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# OPG/RANK/RANKL Axis in Stabilization of Spontaneously Restored Sinus Rhythm in Permanent Atrial Fibrillation Patients after Mitral Valve Surgery

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**For editorial comment see p. 51**

**Key Words**
Atrial fibrillation · Mitral valve, repair, replacement · Extracellular matrix · Pathology · Molecular biology

**Abstract**
***Objective:*** To investigate the expression of the osteoprotegerin (OPG)/receptor activator of nuclear factor-κB (RANK)/RANK ligand (RANKL) axis in the stabilization of spontaneously restored sinus rhythm (SR) in permanent atrial fibrillation (AF) patients after mitral valve (MV) surgery and study its clinical significance. ***Methods:*** Clinical data, biopsies of right atrial appendages were collected from 135 permanent AF patients who spontaneously restored SR after conventional isolated MV replacement. A comparison was made between patients who had recurrence of AF within 7 days and patients with persistent SR for more than 7 days. ***Results:*** AF patients had an increased expression of RANK, RANKL, and the RANKL/OPG ratio compared to SR patients, and the degree of fibrosis was lower in SR compared to AF in the atria. Moreover, the expressions of RANK, RANKL, and the RANKL/OPG ratio were positively correlated with the degree of fibrosis. ***Conclusion:*** These findings suggest that the OPG/RANK/RANKL axis plays important roles in the stabilization of restored SR after MV surgery by stimulating AF-related atrial remodeling in AF patients.


## Introduction

Atrial fibrillation (AF) is present in 40–60% of patients undergoing mitral valve (MV) surgery [1] and has been identified as a risk factor for poor outcomes after MV replacement [2]. Because of the limited success of electrical and/or drug-induced conversion, concomitant maze procedure and intraoperative radiofrequency ablation have been used during the past two decades [1, 3]. However, significant concern remains given the inevitable risk of perioperative complications and uncertainty regarding atrial mechanical function [4, 5]. In fact, most patients with chronic AF experience aspontaneous restoration of sinus rhythm (SR) after MV surgery. However, some patients could have a persistent SR for a long period of time (from weeks to years), while others might have recurrence of AF shortly after the surgery (usually within 1 week) [6–8]. The spontaneous restoration of SR may be attributed to an improved transmitral hemodynamics after the surgery [7, 9], but the mechanism by which patients have a persistent SR remains unknown.

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Osteoprotegerin (OPG) is a member of the tumor necrosis factor (TNF) receptor superfamily, which can function as a decoy receptor by binding to receptor activator of NF-κB (RANK) ligand (RANKL) and competitively inhibiting the interaction between RANKL and RANK [10]. The OPG/RANK/RANKL axis has been identified as a key regulatory system in bone homeostasis [11]. However, recent experimental, clinical, and epidemiological data have also implicated this axis in cardiovascular diseases [12]. Elevated myocardial protein expression of OPG, RANK, RANKL, and the RANKL/OPG ratio has been found in both experimental and clinical heart failure (HF) [13], and our recent studies found that higher atrial expressions of RANK and RANKL and the RANKL/OPG ratio may contribute to the development and progression of AF by regulating atrial remodeling [14, 15]. The increased RANKL/OPG ratio, which reliably reflects the activity of the axis, can aggravate myocardial fibrosis by regulating the matrix metalloproteinase (MMP)/tissue inhibitor of metalloproteinase (TIMP) system [13–15]. More interestingly, a significant association was recently identified between the circulating OPG concentration and AF incidence in a community-based study [16].

In the present study, we hypothesized that the stabilization of spontaneously restored SR in patients with permanent AF after MV surgery is associated with expression of the OPG/RANK/RANKL axis in atria. To this end, we performed a study including 135 patients in order to obtain essential clinical data as well as biopsies of right atrial appendages (RAAs). We measured protein expressions of OPG, RANK, and RANKL and determined the degree of interstitial fibrosis in RAA tissues.

## Materials and Methods

*Patients and Study Design*

We consecutively recruited 135 patients admitted to the First Affiliated Hospital of Nanjing Medical University between November 2007 and June 2010. Inclusion criteria were: (1) preoperative permanent AF (persisting at least 1 year) [17] and (2) spontaneous restoration of SR after conventional isolated MV replacement surgery. Exclusion criteria were: (1) patients who exceeded 65 years of age or had a history of cancer, (2) a history of cardiac arrhythmia other than AF, (3) patients who suffered a bone fracture within the previous half a year, (4) patients with aortic or valvular calcification, (5) patients who suffered bacterial endocarditis or had detected rheumatic activity, (6) patients with complicated diabetes or renal dysfunction (serum creatinine >136 μmol/l), (7) the need for coronary artery bypass grafting (CABG) or other associated procedures at the same time, (8) requirement of redo surgery, (9) severe postoperative complications, and (10) requirement of postoperative pacemaker implantation or treatment with an antiarrhythmic agent (except digitalis) within 7 days. The present study was conducted according to the Helsinki Declaration and was approved by the ethics committee of Nanjing Medical University. All of the patients gave written informed consent before being included in the study.

*Surgery and Tissue Collection*

The same cardiac anesthesiologist, perfusionist, and surgical team performed all of the operations. All of the patients underwent standard cardiopulmonary bypass with moderate hypothermia, antegrade crystalloid cardioplegic arrest, and local hypothermia with ice-slash. The MV was approached through a transseptal approach and replaced with prosthetic valves. The 135 patients returned spontaneously to SR when they were weaned from extracorporeal circulation and remained in SR until the end of the surgical procedure.

About 250 mg of RAA tissue for each patient was obtained from the cannulation site before starting extracorporeal circulation. Fifty milligrams of RAAs were fixed in paraformaldehyde for histology and immunohistochemistry, and the others were immediately snap-frozen in liquid nitrogen for biochemical analysis.

*Recurrence of AF*

After surgery, patients were transferred to the cardiothoracic intensive care unit. The patients were monitored by continuous ECG during a minimum period of 48 h postoperatively, and thereafter it was repeated at least every 8 h for 15 min each time until discharge. In case of rhythm disturbance reported by a nurse or the patient during the interval, a 12-lead ECG recording was conducted and continuous ECG monitoring was re-used. The recurrence of AF was diagnosed on the basis of a minimum of 15 min of AF duration documented by a continuous 12-lead ECG recording [18]. Before discharge, the patients were divided into two groups according to whether (AF group) or not (SR group) AF recurrence took place within 7 days postoperatively [19].

*Western Blot*

Frozen RAAs were used for protein isolation as described previously [15]. Proteins (40 μg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyinylidene fluoride membranes using a Bio-Rad semidry transfer system (Bio-Rad). The membranes were blocked with 5% non-fat dry milk and then probed with rabbit polyclonal anti-OPG (ab9986), mouse monoclonal anti-RANKL (ab45039) and anti-RANK (ab12008; Abcam, USA), and horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-GAPDH (KC-5G5; KangChen Biotech, China). The working dilutions were 1:500 (OPG, RANKL, and RANK) and 1:5,000 (GAPDH). The resulting reaction was visualized using HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Santa-Cruz Biotechnology, The Netherlands), followed by incubation with an ECL Western Blot Detection Kit (Amersham, The Netherlands) for 1 min. The blots were exposed to Kodak film for 5 min and immunoreactive bands developed for quantification using The Discovery Series™ image analysis software (Bio-Rad) normalized by the corresponding value of GAPDH. Experiments were repeated 3 times and the mean was scored.

*Histology and Immunohistochemistry*

After fixation with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 24 h, the tissues were subjected to alcoholic dehydration and embedded in paraffin. Four-micrometer serial sections were sliced and subjected to Masson's trichrome staining to highlight collagen fibers. The collagen volume fraction (CVF) was determined by the HPISA 100 chromatic color pathological analysis system (Olympus, Japan) using 5 random images from each slide and 5 slides per sample, and the mean values of CVF were obtained by one investigator blinded to the groups.

Immunohistochemical staining for detecting OPG, RANKL, and RANK (the same antibodies as Western blot but with working dilution at 1:100) was performed on 4-μm-thick sections from each paraffin-embedded tissue, according to our previous report [19]. A peroxidase-based detection system was used, with diaminobenzidin as the final chromogen.

*Statistical Analysis*

For comparison between the two groups, Student's t test (normally distributed) or the Mann-Whitney test (nonnormally distributed) was used for continuous variables, and the $\chi^2$ test was utilized for categorical variables. Correlation analysis (Pearson or Spearman) was used to assess the association between expression of the OPG/RANK/RANKL axis, the RANKL/OPG ratio, and the degree of interstitial fibrosis in RAAs. $p < 0.05$ (two-sided) was considered statistically significant. Statistical analysis was performed with the GBSTAT statistical analysis package (version 9.0; Dynamic Microsystems, Inc.).

## Results

*Patient Characteristics*

The 135 patients were divided into an SR group (n = 52) and an AF group (n = 83). No significant differences were found in terms of age and gender distribution. The preoperative echocardiographic atrial sizes were smaller in SR than in AF. The preoperative plasma levels of C-reactive protein were lower in SR than in AF. In addition, durations of intensive care unit stay and postoperative hospitalization were shorter in SR than in AF (table 1).

*Expression of OPG, RANKL, and RANK in RAAs*

Both immunohistochemistry and Western blot analysis showed that expression of OPG was comparable between the AF and SR groups (fig. 1a, parts 1–3). It also showed an increased expression of RANKL (fig. 1b, parts 1–3), RANK (fig. 1c, parts 1–3), and the RANKL/OPG ratio (fig. 1d) in AF compared to SR.

*Interstitial Fibrosis in RAAs*

Interstitial collagen, revealed by Masson's trichrome staining and expressed as CVF, was lower in SR than in AF (fig. 2a, b).

**Table 1.** Clinical characteristics

| Variables | AF | SR | p |
|---|---|---|---|
| Patients | 83 | 52 | – |
| Age, years | 48.8 ± 10.2 | 49.0 ± 9.4 | 0.921 |
| Gender (male/female) | 36/47 | 18/34 | 0.312 |
| C-reactive protein, mg/l | 5.80 ± 2.40 | 3.65 ± 1.96 | <0.001 |
| Duration of mitral disease, years | 9.4 ± 4.1 | 9.4 ± 3.7 | 0.806 |
| Duration of AF, years | 2.6 ± 1.5 | 2.6 ± 1.5 | 0.953 |
| NYHA class I/II/III/IV | 3/29/34/17 | 2/16/31/3 | 0.067 |
| Echocardiography | | | |
| Ejection fraction, % | 62.0 ± 5.8 | 62.9 ± 5.2 | 0.107 |
| Left atrial diameter, mm | 60.8 ± 8.2 | 46.9 ± 5.0 | <0.001 |
| Right atrial diameter, mm | 46.3 ± 5.9 | 41.4 ± 3.7 | <0.001 |
| PASP, mm Hg | 41.0 ± 7.6 | 38.9 ± 9.4 | 0.143 |
| Mitral valve disease | | | 0.144 |
| Pure mitral stenosis | 44 | 28 | |
| Pure mitral regurgitation | 10 | 12 | |
| Mitral stenoregurgitation | 29 | 12 | |
| Cause of mitral valve disease | | | 0.121 |
| Rheumatic | 76 | 43 | |
| Degenerative | 7 | 9 | |
| Preoperative length of stay, days | 14.2 ± 6.0 | 14.8 ± 4.4 | 0.297 |
| Preoperative antiarrhythmics | | | |
| Digitalis | 77 | 42 | 0.036 |
| Calcium channel blocker | 13 | 3 | 0.084 |
| β-Blocker | 30 | 12 | 0.110 |
| Left atrial thrombus | 22 | 9 | 0.216 |
| Combined TAP | 56 | 32 | 0.481 |
| CPB duration, min | 97 ± 11 | 97 ± 9 | 0.901 |
| Aortic clamp time, min | 56 ± 9 | 55 ± 7 | 0.504 |
| Duration of ventilation, h | 23.0 ± 6.0 | 22.5 ± 7.4 | 0.315 |
| Intensive care unit stay, h | 39.7 ± 11.9 | 35.8 ± 10.4 | 0.036 |
| Postoperative length of stay, days | 17.1 ± 3.2 | 14.0 ± 2.5 | <0.001 |

Values are presented as means ± SD or numbers of patients.

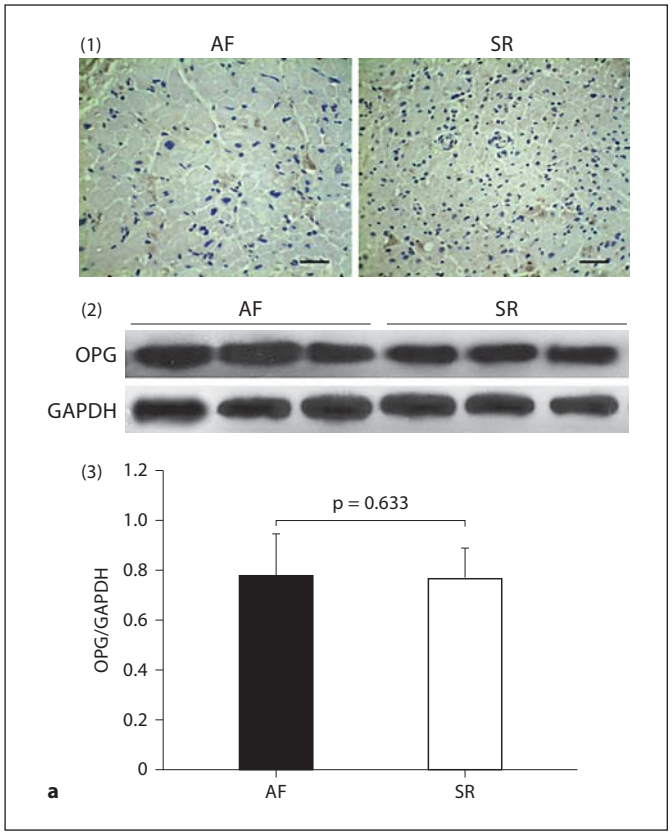
CPB = Cardiopulmonary bypass; NYHA = New York Heart Association; PASP = pulmonary artery systolic pressure; TAP = tricuspid valve annuloplasty.

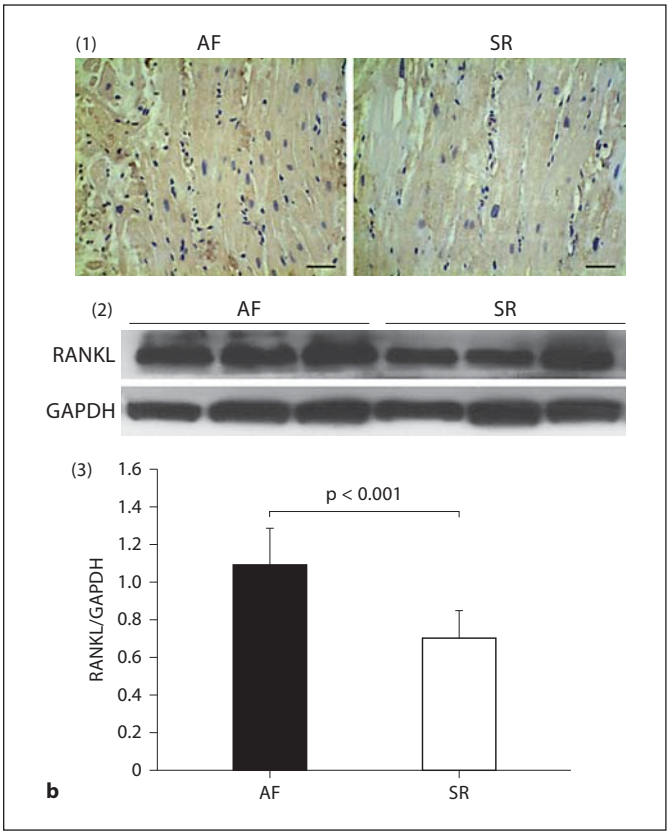
*Correlations between Expression of the OPG/RANK/RANKL Axis, the RANKL/OPG Ratio, and the Degree of Interstitial Fibrosis in RAAs*

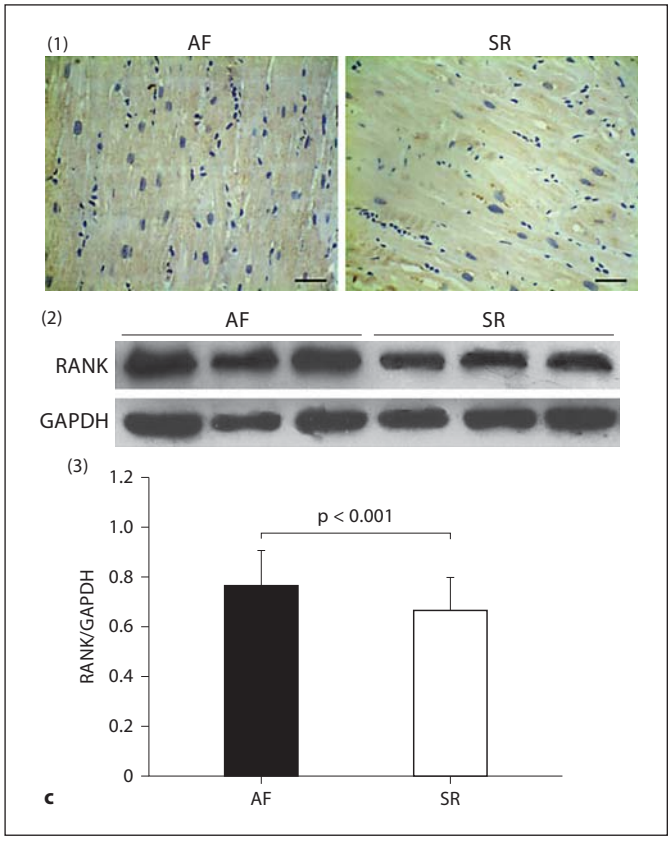
Taking these data together, the correlation test revealed a positive correlation between CVF and atrial expression of RANKL (r = 0.668) and RANK (r = 0.294) and the RANKL/OPG ratio (r = 0.745) (table 2).

## Discussion

Left atrial size was reported reflecting the degree of structural remodeling [20]. It was indicated that patients with a larger left atrial size had more severe interstitial



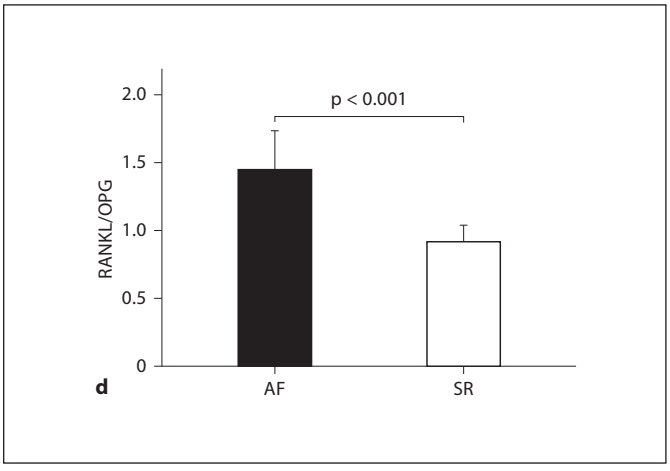




**Fig. 1.** Expression and localization of the OPG/RANK/RANKL axis in RAAs. **a** OPG. **b** RANKL. **c** RANK. **d** RANKL/OPG ratio. The respective parts 1 are representative immunostaining photomicrographs of AF and SR. ×200. Scale bar = 50 μm. Parts 2 and 3 show the results of Western blot analysis. Values are expressed as means ± SD.

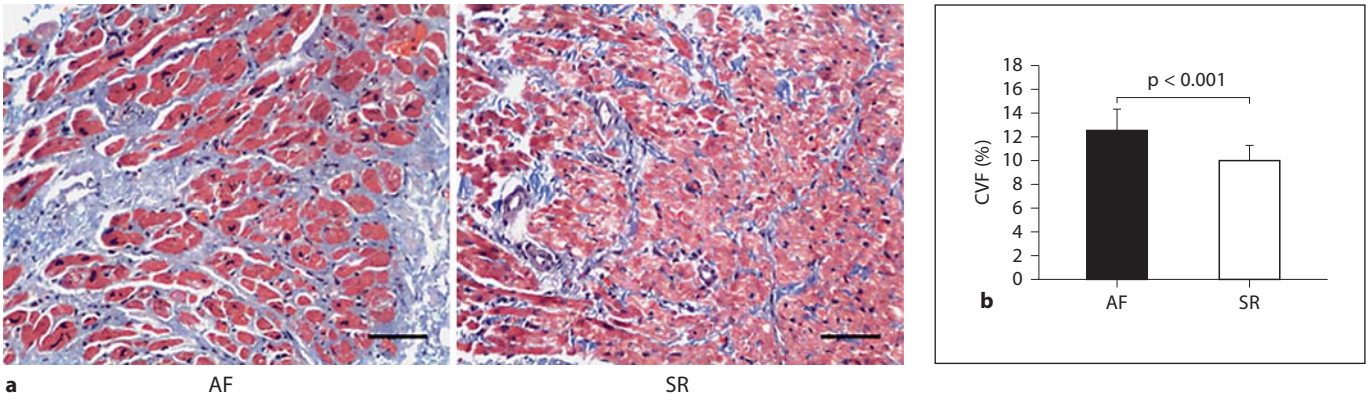



**Fig. 2.** Fibrosis in RAAs. **a** Representative photomicrographs of Masson's trichrome staining showing the interstitial collagen (blue; color refers to the online version). × 200. Scale bar = 50 μm. **b** CVF are expressed as means ± SD.

fibrosis and apoptosis in atria [20]. In agreement with this evidence, herein we identified that left and right atrial diameters were significantly lager in patients with AF recurrence. Of particular interest, herein we found that a significant higher expression and activation of the OPG/RANK/RANKL axis in atrial tissues might stimulate the recurrence of AF in patients after conventional isolated MV replacement. More importantly, we further found that atrial expression and the activity of the axis were statistically correlated with CVF in RAAs.

The expression of both RANK and RANKL molecules is significantly enhanced in monocytes of 'mini-invasive on-pump' cardiac surgical patients [21]. Moreover, a study using a rat model of post-infarction HF showed increased gene expression of OPG, RANK, and RANKL in the ischemic part of the left ventricle (LV) and, for OPG, in the nonischemic part of the LV, with persistently high levels during the observation period that involved both noncardiomyocyte and in particular cardiomyocyte tissue [13]. In patients with AF, the circulating OPG concentration was significantly associated with incident AF [16]. In our recent studies, the expressions of OPG, RANKL, RANK, and the RANKL/OPG ratio in atrial tissues were found to be higher in paroxysmal AF than in persistent AF and SR patients [14, 15]. Also, an increased ratio of RANKL/OPG can regulate the MMP/TIMP system and then aggravate myocardial fibrosis [15, 22]. In the present study, we investigated the expressions of OPG, RANK, and RANKL as well as the RANKL/OPG ratio simultaneously in 135 patients (AF vs. SR), and the expressions of RANK, RANKL, and the RANKL/OPG ratio in atrial tissues were found to be higher in AF patients than in SR patients.

**Table 2.** Correlations between expression of the OPG/RANK/RANKL axis, the RANKL/OPG ratio, and the degree of interstitial fibrosis in RAAs

| | CVF | |
|---|---|---|
| | correlation coefficient | p value |
| OPG | –0.090 | 0.302 |
| RANKL | 0.668 | <0.001 |
| RANK | 0.294 | 0.001 |
| RANKL/OPG | 0.745 | <0.001 |

Recurrence of AF after cardioversion is likely related to a biologic phenomenon known as remodeling, in which the structural properties of atrial tissue and cardiac cells are progressively and irreversibly altered, creating more favorable substrates for AF [23–26]. Interstitial fibrosis of the myocardium is a hallmark of arrhythmogenic structural remodeling. It results from an accumulation of fibrillar collagen deposits, occurring most commonly as a reparative process to replace degenerating myocardial parenchyma with concomitant reactive fibrosis, which causes interstitial expansion. Collagens, which surround and support cardiomyocytes and muscle fibers, are the major extracellular matrix proteins in the heart; the degradation of extracellular matrix is mainly regulated by the MMP/TIMP system. Unrestricted MMP activation appears to be of particular importance for increased matrix degradation in various disorders. Moreover, inhibition of MMP activity in animal models of HF has been found to attenuate the onset of LV dilatation. Cardiac fibroblasts

are the major site for the synthesis of collagen in the myocardium, and these cells also play an important role in the regulation of collagen degradation by MMPs [27]. Nakano et al. [28] demonstrated previously that the expression of the MMP-9 increases in fibrillating atrial tissue, and the alteration in the MMP-9/TIMP-1 may contribute to the atrial structural remodeling and atrial dilation during AF.

In clinical and experimental HF, 2 previous studies demonstrated that upregulation of RANKL contributed to enhanced MMP activity (MMP-2 and MMP-9 in particular), as well as a modest decrease in TIMP expression in human fibroblasts, which suggested a potential mechanism by which activation of the OPG/RANK/RANKL axis might result in matrix degradation, adverse ventricular remodeling, and worsening myocardial function [13, 29]. In light of the ability of RANKL to influence the MMP/TIMP system, decreased activation of RANKL may lead to a decrease in MMPs and TIMPs, followed by inhibition of myocardial fibrosis. Moreover, a recent report showed that OPG could solely increase MMP-9 (2-fold) and MMP-2 (1.5-fold) activity in THP-1 cells [30], speculating that binding RANKL by OPG may inhibit the rapid clearance of RANKL (stabilizing its levels), thus seemingly augmenting it pro-fibrotic actions. More importantly, in our study, we found that atrial expression and activity of the axis were statistically correlated with the CVF in RAAs. It supported the findings by Schnabel et al. [12] and reinforced the growing evidence that the OPG/RANK/RANKL axis might be one of regulatory systems in atrial remodeling of AF.

In an experimental rat model of post-autoimmune myocarditis remodeling, Liu et al. [27] recently revealed that the RANK-RANKL interaction is the gatekeeper of fibroblast differentiation and activation, suggesting that the OPG/RANK/RANKL axis may be involved in cardiac remodeling in immunoinflammatory myocardial diseases and progression of chronic HF [27]. Convincingly, they further found that anti-RANKL or OPG treatment could completely inhibit the induction of IL-17 on the upregulation of MMP-1 in cardiac fibroblasts, followed by inhibition of myocardial fibrosis [22]. Indeed, several pathways are activated by the RANK-RANKL interaction, but of particular importance are the NF-κB and activator protein-1 (AP-1) pathways, whose common response elements are contained in the promoter region of the gelatin MMP genes [31, 32]. In experimental and human HF, both NF-kB and AP-1 are chronically activated in cardiac myocytes. These findings point to an important involvement of NF-kB and AP-1 in the cardiac remodeling process [33], suggesting that reduced the activation of RANK-RANKL pathway may attribute to the stabilization of restored SR.

*Potential Clinical Perspective*

Because the stabilization of restored SR after MV surgery is associated with restraint of the activity of the OPG/RANK/RANKL axis, interventions that suppress these proteins may prevent the atrial remodeling that leads to AF development and recurrence, thereby affording a new possible therapeutic modality in managing this common complication. Patients with early recurrence of AF have more advanced pathological changes in the atria and may be good candidates for concomitant anti-AF procedures. Therefore, it is valuable in predicting which patients in AF undergoing MV surgery are prone to having the early recurrence of AF postoperatively.

*Study Limitations*

First, we could not exclude the patients who had asymptomatic recurrences of AF for more than 15 min but spontaneously restored SR in the interval of 12-lead ECG monitoring at least every 8 h. Therefore, it is possible that some asymptomatic early recurrences occurred. Second, owing to a lack of information regarding left atria, it is unknown if the left atria could be a more ideal material in the patients studied. Thus, further evaluation is warranted.

## Conclusions

Significant activation of the OPG/RANK/RANKL axis in atrial tissue integrity culminates in excessive atrial fibrosis and maintenance of AF remodeling, which may stimulate the recurrence of AF in patients after conventional isolated MV replacement. This suggests a potential role for known mediators of bone homeostasis in the pathogenesis of AF and possibly represents new targets for therapeutic intervention in this disorder.

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## Conflict of Interest

None declared.

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