intervention along with the second interview and the tests at the end of the study. Among them 148 in the robust group (93%) and 159 in the frail group (89%) completed the study.

All 100 scheduled intervention sessions were completed. The median relative adherence was 92% (25–75th percentile, 85–95%) for the robust group and 92% (85–95%) for the frail group. No health problems, such as cardiovascular and musculoskeletal complications, occurred during the training sessions or testing. Minor problems were observed in both groups such as aching muscles after the first training session and fatigue. All the problems were managed easily by adjustment of the intervention and were improved during subsequent interventions.

Effect of the resistance training on outcome measures

LLM after resistance training in the robust and frail groups was significantly increased from the baseline ($P < 0.05$) (Table 1, Figure 1B). Pre- and post-intervention group statistics and group × time interactions are summarised in Table 1. A statistically significant group × time interaction was observed for TUG, FR and fear of falling ($P < 0.05$) (Figure 1C). Bonferroni-corrected paired-sample t-tests demonstrated a significant effect of the resistance training on TUG, FR and fear of falling in the frail group ($P < 0.025$).

Discussion

In this study, we showed that LLM was improved by the resistance training programme in both groups. However, the effect on physical function was limited to frail elderly defined by TUG. The role of muscle strength on physical function is supported by numerous cross-sectional studies that have shown a strong association between low muscle strength and decreased mobility in elderly [18]. On the

Table 1. Functional fitness items by group at pre- and post-intervention

	Robust group ($n = 148$)		E/S	P-value[a]	Frail group ($n = 159$)		E/S	P-value[a]	P-value[b]	F-value 1. Time effect 2. Group × Time
	Mean	SD			mean	SD				
Age, years	75.4	7.7			76.1	8.3			0.440	
Height, cm	157.7	10.1			156.7	9.1			0.266	
Weight, kg	58.2	11.1			56.8	10.9			0.280	
Gender, female n (%)	74 (50.0%)				82 (51.5%)				0.436	
Fall incidence, n (%)	48 (32.4%)				77 (48.4%)				0.003	
Leg lean mass, kg/weight										
Pre	0.160	0.024	0.39	<0.001	0.162	0.024	0.27	0.002	0.448	32.1**
Post	0.167	0.024			0.167	0.021				1.1
Percent change, %	0.05	0.09			0.04	0.11				
Walking time, s										
Pre	10.0	1.9	0.11	0.294	16.1	3.8	0.16	0.130	0.017	1.1
Post	10.2	2.1			15.5	4.1				3.6
Percent change, %	0.3	15.5			−7.7	27.5				
Timed up and go test, sec										
Pre	9.9	1.8	0.09	0.374	17.4	3.0	0.32	0.004	0.002	6.1*
Post	10.1	2.5			16.1	3.9				10.5**
Percent change, %	0.9	18.1			−14.5	37.6				
One leg standing, s										
Pre	9.8	11.8	0.06	0.567	1.7	1.9	0.16	0.160	0.987	0.1
post	9.2	13.9			2.6	5.4				1.4
Percent change, %	−47.3	173.4			46.8	248.3				
Functional reach, cm										
Pre	23.5	5.9	0.01	0.948	18.0	5.6	0.46	<0.001	0.029	7.5**
Post	23.4	5.9			20.9	6.8				8.0**
Percent change, %	−7.2	46.4			23.6	48.1				
Five chair stand, s										
Pre	11.2	3.2	0.07	0.498	16.8	5.2	0.17	0.144	0.004	1.6
Post	11.5	4.7			15.1	8.6				3.1
Percent change, %	5.0	31.3			−29.9	72.8				
Fear of falling, points										
Pre	36.6	4.4	0.18	0.081	32.9	6.2	0.51	<0.001	<0.001	26.2**
Post	37.1	3.9			35.9	3.5				15.4**
Percent change, %	1.5	7.3			12.9	23.3				

E/S, effect size.

[a]As calculated by comparing pre- and post-intervention.

[b]As calculated by group comparison.

*$P < 0.05$.

**$P < 0.01$.

other hand, muscle strength does not depend solely on muscle mass, and the relationship between strength and mass is not linear [19]. Rantanen *et al.* reported that the relationship between muscle strength and physical disability in older adults is non-linear [20]. The discrepancy between these results may stem from the heterogeneity of subjects. In this study, we stratified subjects into robust and fail elderly groups. In frail elderly, the 50-week resistance training programme was effective for the improvement of LLM and physical performance. In contrast, there was no correlation between the change in LLM and physical performance in robust elderly undergoing the resistance training programme. These results suggested that our resistance training programme is not effective for the improvement of physical performance in robust elderly. Furthermore, resistance training improved muscle strength, but did not improve physical performance in the relatively healthy elderly [21]. On the other hand, in frail elderly, improvements in leg power, independent of strength, appear to make an important contribution to clinically meaningful improvements in physical performance [22].

Resistance training improved balance function, such as FR in frail elderly. Improved balance function with resistance training is hypothesised to occur by reduced motor-unit discharge variability [23]. However, SLS was not improved. These results suggested that balance improvement after power training may be explained, in part, by adaptations in force control. However, resistance training *per se* is not effective for balance function. For the improvement of balance function, it is useful to add not only the resistance training but also balance training, such as Tai Chi Chuan [24].

In addition to improving physical performance, the resistance training programme was effective for decreasing fear of falling, but only in the frail group. It is considered important to reduce fear of falling by targeting downstream factors such as physical functioning [25] or predictors of those factors [26]. Thus, our study has an important implication for the reduction in fear of falling in frail elderly.

There are several limitations to this study that warrant mention. First, although we used only TUG to define frailty, TUG may not be enough to define frailty. For example, the short physical performance battery evaluates balance, gait, strength and endurance by examining an individual's ability [27]. It has been recently recommended by an international working group to use a functional outcome measure in clinical trials in frail older adults [28]. Second, we did not measure muscle force. The relationship between LLM and muscle strength is still unclear and needs to be addressed in future studies. Third, no follow-up was conducted. Evidence regarding the long-term effect of exercise on fall prevention is limited, and, therefore, this issue also needs to be addressed. Finally, a control group was lacking. The participants in both groups may have had higher motivation and interest in health issues than the general elderly population.

This is the first study to demonstrate that the effects of a resistance training programme on physical performance differed according to the level of physical well-being. Future work should determine whether tailor-made interventions can effectively improve physical function in both robust and frail elderly.

Key points

- The current trial compared the effects of resistance training between robust and frail elderly on skeletal muscle mass, physical performance and fear of falling.
- Skeletal muscle mass after resistance training was significantly increased from the baseline in both groups.
- The resistance training programme was more effective for the improvement of physical performance and fear of falling in frail elderly than in robust elderly.

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Conflicts of interest

None declared.

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Transient ischaemic attack, vascular risk factors and cognitive impairment: a case–controlled study

SIR—Cognitive impairment, especially difficulties with temporal orientation and verbal recall, is associated with the increasing number and severity of vascular risk factors (VRFs) such as hypertension and diabetes [1–3] which can result in an associated impairment of the cerebral microcirculation causing white matter volume changes linked to large artery stiffness [4, 5]. These cognitive deficits can be detected by using simple standard screening tools [6] such as the Mini Mental State Examination [7], Montreal Cognitive Assessment (MoCA) [8] and the DemTec [9], and have been shown to be related to the development of both subclinical (mild) or established vascular disorders [7–12].

However, our understanding of the relation between transient ischaemic attacks (TIAs) and cognitive status is incomplete. We hypothesised that subjects with newly diagnosed TIA would have evidence of an associated mild cognitive impairment; this being a manifestation of the same pathological process underlying the pathogenesis of the vascular event being initiated and accelerated by VRFs. The aims of the current study were, therefore, (i) to examine whether patients with first ever TIA and no history of stroke have evidence of cognitive impairment and, if so, whether the extent of the impairment was greater than expected compared with an age-, sex-matched control populations without VRFs and (ii) to determine which VRFs are associated with cognitive impairment.

Methodology

We conducted a case–controlled study between August and November 2008 in a University Hospital in UK (catchment population 750,000). Cases were defined as those patients with first ever TIA aged ≥45 years, assessed in a

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Original article

Differential determinants of physical daily activities in frail and nonfrail community-dwelling older adults

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ABSTRACT

Background/Purpose: The purpose of this study was to determine whether or not daily activities determined by average daily steps are associated with age, gender, body mass index, fear of falling, and physical functions (locomotive function, balance function, and muscle power) in community-dwelling nonfrail and frail older adults.

Methods: This is a cross-sectional study conducted in community-dwelling older adults in Japan. Based on the Timed Up and Go (TUG) test, 629 elderly adults were divided into two groups: 515 were grouped to nonfrail elderly (TUG time less than 13.5 seconds, mean age 77.0 ± 7.2 years) and 114 to frail elderly (TUG time of 13.5 seconds or more, mean age 76.1 ± 7.5 years). Daily physical activities were determined by average daily steps measured by pedometer and four other physical function tests (10-m walk test, single-leg standing, functional reach, and five-chair stand test) were performed along with the assessment of fear of falling.

Results: Stepwise regression analysis revealed that age, gender, 10-m walk test, and single-leg standing were significant and independent determinants of the average step counts in the nonfrail elderly ($R^2 = 0.282$, $p < 0.001$), whereas fear of falling was the only significant and independent determinant of the average step counts in the frail elderly ($R^2 = 0.119$, $p < 0.001$).

Conclusion: These results indicate that differential factors may be related to daily activities depending on the level of frailty in community-dwelling older adults.

1. Introduction

Physical activities show positive associations with various components of physical functions, such as walking speed, lower-limb strength, and balance and negative associations with the incidence of coronary artery disease, obesity, osteoporosis, and other causes of morbidity and mortality in elderly.[1–4]

Higher physical activities can also improve quality of life and physical and psychological functions, facilitate independent living, and reduce the risk of dementia in older adults.[5–8] Physical Activity Guidelines for Americans concluded that, for older adults, in addition to the well-known health benefits of a physically active lifestyle, "strong evidence indicates that being physically active is associated with higher levels of functional health and a lower risk of falling."[9]

However, Yoshida et al[10] showed that the association between physical fitness and ambulatory activity is affected by the level of instrumental activity of daily life in elderly women, suggesting the effect of frailty on the association. We demonstrated that the resistance training program is effective at decreasing the fear of falling in frail elderly but not in nonfrail elderly (Yamada et al, present study), indicating the difference of the effect of physical training in elderly with different physical fitness. We hypothesized, therefore, that differential factors could affect the level of physical daily activities in the presence or absence of frailty. The purpose of this study was to determine whether or not physical activities determined by average daily steps are associated with age, gender, body mass index (BMI), fear of falling, and physical function (locomotive function, balance function, and muscle power) in community-dwelling nonfrail and frail older adults.

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2. Methods

2.1. Participants

Participants were recruited by an advertisement in a local press. We used the following criteria to screen participants in the initial interview and invited to participate in this study if he or she was aged 65 years or older , was community-dwelling, had a score of eight or more by Rapid Dementia Screening Test,[11] and was able to walk independently.

We excluded participants based on the following exclusion criteria: the presence of severe cardiac, pulmonary, or musculoskeletal disorders; comorbidities associated with an increased risk of falls (i.e., Parkinson's disease or stroke); and use of psychotropic drugs. We obtained written informed consent from each participant in accordance with the guidelines approved by the Kyoto University Graduate School of Medicine and the Declaration of Human Rights, Helsinki, 1975.

2.2. Definition of frailty

The definition of frailty is based on the results of previous study. The Timed Up and Go (TUG) is a simple test developed to screen basic mobility performance and has been shown to be significantly associated with activities of daily living function in frail older adults.[12] It has been reported that elderly with a TUG score greater than 13.5 seconds have an increased risk of falls.[13] Therefore, frailty was defined as a TUG score greater than 13.5 seconds. Based on key components of the screening examination (TUG score greater than 13.5 seconds), 114 elderly were classified as frail, whereas 515 elderly as nonfrail.

2.3. Measurement of physical activities

A valid, accurate, and reliable pedometer, Yamax PowerWalker EX-510 (Yamax Corp., Tokyo, Japan), was used to measure free-living step counts.[14] Measurement of step counts was conducted between October and November 2010. Participants were instructed to wear the pedometer in their pocket of dominant leg for 14 consecutive days except during bathing, sleeping, and performing water-based activities. This pedometer has a 30-day data storage capacity. We calculated the averages of their daily step counts for 2 weeks.

2.4. Measurement of fear of falling

We assessed fear of falling by asking a single yes or no question, "Are you afraid of falling,?" which has a high test-retest reliability.[15] The test-retest reliability using the Kappa coefficient was 0.960.

2.5. Measurement of physical function

The participants received four other physical function tests that are widely used to identify high-risk elderly: 10-m walk test, single-leg standing, functional reach, and five-chair stand. In 10-m walk test, the participants were asked to walk as fast as possible along a 10-m straight line, with a 1 m approach at both ends, making a total length of 12 m. The time required was taken as the measured value. In single-leg standing, the length of time for which participants were able to stand on one leg with their hands placed on their waist was measured. The time was measured twice for each leg and the maximum length of time was taken. Functional reach was measured using the simple clinical apparatus consisting of a leveled yardstick secured to the wall at right acromion height as previously described.[16] In five-chair stand, participants were asked to stand up and sit down five times as quickly as possible and were timed from the initial sitting position to the final standing position at the end of the fifth stand.[17] For each function test, the participants performed twice, and the average score was then calculated. All test measurements were completed before the daily step measurement.

2.6. Statistical analysis

The relationship between the average daily steps and physical function was investigated with the Pearson correlation coefficient. The t test and χ^2 test were used to compare the results of measurements between frail and nonfrail groups.

A multivariate analysis by means of multiple regression using a stepwise method was performed to investigate which of the age, gender, BMI, fear of falling, and five measures of physical function (i.e., 10-m walk test, TUG, single-leg standing, functional reach, and five-chair stand test) were independently associated with the average daily steps in each group.

Data were analyzed using the Statistical Package for Social Science (Windows version 18.0; SPSS Inc., Chicago, IL, USA).

3. Results

There were no significant differences in age (nonfrail = 77.0 ± 7.2, frail = 76.1 ± 7.5, $p = 0.241$), gender (nonfrail = 67.5%, frail = 67.5%, $p = 0.541$), height (nonfrail = 153.5 ± 7.6 cm, frail = 153.7 ± 6.1 cm, $p = 0.743$), weight (nonfrail = 53.0 ± 9.6 kg, frail = 53.6 ± 4.5 kg, $p = 0.576$), and BMI (nonfrail = 22.4 ± 3.2, frail = 22.7 ± 1.9, $p = 0.393$) between the two groups (Table 1). However, all physical function tests and average daily steps were significantly different between the two groups. More fear of falling was observed (nonfrail = 39.1%, frail = 73.6%, $p < 0.001$), longer time was required for 10-m walk test (nonfrail = 9.9 ± 2.2 seconds, frail = 17.1 ± 6.6 seconds, $p < 0.001$), single-leg standing (nonfrail = 13.3 ± 12.1 seconds, frail = 3.1 ± 6.0 seconds, $p < 0.001$), and five-chair stand (nonfrail = 8.9 ± 3.6 seconds, frail = 17.6 ± 8.5 seconds, $p < 0.001$) in frail elderly. Less functional reach (nonfrail = 25.0 ± 8.2 cm, frail = 17.9 ± 8.4 cm, $p < 0.001$), and average daily steps (nonfrail = 4414 ± 2726 steps, frail = 1585 ± 1013 steps, $p < 0.001$) were observed in frail elderly.

To determine the association of average step counts with physical functions and demography, we analyzed Pearson's correlation coefficients in frail and nonfrail elderly. Table 2 shows that average step counts in the nonfrail group were correlated with age ($r = -0.311$, $p < 0.001$), BMI ($r = 0.167$, $p < 0.001$), 10-m walk test ($r = -0.475$, $p < 0.001$), TUG ($r = -0.412$, $p < 0.001$), functional

Table 1
Comparison of demography, fear of falling, and physical function and activities between nonfrail and frail elderly

Items	Nonfrail group ($n = 515$)		Frail group ($n = 114$)		p
	Mean	SD	Mean	SD	
Age (yr)	77.0	7.2	76.1	7.5	0.241
Gender (male = 0, female = 1)	67.5		67.5		0.541[a]
Height	153.5	7.6	153.7	6.1	0.743
Weight	53.0	9.6	53.6	4.5	0.576
BMI (kg/m^2)	22.4	3.2	22.7	1.9	0.393
Fear of falling (yes = 1, no = 0)	39.1		73.6		<0.001[a]
10-m walking time (s)	9.9	2.2	17.1	6.6	<0.001
Timed up & go test (s)	8.8	2.1	20.2	6.8	<0.001
Single leg standing (s)	13.3	12.1	3.1	6.0	<0.001
Functional reach (cm)	25.0	8.2	17.9	8.4	<0.001
Five chair stand (s)	8.9	3.6	17.6	8.5	<0.001
Average daily step (step)	4414.4	2726.3	1585.0	1012.6	<0.001

BMI = body mass index; SD = standard deviation.
[a] χ^2 test.

Table 2
Pearson's correlation coefficients (r) between average daily steps and physical functions, age, and BMI

Items	Nonfrail group ($n = 515$)	Frail group ($n = 114$)	Overall ($n = 629$)
Age (yr)	−0.311**	−0.109	−0.241**
BMI (kg/m^2)	0.167**	−0.013	0.130**
10-m walking time (s)	−0.475**	−0.047	−0.448**
Timed up & go test (s)	−0.412**	−0.131	−0.450**
Functional reach (cm)	0.348**	0.175	0.406**
Five-chair stand (s)	−0.297**	−0.226*	−0.397**
Single-leg standing (s)	0.440**	0.077	0.502**

BMI = body mass index.
*$p < 0.05$; **$p < 0.01$.

reach ($r = 0.348$, $p < 0.001$), five chair stand test ($r = -0.297$, $p < 0.001$), and single-leg standing test ($r = 0.440$, $p < 0.001$). In the frail group, however, a significant association was found only with five-chair stand test ($r = -0.226$, $p < 0.001$). Figure 1 shows linear regressions between physical functions and average step counts in nonfrail and frail elderly. Average step counts had a positive association with functional reach (Fig. 1C) and negative associations with 10-m walk test (Fig. 1A) and TUG (Fig. 1B) only in nonfrail elderly. However, step counts had a negative association with five-chair stand (Fig. 1D) both in nonfrail and frail elderly.

Stepwise regression analysis revealed that age ($\beta = -0.108$, $p = 0.03$), gender ($\beta = 0.255$, $p < 0.001$), 10-m walk test ($\beta = -0.202$, $p < 0.001$) and single-leg standing ($\beta = 0.306$, $p < 0.001$) were

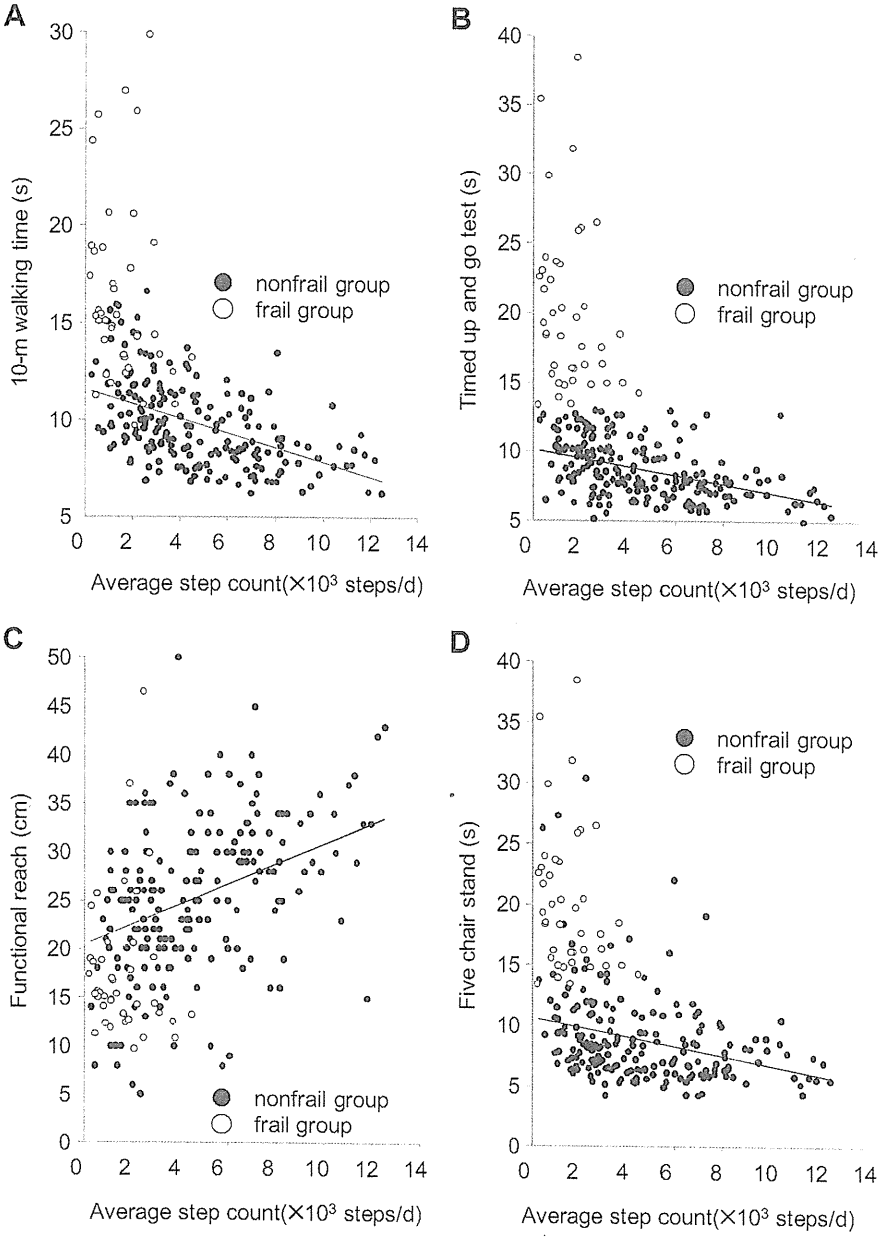

Fig. 1. Relationships between average daily steps and physical function. The physical function was associated with physical activities in nonfrail but not in frail elderly. (A) 10-m walk test; (B) Timed up and go test; (C) Functional reach; (D) Five-chair stand test.

Table 3
Multiple stepwise regression analysis

Independent variables	Nonfrail group Adjusted R^2 value = 0.282 standard regression value	Frail group Adjusted R^2 value = 0.119 standard regression value	Overall Adjusted R^2 value = 0.345 standard regression value
Age (yr)	−0.108*		−0.137**
BMI (kg/m^2)			
Gender (male = 0, female = 1)	0.255**		0.238**
Fear of falling (yes = 1, no = 0)		−0.356**	−0.089*
10-m walking time (s)	−0.202**		−0.172**
Timed up & go test (s)			
Functional reach (cm)			
Five chair stand (s)			−0.147**
Single leg standing (s)	0.306**		0.314**

*$p < 0.05$; **$p < 0.01$.

significant and independent determinants of the average step counts in nonfrail elderly ($R^2 = 0.282$, $p < 0.001$) (Table 3). Stepwise regression analysis also revealed that fear of falling ($\beta = -0.356$, $p < 0.001$) was the only significant and independent determinant of the average step counts in frail elderly ($R^2 = 0.119$, $p < 0.001$) (Table 3).

4. Discussion

In the present study, we showed that the differential factors of physical functions may relate to the daily activities in frail and nonfrail community-dwelling elderly Japanese. Our data implicate that physical daily activities can be maintained in the robust elderly with high physical function, whereas fear of falling plays a more important role for the maintenance of physical daily activities if an older adult becomes functionally impaired and frail. Previous studies also indicated that the low self-efficacy for daily activities reduces physical activity, and psychological well-being is an important predictor for staying physically active.[18,19] Thus, differential approaches should be taken to keep the daily activities depending on their physical fitness in elderly.

The physical functions, age, and gender were associated with daily activities in nonfrail elderly but not in frail elderly. Rantanen et al[20] also reported that the relationship between muscle strength and physical disability in older adults is nonlinear. Moreover, in most of previous reports, the participants were nonfrail older adults.[1–4] Therefore, it has been assumed that there is an association between daily activities and physical functions. In addition, daily activities tended to be greater in women than in men. The reason for greater daily activities in women is often ascribed to activities, such as housework and gardening.[20]

On the other hand, we demonstrated that fear of falling was associated with physical daily activities in frail elderly but not in nonfrail elderly. Fear of falling is shown to be associated with frailty.[21,22] Several studies have indicated that people who are afraid of falling appear to enter a debilitating spiral of loss of confidence, restriction of physical activities, physical frailty, lack of social participation, falls, and loss of independence.[23–28] However, Wolf et al[29] reported that increased core and lower extremity strength with exercise decreases the fear of falling. Moreover, cognitive behavioral therapy has been shown to reduce fear of falling.[30–32]

There were several limitations of this study that warrant mention. First, although we used TUG to define frailty, TUG may not be enough to define frailty. Edmonton frail scale adopts eight other domains, such as cognition, general health status, functional independence, social support, medication use, nutrition, mood, and continence other than TUG.[33] Further study is required to test the levels of these domains in this cohort. Second, participants have used pedometer measurements limited to only 2 weeks. If seasonal changes in activity pattern were taken into consideration, long-term use would be more appropriate. Third, the participant's community was not in the rural area. The present study is the result of being restricted to older adults in the urban area.

This is the first study to demonstrate that differential factors affect daily activities depending on the level of frailty. Future work should determine whether individualized intervention can effectively improve physical activity in both nonfrail and frail elderly.

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Activation of Src Mediates PDGF-Induced Smad1 Phosphorylation and Contributes to the Progression of Glomerulosclerosis in Glomerulonephritis

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Abstract

Platelet-derived growth factor (PDGF) plays critical roles in mesangial cell (MC) proliferation in mesangial proliferative glomerulonephritis. We showed previously that Smad1 contributes to PDGF-dependent proliferation of MCs, but the mechanism by which Smad1 is activated by PDGF is not precisely known. Here we examined the role of c-Src tyrosine kinase in the proliferative change of MCs. Experimental mesangial proliferative glomerulonephritis (Thy1 GN) was induced by a single intravenous injection of anti-rat Thy-1.1 monoclonal antibody. In Thy1 GN, MC proliferation and type IV collagen (Col4) expression peaked on day 6. Immunohistochemical staining for the expression of phospho-Src (pSrc), phospho-Smad1 (pSmad1), Col4, and smooth muscle α-actin (SMA) revealed that the activation of c-Src and Smad1 signals in glomeruli peaked on day 6, consistent with the peak of mesangial proliferation. When treated with PP2, a Src inhibitor, both mesangial proliferation and sclerosis were significantly reduced. PP2 administration also significantly reduced pSmad1, Col4, and SMA expression. PDGF induced Col4 synthesis in association with increased expression of pSrc and pSmad1 in cultured MCs. In addition, PP2 reduced Col4 synthesis along with decreased pSrc and pSmad1 protein expression in vitro. Moreover, the addition of siRNA against c-Src significantly reduced the phosphorylation of Smad1 and the overproduction of Col4. These results provide new evidence that the activation of Src/Smad1 signaling pathway plays a key role in the development of glomerulosclerosis in experimental glomerulonephritis.

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Introduction

Glomerulonephritis is usually progressive and remains an important cause of end stage renal disease. In sclerosing glomerulonephritis, accumulation of the extracellular matrix (ECM) is a critical process in progressive glomerular injuries [1,2]. Type IV collagen (Col4) is one of the most important components of the expanded ECM [3]. Moreover, smooth muscle α actin (SMA) is a known common molecular marker of phenotypic changes of mesangial cells (MCs) in many glomerular diseases. We previously reported that Smad1 participates in the development of glomerulosclerosis in experimental glomerulonephritis [4]. We also reported that Smad1 transcriptionally regulates the expression of Col4 and SMA [5,6]. However, the mechanisms by which Smad1 is activated in glomerulonephritis have not been fully elucidated.

Platelet-derived growth factor (PDGF) is known to be a critical mitogen for MCs in vitro and in vivo [1,7]. It is noteworthy that mice deficient for PDGF B or PDGF receptor show abnormal glomeruli due to a lack of MC development [8–11]. Several lines of evidence indicate that PDGF plays a key role in the development of glomerulosclerosis not only in experimental models but also in human glomerular diseases [12,13]. The introduction of a neutralizing anti-PDGF antibody has shown that both mesangial proliferation and glomerulosclerosis can be markedly ameliorated in a rat glomerulonephritis model [14]. Moreover, we previously showed that the development of glomerulosclerosis from mesangial proliferation is dependent on PDGF-induced Smad1 activation [4], but little is known concerning the regulatory mechanisms of Smad1 activation by PDGF in glomerulonephritis. c-Src is a ubiquitously expressed non-receptor protein-tyrosine kinase [15] that is involved in multiple pathways regulating cell growth, migration, and survival [16]. c-Src is also an important component of the PDGF signal transduction pathway [17]. Several reports have demonstrated that PDGF plays a key role in MC proliferation and glomerulopathy *in vivo* and *in vitro* [7,18,19]. Previously we demonstrated that Smad1 is phosphorylated by PDGF in MCs [4]. However, the exact role of c-Src in MCs as well as in glomerulonephritis remains unclear.

In the present study, we demonstrated that c-Src is activated in experimental proliferative glomerulonephritis and that the reduction of c-Src ameliorates the development of glomerulosclerosis by blocking of the Smad1 signal transduction pathway. We further

showed that c-Src plays an important role as a switch molecule for the activation of Smad1 downstream of PDGF signaling. These findings unveil the molecular mechanisms underlying the induction of MC proliferation and MC phenotype alteration, resulting in proliferative glomerulonephritis. Taking these results together, we hypothesized that the Src/Smad1 pathway may be critical in the pathogenesis of proliferative glomerulonephritis.

Materials and Methods

Animals

Full details of the animal experimental protocols were approved and ethical permission was granted by the Review Board of Kyoto University (Permit Number: Med Kyo 08508). We used age-matched male Wistar rats (8 to 12 weeks old, 180 to 200 g) bred at the Shimizu Laboratory Animal Center (Hamamatsu, Japan). The animals were housed under specific pathogen-free conditions at the Animal Facility of Kyoto University. Levels of serum creatinine and blood urea nitrogen were measured using a Hitachi Mode 736 autoanalyzer. The urinary albumin concentrations were measured from 24-h urine collections by Nephrat and Albuwell (Exocell), according to the manufacturer's protocols.

Cell culture experiments

A glomerular mesangial cell line was established from glomeruli isolated from normal 4-week-old mice (C57BL/6JxSJL/J) and was identified according to a method described previously [7]. The MCs were plated on 100-mm plastic dishes (Nunc) that were maintained in B medium (a 3:1 mixture of minimal essential medium/F12 modified with trace elements) supplemented with 1 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 μg/ml, and 10% fetal calf serum (Irvine Scientific). The cells were passaged weekly with trypsin-EDTA. The cultured cells fulfilled the previously described criteria generally accepted for glomerular mesangial cells [20]. Stimulation with angiotensin II (Ang II) (Sigma), PDGF, PP2 (Calbiochem, Darmstadt, Germany), or olmesartan (Cosmo Bio, Tokyo, Japan) was carried out in DMEM containing 0.5% FCS at 37°C for the indicated times. A rat monoclonal anti-PDGFβ-receptor antibody (APB5) and its antagonistic effects on the PDGFβ-R signal transduction pathway in vitro have been described previously [4].

Constructs, transfection, and co-immunoprecipitation

Src cDNAs (pUSE Src wild type, pUSE Src kinase mutant, and empty vector) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). MCs were transfected using FuGene6 (Roche, Mannheim, Germany) according to the manufacturer's protocol. After 48 h of transfection, the cells were washed with PBS, and 1 ml ice-cold lysis buffer (25 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, Complete protease inhibitors cocktail; Roche) was added. For co-immunoprecipitation assay, whole cell lysates were first pre-cleared with protein G–Sepharose (Amersham) and followed by incubation with anti-PDGFR antibody (Santa Cruz) for 3 h at 4°C. The immune complex was isolated and separated by SDS–PAGE and analyzed by Western bot analysis. Protein was detected using polyclonal rabbit anti-Src antibody (Cell Signaling Technology).

Histology and Immunohistochemistry

Tissues were fixed in Methyl Carnoy's solution and were paraffin-embedded. Multiple sections were prepared and stained with periodic acid silver methenamine (PASM) and periodic acid-Schiff's reagent (PAS). Immunohistochemical staining was performed with antibodies specific to Col4 (Progen) or SMA (Abcam), using an established avidin-biotin detection method (Vector Laboratories). Frozen sections were used for the detection of pSrc and pSmad1 (Cell Signaling Technology). Glomerular morphometry was evaluated in PASM-stained tissues. The glomerular surface area and the PASM-positive area/glomerular area (%) were measured using an image analyzer with a microscope (IPAP, Sumitomo Chemical, Osaka, Japan) as previously described [21–24]. To quantitatively measure the expression of pSrc and pSmad1, pSrc-positive or pSmad1-positive cells/DAPI-positive nuclei were counted, and the mean percentages of pSrc-positive or pSmad1-positive cells were calculated. An investigator scored sections in a blinded fashion, according to an established scoring system (range 0–4; 0, no ECM deposition; 4, ECM deposition in all sections of the glomeruli) to semiquantify the localization of Col4 and SMA.

Small-interfering RNA

MCs (0.5×10^5) were seeded into 12-well plates (Nunc) and were grown until they were 60% to 80% confluent. The small-interfering RNAs (siRNAs) for c-Src, Smad1, and LRP1 (Dharmacon) or control scrambled siRNA (Dharmacon) were combined with DharmaFECT transfection reagent (Dharmacon), and the cells were transfected according to the recommended protocol with siRNA (100 nM final concentration). After 48 h of transfection, cells were starved in DMEM containing 0.5% BSA before treatment. After 48 h of incubation, the cells were stimulated with or without PDGF (Calbiochem).

TGFβ-neutralizing antibody assay

MCs were resuspended at a concentration of 1×10^6 cells/ml and plated onto 100-mm dish either in the presence of 10 μg/ml TGFβ-neutralizing antibody (R&D Systems) or a control normal chicken IgY. After 24 h of incubation, the cells were treated with PDGF for additional 12 h and were harvested and underwent protein extraction on Western blotting.

Western blotting

Isolated glomerular MCs were suspended in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% SDS, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml of aprotinin) and incubated for 1 h at 4°C. After centrifugation, the supernatants were used as total cell lysates. Twenty micrograms of each sample was applied to SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose filters (Schleicher & Schuell). The blots were subsequently incubated with anti-phospho-Smad1, anti-phspho-Src (Cell Signaling Technology), anti-SMA, anti-LRP1 (Abcam) or anti-Col4 antibody (Progen), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG and sheep anti-mouse IgG (Amersham). The immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescent system (Amersham). These bands were quantified using an imaging densitometer (Science Lab 99 Image Gauge, Fujifilm, Tokyo, Japan).

Data analysis

The data are expressed as the mean ± S.D. Comparison among more than two groups was performed by one-way analysis of variance (ANOVA), followed by post hoc analysis (Bonferroni/Dunn test) to evaluate the statistical significance between the two groups. All analyses were performed using StatView (SAS Institute, Cary, NC). Statistical significance was defined as $P<0.05$.

Results

Glomerular phosphorylation of c-Src and Smad1 parallels the progress of glomerulosclerosis in rat Thy1 GN

We utilized a model of mesangial proliferative glomerulonephritis, known as anti-Thy1-induced glomerulonephritis (Thy1 GN), which exhibits sclerosis in the glomeruli. The renal function of Thy1 GN on day 6 was significantly decreased (Figure S1A). MC proliferation began on day 3 and glomerulosclerosis began on day 6. Renal damage clearly regressed until day 15. Sclerosis in the kidney peaked on day 6 and sclerotic changes subsided until day 15 (Figure 1A and B). Localization of phospho-Src (pSrc) and phospho-

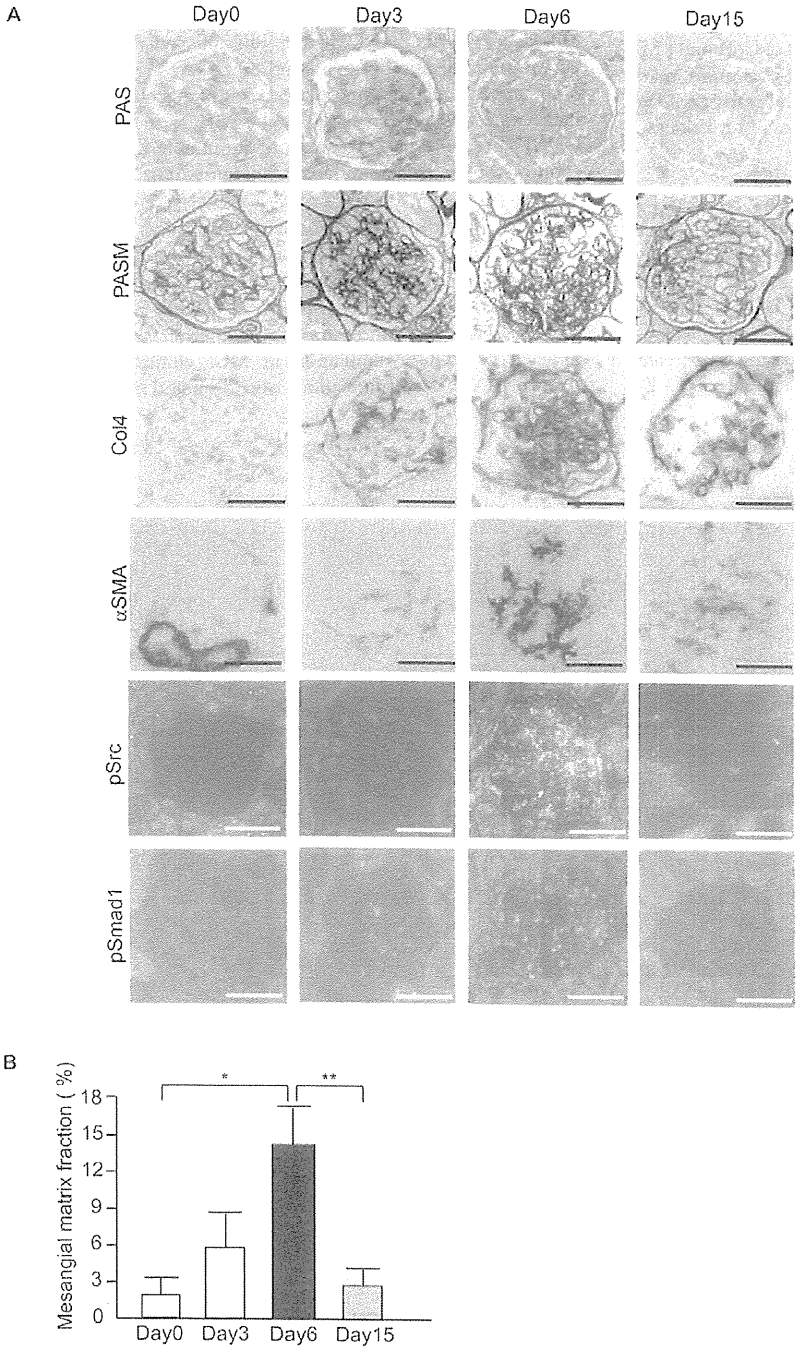

Figure 1. Induction and activation of c-Src and Smad1 in proliferative glomerulonephritis. (A) Representative light-microscopic appearance and immunohistochemistry of glomeruli in Thy1 GN. Scale bars = 100 μm. (B) Quantitative assessment of PASM staining in Thy1 GN. *P = 0.002, **P = 0.002).
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Smad1 (pSmad1) in the nuclei was scant on day 0. On day 3, phosphorylation began in c-Src and Smad1 proteins. The level of phosphorylation gradually increased and positively stained nuclei in parallel with the activity of mesangial proliferation during the development of glomerulosclerosis. Phosphorylation peaked on day 6 and then decreased towards day 15 (Figure 2, C, D and E). Phosphorylation of c-Src and Smad1 was almost undetectable on day 0 but became prominent during the proliferative stages in Thy1 GN, peaked on day 6, and then decreased towards day 15 (Figure 2C, D and E). In addition, the expression of Col4 and SMA changed in parallel with the activation of c-Src and Smad1 (Figure 2A, B and E). These data suggest that both Smad1 and c-Src are activated in the course of proliferative injuries in rat kidneys.

PP2 preserves renal function and attenuates glomerulosclerosis in rat glomerulonephritis

To investigate whether the c-Src/Smad1 pathway plays a pivotal role in developing glomerulosclerosis, we administered a Src specific inhibitor, PP2, to Thy1 GN rats from days 0 to 6 and assessed glomerulosclerosis on day 6. Untreated Thy1 GN rats showed an increased degree of glomerulosclerosis, whereas glomerulosclerosis was significantly decreased in the PP2-treated group (Figure 3A, B), along with renal function (Figure 3, C–E).

PP2 represses the activation of Smad1 and the expression of both Col4 and SMA in rat glomerulonephritis

Next, to examine the effect of PP2 on the morphological changes seen in Thy1 GN glomerulosclerosis, we examined Col4 and SMA expression in the two groups. PP2 treatment significantly inhibited Col4 and SMA expression, whereas expression was increased in the non-treatment group (Figure 3F). Moreover, we examined whether PP2 affected the phosphorylation and translocation of c-Src and Smad1 in Thy1 GN rats. PP2 treatment inhibited the phosphorylation of c-Src and Smad1, and their expression was localized in the nucleus in untreated Thy1 GN (Figure 3F). These data from immunohistochemistry were confirmed by Western blot analysis (Figure 3G).

Effect of PP2 on PDGF-mediated signaling in MCs

Because PDGF is well known to play a key role in the development of glomerulosclerosis, we investigated whether PDGF can activate c-Src/Smad1 signal transduction and increase the synthesis of Col4. Expression of Col4, pSrc, and pSmad1 was induced by PDGF stimulation in MCs cultured for 12 hours (Figure 4A–D). These inductions were inhibited by PP2 treatment

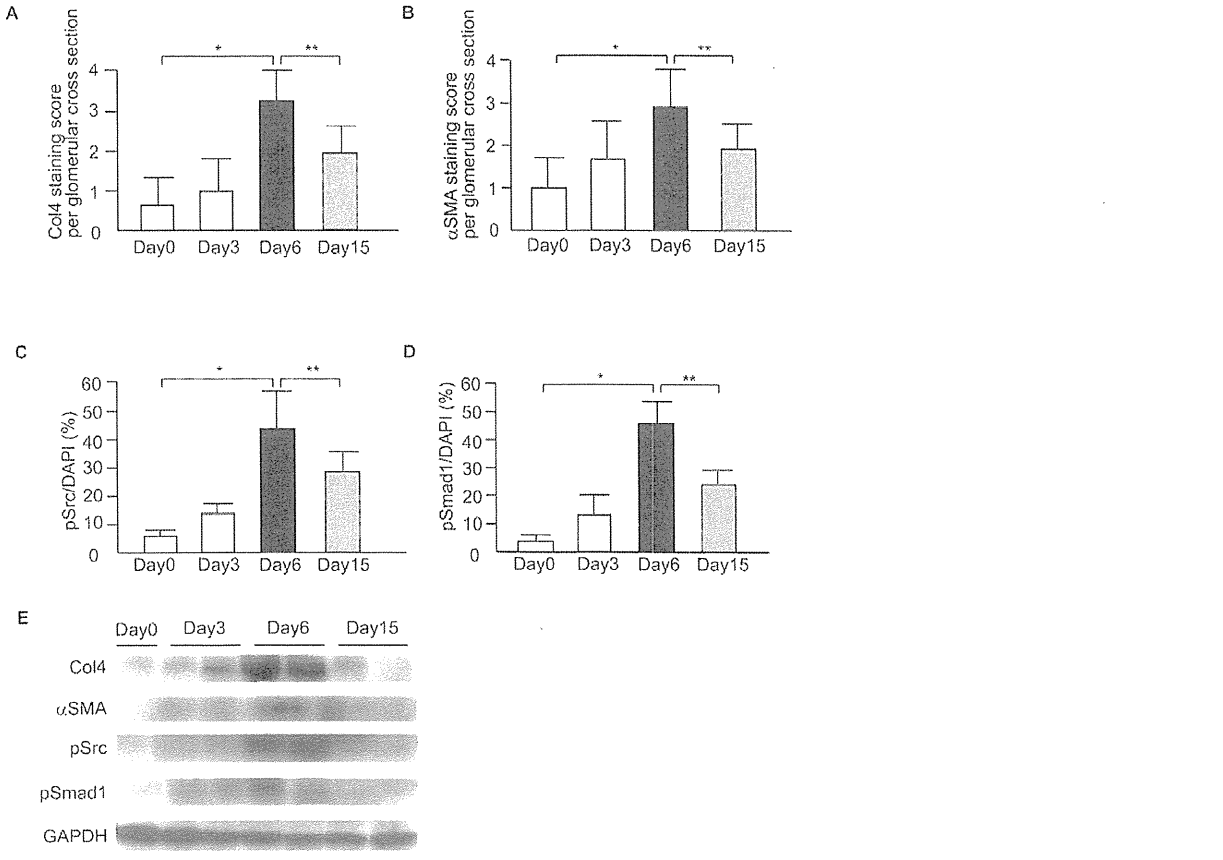

Figure 2. Time course of glomerular expression of Col4, SMA, pSrc and pSmad1 in Thy1 GN. (A, B) Staining scores per glomerular cross-section for Col4 (*$P<0.001$, **$P<0.001$) and SMA (*$P<0.001$ and **$P=0.009$) were calculated. Data represent mean values ± S.D. of at least three independent experiments; n = 6 for each experimental group. (C, D) Quantification of glomerular pSrc and pSmad1 by optical densitometry. The pSrc-positive nuclei and pSmad1-positive nuclei were counted in 10 consecutive fields in each specimen and normalized by the number of DAPI-positive nuclei. *$P<0.001$, **$P<0.001$. (E) Western blot for the glomerular lysates from each group. Data represent mean values ± S.D. of at least three independent experiments; n = 6 for each experimental group.
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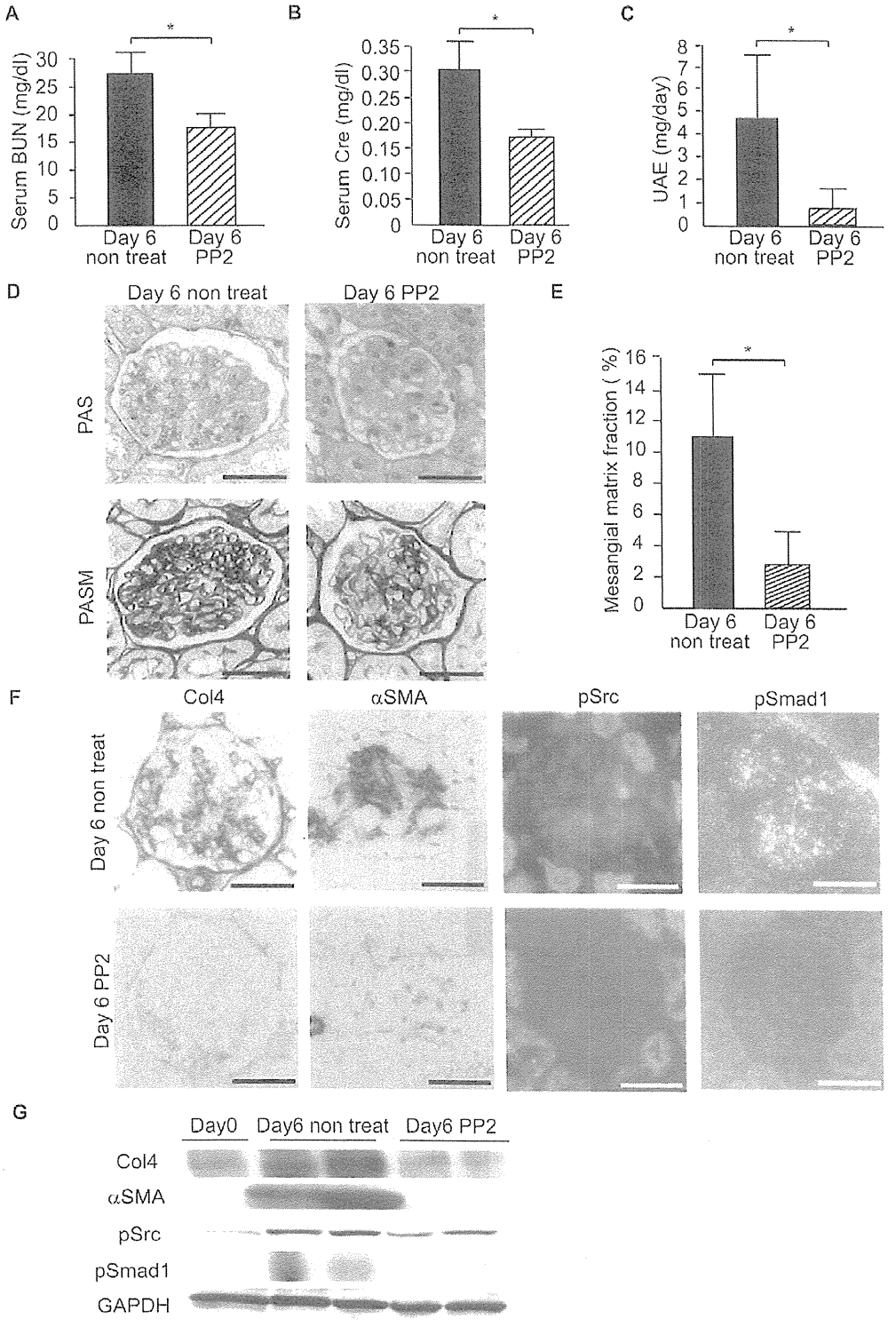

Figure 3. Src-specific inhibitor PP2 inhibits glomerulosclerosis and glomerular expression of pSrc and pSmad1 in Thy1 GN. (A–C) Serum blood urine nitrogen (BUN), serum creatinine (Cre), and UAE in the nontreatment and PP2 groups. *P* values were 0.001, 0.001 and 0.017, respectively. (D, E) Representative light-microscopic appearance of glomeruli (PAS and PASM staining) and quantitative assessment of PASM staining in Thy1 GN with or without PP2 on day 6. Scale bars = 100 μm. *$P<0.001$. (F) Immunohistochemistry of glomeruli (Col4, SMA, pSrc and pSmad1) in Thy1 GN with or without PP2 on day 6. Scale bars = 100 μm; n = 6 for each experimental group. (G) Western blot for the glomerular lysates from each group. Data represent mean values ± S.D. of at least three independent experiments; n = 6 for each experimental group on day 6.
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(Figure 4A–D). These results indicate that PDGF induced the expression of Col4 through the activation of Src/Smad1 signal transduction.

Silencing of c-Src in MCs inhibits PDGF-mediated phosphorylation of Smad1 and synthesis of Col4

To further confirm the role of c-Src in PDGF-induced upregulation of Smad1 and Col4 expression, c-Src gene silencing by siRNA was performed. c-Src silencing suppressed the PDGF-induced phosphorylation of Smad1 and the synthesis of Col4. In contrast, GAPDH protein levels, used as a loading control, were not affected across the samples (Figure 4E–H). We confirmed the result of knockdown experiments with PDGF stimulation by using three c-Src siRNAs (Src siRNA-1, -2, and -3) (Figure S2). We showed the representative data from using Src siRNA-3 in Figure 4E–H. From these results, c-Src may be significantly involved in PDGF-mediated Col4 expression.

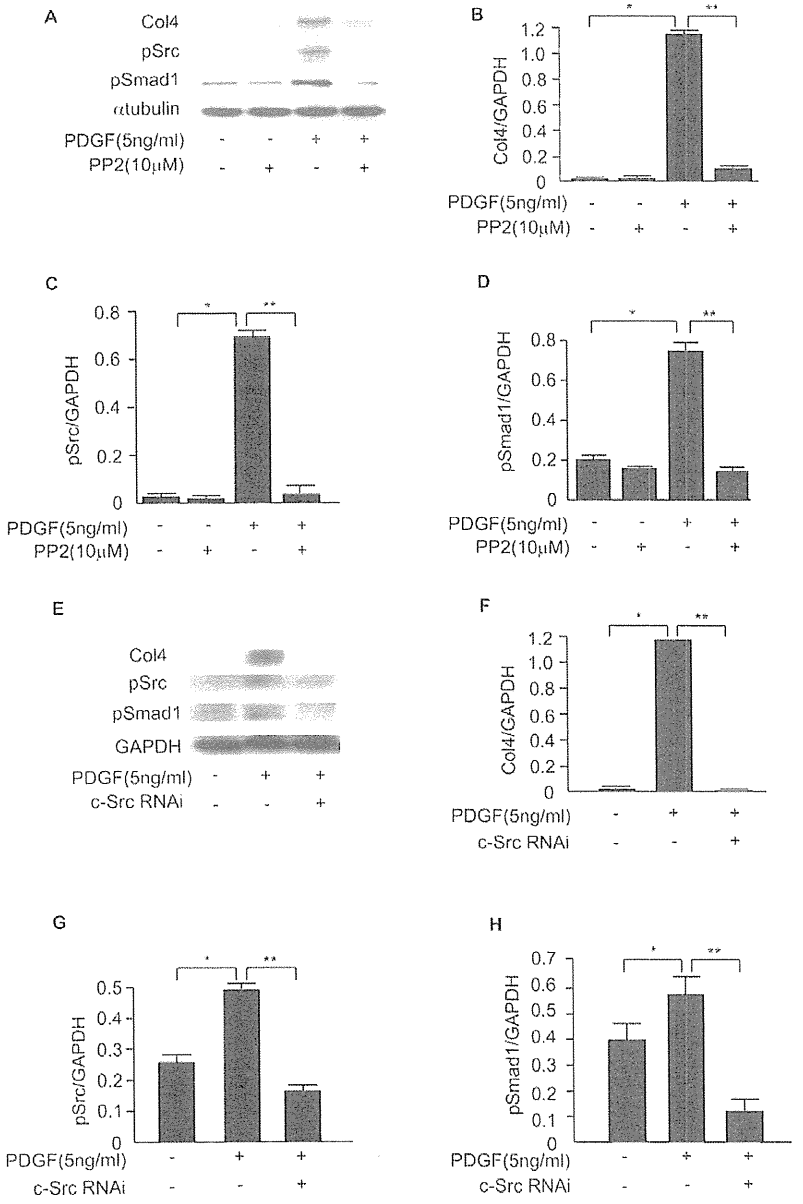

Figure 4. Activation of c-Src and Smad1 is regulated by PDGF in MCs. (A) Effect of PP2 on pSrc, pSmad1 and Col4. MCs were preincubated with PP2 (10 μM) or DMSO for 48 h before exposure to PDGF (5 ng/ml, 12 h). (B) Optical densitometry of Col4 in western blot. *$P<0.001$ and **$P<0.001$. (C, D) Optical densitometry of pSrc (*$P<0.001$ and **$P=0.003$) and pSmad1 (*$P=0.002$, **$P=0.002$) in western blot analyses. (E) Effects of RNAi-mediated silencing of c-Src on pSrc, pSmad1 and Col4 under stimulation of PDGF (5 ng/ml, 12 h). (F–H) Optical densitometry of Col4 (*$P<0.001$, **$P<0.001$), pSrc (*$P<0.001$, **$P<0.001$), and pSmad1 (*$P=0.02$, **$P=0.002$) in western blot. Data represent mean values ± S.D. of at least three independent experiments.
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Activated c-Src is associated with PDGFR in MCs

To clarify the intracellular interaction between PDGF signaling pathway and c-Src/Smad1 axis, the effects of constitutively active form of c-Src (caSrc) transfected in MCs was examined. Transient transfection of MCs with caSrc could induce phosphorylation of Smad1 wihtout stimulation of PDGF, and subsequently upregulated Col4 expression (Figure 5A). In contrast, transfection of the dominant negative Src (dnSrc) did not show these regulations. Moreover, we performed knockdown analysis using Smad1 siRNAs to confirm the role of Smad1 in the regulatory effect of PDGF-induced Col4 expression. Knockdown study revealed that Smad1 acts downstream of PDGF-c-Src signaling pathway in the induction of Col4 (Figure 5B). Furthermore we have explored the possibility that c-Src, while interacting directly with PDGF receptor, could transduce the PDGF signals in MCs. For this purpose, PDGF receptor was immunoprecipitated from whole cell lysates after PDGF stimulation. Anti-c-Src immunoblot revealed that c-Src really associates with PDGFR only when stimulated by PDGF (Figure 5C).

TGFβ signaling pathway partially mediated PDGF-induced Smad1/Col4 expression in MCs

Transforming growth factor beta (TGFβ) is an important growth factor in the modulation of cell proliferation as well as PDGF in a variety of cells. In addition, several studies reported that PDGF may increase the production of TGFβ and the expression of TGFβ type I receptor [25,26]. To elucidate the the molecular basis of the influence of PDGF on TGFβ signaling pathway, we performed TGFβ-neutralizing antibody assay for PDGF-stimulated MCs. PDGF increased the expressions of TGFβ and activin receptor-like kinase 5 (ALK5) and activated Smad1. However, these changes by PDGF could not be inhibited by neutralizing anti-TGFβ antibody (Figure 6A), indicating that PDGF, but not TGFβ, upregulates expression of ALK5, pSmad1, pSrc, and Col4. In particular, pSmad1 is phosphorylated by ALK1, but not by ALK5, therefore, we investigated the effects of high concentration of PDGF on MCs. At concentration of 50 ng/ml, PDGF increased the expressions of ALK1 as well as other proteins (Figure 6B). Interestingly, an addition of neutralizing anti-TGFβ antibody suppressed not only ALK1 expression, but also expressions of pSmad1 and Col4 (Figure 6B). These results suggest that PDGF has the potential to enhance TGFβ signal transduction through ALK1 as well as ALK5.

TGFβ signaling pathway partially mediated PDGF-induced Smad1/Col4 expression in MCs

To further elucidate the regulatory mechanisms controlling the cross-talk between PDGF and TGFβ in the activation of Smad1

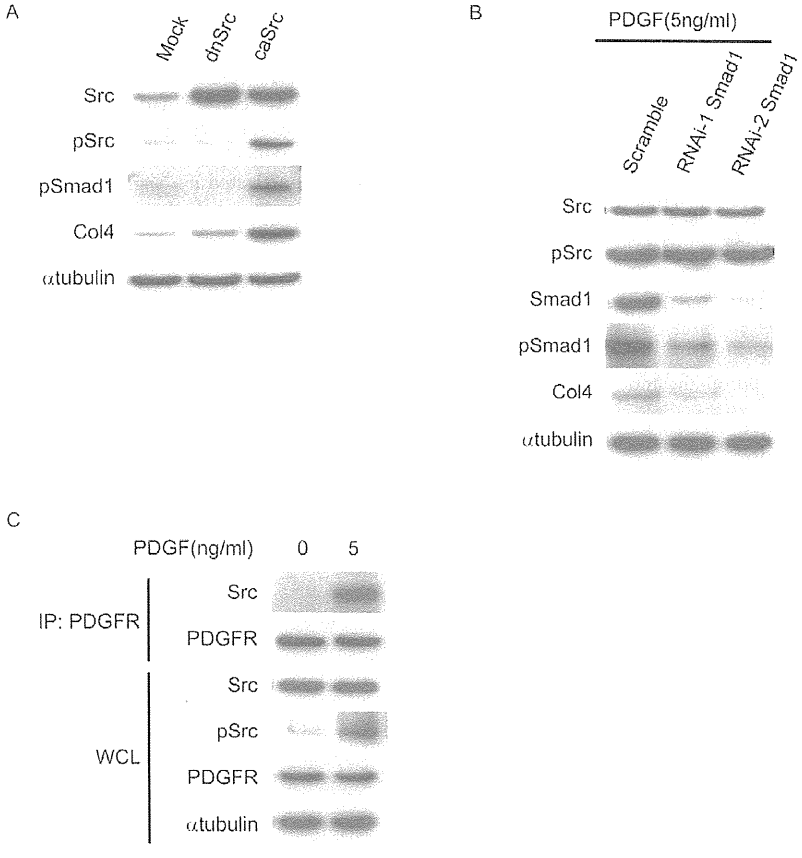

Figure 5. Activated c-Src is associated with PDGF Receptor (PDGFR) in MCs. (A) Western blot analyses of MCs transfected with constitutively active c-Src (caSrc), dominant negative c-Src (dnSrc), and empty vector (Mock). One of three independent experiments is shown. (B) Effects of RNAi-mediated silencing of Smad1 on pSmad1 and Col4 after 5 h stimulation of PDGF (5 ng/ml). Scrambled siRNA (Scramble) was used as a control. One of three independent experiments is shown. (C) MCs were serum-starved for 10 h and then incubated with 5 ng/ml of PDGF for 5 min. Whole cell lysates (WCL) were immunoprecipitated with polyclonal anti-PDGFR antibody and subjected to anti-Src immunoblot.
doi:10.1371/journal.pone.0017929.g005

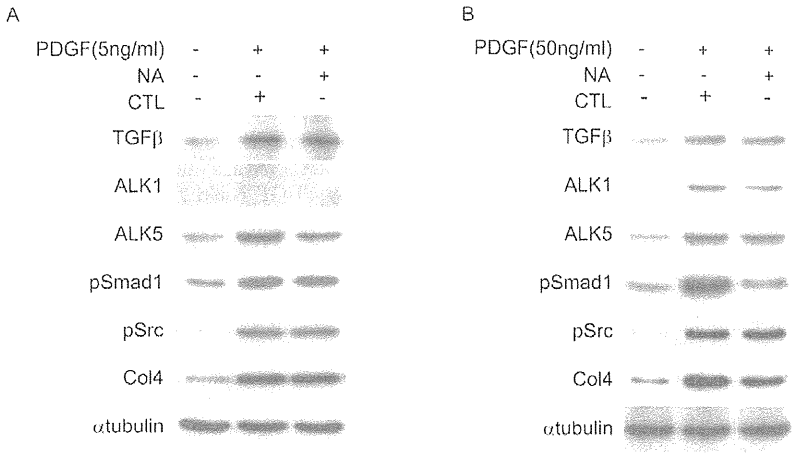

Figure 6. PDGF modulated TGFβ-Activin Receptor-like Kinases (ALKs) signaling pathways in MCs. (A, B) MCs were treated with neutralizing antibody for TGFβ (10 μg/ml) (NA) or control normal IgY (CTL) for 24 h prior to treatment with PDGF at indicated concentrations for 24 h. Equal amounts of cell lysates were subjected to Western blot. One of three independent experiments is shown.
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and induction of Col4 in MCs, we examined whether LDL receptor related protein-1 (LRP1) is involved in the signal pathways. Because Boucher et al. reported that LRP1 is tightly involved in the pathogenesis of atherosclerosis by regulating signaling of TGFβ and PDGF, and their receptors [27,28], knockdown analysis using LRP1 siRNAs was perfomed to examine the role of LRP1 in the regulatory effect of PDGF-induced Col4 expression and PDGF-activated TGFβ signaling pathway in MCs. Knockdown of LRP1 enhanced the downstream pathway of PDGF (Figure 7A) with the exception of ALK1 (Figure 7B). These results suggest that LRP1 has a significant inhibitory effect on PDGF signaling pathway leading to production of Col4 in MCs.

PDGF signaling pathway is partially involved in the AngII-induced c-Src/Smad1 signal activation in MCs

We previously reported that AngII activates the c-Src/Smad1 signaling pathway in the development of diabetic nephropathy and cultured MCs [23]. To investigate whether AngII signals influence the regulatory mechanisms of PDGF-induced c-Src/Smad1 signal transduction, we examined the inhibitory effects of APB5 and AngII receptor blocker (ARB) on the activation of c-Src, Smad1, and Col4 by AngII and PDGF, respectively. APB5 clearly attenuated the AngII-induced c-Src/Smad1/Col4 signal (Figure 8A). In contrast, ARB treatment slightly reduced PDGF-induced activation of the signal (Figure 8B). These data suggest that PDGF signaling pathway is activated by AngII in MCs.

Discussion

Cellular proliferation and extracellular matrix accumulation are characteristic features of progressive glomerular diseases, a major cause of end-stage renal failure in humans throughout much of the world. Glomerulosclerosis followed by mesangial proliferative glomerulonephritis is characterized by mesangial matrix expansion

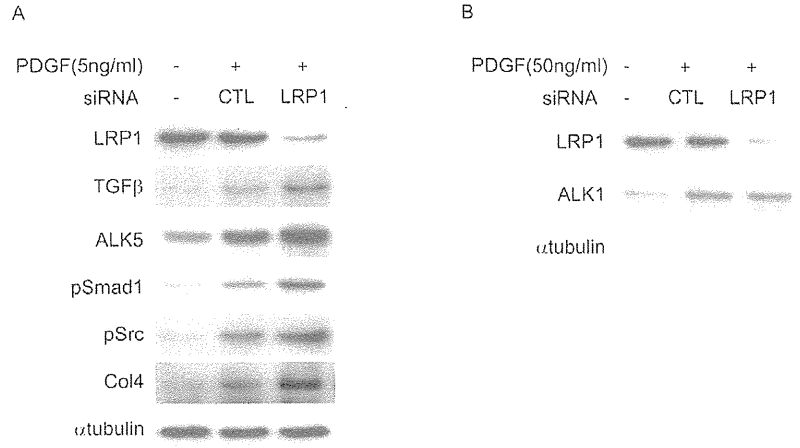

Figure 7. LRP1 modulated both PDGF and TGFβ signaling pathways in MCs. (A, B) Effects of PDGF stimulation and RNAi-mediated silencing of LRP1 after 5 h stimulation of PDGF at indicated concentrations on MCs. Scrambled siRNA (Scramble) was used as a control (CTL). Equal amounts of cell lysates were subjected to Western blot. One of three independent experiments is shown.
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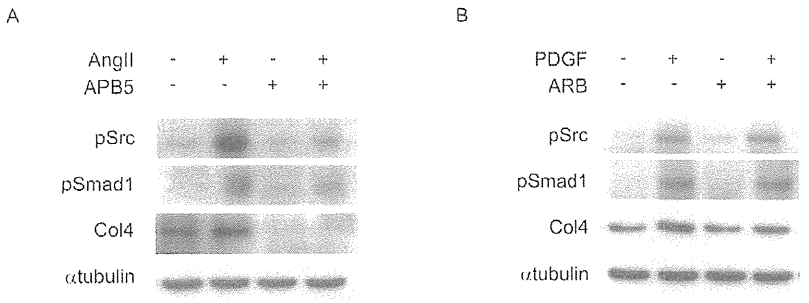

Figure 8. Molecular cross-talk between PDGF and AngII signaling pathways in MCs. (A) Effects of APB5 on pSrc, pSmad1 and Col4. MCs were preincubated with APB5 (100 ng/ml) or control rat IgG for 24 h before exposure to AngII (0.1 μM, 30 min). (B) Effects of olmesartan (ARB) on pSrc, pSmad1 and Col4. MCs were preincubated with olmesartan (10 μM) or methanol for 48 h before exposure to PDGF (5 ng/ml, 12 h). Equal amounts of cell lysates were subjected to Western blot. One of three independent experiments is shown.
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and phenotypic change of MCs [3]. In the expanded mesangial matrix, Col4 is a major component of ECM and is overproduced in glomerulosclerosis [6]. In addition, phenotypic modulation is a commonly observed feature in the progression of many renal diseases leading to CKD and ESRD. Expression of SMA is a well-known marker for the activation of MCs in most glomerular diseases. We previously reported that Smad1 upregulated the expression of Col4 and SMA [5,6] and thereby participates in the development of glomerulosclerosis in experimental glomerulonephritis [4]. However, the molecule that activates Smad1 in glomerulonephritis has not been fully elucidated. Since PDGF has been consistently implicated in cell proliferation and extracellular matrix accumulation, which characterize progressive glomerular disease [29], and since c-Src is an important component of the PDGF signaling pathway [30], we first investigated whether c-Src is induced in glomeruli of proliferative glomerulonephritis. In Thy1 GN, Col4 is strongly expressed in the sclerotic lesions of glomeruli, as previously described [4,21]. We show here that c-Src and Smad1 are heavily phosphorylated in the nuclei of glomerular cells in Thy1 GN. This phosphorylation parallels the progress of glomerulosclerosis and peaks on day 6, when Col4 and SMA expression levels have peaked. These results suggest that c-Src has a potential to be involved in the development of glomerulosclerosis in mesangial proliferative glomerulonephritis.

c-Src was identified as the first proto-oncogene, and a great deal of work has been carried out to elucidate its role in biological systems [31–33]. The two main areas in which Src inhibitors have been applied are regulating bone resorption [34,35] and both tumor growth and metastasis [36,37]. Most previous studies have shown that the role of Src family members is related to inflammatory responses. Additionally, the small chemical inhibitors that effectively and specifically block Src kinases could have great clinical implications for diseases with acute inflammatory responses [38,39]. In a rat renal ischemia-reperfusion injury model, increased active Src expression was found in the injured rat kidney after reperfusion [40]. To our knowledge, however, no report has demonstrated that c-Src is involved in the development of glomerulosclerosis in glomerular diseases. In the rat proliferative glomerulonephritis model, administration of PP2 completely abolished the phosphorylation of c-Src and Smad1 and resulted in the amelioration of glomerulosclerosis. Therefore, the activation of c-Src signal transduction plays a pivotal role in glomerulosclerosis, implicating it as a novel target of the therapeutic strategies for glomerulonephritis. Moreover, our findings show a new side of PP2 as an anti-glomerular disease agent.

In addition, PDGF is known to contribute to the development of both experimental and human glomerulonephritis [12,13]. Src kinase activation has been reported to contribute to PDGF-dependent cell-cycle proliferation, mitogenesis, and chemotaxis [24,29,30]. Thus, to investigate the molecular mechanisms underlying the progression of proliferative glomerulonephritis, we used cultured MCs under PDGF stimulation. PDGF induced phosphorylation of c-Src and Smad1 as well as Col4 expression, and these changes were blocked by PP2. The interaction between PDGFR and c-Src may be important for the phosphorylation of c-Src. In addition, the siRNA silencing experiments confirmed that c-Src regulated Smad1 activation. These findings suggest that c-Src activation is a key event in the PDGF-induced phosphorylation of Smad1, followed by the subsequent overproduction of Col4 in proliferative glomerulonephritis. In addition, PDGF activated TGFβ signaling pathways by induction of TGFβ and its type I receptors, ALK1 and ALK5. In particular, the induction of ALK1 may be an important event, because ALK1 transduce TGFβ signals to Smad1. Furthermore, several recent reports demonstrated that LRP1 has an inhibitory effect on TGFβ signaling pathway as well as PDGF signaling pathway [27,28]. As expected, LRP1 silencing exhibited additional effect on the activation of TGFβ signals by PDGF. Hence, LRP1 represents a promising new therapeutic target for the control of proliferative glomerular diseases. Moreover, our previous study demonstrated that AngII stimulated this Src-Smad1 axis independent of p44/42 MAP kinase activation and that the AngII receptor blocker ARB blocked this pathway. Because it is generally accepted that the AngII blockade significantly delays the progression of proliferative glomerulonephritis [41,42], our previous findings implied that the inhibition of the Src-Smad1 axis may partially explain the AngII-induced progression of proliferative glomerunonephritis. PDGF-induced activation of c-Src/Smad1 signaling pathway leading to Col4 production also plays an important role downstream of AngII stimulation, whereas ARB treatment did not fully suppressed the effect of PDGF. Chemical inhibitors directly or indirectly targeting Src kinases have been developed as potential drugs for the treatment of cancer [43]. It was recently reported that the inhibition of c-Src by these chemical inhibitors helps to prevent ischemia-reperfusion-induced injury in organs [38,39]. The present study raises the possibility that using these chemical inhibitors to block Src signal transduction could be a promising option for ameliorating proliferative glomerulonephritis as well as for the already reported effects of these inhibitors on excessive inflammatory cells, monocytes and macrophages [44,45]. Another report by Severgnini et al. demonstrated that c-Src controls

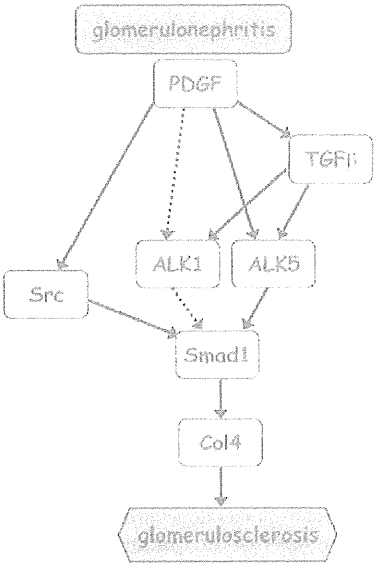

Figure 9. Proposed model for PDGF effects on Smad1 activation and Col4 expression in glomerulonephritis. Activation of Smad1 by PDGF mediates at least two different signal transduction pathways, TGFβ-ALK5-Smad1 and Src-Smad1. ALK1 may potentially activate Smad1 when exposed to high concentration of PDGF (broken arrows). The expression of ALK5 is induced by PDGF and is largely independent of TGFβ. Excessive activation of these signaling pathways may result in Col4 overproduction leading to the development of glomerulosclerosis in glomerulonephritis.
doi:10.1371/journal.pone.0017929.g009

STAT3 activation in acute lung injury [46]. In addition, we previously reported that STAT3 is involved in the development of glomerulosclerosis in experimental proliferative glomerulonephritis [4]. In light of these previous findings, our results highlight the importance of c-Src in the development of glomerulosclerosis in glomerulonephritis. Combining with our overall findings summarized in Figure 9, we can speculate that Smad1-mediated production of Col4 leading to mesangial expansion is a critical event in the development of glomerulosclerosis.

In conclusion, our present study indicates that c-Src activates Smad1-induced ECM production and phenotypic alteration, and is involving in the progression of proliferative glomerulonephritis leading to glomerulosclerosis. Further understanding of the Src/Smad1 pathway and the molecules involve in this pathway is critical for the clarification of glomerulosclerosis and to pave the way for a strategy to treat progressive glomerulonephritis.

Supporting Information

Figure S1 Time course of renal function in Thy1 GN. Urine volume (*$P = 0.042$) (A), serum BUN (*$P = 0.014$) (B), and UAE (*$P = 0.017$) (C) in Thy1 GN. Data represent mean values ± S.D. of at least three independent experiments; n = 6 for each experimental group.
(TIF)

Figure S2 Knockdown of c-Src expression. MCs were transfected with three different siRNAs specific for c-Src and with scrambled siRNA with or without PDGF stimulation. Effects of RNAi-mediated silencing of c-Src on pSrc, pSmad1 and Col4 under stimulation of PDGF (5 ng/ml, 12 h) were analyzed by Western blot. GAPDH served as a loading control.
(TIF)

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Author Contributions

Conceived and designed the experiments: TD H. Abe. Performed the experiments: AM H. Abe KN TM MA KT TT. Analyzed the data: H. Abe H. Arai NI AF TK TD. Wrote the paper: AM H. Abe.

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DUAL-TASK WALK IS A RELIABLE PREDICTOR OF FALLS IN ROBUST ELDERLY ADULTS

To the Editor: Falls are relatively common in elderly people, with approximately 30% of individuals aged 65 and older falling at least once a year and approximately half experiencing repeated falls.[1] In daily-life situations, locomotion occurs under complicated circumstances with cognitive attention focused on a particular task, such as watching traffic or reading street signs, rather than performing the specific motor task of walking. A seminal study demonstrating that the characteristic "stops walking when talking" could serve as a predictor of falls introduced a novel method for fall prediction based on dual-task (DT) performance.[2] Recently, a number of studies have evaluated DT walking in elderly people, but one found that reliable conclusions based on DT results for fall prediction cannot be made because of the lack of standardization in DT paradigms.[3] The aim of the current study was therefore to examine prospectively whether two kinds of DT walking (cognitive task (CT) and manual task (MT)) could predict the risk of falls in a community-dwelling elderly population according to physical function.

The study population consisted of 1,038 community-dwelling elderly Japanese people aged 65 and older (401 men, 637 women, mean age 77 ± 8) in 2009. Six items of physical function were assessed: single-task (ST) 10-m walking time, DT (CT and MT) 10-m walking time, Timed Up and Go (TUG) Test,[4] functional reach, and five-chair stand test (Table 1). In CT walking, participants walked 15 m at the most comfortable speed while counting numbers aloud in reverse order starting at 100. In MT walking, participants walked 15 m at the most comfortable speed while carrying a ball (7 cm in diameter, 150 g in weight) on a tray (17 cm in diameter, 50 g in weight). The DT cost (CT and MT) was then calculated as follows:

$$\text{DT cost}[\%] = 100 \times (\text{DT walking time} - \text{ST walking time}) / ((\text{ST walking time} + \text{DT walking time})/2)$$

Information on the incidence of falls during the following year was collected from participants in a monthly

Table 1. Characteristics of 1,038 Individuals Aged 65 to 97 According to Quartiles of Timed Up and Go Test Results (Seconds)

	Mean ± Standard Deviation							
	Fastest (≤ 8.3) (n = 230)		Faster (8.4–11.0) (n = 258)		Slower (11.1–14.9) (n = 264)		Slowest (≥ 15) (n = 286)	
Characteristic	Faller, 46 (20.0%)	Nonfaller,	Faller, 47 (18.2%)	Nonfaller	Faller, 90 (34.1%)	Nonfaller	Faller, 126 (44.1%)	Nonfaller
Age	77.9 ± 7.9	78.4 ± 6.6	77.4 ± 7.3	78.2 ± 8.0	77.5 ± 8.1	78.2 ± 8.8	77.6 ± 9.3	77.3 ± 8.3
Height, cm	154.4 ± 8.4	153.3 ± 6.8	156.5 ± 9.5	154.7 ± 9.4	157.6 ± 8.3	156.3 ± 11.1	153.6 ± 10.2	154.2 ± 9.6
Body, kg	55.6 ± 11.0	53.6 ± 8.3	50.1 ± 22.9	48.9 ± 16.8	51.7 ± 14.7	53.3 ± 9.3	50.4 ± 17.1	49.7 ± 26.1
Locomotive function, seconds*	9.6 ± 2.0	9.2 ± 2.0	10.5 ± 1.9	10.5 ± 2.5	11.4 ± 2.7	11.2 ± 3.6	17.5 ± 7.1	16.8 ± 7.3
Balance function, cm†	27.1 ± 5.5	25.0 ± 5.4	24.3 ± 7.2	22.6 ± 6.4	21.4 ± 7.9	21.6 ± 7.6	16.6 ± 7.0	18.6 ± 7.0
Muscle power, seconds‡	7.7 ± 1.7	7.5 ± 1.9	9.7 ± 2.8	9.9 ± 2.4	12.8 ± 4.7	11.4 ± 3.5§	17.4 ± 9.8	14.9 ± 5.9§
Cognitive task costs, %	18.7 ± 29.7	16.4 ± 25.5	21.8 ± 23.6	10.6 ± 19.1§	20.2 ± 17.2	20.1 ± 22.2	20.8 ± 20.9	23.1 ± 23.6
Manual task costs, %	8.5 ± 15.8	0.2 ± 11.0§	2.2 ± 14.0	5.8 ± 14.7	12.8 ± 14.0	14.5 ± 16.5	14.5 ± 19.7	16.3 ± 20.7

*Time to complete single-task 10-m walk.
†Distance of functional reach.
‡Time to complete five-chair stand.
§Independent variable that remained in the final step of the regression model.