ORIGINAL RESEARCH

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Elevated Circulating Fibrocytes Is a Marker of Left Atrial Fibrosis and Recurrence of Persistent Atrial Fibrillation

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Background—In atrial fibrillation (AF), a more extensively fibrotic left atrium (LA) provides a substrate for arrhythmias and increases risk of relapse following ablation. Fibrocytes are bone marrow–derived circulating mesenchymal progenitors that have been identified in the atrium of patients with AF who have valvular diseases. The present study investigates the associations between circulating fibrocytes and LA fibrosis or the prevalence of recurrence after ablation in patients with persistent AF.

Methods and Results—We measured the proportion, differentiation, and migration of circulating fibrocytes from patients with persistent AF (n=40), those with paroxysmal AF (n=30), and sinus rhythm controls (n=30). LA low-voltage (fibrosis) area was identified by an electroanatomic mapping system, and patients were followed up for 1 year after ablation. The relationship between circulating fibrocyte percentage and LA low-voltage area or recurrence was assessed by multivariate regression analysis. Circulating fibrocyte percentage positively associated with LA low-voltage area in the persistent AF group, and circulating fibrocyte (≥4.05%) was a significant predictor of 1-year recurrence after ablation. Cultured fibrocytes exhibited enhanced potential of differentiation in the persistent AF group (67.58±1.54%) versus the paroxysmal AF group (56.67±1.52%) and sinus rhythm controls (48.43±1.79%). Furthermore, expression of fibroblast activation markers and cell migratory ability were also elevated in differentiated fibrocytes from patients with persistent AF. Transforming growth factor β1 and stromal cell–derived factor 1 were elevated in the plasma of patients with persistent AF and were shown to promote fibrocyte differentiation and migration, respectively.

Conclusions—In patients with persistent AF, increased circulating fibrocytes served as a marker of LA fibrosis and recurrence. ***(J Am Heart Assoc.* 2018;7:e008083. DOI: 10.1161/JAHA.117.008083.)**

Key Words: atrial fibrillation • atrial fibrosis • fibrocyte • differentiation • biomarker

Atrial fibrillation (AF) is globally the most common arrhythmia contributing significantly to mortality and morbidity.[1] Large clinical trials have shown lack of significant benefits of using antiarrhythmic medications in the treatment of AF.[2,3] While catheter ablation has become the routine intervention for AF, recurrence rates remain high, ranging from 30% to 60% across reports.[4,5] Atrial structural remodeling together with fibrosis is an important mechanism for AF persistence. A more extensively fibrotic left atrium (LA) increases the rate of relapse after ablation.[6,7] To date, a reliable circulating marker that can predict atrial fibrosis and recurrence following ablation is limited.

Resident cardiac fibroblasts are a primary source of collagen production during both homeostatic maintenance and the development of fibrosis. In addition, recent studies have implicated fibrocytes as another important cellular

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Accompanying Tables S1 through S5 are available at http://jaha.ahajournals.org/content/7/6/e008083/DC1/embed/inline-supplementary-material-1.pdf

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Received November 9, 2017; accepted February 5, 2018.

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Clinical Perspective

What Is New?

- Circulating fibrocytes were able to predict left atrial fibrosis status and atrial fibrillation (AF) recurrence after first-time ablation in patients with persistent AF.
- Circulating fibrocytes from patients with persistent AF exhibited a hyperactivated status and had an enhanced capacity to differentiate into myofibroblasts.
- Transforming growth factor β1 and stromal cell–derived factor 1 were the causative mediators that promoted fibrocyte differentiation and migration, respectively.

What Are the Clinical Implications?

- In patients with persistent AF, circulating fibrocytes could serve as a useful cellular marker both for the degree of left atrial fibrosis and the recurrence after single ablation.
- Inhibiting fibrocytes during AF progression might be useful as an adjuvant treatment to improve outcomes of catheter ablation for persistent AF.

source of collagen, contributing to cardiac fibrosis.[8] Fibrocytes are circulating progenitor cells and are able to migrate to sites of tissue injury where they differentiate into fibroblasts and produce extracellular matrix proteins upon stimulation.[8] Fibrocytes express hematopoietic stem cell marker CD34, leukocyte marker CD45, and a variety of mesenchymal markers, including collagen I.[8] Recruitment of fibrocytes into tissue is associated with CD34 downregulation and α-smooth muscle actin upregulation.[9] Fibrocytes are involved in various fibrotic disorders, including asthma, systemic sclerosis, and idiopathic pulmonary fibrosis.[10–13] In addition, fibrocytes have been identified to contribute to myocardial infarction or heart failure–induced cardiac fibrosis in animal models.[14–16] Recently, fibrocytes were found in the atrium of patients with AF who had valvular heart diseases,[17] which prompted us to investigate the diagnostic value and functions of circulating fibrocytes in the setting of AF.

In the present study, our hypothesis is that circulating fibrocytes are increased and activated in the setting of AF and could be an important indicator for LA fibrosis and AF recurrence following ablation.

Methods

Study Patients and Blood Collection

The data, analytic methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure. We recruited 70 consecutive patients with AF (30 paroxysmal AF and 40 persistent AF) who were admitted to the First Affiliated Hospital of Dalian Medical University and scheduled for first-time catheter ablation (2014–2015). Patients with AF were categorized as having paroxysmal AF when episodes self-terminated within 7 days or persistent AF when episodes lasted over 7 days or required electrical cardioversion. Sex, age, and hypertension proportion–matched participants with sinus rhythm (SR) were defined as controls (n=30). Clinical examinations for SR controls included a 12-lead ECG, echocardiograph, blood pressure measurement, and detailed history of medications. Hypertension was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg without any drug interventions. To avoid ventricular structural remodeling, participants with left ventricular ejection fraction <50% or B-type natriuretic peptide levels >400 ng/mL were excluded. Exclusion criteria for all participants also included cardiovascular diseases (except hypertension), cerebrovascular diseases, other vascular diseases, diabetes mellitus, surgery or trauma within 2 years, fibrotic or inflammatory diseases, cancer, and significant renal dysfunction (estimated glomerular filtration rate <30 mL/min per 1.73 m^2). Fresh blood samples (40 mL) were collected from SR controls or patients with AF before ablation for routine hematological test. The study was approved by the ethics committee of the First Affiliated Hospital of Dalian Medical University, and all participants gave written informed consent.

Clinical Follow-Up

All patients with AF were followed up for 12 months. Telephone contact was maintained with all patients following ablation. Patients were evaluated by 24-hour Holter monitor if symptoms of arrhythmia occurred. Routine medical examination, including the 24-hour Holter monitoring, was performed for patients without any symptoms at 3, 6, 9, and 12 months postablation. Recurrence was defined as AF, atrial tachycardia, or atrial flutter >30 seconds in duration after a 90-day blanking period according to the 2012 Heart Rhythm Society consensus document.

Three-Dimensional Electroanatomic Mapping and Definition of Low-Voltage Area

Before ablation, all patients underwent LA voltage mapping. Mapping was performed under SR, and patients not in SR were converted by direct current cardioversion. The entire LA, including the posterior wall, anterior wall, inferior wall and roof, and pulmonary vein (PV) ostia was mapped by a 10-pole circular catheter (Lasso) in conjugation with 3-dimensional (3D) electroanatomic mapping system (CARTO, Johnson). The average acquisition points for mapping sites in the LA were about 1700 points for each patient.

The contribution of low-voltage area to total LA surface area was evaluated using CARTO 3 software (Biosense Webstar) incorporated with the CARTO system offline. During the calculation, PV and valve regions were carefully excluded from LA surface by manually contouring these areas. Reference voltage values were used to distinguish the normal- and low-voltage tissues. The region within the voltage value >0.5 mV was defined as normal tissues,[18] whereas <0.1 mV was considered the low-voltage area.[18] The region between 0.1 and 0.5 mV was called the border zone.[18] There is a significant correlation between LA fibrosis imaged by MRI and the low-voltage area by the CARTO system.[18–21] Measurement was operated by 2 independent technicians.

Radiofrequency Ablation

Antiarrhythmic drugs were stopped on the day of ablation and then restored on the next day following ablation, until 2 months. Oral antiplatelet or anticoagulant drugs were stopped 3 days before ablation. After transseptal puncture, a single bolus of 100 IU/kg heparin was administered to maintain an activated clotting time within a range of 250 to 300 seconds. An anticoagulant was restored after ablation and continued for at least 3 months. Patients with a CHA_2DS_2-VASc score ≥2 received anticoagulant therapy continuously.

All patients underwent PV isolation by radiofrequency ablation. In brief, PV isolation was performed using 2 catheters, a circumferential PV mapping catheter (lasso TM, Biosense and Webster, Inc) and an irrigated ablation catheter Navistar Thermocool 3.5-mm D-F curve with SmartTouch technology (Biosense Webster). The circular mapping catheter was positioned close to the PV ostium, and point-by-point radiofrequency energy was performed to encircle the right and left PV. Radiofrequency energy was applied in a power-controlled mode with a power limit of 35 W and a maximal temperature of 45°C. Radiofrequency current was applied until a voltage of <0.1 mV was achieved at each point, with a maximum of 30 seconds.

Measurement of Circulating Fibrocyte Percentage by Flow Cytometry

Circulating fibrocytes were analyzed using flow cytometry. After red blood cell lysing, white cell pellet was resuspended in 100 µL PBS. Fibrocytes were labeled by peridinin chlorophyll protein complex–conjugated CD45 (BD Biosciences), phycoerythrin-conjugated CD34 (BD Biosciences), and fluorescein isothiocyanate–conjugated collagen I (Millipore) for 30 minutes after permeabilization in the dark.[8] Cells were analyzed with a flow cytometer (BD Canto II) and expressed as a percentage of the whole population of leukocytes ($CD45^+$).

Measurement of Fibrocyte Differentiation in Cell Culture

Plasma was first collected following centrifugation. After lysing red blood cells, the remaining white blood cell pellet was suspended in DMEM supplemented with 10% FBS (Gibco),

Table 1. Clinical Characteristics

	SR	Paroxysmal AF	Persistent AF
Demographics			
No.	30	30	40
Male sex, %	53	53	67
Age, y	56±11	59±10	61±11
Hypertension, %	53	53	55
CHA_2DS_2-VAS_C	NA	1.3±1.0	1.3±1.1
Hemodynamic variables			
Resting heart rate, beats per min	62.8±8.7	76.8±19.9*	87.4±21.1*,†
SBP, mm Hg	128.5±11.9	127.2±13.7	131.9±16.8
DBP, mm Hg	82.2±8.1	81.5±9.3	82.3±10.7
Echocardiography			
LVEF, %	59.0±2.1	58.7±2.1	57.1±5.4
LA volume, mL	68.4±6.4	70.2±7.5*	80.5±10.8*,†
Blood test			
Platelet count, 10^9/L	210.9±35.6	202.6±45.5	197.7±49.9
White blood cells, 10^9/L	6.3±1.6	6.5±1.8	6.0±1.5
Monocytes, 10^9/L	0.5±0.1	0.5±0.2	0.5±0.2
Neutrophils, 10^9/L	3.3±2.4	3.8±1.9	3.3±1.0
Lymphocytes, 10^9/L	2.0±0.7	2.1±0.5	2.1±0.6
LDL, mmol/L	NA	2.4±0.6	2.6±0.9
BNP, pg/mL	NA	92.9±35.0	72.6±46.9
Medications, %			
β-Blockers, %	33	33	45
Calcium channel blockers	0	20*	13*
Angiotensin-converting enzyme inhibitors	6.5	10	8
Angiotensin receptor blockers	6	10*	13*

Data are presented as mean±SD. BNP indicates B-type natriuretic peptide; DBP, diastolic blood pressure; LA, left atrium; LDL, low-density lipoprotein; LVEF, left ventricular ejection fraction; NA, not available; SBP, systolic blood pressure.
**P*<0.05 vs sinus rhythm (SR).
†*P*<0.05 vs paroxysmal atrial fibrillation (AF).

10% of the patient (or control) plasma, and plated at a density of 1×10^6 cells on a fibronectin precoated 6-well plate (Sigma Aldrich) at 37°C with 5% CO_2. After 3 days, nonadherent cells were removed and the remaining adherent cells were cultured until day 12. Differentiated fibrocytes were then detached with trypsin (Gibco) and measured or sorted by flow cytometry using CD45 and collagen I (since cultured cells gradually lost CD34 expression) as described above.[8]

Immunofluorescence

Differentiated fibrocytes were plated onto fibronectin (Sigma Aldrich) precoated slides after sorting by flow cytometry. Cells were fixed by 100% cold menthol and permeabilized with 1% Triton X-100. The slides were blocked with 10% normal goat serum for 30 minutes. After blocking, 50 μL of diluted α-smooth muscle actin antibody (1/200, Abcam) was applied overnight at 4°C. The secondary antibody, goat anti-rabbit conjugated with Alex Fluro 488 (Invitrogen), was applied for 30 minutes in the dark. After washing, the slides were mounted with Prolong Gold Antifade reagent (Invitrogen).

Migration Assay

After purification of differentiated fibrocytes by flow cytometry, as described above, 5×10^4 cells in serum-free DMEM (100 μL) were added to the upper chamber of a transwell chamber (8.0 μm diameter pore, Corning). DMEM containing 20% FBS was added in the lower chamber. After 36 hours, transwell membranes were fixed with methanol and stained with 4′,6-diamidino-2-phenylindole to detect nuclei. Cells accumulating on the lower side of the transwell membrane were acquired by microscopy under high magnification.

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction was used to identify the differentially expressed genes selected based on literature,

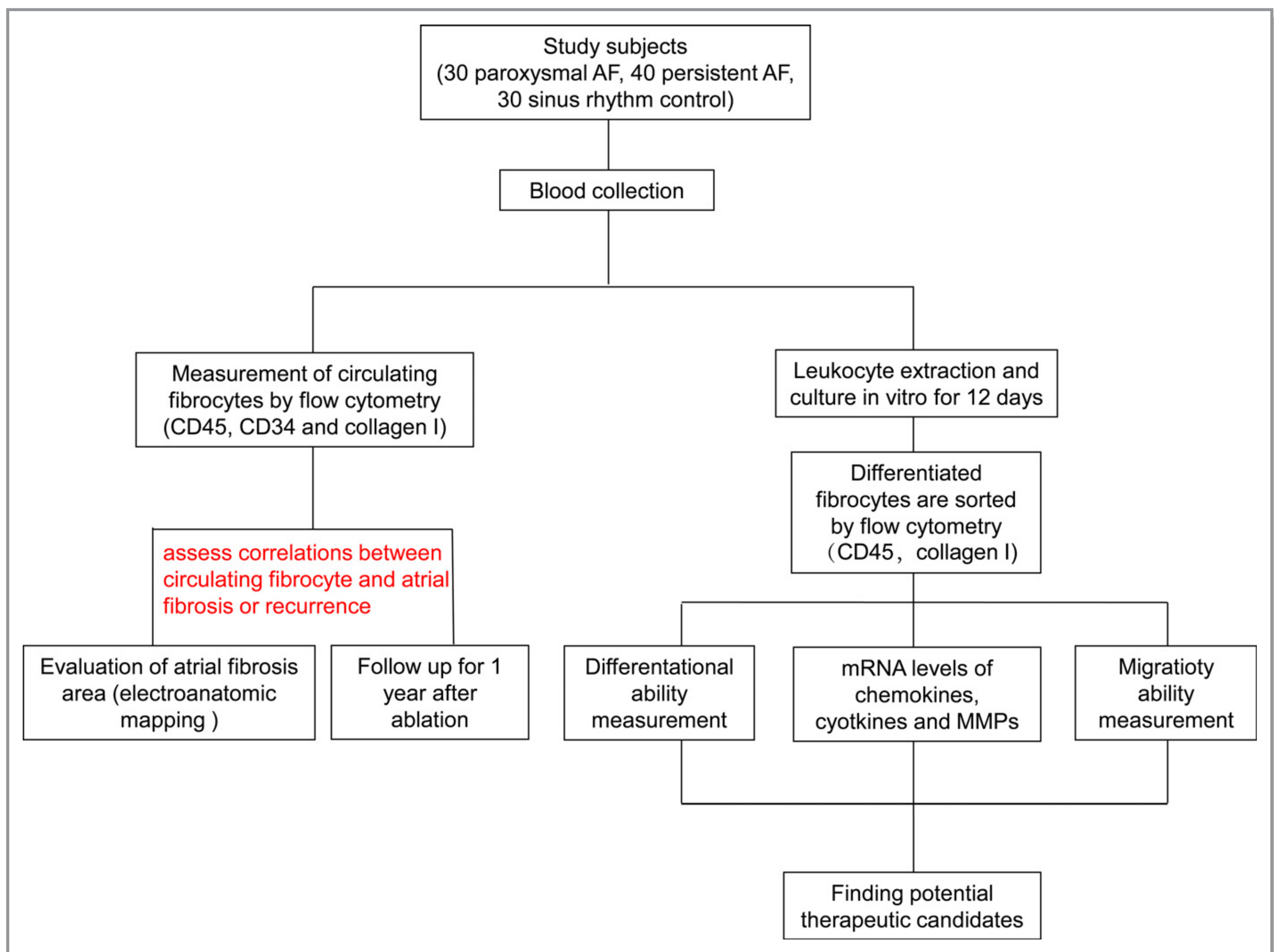

Figure 1. Overview of the workflow for the present study. AF indicates atrial fibrillation; MMPs, matrix metalloproteinases.

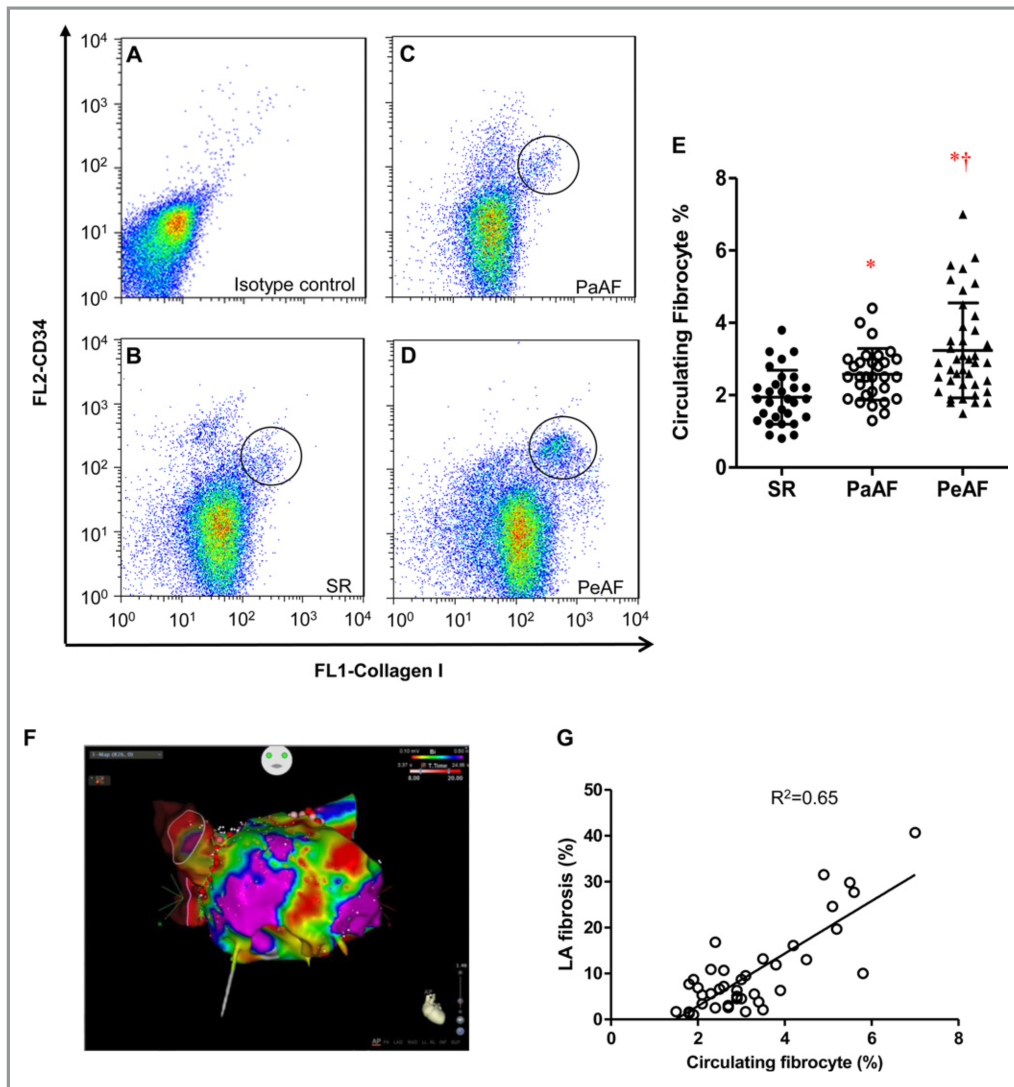

Figure 2. The proportion of circulating fibrocytes linked to the degree of left atrium (LA) low-voltage area and recurrence rate after ablation in patients with persistent disease. All CD45$^+$ leukocytes were gated for analysis. Representative images of flow cytometry displaying circulating fibrocytes, determined by the population of cells expressing CD45, CD34, and collagen I relative to the whole population of leukocytes (CD45$^+$) in the fresh blood from sinus rhythm (SR) controls (B), patients with paroxysmal AF (PaAF; C), or patients with persistent atrial fibrillation (PeAF; D), respectively. Isotype negative control is shown in (A). Patients with persistent atrial fibrillation (AF) displayed a higher percentage of circulating fibrocytes compared with patients with paroxysmal AF or SR controls (E). Representative images of 3-dimensional CARTO showing the electroanatomic map in the LA (F). The red color indicates low-voltage tissues, while the purple color indicates healthy tissues (F). Univariate linear regression analyzed the correlation between circulating fibrocyte percentage and LA fibrosis (G). The percentage of low-voltage area was calculated by the area of red color relative to the whole area of the LA. Receiver operating characteristic curve analysis revealed that the cutoff value for circulating fibrocytes percentage was 4.05%, with a sensitivity of 62% and specificity of 96% (H). Recurrence rate after ablation was examined by Kaplan–Meier analysis (I). Data are presented as mean±SD. *$P<0.05$ vs SR controls, †$P<0.05$ vs PaAF. *$P<0.05$ vs ≥4.05%.

including chemokine (C-C motif) ligand 2, C-C chemokine receptor type 7, CXC chemokine receptor type 4 (CXCR4), CX3C chemokine receptor 1, epidermal growth factor, epidermal growth factor receptor, fibroblast growth factor receptor 1, interleukin (IL) 6, IL-8, matrix metalloproteinase (MMP) 2, MMP-8, platelet-derived growth factor receptor A, platelet-derived growth factor receptor B, transforming growth factor β1 (TGF-β1), transforming growth factor β receptor 1 (TGF-βR1), and vascular endothelial growth factor A. Real-time polymerase chain reaction analysis was performed with a

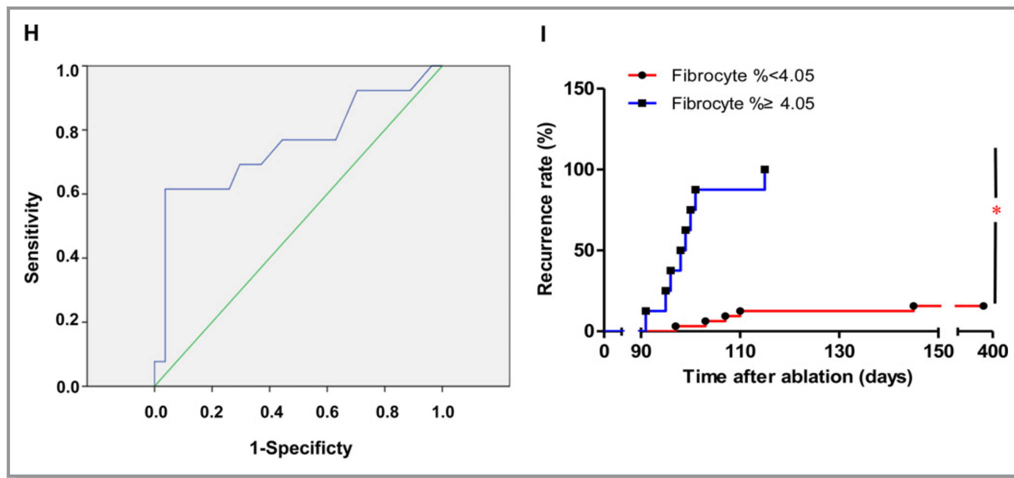

Figure 2. Continued.

7500 system (Applied Biosystems). The primers used are listed in Table S1. The cycling conditions consisted of an initial single cycle of 5 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 54°C, and 15 seconds at 72°C. The gene expression levels were quantified relative to the expression of 18s.

Enzyme-Linked Immunosorbent Assay

To determine the potential cytokines or chemokines that mediated fibrocyte activation, plasma levels of cytokines and chemokines including C-C chemokine ligand 2, C-C chemokine ligand 4, IL-1β, IL-8, MMP-2, MMP-8, serum amyloid P, stromal cell–derived factor 1 (SDF-1), and TGF-β1 from patients with AF and SR controls were measured by ELISA according to manufacturer's instructions.

Recombined Protein Interventions

In order to determine which cytokines or chemokines might promote differentiation of fibrocyte, we added recombined proteins to the medium during fibrocyte culture from SR controls. Fresh leukocytes were suspended in DMEM (Gibco) supplemented with 10% FBS (Gibco), 10% of control plasma, and recombined SDF-1 (100 ng/mL), TGF-β1 (10 ng/mL), and IL-8 (100 ng/mL), respectively. About 1×10^6 cells were seeded on

Table 2. Univariate Linear Regression Analyzed the Correlations Between Circulating Fibrocyte Percentage and LA Low-Voltage Area in Patients With Persistent AF

	Unstandardized Coefficients		Standardized Coefficients			95% CI for B	
Variables	B	SE	B	*t*	*P* Value	Lower	Upper
Sex				−1.463	0.164	−13.495	2.492
Age	−0.135	0.136	−0.159	−0.993	0.327	−0.409	−0.140
Hypertension				2.586	0.051*	−4.309	7.820
Resting heart rate, beats per min	0.097	0.074	0.208	1.309	0.198	−0.053	0.257
LA volume, mL	0.343	0.129	0.396	2.656	0.011*	0.082	0.605
LDL, mmol/L	0.880	1.731	0.082	0.508	0.614	−2.624	4.384
β-Blockers				0.349	0.729	−5.034	7.131
Angiotensin-converting enzyme inhibitors				1.024	0.312	−5.611	17.092
Angiotensin receptor blockers				−0.022	0.827	−10.153	8.164
Calcium channel blockers				−0.632	0.531	−11.962	6.271
Circulating fibrocytes, %	5.718	0.685	0.805	8.352	<0.001*	4.332	7.104

AF indicates atrial fibrillation; CI, confidence interval; LA, left atrium, LDL, low-density lipoprotein.
*$P<0.05$.

Table 3. Multivariate Linear Regressions for the Correlations Between Circulating Fibrocyte Percentage and LA Low-Voltage Area in Patients With Persistent AF

Models	Adjusted R^2	Circulating Fibrocytes, %			95% CI for B
		Unstandardized Coefficients	Standardized Coefficients	*P* Value	
Model 1	0.661	5.997	0.844	<0.001*	3.978–8.061
Model 2	0.683	5.631	0.792	<0.001*	4.244–7.018

AF indicates atrial fibrillation; CI, confidence interval; LDL, low-density lipoprotein. Model 1 included all covariates that may be associated with left atrium (LA) fibrosis. Model 2 included only variables that reached a *P* value <0.05 during the single linear regression analysis.
**P*<0.05.

a fibronectin precoated 6-well plate (Sigma Aldrich) at 37°C with 5% CO_2. After 3 days, nonadherent cells were removed and the remaining adherent cells were cultured until day 12. Differentiated fibrocytes were then detached with trypsin (Gibco) and measured or sorted by flow cytometry as described above. In the transwell assay, we also added these recombined proteins to the lower chamber of transwell, and then loaded purified differentiated fibrocytes (5×10^4) to the upper chamber.

Lentiviral Transduction

The lentiviral-based short hairpin RNA vectors were used for silencing CXCR4 (shCXCR4) and TGF-β1 receptor (shTGFβR1). The scramble shRNA was used as control (shControl). Briefly, 1×10^7 fresh leukocytes were cultured in a fibronectin precoated 6-well plate. After 24 hours, medium with polybrene (Sigma, 6 μg/mL) was added, followed by lentiviral solutions. After 24 hours, medium with lentivirus was replaced with fresh medium. After 3 days, nonadherent cells were removed and the remaining adherent cells were cultured until day 12. Differentiated fibrocytes were then detached with trypsin (Gibco) and measured or sorted by flow cytometry as described above. Further, sorted fibrocytes were assessed to the migration assay.

Statistical Analysis

Continuous data are presented as mean±SD. Chi-square test was used for categorical variables. For continuous variables, comparisons between 2 groups were made using Student *t* test, and comparisons among multiple groups were made using 1-way ANOVA with Tukey multiple comparison test if data were determined to be normal distribution. For nonparametric data, Mann–Whitney *U* test with exact method was used to analyze the differences between 2 groups. A Kruskal–Wallis ANOVA combined with post hoc Dunn multiple comparison test was performed when >2 groups were examined. Correlation was assessed using a Pearson

Table 4. Predictors of 1-Year Recurrence of in Patients With Persistent AF Following Ablation Assessed by Multivariate Cox Regression Analysis

Variables	B	*P* Value	Exp(B)	95% CI	
				Lower	Upper
Sex	1.184	0.131	3.269	0.702	15.218
Age	−0.071	0.057	0.932	0.866	1.002
Hypertension	−0.339	0.632	0.712	0.184	2.752
β-Blockers	1.253	0.185	3.499	0.549	22.312
Angiotensin-converting enzyme inhibitors	0.612	0.642	1.844	0.140	24.350
Angiotensin receptor blockers	0.920	0.457	2.510	0.222	28.336
Calcium channel blockers	1.493	0.305	4.452	0.257	77.254
LA volume, mL	0.081	0.094	1.084	0.986	1.191
Circulating fibrocytes, %	0.611	0.050*	1.842	1.001	3.388

All covariates were entered into the multivariate Cox regression models. Low circulating fibrocyte percentage <4.05 is defined as "0" and ≥4.05 as "1"according to receiver operating characteristic curve. AF indicates atrial fibrillation, CI, confidence interval, LA, left atrium.
**P*<0.05.

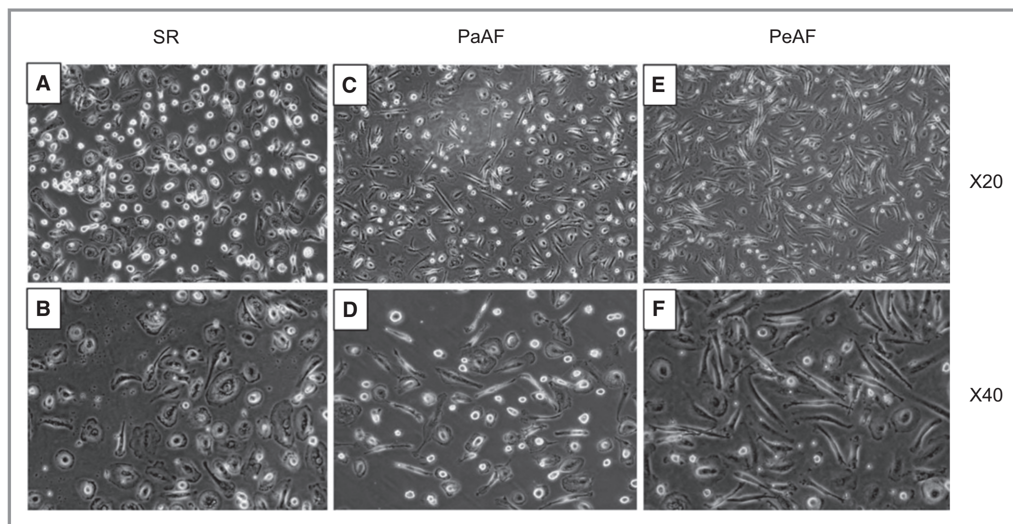

Figure 3. Circulating fibrocyte differentiation in the cell culture. Representative images of differentiated fibrocytes cultured from sinus rhythm (SR) controls (A and B), patients with paroxysmal atrial fibrillation (PaAF; C and D), or patients with persistent atrial fibrillation (PeAF; E and F) at a magnification of ×20 and ×40. Spindle-shaped cells were myofibroblasts that were differentiated from circulating fibrocytes.

coefficient. Associations between circulating fibrocyte percentage and LA low-voltage regions were assessed by multivariable linear regression models adjusting for covariables, including age, sex, hypertension, resting heart rate, LA volume, and low-density lipoprotein. The recurrence rate in patients with AF following ablation was visualized using Kaplan–Meier survival curves and log-rank tests. Multivariable Cox regression was performed to analyze the influence of circulating fibrocytes on AF recurrence after ablation. Variables expected to influence AF recurrence from previous publications, including age, sex, hypertension, LA volume, and circulating fibrocyte percentage were entered into the regression model. Low circulating fibrocyte percentage was defined as <4.05 and high circulating fibrocyte percentage as ≥4.05 according to receiver operating characteristic curve.

Results

Baseline Characteristics of Study Patients

Table 1 summarizes the baseline characteristics of the study participants. The occurrence of persistent AF was predominant in men. Resting heart rate and LA volume were higher in the 2 AF groups compared with the SR group. No significant difference in prevalence of other known risk factors, including systolic blood pressure, diastolic blood pressure, LA diameter, left ventricular ejection fraction, low-density lipoprotein, and B-type natriuretic peptide levels was observed between the paroxysmal and persistent AF groups. Overview of the workflow for the present study is summarized in Figure 1.

Circulating Fibrocyte Percentage Was Increased in Patients With Persistent AF

Circulating fibrocytes as percentage of the whole population of leukocytes ($CD45^+$) in peripheral blood were measured by flow cytometry using 3 markers (CD45, CD34, and collagen I) (Figure 2A through 2D). Patients with persistent AF displayed a high percentage of circulating fibrocytes (3.24±0.51%) compared with paroxysmal AF (2.58±0.16%) and SR controls (1.94±0.17%). The percentage of circulating fibrocytes was also significantly higher in patients with paroxysmal AF than in SR controls (Figure 2E).

Circulating Fibrocyte Percentage Correlated With Low-Voltage Region in LA and AF Recurrence After Ablation in Patients With Persistent AF

There is a reliable association between LA fibrosis imaged by MRI and low-voltage area by the CARTO system.[18–21] In the present study, LA low-voltage area and the complete LA area were measured by the 3D CARTO system (Figure 2F). Both univariate and multivariate linear regression models were used to evaluate relationships between circulating fibrocyte percentage and the extent of LA low-voltage area in patients with persistent AF (Figure 2G, Tables 2 and 3). Two models were selected for multivariate linear regression analysis. Model 1 included all covariates that may have associated with LA fibrosis and model 2 included only variables that reached a *P* value <0.05 during the univariate linear regression analysis. Circulating fibrocyte percentage was strongly associated with LA low-voltage area when age, sex,

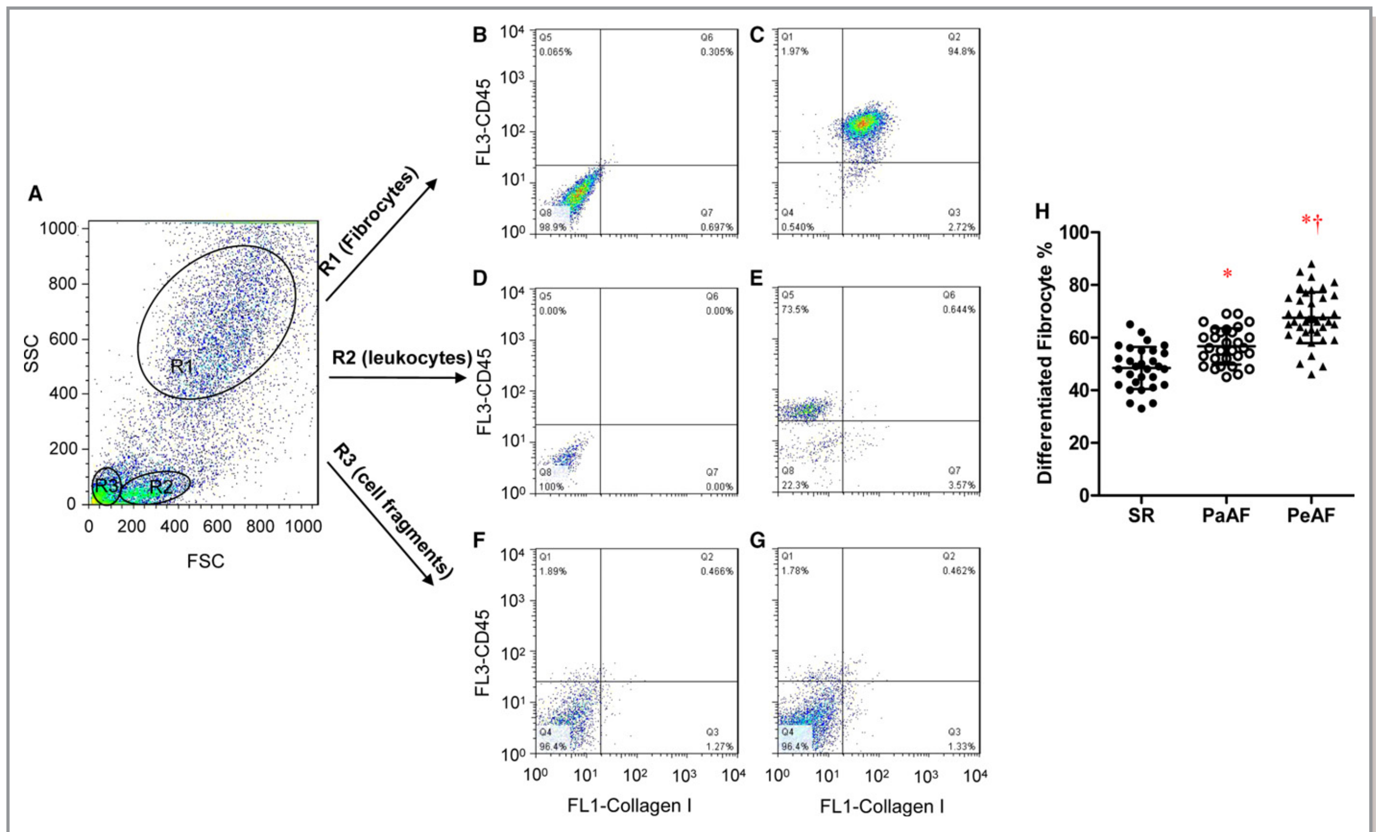

Figure 4. Quantitation and purification of differentiated fibrocytes following cell culture. Differentiated fibrocytes were determined by the population of cells expressing CD45 and collagen I relative to the whole population of leukocytes ($CD45^+$) in the cultured cells from sinus rhythm (SR) controls, patients with paroxysmal atrial fibrillation (PaAF), or patients with persistent atrial fibrillation (PeAF). Representative forward and side scatter properties of adherent cells after 12 days of culture by flow cytometry was used to separate the 3 populations (A: R1, R2, and R3). Isotype controls are presented in the left panels (B, D, and F). Double positive cells were identified as differentiated fibrocytes (R1, C), CD45-positive cells were leukocytes (R2, E), and double negative cells were accepted as cell fragments (R3, G). Quantitation of differentiated fibrocytes in the group, patients with paroxysmal atrial fibrillation (PaAF), and patients with persistent atrial fibrillation (PeAF) are presented in (H). Data are presented as mean±SD. *$P<0.05$ vs SR controls, †$P<0.05$ vs PaAF.

hypertension, resting heart rate, LA volume, low-density lipoprotein, and/or medication use were adjusted in 2 different models (Table 3). The relationship between circulating fibrocyte percentage and LA low-voltage area was also evaluated in patients with paroxysmal AF by using both univariate and multivariate linear regression models (not significant, Tables S2 and S3).

During 12 months of follow-up after a single ablation, 67.5% (27/40) of patients with persistent AF successfully maintained SR. Receiver operating characteristic curve analysis demonstrated that a circulating fibrocyte portion ≥4.05% was predictive of recurrence after a single ablation, with 62% sensitivity and 96% specificity (Figure 2H). Kaplan–Meier analysis showed a significantly higher rate of recurrent AF in the high fibrocyte group (8/8, 100%) versus the low fibrocyte group (5/32, 15.6% [Figure 2I]). To further identify whether circulating fibrocyte portion is a risk factor for recurrence, all variables that were expected to influence relapse, including age, sex, hypertension, LA volume, medications, and circulating fibrocyte percentage, were entered into the multivariable Cox regression model. According to our analysis, circulating fibrocyte ≥4.05% is a significant predictor of 1-year recurrence after ablation in patients with persistent AF (P=0.05, Table 4).

In the group with paroxysmal AF, 90% (27/30) of patients were successfully converted to SR. In order to further identify whether circulating fibrocyte portion is a risk factor for paroxysmal AF recurrence, all variables including age, sex, hypertension, LA volume, medications, and circulating fibrocyte percentage were entered into the multivariable Cox regression model. Our results showed that circulating fibrocyte percentage was not a significant predictor of 1-year recurrence after ablation in patients with paroxysmal AF (Table S4). When combining paroxysmal AF and persistent AF, circulating fibrocyte ≥4.10% was a significant predictor of 1-year recurrence after ablation for all recruited patients with AF (Table S5). Notably, all patients with recurrent AF had persistent, not paroxysmal, disease. Thus, we believe that circulating fibrocyte

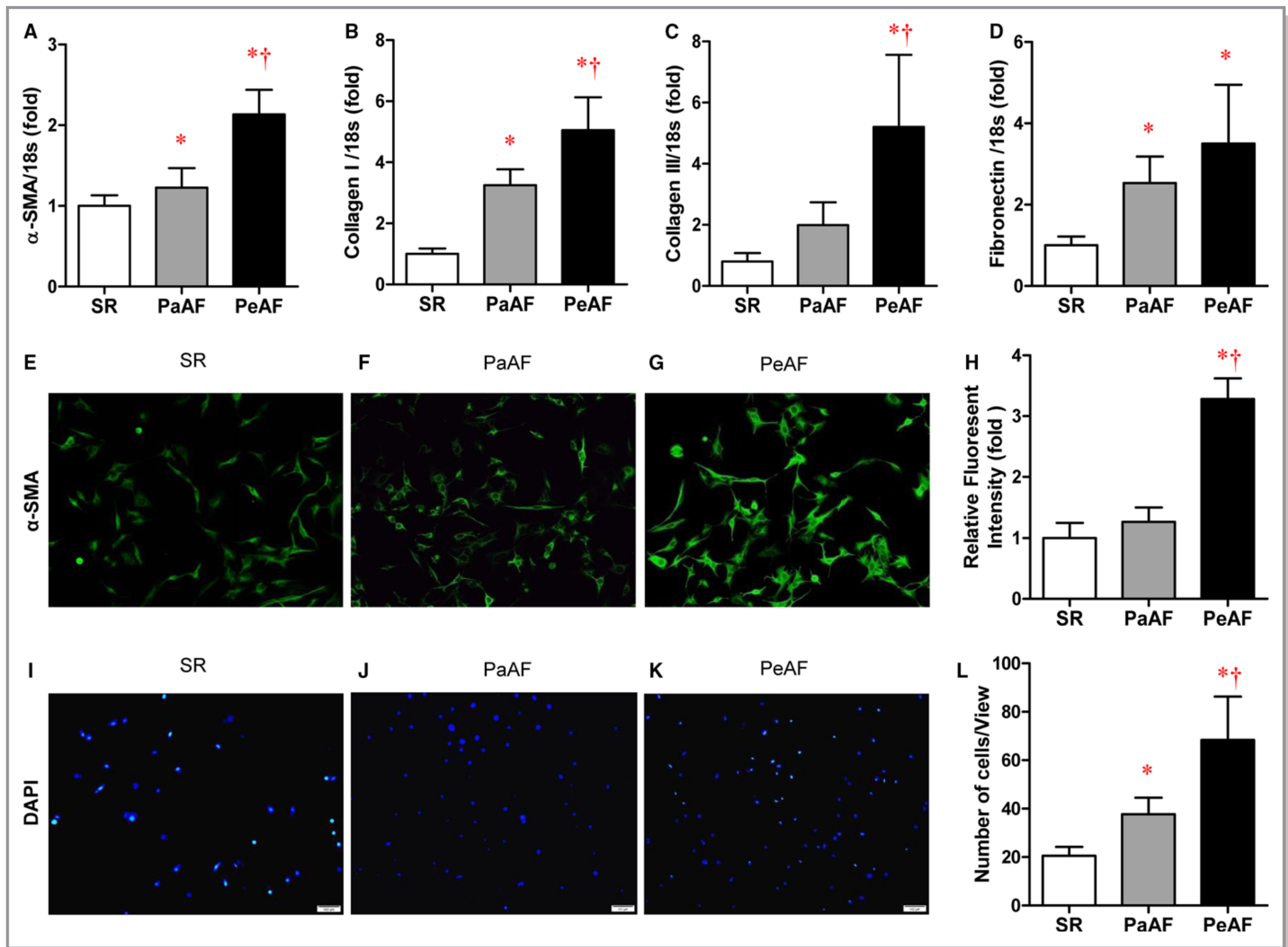

Figure 5. Differentiated fibrocytes from patients with atrial fibrillation (AF) were in a hyperactivated status. The mRNA levels of α-smooth muscle actin (α-SMA) (A), collagen I (B), collagen III (C), and fibronectin (D) of sorted differentiated fibrocytes from sinus rhythm (SR) controls, patients with paroxysmal atrial fibrillation (PaAF), and patients with persistent atrial fibrillation (PeAF) were measured by real-time polymerase chain reaction analysis. Representative immunofluorescence showed protein levels of α-SMA in differentiated fibrocytes from SR controls (E), patients with PaAF (F) or PeAF (G). Quantitation of total α-SMA fluorescent intensity is presented in (H). Migrated cells in transwell assays were stained by 4′,6-diamidino-2-phenylindole (DAPI; I through K). Differentiated fibrocytes from patients with PeAF (K) presented an enhanced migratory ability at 36 hours compared with patients with PaAF (J) and SR controls (I). Quantitation of migrated cell number is presented in (L). Data are presented as mean±SD (n=4–6 per group). **P*<0.05 vs SR controls, †*P*<0.05 vs PaAF.

percentage was more important for persistent AF recurrence predication.

Circulating Fibrocytes From Patients With Persistent AF Had an Enhanced Ability to Differentiate Into Myofibroblasts

To test whether fibrocytes were able to differentiate into myofibrosblasts, the morphology of cultured leukocytes from the 3 groups was microscopically examined after 12 days in culture. Differentiated fibrocytes appeared as spindle-shaped cells resembling tissue fibroblasts. We found that the number of spindle-shaped cells per field was markedly increased in the persistent AF group compared with the paroxysmal AF group or SR control group (Figure 3). Flow cytometry further revealed an increased proportion of differentiated fibrocytes (CD45$^+$/collagen I$^+$ cells, since CD34 is gradually disappeared during the differentiation) in patients with persistent AF (67.58±1.54%) compared with those with paroxysmal AF (56.67±1.52%) and SR controls (48.43±1.79%, Figure 4H).

Differentiated Fibrocytes From Patients With AF Were in a Hyperactivated Status

Differentiated fibrocytes from patients with AF were more activated compared with those in the paroxysmal AF group and SR controls, as measured by the content of α-smooth

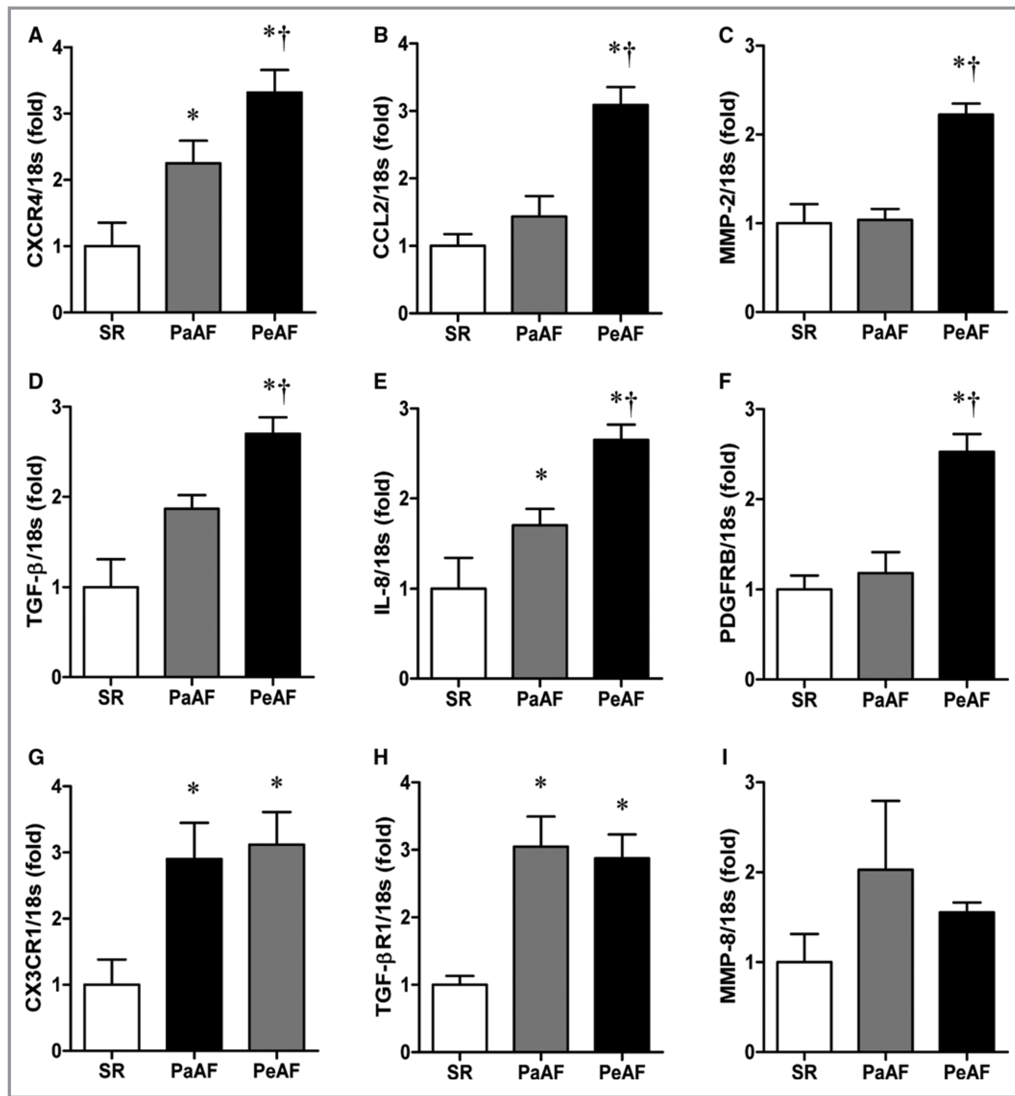

Figure 6. Differentially expressed genes in differentiated fibrocytes among 3 groups. The mRNA levels of 20 candidate genes in the differentiated fibrocytes (myofibroblasts) were examined using real-time polymerase chain reaction analysis. Relative to the sinus rhythm (SR) groups, levels of CXC chemokine receptor type 4 (CXCR4), chemokine (C-C motif) ligand 2 (CCL2), matrix metalloproteinase (MMP) 2, transforming growth factor β1 (TGF-β1), interleukin (IL) 8, platelet-derived growth factor receptor B (PDGFRB), CX3C chemokine receptor 1 (CX3CR1), transforming growth factor β receptor 1 (TGF-βR1), and MMP-8 in SR controls, patients with paroxysmal atrial fibrillation (PaAF), and patients with persistent atrial fibrillation (PeAF) are shown in (A through I). Data are presented as mean±SD (n=4–6 per group). *$P<0.05$ vs SR controls, †$P<0.05$ vs PaAF.

muscle actin, collagen I, collagen III, and fibronectin at mRNA (Figure 5A through 5D) or α-smooth muscle actin at protein levels (Figure 5E through 5G), and by a significantly higher migratory ability (Figure 5I through 5L).

Differentially Expressed Genes in Differentiated Fibrocytes Among 3 Groups

To investigate which mediators might be involved in fibrocyte differentiation, differentiated fibrocytes after purification were assessed by real-time polymerase chain reaction. According to the literature, genes that were expected to influence fibrocyte differentiation, such as cytokines, chemokines, MMPs, and growth factors, were examined. Relative to the SR control group and the paroxysmal AF group, the expression levels of CXCR4, C-C chemokine ligand 2, MMP-2, TGF-β1, IL-8, and platelet-derived growth factor receptor B were significantly upregulated in the persistent AF group (Figure 6A through 6F). The expression levels of CX3C chemokine receptor 1 and TGF-βR1 were enhanced in both AF groups

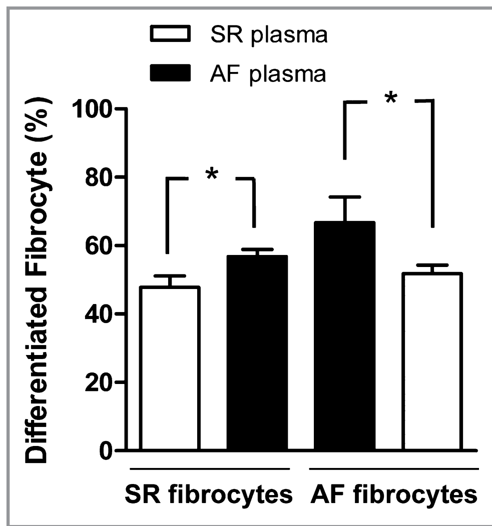

Figure 7. Circulating fibrocytes showed variations of differentiation in response to different sources of plasma. Leukocytes from patients with sinus rhythm (SR) or persistent atrial fibrillation were cultured in DMEM supplemented with plasma from SR controls or patients with persistent atrial fibrillation (AF). Plasma from patients with AF enhanced differentiational ability of SR fibrocytes compared with culture in its own plasma, while AF fibrocytes showed a reduced capacity to differentiate in response to SR plasma. Data are presented as mean±SD (n=4–6 per group). **P*<0.05 vs AF plasma.

compared with the SR control group, but no significant difference was observed between the paroxysmal and persistent AF groups (Figure 6G and 6H). There was no significant difference in the gene expression of MMP-8 (Figure 6I), platelet-derived growth factor receptor A, IL-6, epidermal growth factor, and epidermal growth factor receptor among 3 groups (data not shown). Notably, C-C chemokine receptor type 7, fibroblast growth factor receptor 1, and vascular endothelial growth factor A were weakly expressed (data not shown).

Changes in Plasma Cytokines and Chemokines

We observed that circulating fibrocytes showed diverse ability to differentiate, depending on the sources of plasma added to the culture medium. Plasma from patients with persistent AF increased the ability of SR control–derived fibrocytes to differentiate into fibroblasts, as compared with cultured SR control plasma. Conversely, when AF fibrocytes were cultured in SR control plasma, fewer fibroblasts were detected than cultured in its own plasma (Figure 7).

To further identify the potential cytokines or chemokines mediating fibrocyte differentiation, we measured plasma concentrations of 10 cytokines and chemokines selected according to the literature.[10,11] Among these, TGF-β1 and SDF-1 levels were significantly elevated in patients with persistent AF compared with those with paroxysmal AF and SR controls (Figure 8A and 8B). The plasma level of IL-8 was increased in both AF groups compared with the SR control group (Figure 8C). There were no significant differences in plasma levels of MMP-2, MMP-8, and serum amyloid P among the 3 groups (Figure 8D through 8F). However, C-C chemokine ligand 4, IL-1β, C-C chemokine ligand 8, and CXCL9 levels were undetectable in most plasma samples (data not shown).

Regulation of Differentiation and Migration of Circulating Fibrocytes

As shown in Figure 7, plasma from patients with persistent AF increased the ability of SR control–derived fibrocytes to differentiate into fibroblasts. Further, we found that plasma levels of TGF-β1, SDF-1, and IL-8 were elevated in patients with persistent AF, thus we speculated that these 3 inflammatory mediators might be potential candidates for regulating fibrocyte differentiation or migration. Treatment of recombined TGF-β1 promoted the ability of SR control–derived fibrocytes to differentiate into fibroblasts as compared with that cultured in the medium alone (Figure 9 through 9E). In contrast, TGF-βR1 knockdown blocked TGF-β1–induced differentiation of fibrocytes (Figure 9F through 9J). Moreover, SDF-1 had less ability to promote the differentiation of SR-derived fibrocytes compared with TGF-β1 (data not shown). IL-8 had no effect on promoting fibrocyte differentiation (data not shown).

We next determined whether these factors affect migration of fibrocytes. Transwell assay revealed that treatment of recombined SDF-1 markedly enhanced the migratory ability of SR control–derived fibrocytes compared with vehicle control (Figure 10A through 10C). Conversely, knockdown of CXCR4 significantly inhibited SDF-1–induced trafficking (Figure 10D through 10F). However, TGF-β1 and IL-8 had no effect on fibrocyte trafficking (data not shown). Overall, these results indicated that TGF-β1 promoted fibrocyte differentiation, while SDF-1 boosted fibrocyte migration.

Discussion

The major findings in the current study were: (1) circulating fibrocytes positively correlated with LA low-voltage area and were able to predict recurrence after ablation in patients with persistent AF; (2) circulating fibrocytes from patients with persistent AF had an enhanced capacity to differentiate into myofibroblasts; and (3) TGF-β1 and SDF-1 were the causative mediators that promoted fibrocyte differentiation and

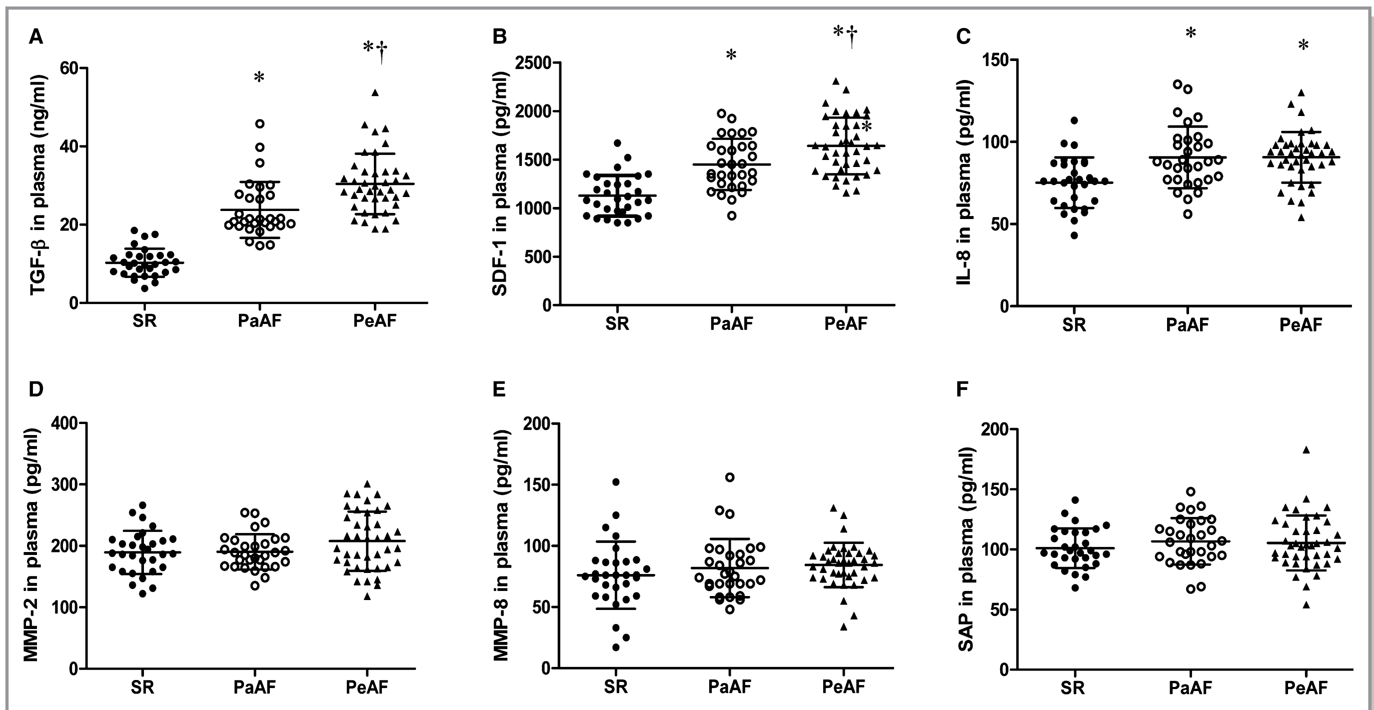

Figure 8. Changes in plasma cytokines and chemokines. Plasma levels of transforming growth factor β1 (TGF-β1), stromal cell–derived factor 1 (SDF-1), interleukin (IL)8, matrix metalloproteinase (MMP) 2, MMP-8, and serum amyloid P (SAP) were measured by ELISA kit and are presented by dot plots (A through F). Data are presented as mean±SD. **P*<0.05 vs sinus rhythm (SR) controls, †*P*<0.05 vs paroxysmal atrial fibrillation (PaAF). PeAF indicates persistent atrial fibrillation.

migration, respectively. Our results provide a circulating marker for assessing LA fibrosis extent and response to catheter ablation and advance our understanding of the mechanisms of LA fibrosis.

The contributions of circulating fibrocytes in cardiac fibrosis have been demonstrated in several studies. Circulating fibrocytes increased in patients with hypertensive heart disease, and there was a strong correlation between left ventricular mass index and total fibrocyte number.[22] Circulating fibrocytes from patients with hypertrophic cardiomyopathy with diffuse fibrosis had an enhanced ability of differentiation compared with patients without fibrosis or healthy controls.[23] In addition to circulation level, high levels of fibrocytes were also found in cardiac tissues under the condition of myocardial infarction, chronic heart failure, or angiotensin II infusion in mice.[15,16,23] Consistent with previous findings, our data showed both counting and activity of fibrocytes were elevated in patients with persistent AF, suggesting that circulating fibrocytes might contribute to AF progression. A previous study showed an elevated number of circulating $CD34^+$ stem cells in patients with persistent AF, which was reversed following cardioversion.[24] However, the functionality of these elevated $CD34^+$ cells was not studied. Our study provided evidence that circulating fibrocytes account, at least in part, for the elevated $CD34^+$ cell pool in patients with persistent AF, albeit mobilization of fibrocytes from bone marrow remains to be investigated.

During the catheter ablation procedure, electroanatomic mapping systems are used to provide a 3D atrial geometry and catheter navigation. Additionally, electroanatomic mapping is able to delineate the fibrotic area by imaging the low-voltage area in LA, which is in agreement with scar tissues imaged by MRI.[18] This finding was further confirmed by several studies.[19–21] By using a 3D CARTO system to quantify LA low-voltage area, we found a positive correlation between the circulating fibrocyte percentage and the extent of low-voltage area in the LA from patients with persistent AF. Thus, circulating fibrocytes could be an easy way to evaluate LA fibrosis for patients with persistent AF. Interestingly, patients with persistent AF who had >4.05% fibrocytes were at high risk of recurrent AF compared with patients with <4.05% fibrocytes, indicating that circulating fibrocytes could also serve as a strong predictor of future events for patients with persistent AF. However, circulating fibrocytes were not suitable for recurrence predication in paroxysmal AF after ablation.

AF is associated with both regional and systemic inflammatory responses.[25] The extent of regional inflammatory responses in the atrium is characterized by leukocyte infiltration and upregulation of inflammatory mediators.[25–27]

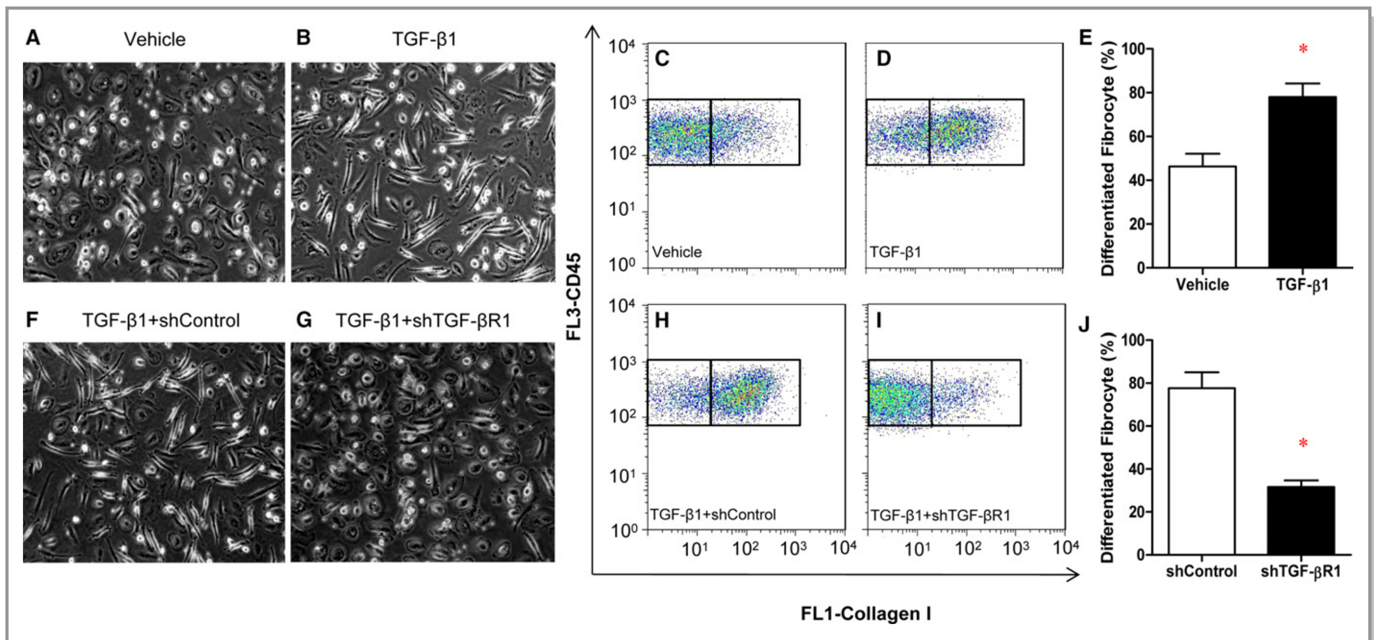

Figure 9. Transforming growth factor β1 (TGF-β1) promoted circulating fibrocytes to differentiate into myofibroblasts. Representative images of differentiated fibrocytes from sinus rhythm (SR) controls cultured with or without TGF-β1 (A and B). Spindle-shaped cells are recognized as myofibroblasts that differentiated from circulating fibrocytes. The portion of myofibroblasts ($CD45^+$/collagen I^+ cells) was examined by flow cytometry (C and D), and quantitation is shown in (E). Representative images of differentiated fibrocytes from SR controls under the condition of TGF-β1 treatment, with or without transforming growth factor β receptor 1 (TGF-βR1) knockdown are shown in (F and G). The portion of $CD45^+$/collagen I^+ cells infected by short hairpin control (shControl) or short hairpin TGF-βRa (shTGF-βR1) was analyzed by flow cytometry and is presented in (H and I). Quantitation of differentiated fibrocytes is shown in (J). Data are presented as mean±SD (n=3–5 per group). *$P<0.05$ vs vehicle or shControl.

In addition to regional levels, plasma levels of inflammatory mediators have also been reported in animals and patients with AF, but the cellular sources have not been well explored.[28] In the present study, we found that differentiated fibrocytes from patients with AF expressed high levels of cytokines, chemokines, and MMPs, which were predominantly involved in the functions of chemotaxis and inflammatory responses. Thus, our data extend previous findings by demonstrating that circulating fibrocytes are likely a cellular source of plasma inflammatory mediators, thereby enhancing inflammatory responses.

Circulating fibrocytes comprise a minor population of the circulating pool of leukocytes in healthy individuals, with limited ability of differentiating.[8] Here, we found that the capacity of plasma in inducing fibrocyte differentiation was more potent for plasma from patients with persistent AF than that of SR controls, suggesting the presence or enrichment of molecules in the plasma of patients with persistent AF that were able to mediate fibrocyte differentiate under culture conditions. In our study, plasma levels of TGF-β1 were significantly increased in patients with persistent AF. TGF-β1 was reported to promote the differentiation of fibrocytes into myofibroblasts via activating Smad2/3 and stress-activated protein kinase/c-Jun N-terminal kinase pathways.[29,30] Further, Shipe el al[31] found that fibrocytes from patients with asthma expressed high levels of phospho-Smad2/3 compared with the control group. Consistently, we demonstrated that activation of TGF-βR1 by TGF-β1 enhanced differentiation of fibrocyte into myofibroblasts.

Given the origin in bone marrow, fibrocytes traffic through the circulation and then are recruited to target tissues via the action of chemokines on fibrocyte surface receptors. SDF-1 (also called CXCR12) is the ligand of CXCR4. The SDF-1/CXCR4 axis has been shown to regulate mobilization and migration of stem cells, including fibrocytes, under disease conditions.[16,32,33] Cardiomyocyte-secreted SDF-1 plays an important role in recruiting fibrocytes into the myocardium in settings of myocardial infarction and angiotensin II infusion.[16,34] Furthermore, compared with SR controls, patients with persistent AF have a significantly higher plasma level of SDF-1, which is restored following cardioversion.[24] Consistently, we confirmed an elevation of SDF-1 in circulation and CXCR4 expression in fibrocytes of patients with AF. Moreover, we found that the SDF-1/CXCR4 axis played a predominant role in promoting the fibrocyte chemotaxis. Thus, targeting TGF-β1/TGF-βR1 or SDF-1/CXCR4 could be a useful treatment for preventing AF progression in the early phase or improving outcomes of catheter ablation in the late phase.

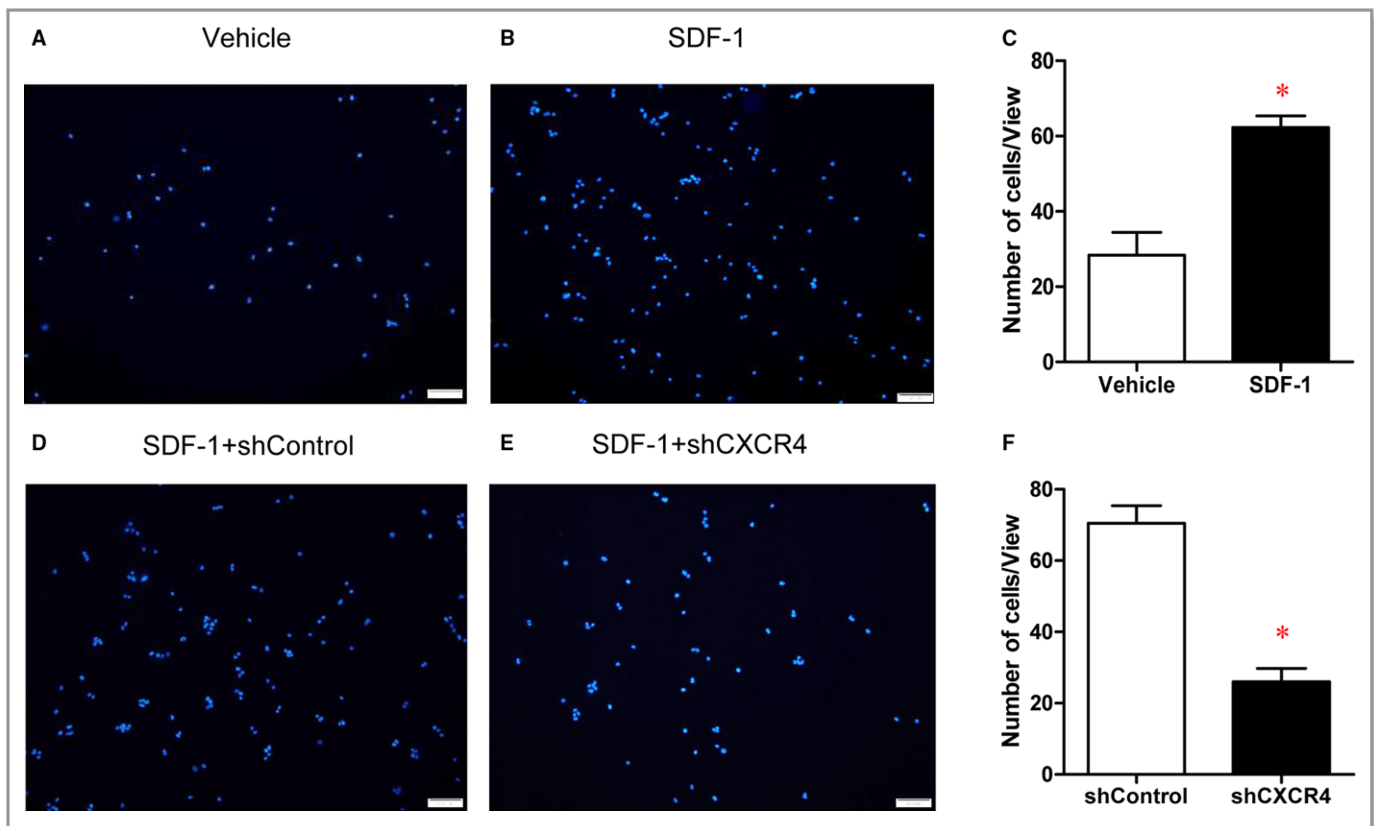

Figure 10. Stromal cell–derived factor 1 (SDF-1) promoted migration of fibrocytes. Circulating fibrocytes from sinus rhythm (SR) controls were loaded into the upper chamber of transwells for 36 hours after addition of SDF-1 or vehicle to the lower chamber of transwell. Representative images of migrated fibrocytes were stained with 4′,6-diamidino-2-phenylindole (DAPI) and are shown in (A and B). Quantitation of migrated cells is shown in (C). Fibrocytes with short hairpin control (shControl) or short hairpin CXC chemokine receptor type 4 (shCXCR4) were assessed to the transwell assay under the stimulation of SDF-1 for 36 hours. Representative images of migrated fibrocytes stained with DAPI are shown in (D and E). Quantitation of migrated cells is presented in (F). Data are presented as mean±SD (n=3–5 per group). *$P<0.05$ vs vehicle or shControl.

Limitations

There are a few limitations in the present study. First, the group size was relatively small. Second, we did not measure the counting and activity of circulating fibrocytes after ablation in the long-term follow-up studies. Third, because of the complex and long medication history, the drug effects on fibrocytes are still unclear.

Conclusions

In the setting of persistent AF, circulating fibrocytes were increased and activated and served as an important biomarker for the degree of low-voltage area in LA. In addition, a circulating fibrocyte portion ≥4.05% was predictive of recurrence after the first-time ablation. Inhibiting fibrocyte differentiation or trafficking might be useful as an adjuvant treatment to improve outcomes of catheter ablation for persistent AF.

Sources of Funding

Y. Liu, Li, Yu, and Xia were supported by a grant from the National Nature Science Foundation of China (No. 81201656 to Y. Liu), (81630009 to Li), (No. 81500276 to X. Yu) and (No. 81570313 to Xia); Li was supported by the Chang Jiang Scholar Program of China.

Disclosures

None.

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SUPPLEMENTAL MATERIAL

Table S1. Sequences of primers for qPCR.

Gene	Primer	Primer sequence
18s	Forward	5’ – TTG ACG GGA AGG GCA CCA CCA G – 3’
	Reverse	5’ – GCA CCA CCA CCC ACG GAA TCG – 3’
α-SMA	Forward	5’ – GGG ATT CCC TGG ACC TAA AG – 3’
	Reverse	5’ – GGA ACA CCT CGC TCT CCA – 3’
CCL2	Forward	5’ – TCGCTCAGCCAGATGCAAT – 3’
	Reverse	5’ – TGGCCACAATGGTCTTGAAG – 3’
CCR7	Forward	5’ – GCT CCA GGC ACG CAA CTT T – 3’
	Reverse	5’ – AGC TCA CAG GTG CTA CTG GTG AT– 3’
CX3CR1	Forward	5’ – AAT GAA AAG GGC CTC CAC AAT– 3’
	Reverse	5’ – GGT CCG GTT GTT CAT GGA GTT – 3’
CXCR4	Forward	5’ – ACT GAC GTT GGC AAA GAT GA – 3’
	Reverse	5’ – GGT GGT CTA TGT TGG CGT CT – 3’
Collagen I	Forward	5’ – GGG ATT CCC TGG ACC TAA AG – 3’
	Reverse	5’ – GGA ACA CCT CGC TCT CCA – 3’
Collagen III	Forward	5’ – TGG ATG GTG GTT TTC AGT TTA GCT A – 3’
	Reverse	5’ – TTT ACA TTT CCA CTG GCC TGA TC– 3’
EGF	Forward	5’ – CAG GGA AGA TGA CCA CCA CTA TT – 3’
	Reverse	5’ – TCC CCG ATG TAG CCA ACA AC – 3’
EGFR	Forward	5’ – CCT TGC CGC AAA GTG TGT AA – 3’
	Reverse	5’ – TGA AGG AGT CAC CCC TAA ATG C – 3’
FGFR1	Forward	5’ – CTG AGT ATG AGC TTC CCGAAGAC – 3’
	Reverse	5’ – CGT CCG ACT TCA ACA TCT TCA C –3’
Fibronectin	Forward	5’ – CTG GCC GAA AAT ACA TTG TAA A – 3’
	Reverse	5’ – CCA CAG TCG GGT CAG GAG – 3’
IL-6	Forward	5’ – TGG CTG AAA AAG ATG GAT GCT – 3’
	Reverse	5’ – TCT GCA CAG CTC TGG CTT GT – 3’
IL-8	Forward	5’ – TTG GCA GCC TTC CTG ATT TC – 3’
	Reverse	5’ – TGG TCC ACT CTC AAT CAC TCT CA –3’

MMP-2	Forward	5’ – TGT GAC GCC ACG TGA CAA G – 3’
	Reverse	5’ – GCT GGC TGA GTA GAT CCA GTA TTC A – 3’
MMP-8	Forward	5’ – CAG GGA GAG GCA GAT ATC AAC ATT – 3’
	Reverse	5’ – CCA TGT TTC TTC GGC ATC AA – 3’
PDGFRA	Forward	5’ – AAA TTG TGT CCA CCG TGA TCT G – 3’
	Reverse	5’ – CTC AGT GTG GTG TAG AGG TTG TCA A – 3’
PDGFRB	Forward	5’ – CCT GGC GGC TAG GAA CGT – 3’
	Reverse	5’ – GTG GTG TAG AGG CTG TTG AAG ATG – 3’
TGF-β1	Forward	5’ – CGC CAG AGT GGT TAT CTT TTG A – 3’
	Reverse	5’ – CGG TAG TGA ACC CGT TGA TGT– 3’
TGF-βR1	Forward	5’ – CCG TGG AGG GGA AAT TGA GG – 3’
	Reverse	5’ – GTA GTG TTC CCC ACT GGT CC– 3’
VEGFA	Forward	5’ – AGG AGT ACC CTG ATG AGA TCG AGT A – 3’
	Reverse	5’ – TGG TGA GGT TTG ATC CGC ATA– 3’

Table S2. Univariate linear regression analyzed the correlations between circulating fibrocyte percentage and LA low voltage area in paroxysmal AF patients.

Variables	Unstandardized Coefficients		Standardized Coefficients	t	*P*	95% CI for B	
	B	SE	B			Lower	Upper
Sex				-0.240	0.812	-3.608	2.851
Age	-0.011	0.083	-0.025	-0.135	0.894	-0.182	-0.159
Hypertension				-0.236	0.815	-3.601	2.858
Resting heart rate (beats/min)	-0.042	0.036	-0.215	-1.163	0.255	-0.115	0.032
LA volume (ml)	-0.027	0.107	-0.047	-0.250	0.805	-0.246	0.192
LDL (mmol/L)	1.550	1.322	0.216	1.173	0.251	-1.158	4.258
β-receptor blockers				-1.870	0.072	-6.171	0.281
Angiotensin convert enzyme inhibitor				1.843	0.076	-0.510	9.644
Angiotensin receptor blockers				-1.651	0.110	-9.269	0.995
Calcium channel blockers				-0.110	0.931	-4.248	3.814
Circulating Fibrocytes (%)	1.633	1.077	0.276	1.517	0.141	-5.73	3.840

*P<0.05

Table S3. Multivariate linear regressions for the correlations between circulating fibrocyte percentage and LA low voltage area in paroxysmal AF patients.

Variables	Adjusted R^2	Circulating Fibrocyte%			95% CI for B
		Unstandardized Coefficients	Standardized Coefficients	*P*	
Circulating Fibrocytes	-0.123	0.228	0.038	0.900	-3.545-4.001

The multivariate linear model included all covariates which may associate with LA fibrosis.

*P<0.05

Table S4. Predictors of 1-year recurrence of paroxysmal AF following ablation assessed by multivariate Cox regression analysis.

				95% CI	
	B	*P*	Exp(B)	Lower	Upper
Sex	-6.417	0.894	0.002	0.000	2E+038
Age	0.466	0.942	1.593	0.000	451915.4
Hypertension	-2.014	0.967	0.133	0.000	1E+041
β-receptor blockers	-21.046	0.946	0.000	0.000	3E+256
Angiotensin convert enzyme inhibitor	0.957	0.998	2.603	0.000	.
Angiotensin receptor blockers	9.773	0.884	17557.297	0.000	2E+061
Calcium channel blockers	27.467	0.925	9E+011	0.000	4E+259
LA volume (ml)	-2.234	0.577	0.107	0.000	275.827
Circulating Fibrocyte (%)	5.656	0.978	286.099	0.000	2E+173

All covariates were entered into the multivariate Cox regression models. Low circulating fibrocyte percentage < 2.85 is defined as “0” and ≥ 2.85 as “1”according to ROC curve. *$P<0.05$

Table S5. Predictors of 1-year AF (paroxysmal+persistent) recurrence following ablation assessed by multivariate Cox regression analysis.

				95% CI	
	B	*P*	Exp(B)	Lower	Upper
Sex	1.164	0.082	3.203	0.862	11.897
Age	-0.049	0.161	0.952	0.888	1.020
Hypertension	-0.176	0.808	0.839	0.204	3.449
β-receptor blockers	-0.298	0.632	1.347	0.398	4.565
Angiotensin convert enzyme inhibitor	-0.320	0.780	0.726	0.077	6.869
Angiotensin receptor blockers	0.049	0.959	1.050	0.161	6.828
Calcium channel blockers	0.593	0.472	1.809	0.359	9.101
LA volume (ml)	0.053	0.044	1.054	1.001	1.110
Circulating Fibrocyte (%)	2.715	0.000	15.110	3.956	57.718

All covariates were entered into the multivariate Cox regression models. Low circulating fibrocyte percentage < 4.10 is defined as "0" and ≥ 4.10 as "1"according to ROC curve.

*P<0.05