CYR61/TGF-β axis promotes adventitial fibrosis of Takayasu's arteritis in the IL-17 mediated inflammatory microenvironment

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Abstract

Objective

Takayasu's arteritis (TAK) is characterised by inflammation and fibrosis in the aortas, but its pathogenesis remains unclear. The aim of the study is to demonstrate the role of cysteine-rich protein 61 (CYR61), a novel proinflammatory factor, in the inflammation and fibrosis of TAK vessels.

Methods

CYR61 expression in the aortic vessel was compared between TA tissues and healthy samples by immunohistochemistry staining. The effect of CYR61 on the proliferation, migration and activation of adventitial fibroblasts (AFs) in the IL-17-mediated inflammatory microenvironment was studied in vitro.

Results

Here we found higher expression of CYR61 in the aortic adventitia in TAK patients than in healthy donors by immunohistochemistry staining. In vitro, recombinant human CYR61 (rhCYR61) significantly upregulated the proliferation of primary human aortic adventitial fibroblasts (AFs) and their expression of extracellular matrix (ECM) proteins such as collagen I, collagen III and fibronectin at the mRNA and protein levels, but rhCYR61 partly inhibited the migration of AFs. The integrin αvβ1 was identified as a membrane receptor of CYR61 in AFs, and its downstream Erk1/2 pathway was found activated by detecting its phosphorylation level. Pretreatment with PD98059, an inhibitor of Erk1/2, down-regulated the mRNA and protein expression of ECM proteins in the rhCYR61-stimulated AFs. Furthermore, rhCYR61 up-regulated the expression of TGF-β, and TGF-β siRNA transfection obviously attenuated the profibrotic effect of rhCYR61. Finally, to clarify the cooperation between CYR61 and classical proinflammatory factors, IL-17 was chosen as a co-stimulator in the culture of AFs. rhIL-17 promoted the mRNA and protein expression of CYR61 in AFs, and the collaboration of rhIL-17 and rhCYR61 dramatically boosted the synthesis of ECM and TGF-β.

Conclusion

Our findings suggest that CYR61 played a profibrotic role through the TGF-β pathway and it enhanced IL-17-mediated inflammation and fibrosis in the mechanism of vascular impairment in TAK.

Key words

cysteine-rich protein 61, Takayasu's arteritis, vascular fibrosis, inflammation

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Introduction

Takayasu's arteritis (TAK) is a chronic inflammatory disease that mainly involves the aorta and its primary branches. Vascular remodelling as a prominent feature of this disease is characterised by the vessel wall thickness, lumen stenosis and obliteration, or lumen dilation (1-2). These kinds of pathological remodelling in aorta directly impair blood supply to vital organs, such as brain, heart, lung, and kidney, and this increases the incidence of organ failure and sudden death in the patients with severe TAK (3-4). Of note, the pathogenesis of TAK has not been elucidated, which resulting in the few effective interventions.

The adventitia has been reported as the origin of vascular impairment in TAK, because it exhibits the excess deposition of collagens and increasing infiltration of a variety of inflammatory cells including macrophages, $CD4^+$ T cells, $CD8^+$ T cells, γδ T cells, natural killer (NK) cells and neutrophils during early phase, while the intima appears completely normal (1-2). With the progression of such disease, the loose connective tissue in adventitia is gradually replaced by dense collagen fibres, and finally the whole layers of aorta turn into thickness and stiffness. This pathophysiological change is known as vascular fibrosis outside in (5). Recent treatments including glucocorticoid (GC), leflunomide (LEF) and cyclophosphamide (CTX) show their obvious effects on the control of inflammation but not vascular fibrosis (6-7).

The intensive expression of alpha smooth muscle actin (α-SMA) in the aortic wall implies that myofibroblasts act as a central cell population in the fibrotic adventitia (8). Fibrosis related proteins including profibrotic factors (*e.g.* collagen I/III, fibronectin and TGF-β) are mainly synthetized by myofibroblasts and the excess production of any these proteins would be inclined to aggravate adventitial fibrosis (2, 8). As the maximum subgroup of cells in adventitia, adventitial fibroblasts (AFs) are the principle source of myofibroblasts and they are extremely sensitive to inside or outside stimuli resulting in the rapid increase of fibrosis related proteins expression (5, 9-10). For example, it is believed that via strongly stimulating AFs, IL-6 played a key role in the inflammation and adventitial fibrosis of TAK (11); however, some reports from clinical studies showed that the application of IL-6 receptor antagonist did not alleviate vascular fibrosis (12-13), indicating the existence of new mechanisms beyond IL-6. Thus, it is important to search for these new mechanisms underlying the occurrence and development of vascular remodelling, especially adventitial fibrosis.

Cysteine-rich protein 61 (CYR61), a multifunctional but non-structural protein found in the extracellular matrix (ECM), displays multiple cellular functions, for instance, regulation of cell proliferation, apoptosis, motility, adhesion, chemotaxis and ECM synthesis (14). In *CYR61*$^{-/-}$ knockout foetal mice, CYR61 acts as a key regulator in the progress of aortic angiogenesis for discovering the fatal dilation of dorsal aorta (15). In adults, the profibrogenesis of CYR61 have been evidenced by promoting the proliferation of tissue cells, fibroblasts, keratinocytes etc., during wound healing and tissue remodelling (16-18). More importantly, ten years ago, several studies showed that CYR61 involved in the inflammation of rheumatoid arthritis (RA) (19-21), which initiated the CYR61 targeted researches in inflammatory and autoimmune diseases. So far, as a proinflammatory factor, CYR61 is involved in the pathogenesis of RA, psoriasis, systemic lupus erythematosus and Sjögren's syndrome (22-23). However, the role of CYR61 in the aortic diseases such as TAK has not been clarified.

Additionally, in TAK, the progression of vascular fibrosis is closely related to the inflammatory microenvironment. Prior studies have indicated that IL-6-Th17-IL-17 is a key axis in the systematic inflammation and vascular fibrosis of TAK (24). Our previous studies demonstrated that IL-6 and IL-17 strongly expressed in the wall of TAK aorta and IL-6 aggravated adventitial fibrosis mainly via promoting the activity of AFs and the expression of ECM proteins (8, 11). But our preliminary experiments found that the

mRNA expression of CYR61 had not been enhanced by IL-6 in AFs (unpublished data), indicating that some other proinflammatory factors may activate CYR61 expression in the fibrotic process. Given that IL-17 promoted CYR61 expression significantly in RA synovial fibroblasts, we speculated that the upregulation of CYR61 might be induced by IL-17 not IL-6 in the progression of vascular fibrosis in TAK.

Although CYR61 has been regarded as a vital ECM protein involved in the angiogenesis (15, 25), it is unclear whether CYR61 would influence on the pathogenesis of vasculitis, especially TAK. Therefore, in the present study, we hypothesised that CYR61 would play a role in the pathogenesis of TAK by promoting AF activation and collagen synthesis. We have also investigated the CYR61-IL-17 interactions in adventitial fibrosis.

Materials and methods

Patients

Six patients classified as TAK according to the 1990 ACR classification criteria were enrolled in the study and their corresponding histopathological specimens were obtained in the department of cardiac surgery. The clinical data were recorded at detail and the activity of disease was evaluated by NIH criteria (26). Three healthy aortas from organ donors were used as control. The study was approved by the Ethics Committee of Zhongshan Hospital [B2013-115 (3)], and the written informed consent was obtained prior to the experiment.

Immunohistochemistry

The immunohistochemistry was performed as previously described (11). Briefly, the sections from the lesioned vessels of TAK patients and normal aortas were dewaxed and incubated with rabbit anti-human CYR61, TGF-β, COL1A1 (Abcam, Cambridge Science Park, Cambridge, UK) at 4°C overnight. The next day, Horseradish peroxidase-labelled goat anti-rabbit IgG (Abcam) as secondary antibody was added to each slide which had been rewarmed to the room temperature. After the colour-producing reaction with DAB (Abcam), the stained sections were evaluated by two independent pathologists. For each specific antigen, the strength of its positive staining under 2-fold magnification was recorded semi-quantitatively according to the percentage of its positive expression area covering the whole aortic adventitia: the strong was more than 50%, the moderate within 20~50% and the mild less than 20%.

Cell culture and treatment

The primary human aortic adventitial fibroblasts (AFs) (Sciencell, Corte Del Cedro, CA, USA) were cultured as per the manufacturer's instruction. The complete medium was also provided by Sciencell containing fibroblast culture solution and 5% fetal bovine serum (FBS). The recombinant human CYR61 (rhCYR61, Novus Biologicals, Centennial, CO, USA) and the recombinant human IL-17 (rhIL-17, R&D, Minneapolis, MN, USA) as stimulators were added into AF culture system. In the intervention studies, AFs were pretreated by inhibitors one hour prior to the stimulators.

The concentration- and time-dependent stimulation of rhCYR61 on the proliferation and activation of AFs were conducted to choose the best working dose and duration of rhCYR61 and the optimal dose was detected at 10 μg/ml. The phosphorylation levels of signalling molecules were detected by Western blot every 15 minutes up to 2 hours after the induction of 10 μg/ml rhCYR61. The inhibitors (Cell Signaling Technology, Danvers, MA, USA) of signalling molecules were used to confirm the activated pathway. After the stimulation of 50ng/ml rhIL-17 on AFs, the gene and protein expression of CYR61 were measured by real-time qPCR and Western blot. The comparison among 3 different stimulated groups with 10 μg/ml rhCYR61 alone, 50ng/ml rhIL-17 alone and both was performed to demonstrate the synergistic effect of CYR61 and IL-17. Finally, to evaluate the role of CYR61 (10 μg/ml) in the IL-17 (50ng/ml) mediated inflammatory environment, 20 μg/ml monoclonal antibody of CYR61 (CYR61ab 093G9, given by Prof. Ningli Li, Jiao Tong University, Shanghai, China) was applied as neutraliser (27). Simultaneously, the cells were pretreated with the drugs commonly used in TAK such as 1 μg/ml dexamethasone (DEX, TOCRIS Bioscience, Ellisville, Missouri, USA), 100 μmol/L leflunomide (LEF, TOCRIS) and 5 mg/ml cyclophosphamide (CTX, TOCRIS) as positive controls in order to detect the degree of CYR61ab neutralisation.

Cell proliferation assay

With regard to the effect of CYR61 on the cellular proliferation, AFs were divided into four groups: the control group, the 2.5 g/ml, 5 μg/ml and 10 μg/ml rhCYR61 groups. During the entire 48-hours observation period, the proliferation of AFs was detected by cell counting kit-8 (DOJINDO laboratories, Kumamoto, Japan) following the manual every 6 hours. For each time point in different CYR61 treatment groups, six replicate wells were set. The cell proliferation curve of each group was drawn according to the absorbance values at 450 nm, as measured with FlexStation3 Multi-Mode Microplate Reader (Molecular Devices, Silicon Valley, CA, USA).

Cell migration assay

The AFs were suspended at the concentration of 1×10^5 cells/ml, and 100μl (1×10^4) of them was seeded into the upper chamber of the Transwell (Corning Costar, Cambridge, MA, USA). After 24 hours of starvation, 600μl complete medium supplemented with different concentrations of rhCYR61 (0, 5 and 10 μg/ml) was added into the corresponding lower chambers. AF migration was observed every 12 hours till 48 hours. The measurements were performed in triplicate (three wells) at each time point for every group. AFs that passed through the membrane were fixed in formaldehyde for 30 min and dyed with 0.1% crystal violet, and those that remained in the chamber were gently removed. The number of AFs was counted in five random visual fields under an inverted microscope (400× magnification, Olympus, Tokyo, Japan).

Real-time Quantitative PCR

Total RNA was extracted from AFs using TRIzol reagent (Invitrogen,

Carlsbad, CA, USA), quantified by a spectrophotometer (Denovix DS-11, Wilmington, Delaware, USA) and reversely transcribed into cDNA using PrimeScript RT reagent kit (Takara Biotechnology, Otsu, Japan). The primer sequences (Sangon Biotech, Shanghai, China) are listed in the online Supplementary Table S1. The real-time quantitative PCR was performed with the SYBR-Green PCR Master Mix (Takara) according to the manufacturer's instructions on a real-time PCR system (Eppendorf AG, Hamburg, Germany). GAPDH was used for normalisation and the expression of a specific gene was presented as $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$.

Western blotting

AFs were lysed with RIPA containing phenylmethylsulphonyl fluoride, and 30 μg of total protein was loaded for each sample. The expression of production proteins was detected with the primary antibodies against α-SMA, COL1A1, COL3A1, fibronectin (FN), TGF-β and CYR61 (Abcam). The activation of signal pathway proteins was analysed using specific antibodies against p-PI3K Tyr458, p-Akt Thr308, p-Akt Scr473, p-mTOR Ser2448, p-P70 S6K Thr389, p-P38 MAPK Thr180/Tyr182, p-JNK Thr183/Tyr185, p-Erk1/2 Thr202/Tyr204, t-Erk1/2, p-CREB Ser133, t-CREB, p-FAK Tyr397 and t-FAK from Cell Signaling Technology, and compared among different time points of rhCYR61 stimulation in AFs. The corresponding anti-rabbit or anti-mouse secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) were applied per the primary antibodies. α-Tubulin (Abcam) was chosen as the endogenous control. The blots were developed by Tanon 5200 multi (Tanon Science & Technology, Shanghai, China) and analysed with Gel pro analyzer 4.0 (Media Cybernetics, Silverspring, USA).

Phospho-kinase array

Proteins at baseline and 30 minutes of 10 μg/ml rhCYR61-stimulation were extracted for analysis. Total 400 μg protein from each group was incubated overnight with Human Phospho-Kinase Array (R&D Systems, Minneapolis, MN, USA), which was spotted with antibodies for 43 signal pathway kinases mainly including MAPKs, STATs, AMPK, Akt, mTOR, GSK and 2 related proteins. The phosphorylation levels of signal proteins were detected as per the manufacturer's instructions. The pixel density of each spot on developed x-ray film were captured using Chemi Scope 6300 (Clinx Science Instruments, Shanghai, China) and analysed by ImageJ (NIH, USA).

Transfection

The siRNA targeting TGF-β, whose sequence is listed in Supplementary Table S2, was purchased from GenePharm (Shanghai, China). TGF-β siRNA was transfected by Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) as described previously (6) and TGF-β mRNA was detected after 24 hours to evaluate the knock-down efficiency. To evaluate the intermediate role of TGF-β in the interaction between rhCYR61 and AFs, the TGF-β siRNA was transfected 24 hours prior to the treatment of rhCYR61 (10 μg/ml). The cells were further cultured for 48 hours and the produced proteins level was detected by Western blot.

Statistical analysis

All the quantitative data that met normal distribution were presented as mean ± standard error of mean (SEM). For unpaired two-group comparison, Student's *t*-test was used. The difference among multiple groups were analysed by one-way ANOVA with Tukey's multiple comparisons (parametric tests). The Kappa coefficient was applied to assess the consistency between two independent pathologists. All the experiments were repeated three times unless especially indicated. Graphpad Prism 7.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and significance level was set at $p<0.05$.

Table I. Clinical characteristics of TAK patients.

Patient	Gender	Age(y)	DD(y)	Active	Surgery	IHC		
						CYR61	TGF- β	COL1A1
1	M	43	1	Yes	Bentall	++	ND	ND
2	F	59	3	Yes	Bentall	+	+	+++
3	F	38	3	Yes	Bentall	+	+	++
4	F	39	1	Yes	Bentall	++	++	+++
5	M	32	2	No	Bentall+ TAR	+	+	+++
6	M	45	5	No	Bentall	-	+	+++

M: male; F: Female; DD: disease duration; TAR: total arch replacement; IHC: immunohistochemical; +++: strongly positive; ++: moderately positive; +: mildly positive; -: negative; ND: none done.

Results

Higher CYR61 expression in TAK-affected aortic adventitia

The clinical characteristics and surgical procedures of TAK patients are shown in Table I. All the patients accepted 'Bentall' procedure due to severe aortic insufficiency and dilatation of aortic root. One patient also accepted total arch replacement for the impairment extending to the aortic arch. When the histopathological specimens were obtained, four patients were active with NIH score more than two. All the stained sections were read by two independent pathologists and the Kappa coefficient of their consistency was 0.80.

The adventitia of TAK patients obviously thickened with abundant collagen I deposition in comparison with that of controls. In the continuous sections of TAK, CYR61 and TGF-β also overexpressed in the adventitia, especially the areas of inflammatory cells gathering, but in normal controls, these two cytokines expressed in the junction of media and adventitia. Figure 1 clearly shows these notable differences in the continuous images of TAK-affected tissues from a typical patient (no. 2).

Effect of CYR61 on the proliferation and migration of adventitial fibroblasts

The proliferation of AFs was obviously dependent on the rhCYR61 dose, and the most optional induction concentration was 10 μg/ml, which was used in the following experiments, unless

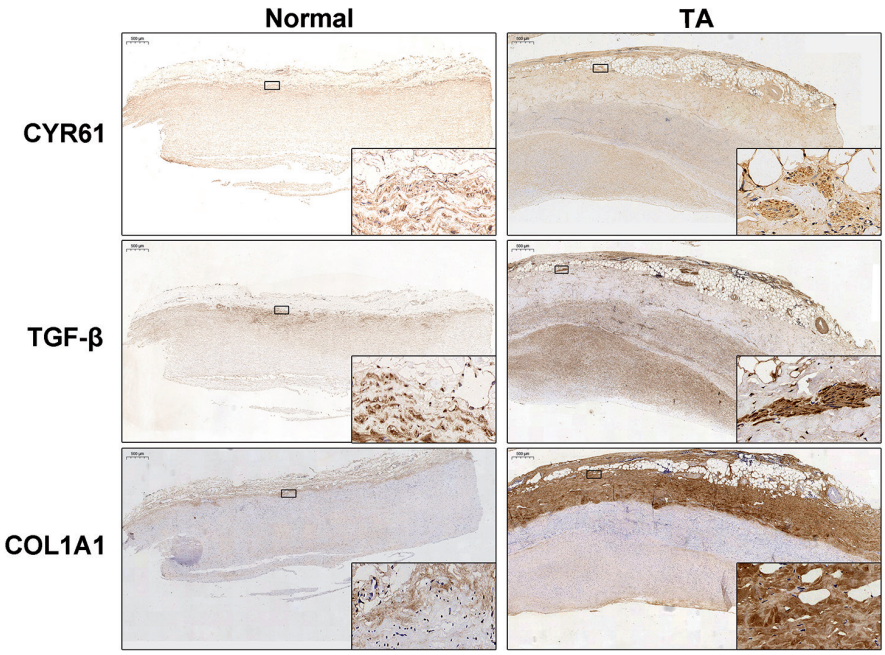

Fig. 1. Increasing expression of CYR61 in the adventitia from patients with TAK. Representative histological sections of normal aorta (right) and lesioned aorta from TAK patient (left) examined by immunohistochemical staining: 1) the overexpression of CYR61, TGF-β and COL1A1 in the adventitia of TAK; 2) the overlap of high CYR61 and TGF-β expression regions. Bar 500μm. Magnification of large pictures ×2 and small pictures at the left bottom corner ×50.

otherwise specified. The proliferation of AFs was confirmed by the elevated expression of the cellular proliferation genes cyclin-1 and PCNA (Fig. 2A-B). CYR61 obviously inhibited the migration of AFs in a time- and concentration-dependent manner (Fig. 2C). Furthermore, after a 6-hour induction with rhCYR61, the relative expression of α-SMA mRNA augmented by 3.56 times in AFs and after 48 hours, the protein expression of α-SMA significantly increased; this was an important indicator of the AF activation (Fig. 2C).

Dose- and time-dependent upregulation of collagen synthesis by CYR61

The mRNA expression of ECM components was dependent on the duration and dose of rhCYR61 treatment (Fig. 3A-F). Consequently, the optimal stimulation time was up to 6 hours and the optimal dose was at 10 μg/ml. The 10 μg/ml rhCYR61 enhanced the synthesis of COL1A1, COL3A1 and fibronectin (FN) by 2.34, 1.93 and 1.28 times, respectively ($p<0.05$ for all, Fig. 3G-H) when comparing with the untreated group at protein level.

TGF-β mediated COL1A1 expression by CYR61 in adventitial fibroblasts

Apart from the ECM proteins, the mRNA expression of various inflammatory factors was also detected in the AFs and rhCYR61 co-culture system. The mRNA expression of TGF-β was remarkably upregulated by 9 times, and its protein synthesis was upregulated by 2.3 times in the rhCYR61-stimulated group ($p<0.001$ for all, Fig. 4A-B). Furthermore, the mRNA expression of IL-10 significantly increased by 2.4 times ($p<0.001$). But other inflammation-related factors, such as CTGF, IL-6, IL-17A, IL-17D, and MCP-1, were not found differentially expressed

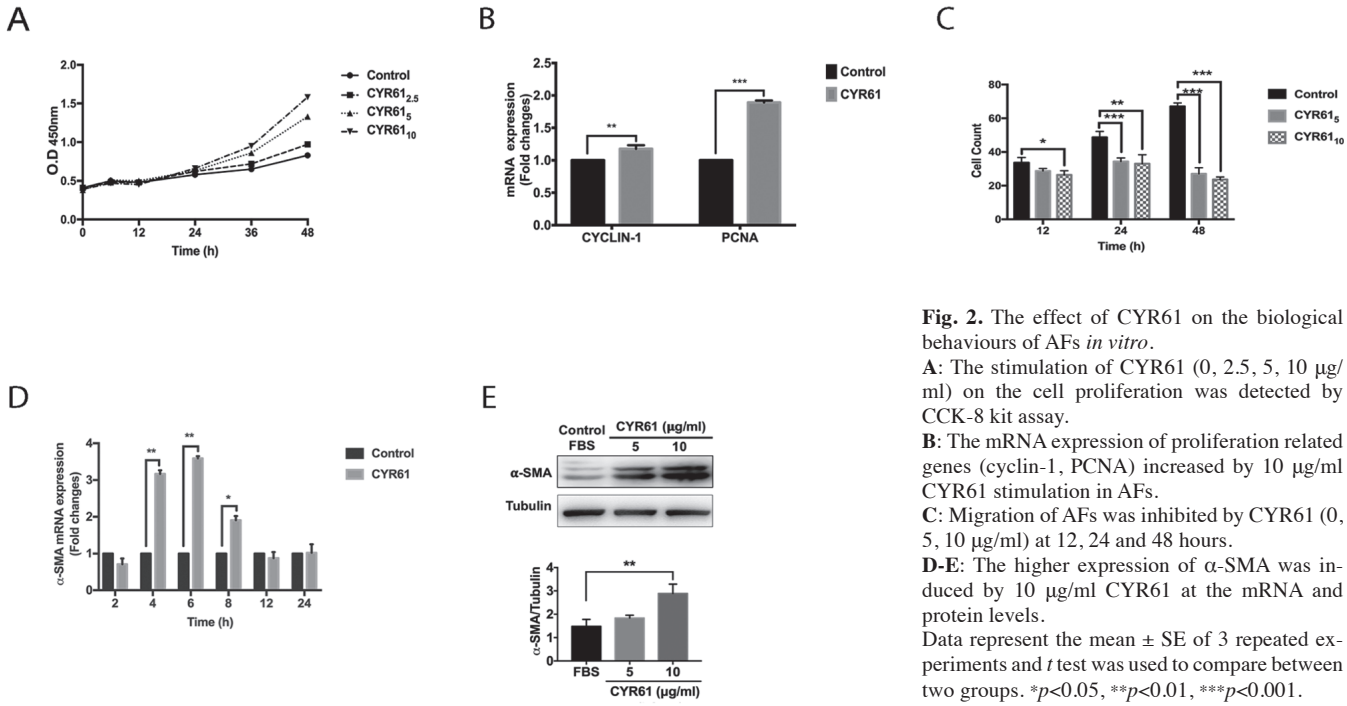

Fig. 2. The effect of CYR61 on the biological behaviours of AFs *in vitro*.
A: The stimulation of CYR61 (0, 2.5, 5, 10 μg/ml) on the cell proliferation was detected by CCK-8 kit assay.
B: The mRNA expression of proliferation related genes (cyclin-1, PCNA) increased by 10 μg/ml CYR61 stimulation in AFs.
C: Migration of AFs was inhibited by CYR61 (0, 5, 10 μg/ml) at 12, 24 and 48 hours.
D-E: The higher expression of α-SMA was induced by 10 μg/ml CYR61 at the mRNA and protein levels.
Data represent the mean ± SE of 3 repeated experiments and *t* test was used to compare between two groups. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

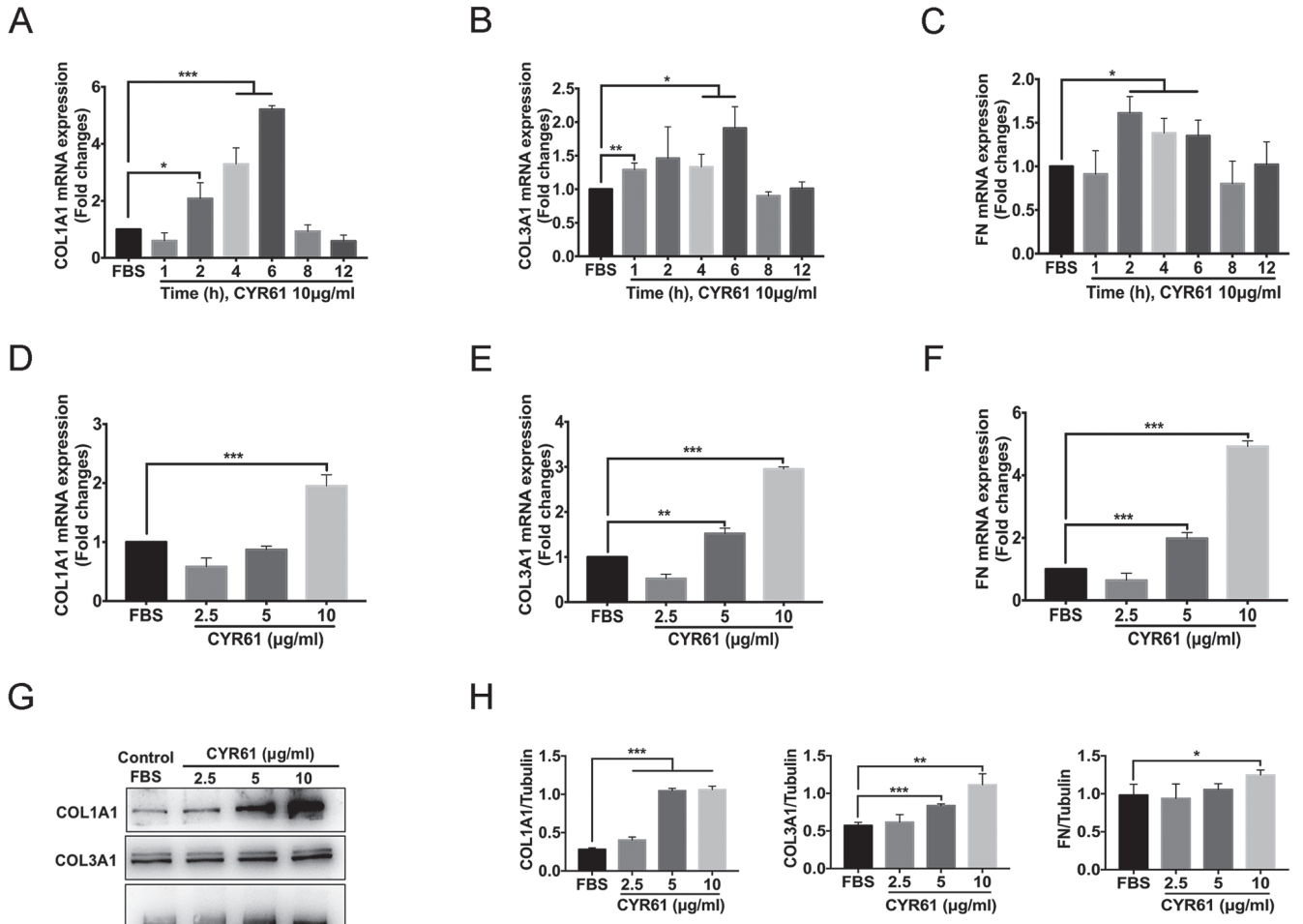

Fig. 3. Dose- and time-dependent upregulation of collagen synthesis by CYR61.
A-F: The CYR61-induced mRNA expression of ECM proteins was time and dose dependent in AFs.
G-H: The CYR61-induced protein synthesis of ECM components was dose dependent and 10 μg/ml was the optimal.
Data represent the mean ± SE of 3 repeated experiments. One-way ANOVA was used to compare three or more groups and for comparison between two groups, *t* test was applied. **p*<0.05, ***p*<0.01, ****p*<0.001.

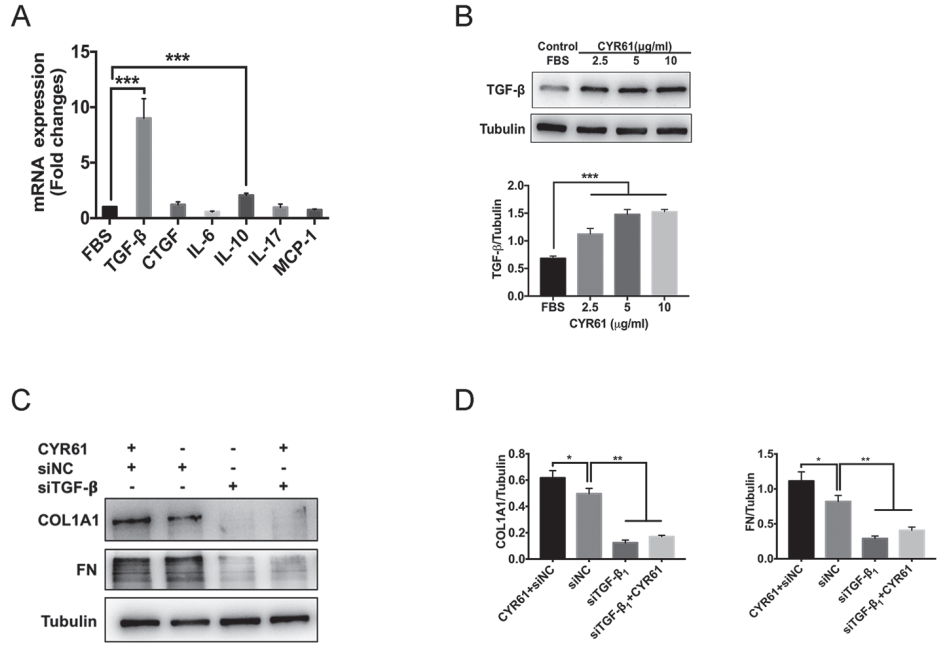

Fig. 4. Induction of CYR61 on the expression of ECM via TGF-β in AFs.
A: The mRNA expression of cytokines in AFs stimulated by CYR61 10μg/ml.
A-B: The expression of TGF-β increased in CYR61-induced AFs at mRNA and protein level.
C-D: The decreasing protein expression of COL1A1 and FN with or without CYR61 stimulation in TGF-β-knockdown AFs was detected by Western blotting.
Data represent the mean ± SE of 3 repeated experiments. One-way ANOVA was used to compare among three or more groups and for comparison between two groups, *t*-test was applied. **p*<0.05, ***p*<0.01, ****p*<0.001.

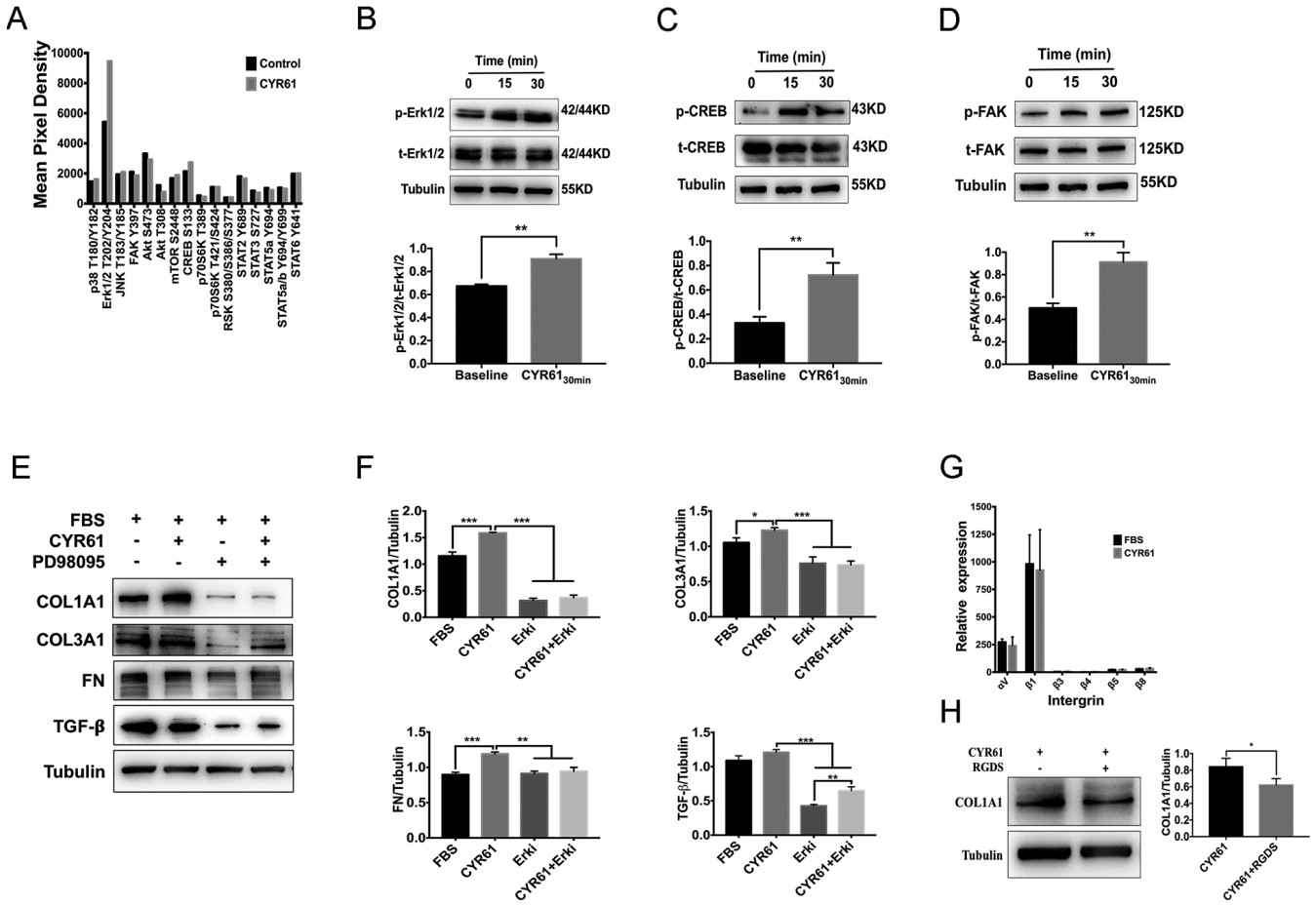

Fig. 5. Signalling pathways involved in regulating Cyr61-induced ECM and TGF-β production in AFs.
A-D: The phosphorylation of signalling pathway proteins after 30 minutes-stimulation of CYR61 were detected by human phosphokinase array and Western blot.
E-F: The pretreatment of Erk1/2 inhibitor, PD98095 attenuated the induction of CYR61 on ECM and TGF-β protein expression in AFs.
G: The mRNA expression of integrin subunits was identified in co-culture of CYR61 and AFs.
H: The decreasing protein expression of COL1A1 was detected by Western blot after the integrin inhibitor, RGDS was applied.
Data represent the mean ± SE of 3 repeated experiments. One-way ANOVA was used to compare among three or more groups and for comparison between two groups, *t* test was applied. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

in comparison with the unstimulated group. TGF-β siRNA experiments were also conducted (Fig. S1), and the transfection of TGF-β_1 siRNA into the AFs-rhCYR61 culture system was found to distinctly inhibit the synthesis of COL1A1 and fibronectin (Fig. 4C-D), indicating that TGF-β mediated COL1A1 expression by CYR61.

Integrin αvβ1/Erk1/2 pathway involved in the stimulation of CYR61 on adventitial fibroblasts

The phosphorylation of Erk1/2 Thr202/Tyr204 (member of the MAPK signalling pathway) significantly increased in AFs after 30-minuntes treatment with rhCYR61. This was confirmed by western blot analysis (Fig. 5A-B and Suppl. Fig. 2). As a Erk1/2 downstream transcription molecule, CREB Ser133 was also found to be activated (Fig. 5C). Additionally, the phosphorylation of FAK Tyr397 was obviously enhanced (Fig. 5D). Application of the Erk1/2 inhibitor PD98059 (50 ng/ml) resulted in a remarkable decrease in AF proliferation and ECM synthesis. Similarly, TGF-β expression was downregulated but partly reversed by rhCYR61 stimulation (Fig. 5E-F).

Further to identify which integrin, a membrane receptor of CYR61, was involved in the CYR61-mediated regulation of AFs, the genetic expression of the common subunits, including αv, β1, β3, β4, β5, β6, and β8, was screened by real-time qPCR in AFs with or without rhCYR61 stimulation. αv and β1 were found to have extremely high expression levels (Fig. 5G). An Arg-Gly-Asp-Ser (RGDS) peptide displaying high affinity with integrin is usually applied to block the binding between integrin and its ligands. In our study, the RGDS peptide could partly diminish the production of CYR61-induced COL1A1 (by 28.02%) in comparison to the untreated group (Fig. 5H).

Upregulation of COL1A1 and FN expression by CYR61 in cooperation with IL-17 in adventitial fibroblasts

With the addition of 50 ng/ml rhIL-7 into the co-culture system, the protein synthesis of COL1A1, COL3A1, FN, TGF-β showed a dramatic increase in comparison with sole stimulation with rhCYR61 ($p<0.01$ for all, Fig. 6A-B). rhIL-17 was also found to distinctly

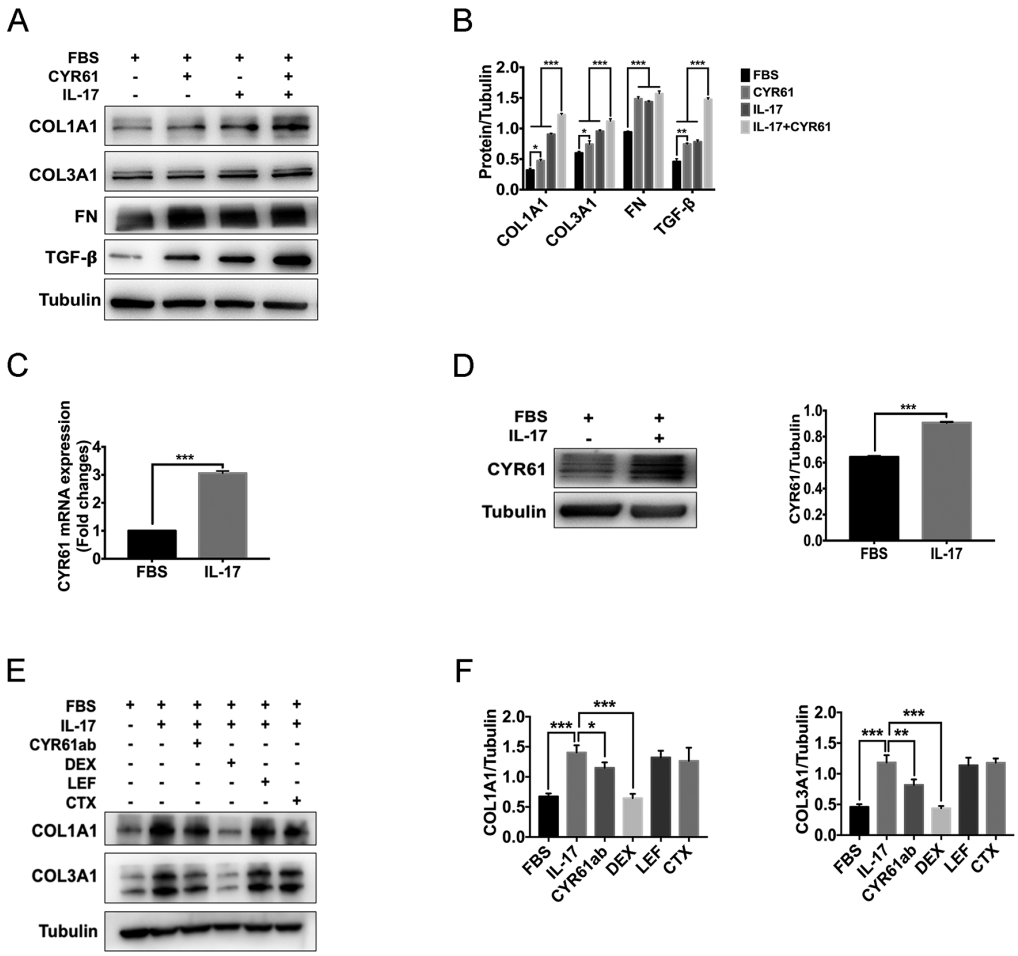

Fig. 6. Collaboration between CYR61 and IL-17 in the regulation of ECM and TGF-β synthesis.
A-B: The increasing expression of COL1A1, COL3A1, FN, TGF-β stimulated strongest
C-D: The increasing mRNA and protein expression of CYR61 induced by IL-17 in **AFs.**
E-F: Anti-Cyr61 mAb 093G9 and immunosuppressors (DEX, LEF and CTX) as positive controls were used to intervene the COL1A1 and COL3A1 protein expression in IL-17-stimulated AFs. Data represent the mean ± SE of 3 repeated experiments and *t* test was used to compare between two groups. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

upregulate the expression of CYR61 in AFs ($p<0.001$, Fig. 6C-D). Pre-treatment of CYR61 monoclonal antibody (CYR61ab 20 μg/ml) and DEX (1 μg/ml) before induction with rhIL-17 resulted in an obvious downregulation in the protein expression levels of COL1A1 and COL3A1, and especially in DEX group, they approached those levels of control group (Fig. 6E-F). However, LEF (100 μmol/L) and CTX (5 mg/ml) did not exhibit such effects on inhibiting the rhIL-17 induced expression of COL1A1 ($p=0.42$ and $p=0.38$, respectively) and COL3A1 ($p=0.66$ and $p=0.94$, respectively). These data exploited that IL-17 upregulated CYR61 expression in AFs in advance before Cyr61 activated TGF-β- COL1A1/FN expression.

Discussion

It is well known that the outstanding features of TAK vessels are thickness and stiffness of aortic wall (1-2). In this study, we found that excessive deposition of ECM such as collagen I replaced the normal loose connective tissue in adventitia. Meanwhile, the high expression site of classical profibrotic cytokine TGF-β and the novel proinflammatory factor CYR61 were found in adventitia, especially the zone of fibrotic progression in TAK. In the lesioned vessels of the active patients, CYR61 and TGF-β mainly overexpressed around the areas where inflammatory cells assembled. The similar distribution of CYR61 and TGF-β made it interesting to clarify the role of CYR61 in the vascular fibrosis and its relationship with TGF-β in TAK. *In vitro*, CYR61 was found to stimulate the phenotypic transformation of primary human AFs to profibrotic myofibroblasts, as evidenced by the higher expression of α-SMA. Further, the effects of CYR61 on cellular proliferation and ECM synthesis were obviously time and dose dependent. CYR61 significantly upregulated the expression of collagen I, collagen III and fibronectin, which are important components of ECM, in the adventitia. Furthermore, CYR61 could significantly enhance the mRNA expression of TGF-β and IL-10. These indicated that CYR61 played a profibrotic role in the remodelling of the aorta. The following findings indicated that the effect of CYR61 on the adventitial fibrosis majorly involved the TGF-β pathway. CYR61 could upregulate the expression of TGF-β at the mRNA and protein levels. Addition of TGF-β siRNA into the co-culture system of AFs and CYR61 inhibiting the production of TGF-β resulted in the attenuation of the ECM synthesis. Collectively, the profibrotic effect of CYR61 was dependent on the activity of TGF-β pathway; this is in agreement with the findings reported by Kurundkar on the fibrogenic responses to lung injury (28).

Since integrin αvβ3 was firstly found as the membrane receptor for CYR61, CYR61 mainly binds a variety of integrins to regulate cellular bioactivities (29-30). In the present study, through

a screening test and blocking with an RGDS peptide, the integrin αvβ1 was identified as a cytomembrane receptor of CYR61 in AFs. After the binding of CYR61 to αvβ1, Erk1/2, a member of the MAPK pathway, was successively activated, as indicated by its increasing phosphorylation. The application of Erk1/2 inhibitor PD98059 significantly downregulated the expression of ECM and TGF-β. To sum up, the integrin αvβ1/Erk1/2 pathway may be a vital signalling pathway for the profibrotic effect of CYR61 in AFs. CYR61 could partly reverse TGF-β inhibition by PD98059; this means that other pathways might be involved in the regulation of TGF-β by CYR61, such as Wnt signalling pathway (31-33). However, investigating this was outside of the scope of this study.

It is reported that CYR61 also activated cAMP-response element binding protein (CREB), a downstream transcription target of the Erk1/2 pathway. CREB has been found to directly bind the DNA promoters of collagens and fibronectin and to indirectly bind SMADs to activate the transcription of genes encoding the ECM proteins (34-36). Furthermore, the phosphorylation of FAK was found increasing in CYR61-induced AFs, and might play a role in the CYR61-mediated inhibition of AF migration. Activated FAK elicits the formation of filopodia and lamellipodia, which contributes to the adherence of mouse fibroblasts (37). Thus, CYR61 may be involved in the localisation of AFs at the aortic lesion and in the production of a large amount of ECM proteins to aggravate local fibrosis.

In the active TAK, overexpression of CYR61 was closely related to the inflammation. Previous studies found that IL-17 strongly expressed in the lesional tissues of TAK (11). IL-17 was mainly produced by Th17 which was one major group of $CD4^+$ T helper cells involved in orchestrating the inflammatory microenvironment of TAK (24). Therefore, IL-17 as a representative cytokine was studied its role in the interaction between CYR61 and fibrosis in the study. The results revealed that rhIL-17 could elicit the mRNA and protein expression of CYR61 in AFs. The increase in ECM synthesis was more prominent in the group treated with rhIL-17 and rhCYR61 together than with solo stimulators. Further, DEX, the first-line drug for TAK, showed strongest inhibition of IL-17-induced ECM synthesis and followed by CYR61 monoclonal antibody. However, CTX and LEF could not relieve this pathway of ECM synthesis, although they have been commonly used as combined immunosupressors in the treatment of TAK (38). These findings implicate that except for glucocorticoid, CYR61 blocker may have a beneficial potential in inhibiting the inflammatory fibrosis in TAK, but it remains to be determined including *in vivo* studies. Consequently, the inflammatory microenvironment could upregulate the production of CYR61, who can further exacerbate the progress of fibrosis in the vascular wall. To block CYR61 may break up this vicious circle (Fig. 7).

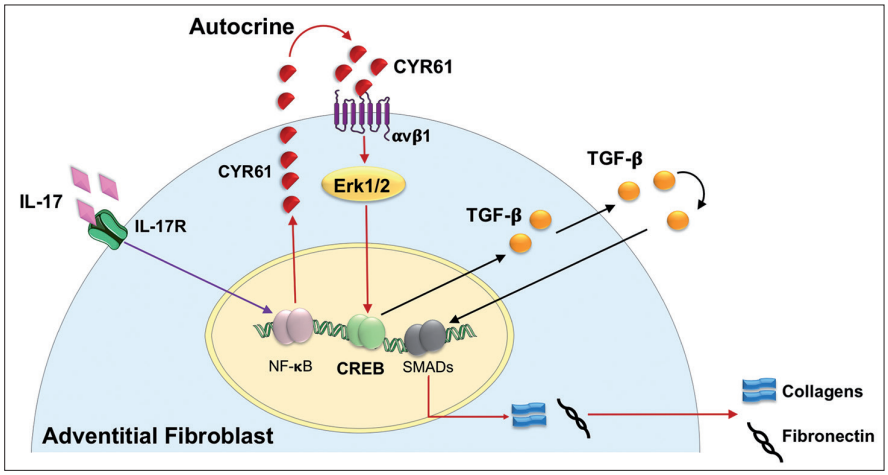

Fig. 7. The schematic model for the role of Cyr61 in the vascular fibrosis of TAK.

A strength of our present study is that it is first time the role of a functional matrix protein, CYR61has been discussed in vascular fibrosis. CYR61 is well known as an angiogenic and proinflammatory factor. In the study, it has been demonstrated its ability to boost the vascular fibrosis via TGF-β pathway. Furthermore, the collaboration of CYR61 and IL-17 evidenced that apart from as effect proteins in the development of vascular inflammation and fibrosis, some matrix proteins might actively regulate these progresses.

Regrettably, all findings on the cellular level could not be verified in the TAK patient-isolated fibroblasts, mainly because of the huge difficulties in collecting the fibroblasts from TAK aortas. The type of vascular lesion was dilation or aneurysm in most TAK patients who accepted surgical treatment. In our pretest, the fibroblasts isolated from the aneurysmal wall were unlikely to survive, and the reason might be the lower proliferation and activity of these cells. In the future, more effort in creating TAK animal models would help to carry out studies *in vivo* and cellular researches from lesioned vessels.

Conclusion

CYR61 might be an important factor in the pathogenesis of diseases characterised by vascular remodelling via promoting vascular fibrosis after inflammation. Our study reveals that the treatment strategy for TAK should consider blocking inflammation and fibrosis simultaneously; the targeting CYR61 may have potential clinical values in the future and is therefore a topic worthy of investigation.

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