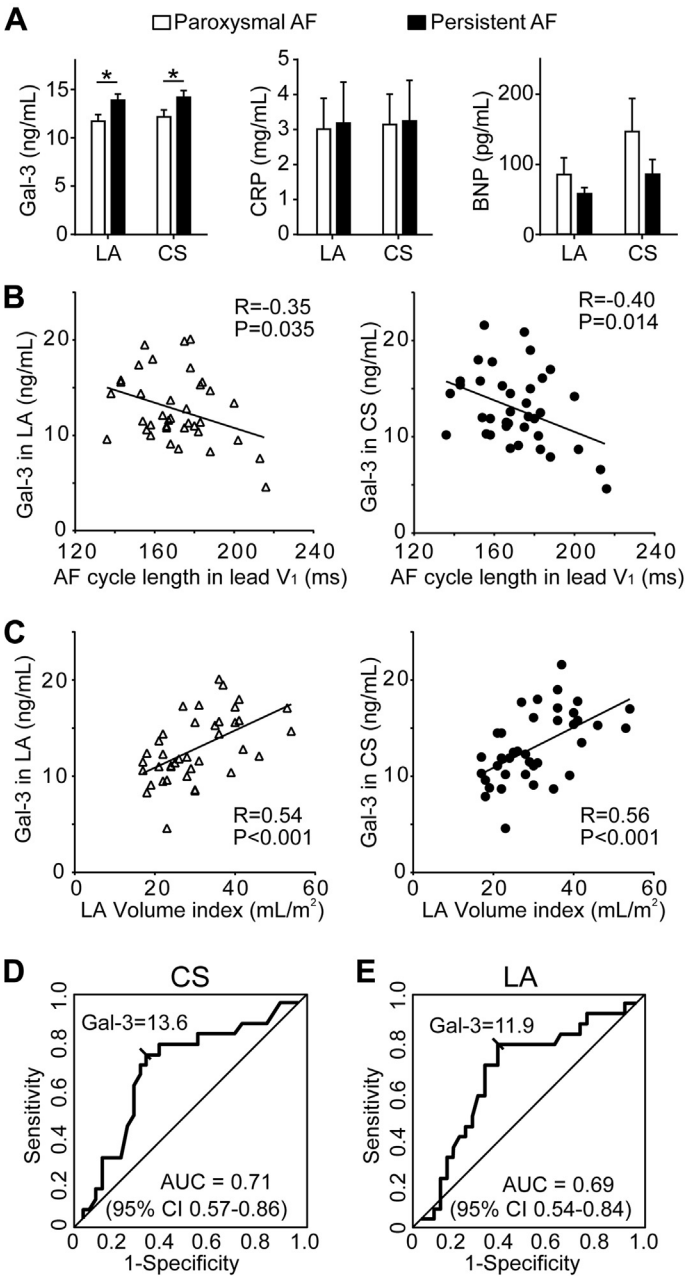
During 12 months of follow-up after a single radiofrequency ablation, 24 of 29 patients with paroxysmal AF and 15 of 26 patients with persistent AF were in sinus rhythm. Gal-3 levels in CS and LA were higher in patients with than without recurrent atrial arrhythmias (CS: 14.9 ± 0.7 ng/ml vs. 12.1 ± 0.6 ng/ml, p = 0.006; and LA: 14.1 ± 0.7 ng/ml vs. 11.9 ± 0.6 ng/ml, p = 0.027). Multivariate analysis revealed Gal-3 levels in CS to be the only independent predictor of recurrent atrial arrhythmias after a single radiofrequency ablation among biomarkers tested (odds ratio: 1.20; 95% confidence interval; 1.01 to 1.45; p = 0.049) (Supplemental Table 2). Receiver-operating characteristic curve analysis demonstrated that a Gal-3 level >13.05 ng/ml in CS and >11.9 ng/ml in LA (**Figure 1D**) was predictive of recurrences after a single ablation with 76% sensitivity and 71% specificity, and 81% sensitivity and 65% specificity, respectively.

PROLIFERATION OF SHEEP ATRIAL MYOFIBROBLASTS IN VITRO. We conducted in vitro experiments to investigate whether Gal-3-regulated profibrotic pathways mediate atrial fibrosis in AF through activation of fibroblasts. The level of transcript of *LGALS3* coding for Gal-3 was similar in sheep lung and LA fibroblasts, and Western blot analysis confirmed Gal-3 protein in atrial myofibroblasts (data not shown). In cultured sheep atrial myofibroblasts, GMCT inhibited Gal-3-induced enhancement of migration and wound healing, a measure of myofibroblast activation (Supplemental Figure 1). Treatment with either recombinant human Gal-3 or TGF-β1 increased the number of atrial myofibroblasts; and the effects were blocked by GMCT (**Figures 2A and 2B**). Gal-3-induced proliferation was fully blocked by TGF-β1 neutralizing antibody (**Figure 2C**). GMCT can also bind to Gal-1 (13), suggesting that Gal-1 might mediate the preventive effects of GMCT on fibroblast proliferation; however, fibroblast proliferation assays demonstrated that Gal-1 increased fibroblast proliferation but GMCT did not inhibit the Gal-1 effects (**Figure 2D**).

STRUCTURAL REMODELING DURING AF PROGRESSION IN SHEEP. We then conducted a blinded, placebo-controlled study in atrially tachypaced sheep. Twenty-four sheep were randomized to intravenous GMCT treatment (12 mg/kg; n = 10) twice per week, intravenous saline treatment (n = 10) twice per week or sham (n = 4). We defined 4 time points from the start of tachypacing: 1) first AF episode (1st-AF); 2) first 1-day-long AF episode (1d-AF); 3) first continuous 7-day stretch of persistent AF (7d-PeAF); and 4) first continuous 35-day stretch of persistent AF (35d-PeAF).

Body weight gain was comparable between GMCT and saline-treated groups. No animals developed heart failure (Supplemental Table 3). The AF-associated LA dilation was significantly attenuated in GMCT-treated compared to saline-treated animals (**Figure 3A**), which matched directly measured atrial

FIGURE 1 Serum Gal-3 in AF Patients and Receiver-Operator Characteristic Curve



(A) Fibrosis and inflammation biomarkers in patients with paroxysmal and persistent atrial fibrillation (AF). **(B)** Mean AF cycle length in lead V_1 is inversely related to galectin (Gal)-3 in both coronary sinus (CS) and left atrium (LA). **(C)** LA volume index is related to Gal-3 in both LA and CS. Receiver-operator characteristic curve of Gal-3 levels in both CS **(D)** and LA **(E)** differentiating patients with and without recurrent atrial arrhythmias after a single ablation procedure. AUC = area under the curve; CI = confidence interval; CRP = C-reactive protein. *$p < 0.05$.

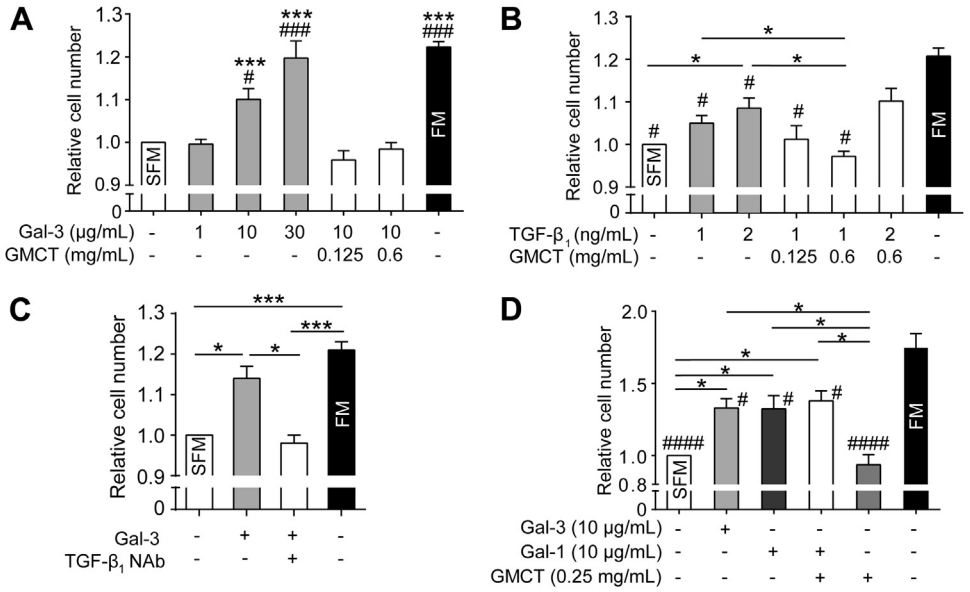
areas (**Figure 3B**, Supplemental Figure 2). At the myocyte level, cell areas from the saline-treated group were significantly increased over sham, but increases were attenuated by GMCT (**Figure 3C**).

Procollagen type III N-terminal peptide, a serum marker for collagen synthesis (14), increased progressively in saline-treated animals, but significantly decreased from baseline with GMCT treatment (**Figure 4A**). In addition, at 35d-PeAF, tissue levels of Gal-3 and TGF-β1 proteins in LA were significantly increased but Gal-1 was decreased; those effects were not affected by GMCT (**Figure 4B**). In keeping with the procollagen III N-terminal propeptide data, fibrotic areas in LA and posterior LA (PLA) and protein levels of α-smooth muscle actin (SMA) from the LA and PLA were significantly lower in GMCT-treated than saline-treated group, and were similar to sham (**Figures 4C and 4D**). GMCT treatment significantly inhibited TGF-β1-mediated signaling pathways as indicated by a decrease in activation of Smad2/3 proteins (**Figure 4E**).

IONIC AND MOLECULAR MECHANISMS OF ELECTRICAL REMODELING. As AF progressed, atrial dominant frequency (DF) increased faster in saline-treated than GMCT-treated groups, and DFs were significantly higher at 35d-PeAF in saline-treated than GMCT-treated animals (**Figure 5A**). Optical mapping was conducted at final evaluation ($n = 12$). Regional maximal DF was significantly reduced in LA and PLA in GMCT-treated compared to saline-treated groups (**Figures 5B and 5C**). Rotors and wavebreaks (singularity points) were counted as described previously (15); the number of rotations, singularity points and areas of singularity point trajectory during AF were significantly reduced in GMCT-treated compared to saline-treated animals (**Figures 5D to 5F**). The results observed in right atrium in vivo and ex vivo were consistent with LA and PLA (data not shown). Altogether, GMCT diminished the electrical and structural substrates that maintain AF. In contrast to atrial effects, GMCT did not alter ventricular function or prolong QT or QTc at final evaluation.

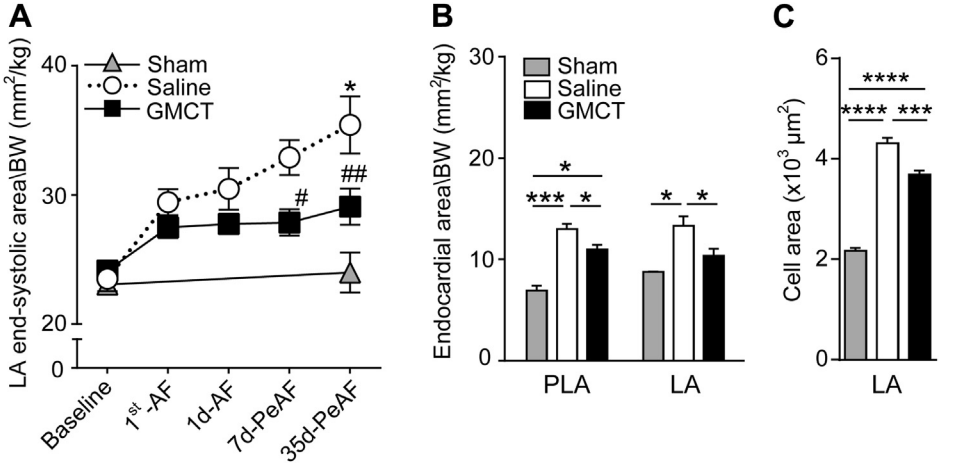
At 35d-PeAF AF-induced shortening of action potential duration at 90% repolarization (APD_{90}) measured optically was significantly attenuated by GMCT (**Figures 6A and 6B**). Conduction velocity during pacing was identical for saline-treated and GMCT-treated groups. Wavelength, the product of APD_{90} and conduction velocity, was longer in GMCT-treated animals in LA and right atrium at cycle length 400 ms (184.8 ± 6.1 mm vs. 161.4 ± 10.6 mm, $p < 0.01$; 241.3 ± 16.7 mm vs. 194.3 ± 14.3 mm, $p < 0.01$, respectively).

FIGURE 2 **In Vitro Proliferation of Sheep Atrial Myofibroblasts**



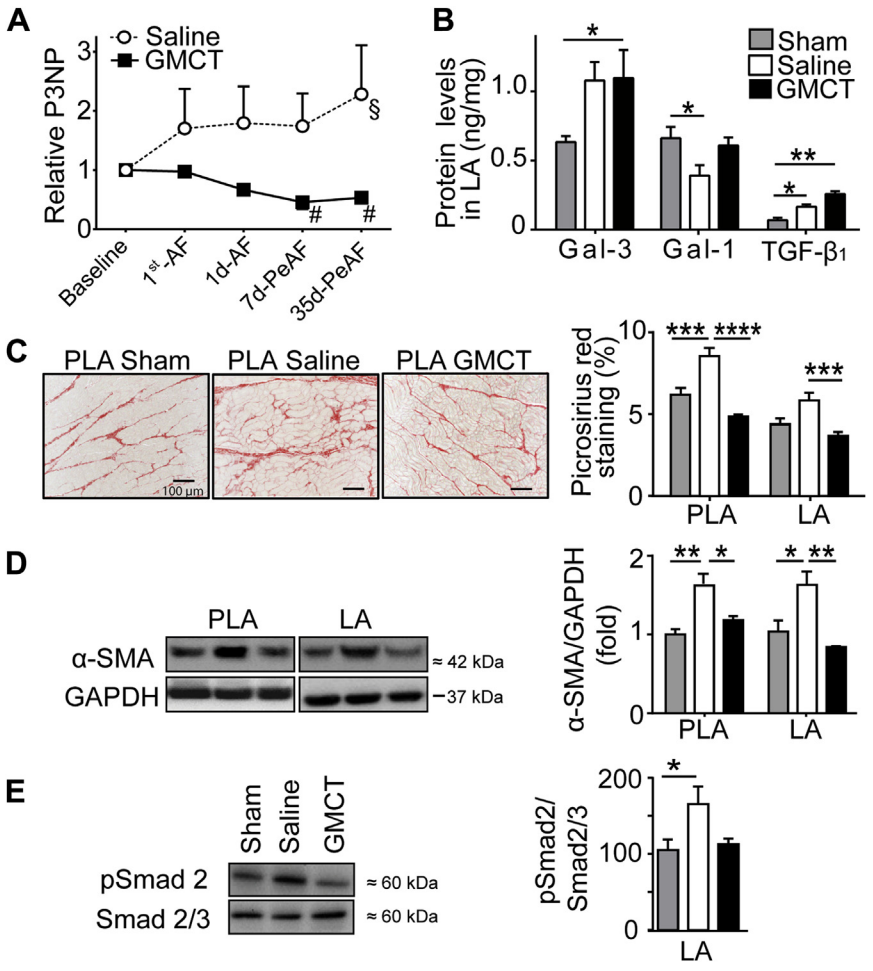
(A) Galectin (Gal)-3 increases proliferation of sheep atrial myofibroblasts; effects are blocked by GM-CT-01 (GMCT). SFM: treatment with serum free medium, negative control; FM: treatment with full media (10% FBS), positive control. N = 2 to 3, n = 4 to 12; ***p < 0.001 versus SFM, Gal-3 (1 μg/ml), Gal-3 (10 μg/ml) + GMCT (0.6 mg/ml); #p < 0.05, ###p < 0.001 versus Gal-3 (10 μg/ml) + GMCT (0.125 mg/ml). **(B)** Transforming growth factor beta (TGF-β1) increases proliferation of sheep atrial myofibroblasts; effects are blocked by GMCT (N = 2 to 3, n = 4 to 12; *p < 0.05, #p < 0.05 vs. FM). **(C)** Pre-treatment with a TGF-β1 neutralizing antibody (TGF-β1 NAb, 1 ng/ml) blocks Gal-3-induced (10 μg/ml) increase in proliferation (*p < 0.05, *** p < 0.001; N = 3, n = 4 to 18). **(D)** Gal-1 increases proliferation of sheep atrial myofibroblasts; effects are not blocked by GMCT (N = 5, n = 13 to 15; *p < 0.05, #p < 0.05, and ####p < 0.0001 vs. FM). N = number of animals; n = number of dishes.

FIGURE 3 **GMCT Mitigates Structural Remodeling During AF Progression**



(A) Echocardiographically obtained adjusted left atrial areas in sham (N = 4), saline-treated (N = 10), and GM-CT-01 (GMCT)–treated groups (N = 10). *p < 0.05 versus sham; #p < 0.05, ##p < 0.01 versus saline-treated group. One-way analysis of variance for each time point. **(B)** Directly measured left atrial areas in sham (n = 4), saline-treated (N = 10), and GMCT-treated groups (N = 10). *p < 0.05, ***p < 0.001. **(C)** Single isolated left atrial cell areas. N/n = 4/192 (sham), N/n = 4/226 (saline), and N/n = 4/234 (GMCT). N = number of animals; n = number of cells. ***p < 0.001, ****p < 0.0001. 1d-AF = first 1-day-long atrial fibrillation episode; 1st-AF = first atrial fibrillation episode; 7d-PeAF = first continuous 7-day stretch of persistent atrial fibrillation; 35d-PeAF = first continuous 35-day stretch of persistent atrial fibrillation; AF = atrial fibrillation; LA = left atrium; PLA = posterior left atrium.

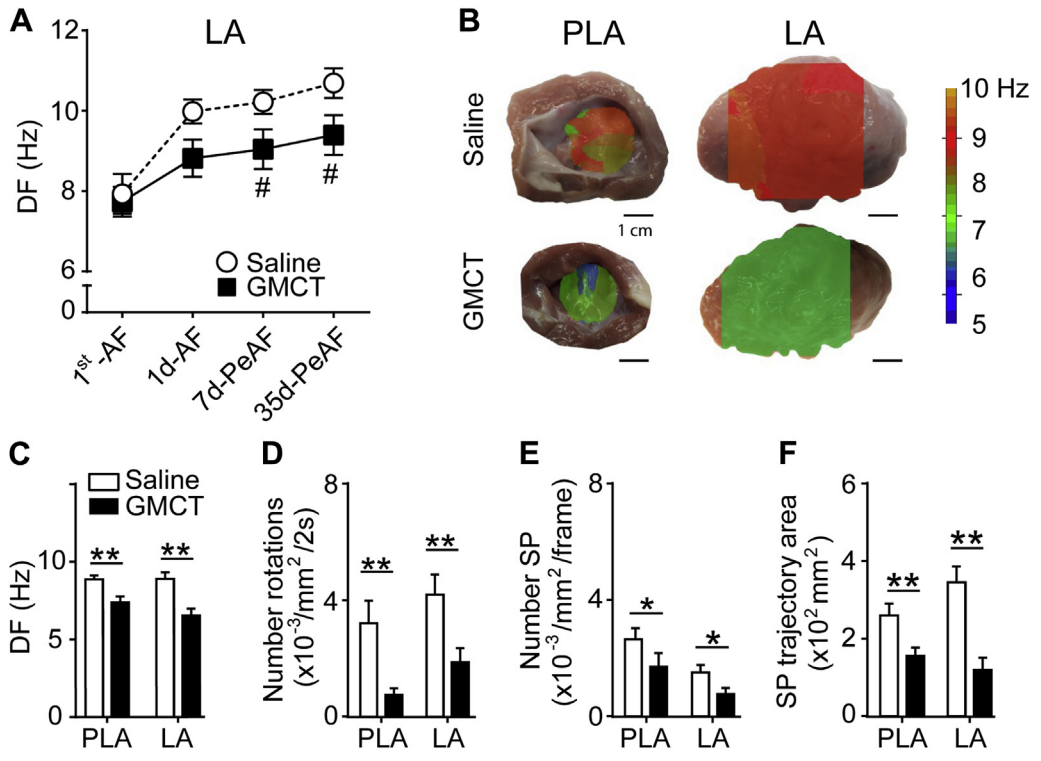
FIGURE 4 GMCT Reduces Atrial Fibrosis



(A) Serum levels of procollagen III N-terminal propeptide (P3NP) during atrial fibrillation (AF) progression in saline-treated and GM-CT-01 (GMCT)-treated (N = 10, respectively) groups. Repeated measures 2-way analysis of variance with Bonferroni multiple comparisons test, §$p < 0.05$ versus GMCT at 35d-PeAF, #$p < 0.05$ versus GMCT at baseline. **(B)** Protein levels of galectin (Gal)-3, Gal-1, and transforming growth factor (TGF)-β1 in left atrium (LA) tissue samples (N = 4, respectively). **(C) Left**: Representative picrosirius red staining of posterior left atrium (PLA). **Right**: Percentage of picrosirius red showing interstitial fibrosis from sham (N = 4), saline-treated (N = 10), and GMCT-treated (N = 10) groups. **(D) (Left)** Representative blots of α-smooth muscle actin (SMA) from **(left lane)** sham, **(middle lane)** saline, and **(right lane)** GMCT. **(Right)** α-SMA in atrial homogenates in sham, saline-treated, and GMCT-treated (N = 4, respectively) groups. **(E)** pSmad2(Ser465/467)/Smad2/3 in LA tissue samples (N = 3 to 4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. 1d-AF = first 1-day-long atrial fibrillation episode; 1st-AF = first atrial fibrillation episode; 7d-PeAF = first continuous 7-day stretch of persistent atrial fibrillation; 35d-PeAF = first continuous 35-day stretch of persistent atrial fibrillation; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

In isolated atrial myocytes from GMCT-treated and saline-treated animals, peak inward sodium current and L-type calcium current were significantly reduced compared to sham (Supplemental Figures 3 and 4); these currents were comparable between GMCT-treated and saline-treated groups. Changes in sodium current and L-type calcium current were consistent with concomitant decreases in expression of $Na_v1.5$ and $Ca_v1.2$ proteins. Inward rectifier potassium current (I_{K1}) increased 2-fold at negative membrane potentials in left and right atria of saline-treated animals, but GMCT attenuated the increase ($p < 0.05$) (**Figure 6C**). Changes in I_{K1} were consistent with concomitant decreases in Kir2.3

FIGURE 5 GMCT Reduces Electrical Remodeling During AF Progression

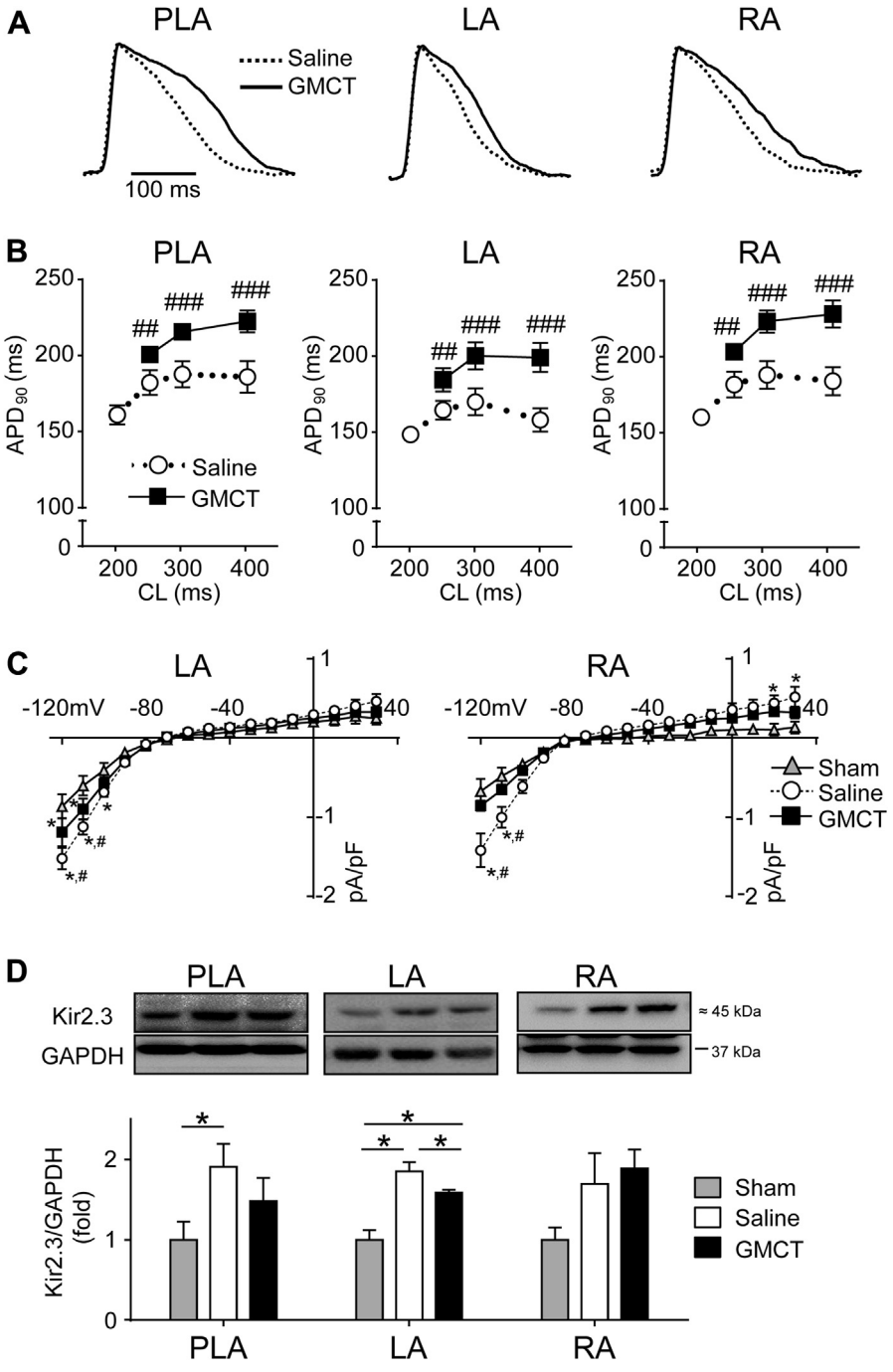


(A) Dominant frequency (DF) in LA (N = 8 for saline- and GMCT-treated groups) measured in vivo (#p < 0.05 vs. saline-treated group). **(B)** Representative dominant frequency (DF) maps during AF optical mapping in saline-treated **(top)** and GMCT-treated hearts **(bottom)**. **(C)** maximal DF, **(D)** number of rotations, **(E)** density of singularity points (SP), and **(F)** SP trajectory area in optical mapping for saline-treated (N = 6) and GMCT-treated hearts (N = 6). *p < 0.05, **p < 0.01. Abbreviations as in Figure 4.

protein in LA and PLA (**Figure 6D**). Therefore, the attenuation of APD shortening by GMCT during AF can be partially attributed to mitigation of I_{K1} increase, which is otherwise responsible for DF acceleration during AF progression (12). To investigate the possibility of additional K^+ channel remodeling, I_{Kr} block was assessed by high-throughput thallium flux assay (16); overnight treatment with GMCT did not affect I_{Kr} (Supplemental Figure 5). Acute GMCT treatment inhibited I_{Kr} only at 12 mg/ml, which is well above concentrations used in in vitro experiments (**Figure 2**, Supplemental Figure 1). Kv1.5 protein (responsible for I_{Kur}) tended to decrease in LA from GMCT-treated animals, and in 24-h cultured acute sheep atrial myocytes, GMCT decreased I_{Kur} density by 30% (Supplemental Figure 6). In addition, during optical mapping of hearts from persistent AF sheep, DPO-1 (1.0 μmol/l) an I_{Kur} blocker (17), acutely prolonged APD_{90} and decreased DF (Supplemental Figure 7).

As expected from previous work (12), ryanodine receptor (RyR2) proteins were reduced and sodium-calcium exchanger (NCX) proteins were increased in AF animals (saline or GMCT) compared to sham (Supplemental Figures 8A to 8D). SERCA, phospholamban, NCX, and RyR2 proteins were similar in saline-treated and GMCT-treated animals. Additionally, caffeine-induced Ca^{2+} transients in isolated sheep LA myocytes demonstrated accelerated decay time constants (tau) in saline-treated animals compared to sham or GMCT-treated animals, consistent with changes in NCX protein levels, suggesting that accelerated Ca^{2+} extrusion in AF was reversed by GMCT-treatment. However, normalized peak Ca^{2+} transient amplitudes were comparable in all groups (Supplemental Figures 8E to 8G). Altogether, similar Ca^{2+} handling properties between saline- and GMCT-treated animals suggest that GMCT-induced amelioration of electrical remodeling occurred independently of altered Ca^{2+} handling.

FIGURE 6 GMCT Mitigates AF-Induced Shortening of APD



(A) Representative action potentials from optical mapping of saline-treated **(dotted line)** and GMCT-treated hearts **(solid line)** at 2.5 Hz pacing. **(B)** Atrial optical mapping data shows cycle length (CL) dependence of action potential duration at 90% repolarization (APD_{90}) from saline-treated (N = 6) and GMCT-treated hearts (N = 6). ##p < 0.01, ###p < 0.001 versus saline-treated group. **(C)** Current-voltage relationships for inward rectifier potassium current (I_{K1}). For right atrium: N/n = 3/8 (sham), N/n = 4/12 (saline), and N/n = 3/9 (GMCT), for LA: N/n = 3/9 (sham), N/n = 3/13 (saline), and N/n = 3/13 (GMCT). *p < 0.05 versus sham, #p < 0.05 versus GMCT. Repeated measures 2-way analysis of variance with Bonferroni multiple comparisons test. **(D)** Representative Kir2.3 Western blots (**left**: sham, **middle**: saline, **right**: GMCT) and quantification of protein expression (N = 4, all groups; *p < 0.05). RA = right atrium; other abbreviations as in Figure 4.

We generated computational action potentials for persistent AF using a human atrial action potential model (Supplemental Figure 9) (18). Ionic changes for modeling persistent AF were based on patch-clamp recordings. Two-dimensional simulations showed that sustained rotor dynamics in the saline-treated model exhibited higher rotational frequency (9.8 Hz) than the GMCT-treated model (7.8 Hz).

AF INDUCIBILITY, BURDEN, AND SPONTANEOUS CARDIOVERSION IN SHEEP. GMCT-treated animals spent longer time in sinus rhythm and had more frequent spontaneous AF terminations than saline-treated animals (**Figures 7A and 7B**). AF inducibility index (inverse of pacing attempts to reinduce AF) after 7d-PeAF was significantly reduced in GMCT-treated compared to saline-treated animals (**Figure 7C**), suggesting GMCT-treated animals were less vulnerable to AF. GMCT significantly reduced AF burden, particularly during time points associated with paroxysmal AF (**Figure 7D**). At later times and after more sustained AF episodes, AF burden, and inducibility index converged.

DISCUSSION

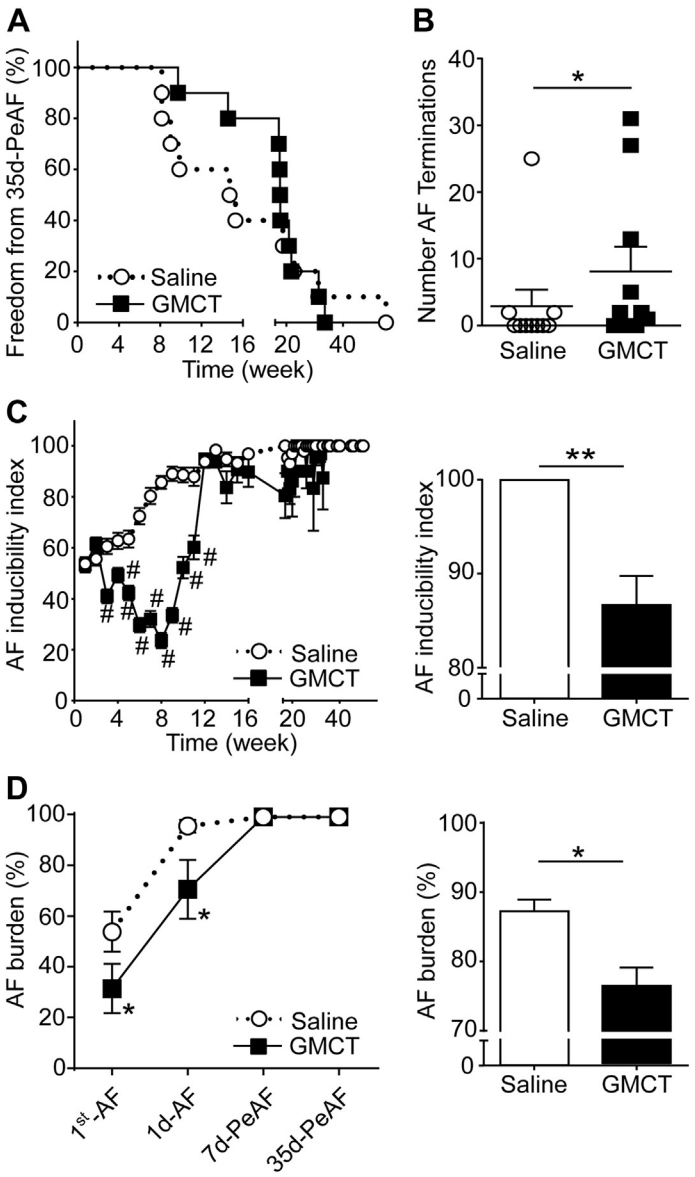
Here we demonstrate for the first time that Gal-3 mediates sustained AF-induced atrial structural and electrical remodeling and contributes to AF perpetuation. In patients, intracardiac serum Gal-3 levels were greater in persistent than paroxysmal AF and the Gal-3 level was an independent predictor of AF recurrences after a single ablation procedure. In a sheep model, the Gal-3 inhibitor GMCT reduced atrial fibroblast proliferation in vitro. In vivo, GMCT mitigated atrial dilation, myocyte hypertrophy, fibrosis, and the expected increase in DF during transition to persistent AF. Ex vivo, GMCT-treated hearts had longer action potential durations, and fewer rotors and wavebreaks during AF than control. GMCT increased the number of spontaneous AF terminations and reduced overall AF burden.

A recent study in patients suggested that serum Gal-3 from peripheral blood was not associated with sinus rhythm maintenance and questioned its usefulness in predicting rhythm outcome of catheter ablation (11). In sharp contrast, our results derived from intracardiac measurements of Gal-3 levels from the CS and LA are significantly higher in patients with persistent than paroxysmal AF, in agreement with another study showing that peripheral plasma Gal-3 predicted clinical outcomes after catheter ablation (10). Moreover, Gal-3 levels correlated positively with left atrial volume index and inversely with AF cycle length (**Figure 1**), supporting a role for profibrotic molecules in the progression of structural and electrical remodeling in patients with persistent AF. These data are consistent with previous clinical findings showing that serum Gal-3 levels are correlated with LA-structural remodeling (19). Atrial size is an important determinant of AF (20) and larger atria have a higher probability for initiation and maintenance of rotor-driven fibrillatory activity (21). Inflammatory markers have been suggested to play a role in AF perpetuation (22). However, inflammatory infiltrates need not be present in the atria of patients with persistent AF (23), which is consistent with our results showing no changes in CRP or BNP (**Figure 1**).

Gal-3 has been shown to play a key role in human diseases associated with fibrosis, including cancer, cirrhosis (24), renal failure (3), lung disease (8), and heart failure (25). However, while there is an urgent need for development of inhibitors that specifically target Gal-3, there is currently no commercially available anti-Gal-3 therapeutic reagent (26). Recently Gal-3 inhibition with GMCT significantly reduced fibrosis and reversed cirrhosis, and reduced Gal-3 expressing portal and septal macrophages in a rat model of liver disease (27). In our sheep model, GMCT mitigated both electrical and structural remodeling during AF progression. Myocyte hypertrophy was also diminished, possibly due to the inhibition of TGF-β1, a key mediator of angiotensin II-induced cardiac fibrosis and hypertrophy (28). Gal-3 is known to retain TGF-β receptors on the myofibroblast cell membrane by binding to the receptor's poly N-acetyl lactosamine residues, which promotes signaling through Smad and Akt pathways (8). By binding to the CRD of Gal-3 (4), GMCT may have reduced activation of the TGF-β/Smad pathway in myocytes and myofibroblasts, leading to reduction of cardiac fibrosis (29). Increased α-SMA protein in the PLA and LA from saline-treated animals may reflect progressive myofibroblast activation evoked by tachypacing that contributed to fibrosis via autocrine and paracrine mechanisms (30). Thus, lower α-SMA expression in GMCT-treated animals likely reflects attenuated myofibroblast activation via inhibition of TGF-β1/Smad pathway (31). Altogether, these findings suggest a mechanistic involvement of Gal-3 via TGF-β mediated pathways in the pathophysiology of atrial fibrosis. Moreover, the results suggest that GMCT interferes with the association between Gal-3 and the TGF-β receptor during AF, which inhibits TGF-β1 mediated signaling and reduces myofibroblast activation leading to prevention of pathological collagen deposition.

Gal-1 increases cardiac myofibroblast proliferation (**Figure 2D**), consistent with known properties

FIGURE 7 GMCT Reduces AF Inducibility and Promotes AF Termination



(A) Kaplan-Meier plot for freedom from 35d-PeAF in saline-treated (N = 10) and GMCT-treated groups (N = 10). p = 0.3254. **(B)** Number of AF terminations after 7d-PeAF for saline-treated (N/n = 3/29) and GMCT-treated groups (N/n = 7/82). **(C) Left**: AF inducibility index for saline-treated (N = 10) and GMCT-treated groups (N = 10) throughout AF progression. #p < 0.0001 versus saline-treated animals. **(Right)** AF inducibility index after 7d-PeAF in GMCT-treated (N/n = 7/77) and saline-treated (N/n = 3/32) animals. **(D) (Left)** AF burden in GMCT-treated (N = 10) and saline-treated (N = 10) animals. **(Right)** Overall AF burden in GMCT-treated (N/n = 10/199) and saline-treated (N/n = 10/187) animals. N = number of animals; n = number of AF episodes. Statistics: **(A)** Gehan-Breslow-Wilcoxon test, **(C, D left)** Mann-Whitney *U* test at each individual time point, **(B, C, D right)** Mann-Whitney *U* test. *p < 0.05, **p < 0.01. Abbreviations as in Figure 4.

of Gal-1 inducing dermal- and gingival-derived myofibroblast activation, migration, and proliferation (32). However, GMCT did not inhibit the effect of Gal-1 at the same dose which completely inhibited Gal-3 proliferation, suggesting that GMCT has higher affinity and more specificity to Gal-3 than Gal-1. Moreover, Gal-1 protein in AF sheep tissue is reduced compared to sham (**Figure 4B**), suggesting that Gal-1 may not be a key regulator of AF. Altogether, these data suggest that the beneficial effects of GMCT are due to Gal-3 inhibition and not Gal-1.

AF progression was characterized by continuously increasing DF until AF became persistent, similar to our previous report (12). Notably, although DF increased progressively as a consequence of electrical remodeling, GMCT reduced DF acceleration, resulting in a lower terminal maximal DF than saline-treated animals at similar time points. Optical mapping showed fewer rotors and wavebreaks in GMCT-treated hearts, likely due to prolonged APDs and refractory periods, as well as a less dense fibrotic substrate where rotors could anchor to maintain AF. Atrial fibroblast proliferation might be responsible for complex fractionated atrial electrograms during persistent/permanent AF (33). This is consistent with our observation of fewer spiral wave breaks in GMCT-treated hearts.

Increased I_{K1} is a major factor in AF maintenance (12,34). Treatment with GMCT attenuated I_{K1} and I_{Kur} remodeling which likely reduced APD prolongation, slowed DF and contributing to fewer rotors than saline-treated animals. Numerical simulations predicted that the effects of GMCT on I_{Kur} and I_{K1} should mitigate AF-induced APD shortening and slow rotor frequency (Supplemental Figure 9). This provides an ionic mechanism for the putative effects of GMCT on DF reduction.

Changes in calcium handling proteins associated with AF were consistent with previous findings (12,35,36). In rabbits, 5-day-sustained high atrial activation rate induced Ca^{2+} signaling silencing through a failure of subcellular propagated Ca^{2+} release (35). In 1-year persistent AF sheep, although NCX was increased in LA, both total RyR2 and phosphorylated RyR2 proteins were decreased, and the ratio of phosphorylated RyR2 to total RyR2 phosphorylation was unaffected (12). CaMKII appeared to facilitate catecholamine-evoked arrhythmias in atrial tissue of patients with sinus rhythm, but it failed to elicit arrhythmias of patients with chronic AF (36). In the current study, we can safely conclude that attenuation of electrical remodeling by GMCT was not due to recovery of Ca^{2+}-handling dysfunction, since

GMCT did not rescue AF-remodeled SERCA, NCX, or RyR2.

In addition to decreasing AF inducibility, GMCT significantly increased the probability of spontaneous cardioversion to sinus rhythm during persistent AF, which may be explained by a combination of effects: first, GMCT ameliorated atrial dilation and fibrosis, both of which are believed to contribute to a substrate for reentry; and second, GMCT significantly mitigated K^+ channel remodeling, APD shortening and DF increase, all of which mark the progression toward persistent AF (12). However, we cannot exclude the possibility that GMCT ameliorated electrical remodeling through other means that we have not explored. The pleiotropic actions of Gal-3 suggest possible roles in cardiomyocytes, including direct modulation of cardiac ion channel function. Gal-3 interacts with beta-galactoside residues of cell surface and matrix glycoproteins through its carbohydrate-binding domain (37). Both voltage-gated Na^+ and some K^+ channels are glycoproteins that contain large amounts of post-translationally attached sialic acids (38). Glycosylation is important for membrane protein folding, cell surface targeting, and function (39). Evidence indicates that in kidney, galectin-N-glycan interaction may contribute to preventing internalization of both inward rectifier (ROMK1) and transient receptor potential cation channels (40). Potassium ion channels, Kir2.3 and Kv1.5, are thought to be internalized by clathrin dependent pathways via endocytosis (41,42). Similar to TGF-β receptors, Gal-3 might act by preventing internalization of such proteins (43). Gal-3 oligomers might bind to and retain Kir2.3 and Kv1.5 channels of atrial myocytes, giving rise to APD shortening and DF increase. GMCT may act by inhibiting binding of Gal-3 to Kir2.3 and $K_v1.5$ channels in atrial myocytes, thus decreasing current density and mitigating APD shortening and DF increase during AF progression. Further studies are required to elucidate better the specific molecular mechanisms in this process.

STUDY LIMITATIONS. We used a tachypacing induced persistent AF sheep model in which continuous sinus rhythm was not permitted, because pacing resumed immediately after AF spontaneous conversion. Thus, it is unknown whether GMCT affects AF induction by modulating pacing-induced focal atrial ectopic depolarizations or PV arrhythmogenesis. Although the findings from the experimental model of AF correlate well with the clinical study, the mechanisms of AF in pacing-induced AF sheep and in humans with AF may have nuanced differences. The intense burst pacing in the sheep model may be more aggressive than intrinsic AF-initiating events during the natural progression of paroxysmal to persistent AF in humans, where instances of AF reinitiation may be much more infrequent. Thus, Gal-3 inhibition only delayed development of AF and did not significantly improve long-term outcomes in the present study. In the human condition where durations between AF paroxysms may last weeks or months, delayed progression of AF may have greater impact on reducing AF burden than in our model. Last, Gal-3 is secreted by macrophages, kidneys, and vasculature (3). Even though blood samples were obtained from CS and LA to assess cardiac-derived Gal-3, it could reflect changes in Gal-3 production in other organs.

CONCLUSIONS

Inhibiting Gal-3 during AF progression might be useful as an adjuvant treatment to improve outcomes of catheter ablation for persistent AF. Gal-3 inhibition may be a potential new upstream therapy for the prevention of AF progression.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Gal-3 represents a common upstream link for both structural and electrical remodeling and a mediator in transition to persistent AF through its effects promoting gene transcription via cytokine mediated signaling pathways.

TRANSLATIONAL OUTLOOK: Should that prove to be true in human AF, then demonstration that one of several types of Gal-3 inhibitors halts AF progression might lead to the first generation of disease-modifying therapies aimed at preventing AF perpetuation and improving AF ablation outcomes.

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KEY WORDS atrial fibrillation, catheter ablation, fibrosis, galectin-3, upstream therapy

APPENDIX For expanded Methods and References sections as well as supplemental tables and figures, please see the supplemental appendix of this article.