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Ageing related periostin expression increase from cardiac fibroblasts promotes cardiomyocytes senescent

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ABSTRACT

Periostin, as an extracellular matrix (ECM) protein, plays a critical role in myocardial fibrosis and also might be involved in the heart inflammatory process since it is a downstream molecule of IL4 and IL13. Considering the possible important role of periostin in heart aging, this study explored periostin expression pattern in both rat and human, the effect of periostin expression on cardiomyocyte senescent and expression of three cytokines (IL13, IL4 and IL6) in different age groups of human. This study found heart aging is associated with increased expression of periostin from cardiac fibroblasts and serum inflammatory cytokines (IL13 and IL6). Excessive periostin expression contributed to cardiomyocyte senescent, which could be alleviated through blocking the Ang-II-TGF β1-MAPK/ERK pathway. Thus, periostin might play an important role in a vicious circle (aging-fibrosis-inflammation-aging) of heart through promoting myocardial fibrosis and cardiomyocyte senescent simultaneously. It is a potential aging marker that could be directly measured in serum.

1. Introduction

The process of aging has accumulative effects on cardiovascular disease and is considered as a key risk factor of cardiac morbidity and mortality [1]. Heart aging is associated with progressive fibrosis and inflammation, two of the most important promoters of cellular senescence [2]. In aging heart, excessive mechanical load and reduced arterial compliance lead to the pathogenesis of cardiac fibrosis [1]. At the same time, both acute and chronic heart tissue injury would trigger a local inflammatory reaction, followed by release of fibrogenic cytokines and growth factors which promote fibrosis as a reparative process, such as TGF-β mediated profibrotic responses [3].

In human heart, extracellular matrix (ECM) is the structural fundamental supporting the organ. Periostin is a 90 kDa ECM protein with four repetitive fasci-clin domains [4]. Previous studies observed that periostin could bind to several other ECM proteins such as collagen I, fibronectin and heparin. Their architectural interactions could directly affect collagen synthesis and maturation and thus facilitate ECM deposition and cardiac remodeling [4]. Periostin expression was positively related to myocardial fibrosis. In animal model, significantly higher periostin expression was observed in mouse heart after transverse aortic constriction [5]. Aging related high periostin expression was also observed in rat heart [6]. In human, up-regulated periostin expression and associated higher level of myocardial fibrosis was observed in individuals with cardiac diseases such as myocardial infarction (MI), myocardial hypertrophy and dilated cardiomyopathy (DCM) [7,8]. Beside the role in myocardial fibrosis, periostin was also related to inflammation and was involved in the inflammatory microenvironment of several disorders including skin inflammation, airway inflammation and atherosclerosis [9]. Previous study observed that periostin was a downstream molecule of interleukin (IL)-4 and IL-13 [10]. In mouse heart, age-related inflammation was related to elevated IL-4 and IL-13 expression, which represents a shift to a Th2 phenotype immune response [11]. Thus, periostin also might be an important mediator in aging related inflammation.

However, although there is solid evidence about the role of periostin in myocardial fibrosis, its effect on cardiomyocytes is still not well recognized. In this study, we firstly reported that ageing related periostin expression increase from cardiac fibroblasts could promote cardiomyocytes senescent and age-dependent periostin expression might be related to increased expression of IL6 and IL13.

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2. Materials and method

2.1. Animals

Sprague–Dawley (SD) rats aged 6–8 months (young) and 24–26 months (old) were maintained in a specific pathogen-free facility. Animal based procedures all followed the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, DC, 1996). Rat serum samples from old and young group were collected to measure serum periostin by using ELISA kit (KAMIYA Biomedical) and used for further study. 10 young rats (6 months) and 10 old rats (24 months) were sacrificed for preparation of rat primary cardiac fibroblasts and cardiomyocytes and myocardial tissues were used for DNA, total RNA and protein extraction.

2.2. Cell culture and preparation of adult rat primary cardiac fibroblasts (CFs) and cardiomyocytes (CMs)

Briefly, ventricles from both young and old SD rats were minced and digested with collagenase II (450 U/mL) (Worthington Biochemical). Cells were pelleted and suspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen) and plated in culture plates. Non-adherent cells were removed, and CFs were cultured and passaged until they reached confluence. CFs were confirmed by immunostaining for vimentin and DDR2. To isolate cardiomyocytes from young rats, Pierce Primary Cardiomyocyte Isolation Kit (Thermo) was used according to recommended manual. Cardiomyoblast cell line H9C2 (ATCC, CRL-1446) was cultured in T75 flasks. All cells were cultured in Dulbecco's modified Eagle's medium (DEME) medium with 10% v/v fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM glutamine in humidified air (5% CO_2) under 37 °C. Cells were cultured in 6-well plates (1×10^5 cells in 2 mL per well) with serum-free essential medium before treated with target agents. To collect conditioned supernatants from CFs of old and young rat, 1×10^6 CFs were cultivated in 2 mL per well in 6-well plates with serum-free essential medium. 24 h after cultivation, conditioned supernatants were harvested with periostin concentration measured by ELISA (KAMIYA Biomedical).

2.3. Human blood collection

Blood samples were collected from physical examination center of the hospital affiliated to Xi'an Jiaotong University with approval from the Human Experimental Ethics Committee of the University. Inclusion criteria of participants included: healthy individuals: 20–35 (young), 36–54 (middle aged) or 55–85 years of age (old); non-current smoker; body mass index <30 kg/m^2; arterial blood pressure (BP) at rest <140/90 mmHg. A total of 74 samples from participants aged from 20 to 85 were randomly selected in the center. Information consent was obtained from participants before taking blood sample. After the blood was coagulated, the samples were centrifuged at 3500 rpm for 10 min to isolate serum. The serum samples were then used to measure serum periostin and inflammatory cytokines, including IL13, IL4 and IL6, with ELISA kits (R&D systems).

2.4. Periostin overexpression and silencing in H9C2 cell line

2.4.1. Stable transfection for periostin overexpression

The full-length periostin gene with 2340 bp (GenBank NM_001135934) was amplified from a human periostin cDNA clone (OriGene) through PCR with following primers: (F) 5′-GACG AAGCTTACCATGATTCCCTTTTTACCC-3′ and (R) 5′-GACGCTCGAGT CACTGAGAACGACCTTC-3′. The PCR products were digested with *Hind III* and *Xho I* and then purified. The purified PCR products were then cloned into the *Hind III/Xho I* sites of the pcDNA3.1(+) expression vector (Invitrogen). The sequence of this recombinant vector (pcDNA3.1-periostin) was verified through sequencing. H9C2 cells were transfected with pcDNA3.1 empty vectors or pcDNA3.1-periostin by using Lipofectamine 2000 (Invitrogen). 600 μg/mL G418 was applied for over two weeks to select stable transfected clones. Stable drug-resistant clones were used for following studies.

2.4.2. Transient transfection for periostin silencing and overexpression

pLenti-C-mGFP vector expressing mGFP-tagged rat periostin, pGFP-C-shLenti vectors expressing rat periostin shRNA and nontarget control shRNA were purchased from OriGene. To produce lentiviral particles, HEK293T cells were co-transfected with pLenti-C-mGFP or pGFP-C-shLenti vectors and Lenti-vpak packaging kit (OriGene) by using Lipofectamine 2000 (Invitrogen). 48 h after transfection, culture supernatants containing lentiviral particles were harvested. H9C2 cells or primary cardiomyocytes were treated culture supernatants containing lentivirus with 5 μg/mL polybrene (Sigma–Aldrich).

2.5. Quantitative real-time PCR

DNA and total RNA of rat heart tissue were extracted separately for telomere length analysis and periostin mRNA quantification respectively. DNA was extracted by using QIAamp DNA mini kit (Qiagen). Total RNA was isolated with using Trizol reagent (Invitrogen). cDNA was generated from total RNA by use of the RevertAid First Strand cDNA Synthesis Kit (Fermentas). The relative length of telomere of both young and aged rat hearts was measured using real-time PCR, according to the method previously described [12]. This method is based on measurement of ratio between the numbers of telomere repeat copy (T) to a single gene copy-albumin (S) in experimental DNA samples and reference DNA samples separately, assuming that both genes had similar amplification efficiency. The relative T/S ratios are proportional to average telomere length [12]. Primers for telomere and albumin followed Cawthon et al.'s recommendation [12]. Periostin mRNA expression was also quantified by real-time PCR. The primers for periostin (F) 5′-TGCCCTGGT-TATATGAGAATGGAAG-3′ and (R) 5′-GATGCCCAG AGTGCCATAAACA-3′. All qRT-PCR was conducted with ABI Prism 7300 Real Time PCR Sequence Detection System (Applied Biosystems) with the QuantiFast SYBR Green PCR kit (Qiagen).

2.6. Confirmation of Ang-II pathway in inducing periostin expression

To confirm Ang-II pathway in inducing periostin expression, cells were pretreated with Lorsatan (Merck) for 30 min and then stimulated with 1 μmol/L angiotensin II (Ang-II) (Sigma–Aldrich) for 48 h. To explore the effect of TGF-β1 on periostin expression, H9C2 cells were pretreated with 1 μmol/L Ang-II alone for 48 h, TGF-β1 (Sigma–Aldrich) (3 ng/mL) for 30 min or anti-TGF-β1 (Biosource) (3 mg/L) for 30 min and then 1 μmol/L Ang-II for 48 h. To explore the effect TGF-β1 signal on angiotensin induced high periostin expression, H9C2 were pretreated with SB202190 (Sigma–Aldrich), PD98059 (Sigma–Aldrich), or SP600125 (Sigma–Aldrich) (all 10 μmol/L) for 30 min and then stimulated with 1 μmol/L Ang-II for 48 h. Expression of periostin and TGF-β1 was detected by Western blot analysis.

2.7. D-Galactose and H_2O_2 treatment and SA β-gal staining

Normal H9C2 cells and primary cardiomyocytes were pretreated with anti-TGF-β1 (Sigma–Aldrich) (3.0 mg/L) or PD98059

(Sigma–Aldrich) (10 μmol/L) for 30 min and the un-pretreated counterparts were also used. Then, selective cell groups were treated with 2 mL essential medium containing 1 mL conditioned culture medium of CFs or serum (20% v/v) from young and old rats for 24 h. Briefly, to induce cells senescence by D-galactose, the DEME medium with 10% v/v fetal calf serum was changed to DMEM supplemented with 5 g/L D-galactose (Sigma–Aldrich) for 48 h incubation. To induce senescence by H_2O_2 (Sigma–Aldrich), cells were cultured in DEME medium containing 25 μmol/L H_2O_2 for 1 h. After the treatment, the medium was changed back to the normal DEME medium for another 72 h. Then, senescence-associated β-gal (SA-β-gal) activity was detected by using the β-gal staining kit (Cell Signaling Technology) at pH 6.0 according to the recommended protocols. Generally, H9C2 cells were washed with phosphate buffered saline (PBS) three times and were fixed with 1 mL fixative solution at room temperature for 10–15 min then were incubated with the staining solution mix at 37 °C overnight. Blue coloration of the cells was observed under microscope with ×400 total magnification.

2.8. Western-blot

Proteins extracted from and rat heart tissues, primary cardiomyocytes and H2C9 cells were used for testing the expression of TGF-β1 and/or periostin. Homogenized rat heart tissues and cells were lysed in ice-cold lysis buffer [50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/L sodium orthovanadate, 10μL/mL protease inhibitor cocktail, and 1 mmol/L PMSF] by incubating for 20 min at 4 °C. The protein concentration was determined by the BCA protein assay (Pierce). Protein sample (30 μg) was fractionated on 10% SDS–PAGE and transferred onto nitrocellulose membrane. The membranes were blocked with 5% nonfat dried milk or bovine serum albumin in 1x TBS buffer containing 0.1% Tween 20. The antibodies used include rabbit anti-periostin (ab14041, Abcam) at a 1:200 dilution, rabbit anti-TGF-β1 antibody (BS1361, Bioworld Technology) at a 1:450 dilution and rabbit anti-α-tubulin (Epitomics) at a 1:5000 dilution. Horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Life Science) was used as the secondary antibody at a dilution of 1:2000. Signal intensities were determined using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

2.9. Statistical analysis

Each experiment was performed in triplicate. Quantitative results were given in the form of means ± standard error of the mean (SEM). Between group difference was compared with two-tailed Student's *t*-test. $p < 0.05$ (one asterisk) and $p < 0.01$ (two asterisks) are considered as significant and highly significant respectively. All statistical analysis was performed with SPSS software (version 17.0; Chicago, IL, USA).

3. Results

3.1. Periostin expression was significantly increased in aged rat hearts and serum

Telomere length of aged rats was only approximately 69.2% of that in young rats ($p < 0.05$) (Fig. 1A). As to periostin expression heart tissues, aged rats had significantly higher periostin expression at both mRNA (2.3-fold, $p = 0.0001$) and protein (2.0-fold, $p = 0.017$) level than that of young rats ($p < 0.05$) (Fig. 1B and C). Serum periostin of aged rats was 1480 ng/mL, which was also significantly higher than 910 ng/mL of young rats ($p = 0.001$) (Fig. 1D).

3.2. Periostin induced senescence of H9C2 cells

To explore the role of periostin in senescent, normal H9C2 cells and H9C2 cells with periostin overexpression or knockdown were used for senescence-associated β-gal (SA-β-gal) activity analysis. Western blot analysis confirmed successful overexpression and knock down through stable transfected cell line selection (Fig. 2A

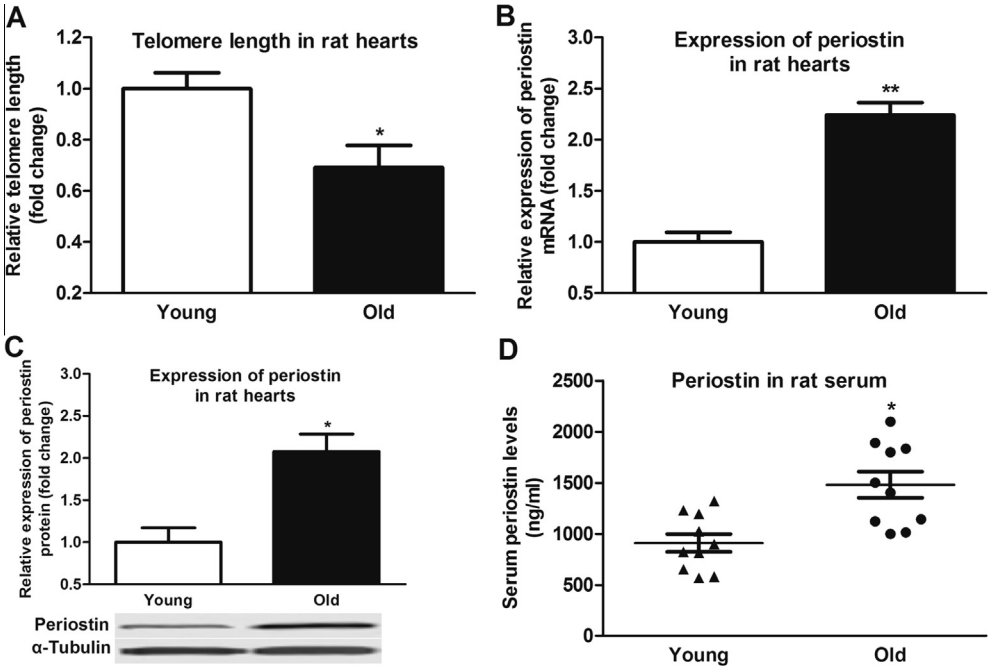

Fig. 1. Periostin expression was increased in aged rats. (A) Real-time PCR-based telomere length measurement of young and aged rat hearts. (B, C) Real-time PCR (B) and Western blot (C) analysis of periostin expression in aged and young rat hearts. (D) Elisa analysis of periostin levels in aged and young rat serum. $^{*}p < 0.05$, $^{**}p < 0.01$. Error bars depict s.e.m.

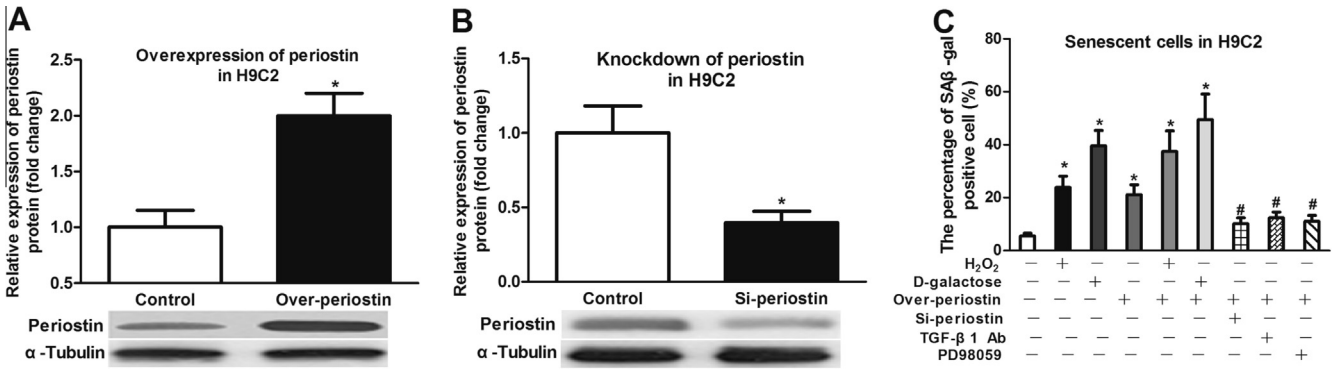

Fig. 2. Periostin induced senescence of H9C2 cells. (A, B) Western blot analysis was performed to confirm the overexpression (A) and knockdown of periostin protein (B). (C) Periostin induced senescent H9C2 cells, evaluated by the number of SA-β-gal-positive cells per microscopic field. $^{*}p < 0.05$ vs. control. $^{\#}p < 0.05$ vs. over-periostin. Error bars depict s.e.m.

and B). Previous studies found Ang-II could mediate periostin expression through ERK1/2/TGF-β1 pathway in mice model [13]. Our study confirmed that aged rats had significantly higher TGF-β1 protein expression than young rats in heart ($p = 0.03$) (Supplement Fig. 1A), while Lorsatan could block the Ang-II induced high periostin expression (Supplement Fig. 1B). TGF-β1 also had similar effect as Ang-II in promoting periostin expression (Supplement Fig. 1C), suggesting TGF-β1 is a critical factor in Ang-II mediated periostin expression. Periostin expression was significant inhibited by the presence of PD98059 (an ERK1/2 upstream kinase inhibitor) and SB202190 (a p38 MAPK inhibitor), but not by SP600125 (a specific c-Jun N-terminal kinase inhibitor) (Supplement Fig. 1D). Thus, PD98059 was chosen as an upstream blocking agent of ERK1/2/TGF-β1 pathway. Overexpression of periostin, similar to both H_2O_2 and D-Galactose treatment, could induce significant higher proportion of SA-β-gal positive cells and also promote senescent synergistically with H_2O_2 and D-Galactose treatment. Adding anti-TGF-β1 or PD98059 had similar effect as periostin interfere, could significantly inhibit periostin overexpression induced cell senescent (Fig. 2C).

3.3. Periostin induced senescence of primary rat cardiomyocytes

To better understand periostin's effect on cardiomyocytes, primary rat cardiomyocytes were isolated for in vitro analysis. Western blot analysis confirmed successful overexpression through transient transfection in primary cardiomyocytes (Fig. 3A). As in H9C2 cell line, overexpression of periostin, had similar effect in inducing senescence as both H_2O_2 and D-Galactose treatment. Adding anti-TGF-β1 or PD98059 had similar effect as

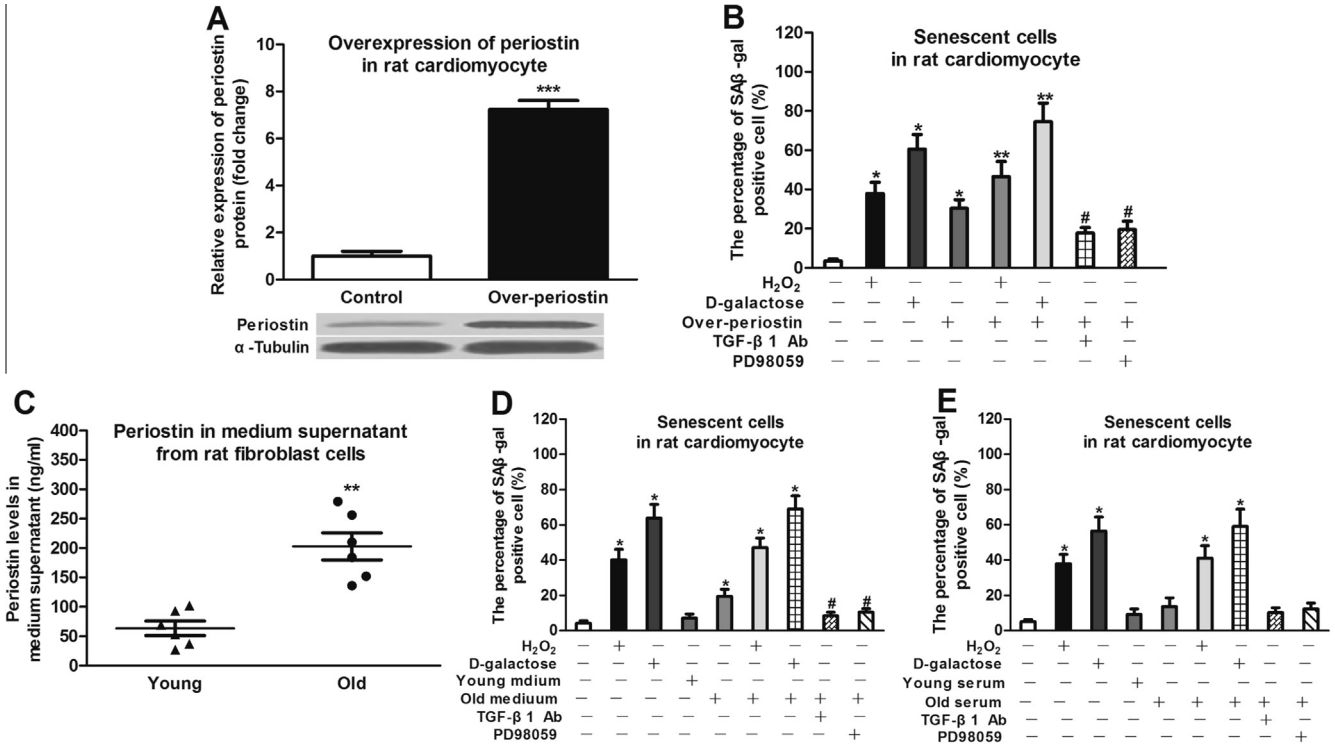

Fig. 3. Periostin induced senescence of primary rat cardiomyocytes. (A) Western blot analysis for overexpression of periostin. (B) Periostin overexpression induced senescence of rat cardiomyocyte cells. (C) Elisa analysis of periostin levels in medium supernatant of old and young rat primary fibroblast cells. (D and E) The effects of medium supernatant from old and young rat primary fibroblast cells (D) and serum from old and young rat (E) on the senescence of rat cardiomyocytes. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control. $^{\#}p < 0.05$ vs. over-periostin or old medium alone. Error bars depict s.e.m.

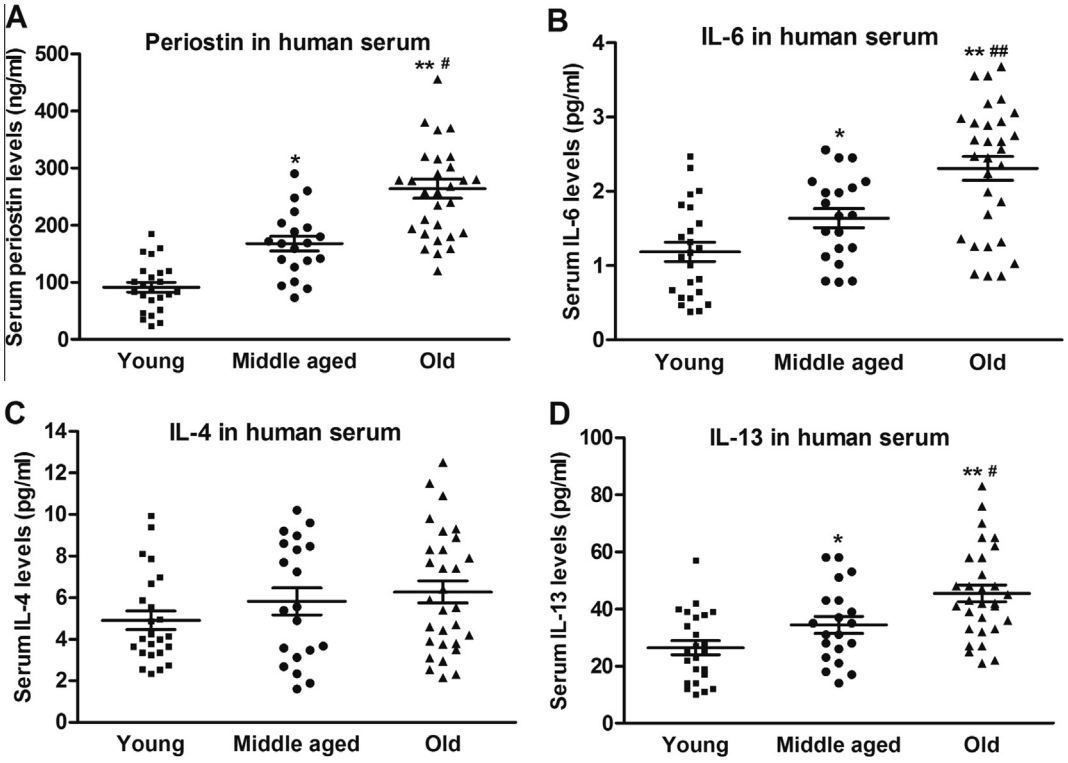

Fig. 4. Periostin expression was increased in old human serum. (A) Elisa analysis of periostin levels in old and young human serum. (B, C) Elisa analysis of IL-6, IL-4 and IL-13 levels in old and young human serum. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. young. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. middle aged. Error bars depict s.e.m.

periostin interfere, could significantly inhibit periostin overexpression induced cell senescent (Fig. 3B). Then, the association between cardiomyocyte senescence and periostin secreted by fibroblasts from young and old rats were further explored. Periostin in the old rat primary fibroblasts culture medium was significantly higher than that in young rat primary fibroblasts culture medium (Fig. 3C). As expected, the old medium had more evident effect in inducing cardiomyocyte senescence (Fig. 3D). However, this trend was not observed in test by applying serum samples from old rat and young rat respectively (Fig. 3E).

3.4. Aging is associated with increased expression periostin and inflammatory factors release in human

ELISA results showed an aged-dependent periostin expression in the three age groups of participants (Fig. 4A). IL-6, which had a well-recognized aged-dependent release pattern, was confirmed in participants' serum samples (Fig. 4B). IL4 and IL13, two upstream cytokines of periostin, presented different expression patterns. IL13 was age-dependent, while IL4 was not (Fig. 4C and D).

4. Discussion

In this study, we observed significant aging-related periostin expression in cardiac fibroblasts. Higher expression of periostin could promote cardiomyocyte senescent. However, through blocking the Ang-II-TGF β1-MAPK/ERK pathway, the level of senescent could be alleviated. Furthermore, the aging-related expression increase was observed in IL6 and IL14.

Aging heart is associated with fibrotic remodeling and accumulation of collagen in the heart, leading to progressive increase ventricular stiffness and impaired diastolic function [14]. Increased periostin in cardiac tissue due to mechanical overload was already confirmed as a key factor mediating fibrosis in both animal and human heart [15]. Actually, increased periostin expression was observed in pathophysiological conditions and recovery of heart, including heart failure and myocardial infarction [16,17]. However, the role of periostin in heart muscle is not well understood. Functionally, Lorts et al. [18] demonstrated that up-regulation and secretion of periostin enhanced muscular dystrophy (MD) in mouse model. However, MD mice with *Postn* gene knockout present significant improved recovery of skeletal muscle structure and function, as well as dramatically decreased fibrosis [18]. Genetic modified mice model with periostin overexpressing in heart present increased hypertrophy, suggesting periostin is involved in cardiac hypertrophic response [16]. Bakay et al. [19] examined periostin mRNA expression in human muscle disease and observed increased periostin expression in all biopsy specimens compared with normal muscle. Interestingly, age dependent periostin expression was found in Duchenne MD muscle [20]. However, these muscle-related studies only demonstrated decreased or overexpression of periostin affected muscle regeneration. No available study showed the impact of periostin overexpression on senescent of cadiomyocytes. In this study, we demonstrated that excessive periostin secreted from cardiac fibroblast promoted senescent of cardiomyocyte, which partly explained how periostin affects muscle regeneration. But the senescent inducing effect of serum was not evident, which might be related to complex components of the serum.

During heart aging, TGF-β1 is a crucial factor mediating fibrosis since it promotes collagen synthesis and transdifferentiation of fibroblast to myofibroblast [13–18]. In heart tissue, periostin expression is closely related to TGF-β1 signal. Exogenous addition of TGF-β1 to cardiomyocytes or elevated TGF-β1 signaling could lead to significantly upregulated periostin expression in both in vitro cell lines and primary cardiac fibroblasts [13–21]. In heart muscle, manipulation of TGF-β through using losartan or neutralizing antibody contributes to muscle function improvement after

myocardial infarction. This effect is mainly achieved by inhibiting periostin [22]. Therefore, periostin might function as a key downstream effector of TGF-β, promoting deleterious effects in heart aging. Our study confirmed the role of Ang-II-TGF β1-MAPK/ERK pathway in mediating periostin expression in myocardial cell line and primary cardiomyocytes. Inhibiting both TGF-β1 and MAPK/ERK could inhibit periostin expression and periostin induced cell senescent.

Tissue injury is always associated with local inflammatory reaction. In this process, fibrogenic cytokines and growth factors released from inflammatory cells are critical triggers of reparative process [23]. In mouse model, the morphological and functional changes of myocardial tissue during aging were associated with increasing release of interleukins 4, 6 and 13 (IL-4, IL-6 and IL-13) and accumulation CD45+ myeloid-derived fibroblasts that are positively related to fibrosis and associated diastolic dysfunction [24]. IL13 was proved a promoter of profibrotic actions *in vivo* [25]. IL-6, which is released in an age-dependent manner, is a strong predictor of coronary risk or stroke in elderly individuals [26]. Aging-associated changes in immune response are characterized as a shift from Th1 (IL-12, IFN-γ) to Th2 (IL-13, IL-4) cytokines in both animal and human studies [11–27]. Thus, these inflammatory cytokines might play important roles in senescence-associated fibrosis of heart. One recent study showed that periostin is a downstream molecule of IL4 and IL13 [28]. In our study based on human serum samples, it was found IL13 and IL6 had similar aged-dependent release as periostin. Considering the available evidence, it is possible that periostin is involved in the aged-related inflammatory process. Therefore, it was hypothesized that aging dependent release of IL13 and IL6, together with increasing periostin expression during heart aging might facilitate senescent of cardiomyocytes. However, future studies are required to confirm this hypothesis. Taking these evidences together, periostin might play an important role in a vicious circle (aging-fibrosis-inflammation-aging) of heart aging through promoting myocardial fibrosis and cardiomyocyte senescent simultaneously.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.109.

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