Cyclin Dependent Kinase 1 (CDK1) Activates Cardiac Fibroblasts via Directly Phosphorylating Paxillin at Ser244

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Summary

Atrial fibrillation has caused severe burden for people worldwide. Differentiation of fibroblasts into myofibroblasts, and consequent progress in atrial structural remodeling have been considered the basis for persistent atrial fibrillation, yet little is known about the molecular mechanisms underlying the process. Here, we show that cyclin-dependent kinase 1 (CDK1) is activated in atrial fibroblasts from patients with atrial fibrillation (AFPAF) and in platelet derived growth factor BB (PDGF-BB)-treated atrial fibroblasts from patients with sinus rhythm (AFPSR). We also demonstrate that inhibition of CDK1 suppresses fibroblast differentiation and focal adhesion (FA) complex formation. The FA protein paxillin is phosphorylated directly at Ser244 by CDK1. Importantly, transfection of a paxillin construct harboring a Ser to Ala mutation causes FA complex disassembly and greatly inhibits fibroblast activation. AFPSRs applied with a lentiviral vector carrying the shRNA sequence of paxillin dramatically prevents PDGF-BB induced functional activation. Taken together, all these results suggest that phosphorylation of paxillin at Ser244 by CDK1 is a key mechanism in fibroblast differentiation and could eventually assist atrial fibrosis.

Key words: Atrial fibrillation, Fibrosis, Platelet derived growth factor BB, Focal adhesion

Atrial fibrillation (AF) is the most common arrhythmia and the major cause of embolic stroke worldwide. The overall morbidity of AF is 0.9%, and the population affected is projected to double over the next 2 decades. However, early recurrence (ER) of AF is common even after ablation of longstanding persistent AF. Myofibroblasts are an active form of fibroblasts and are characterized by enhanced proliferation, migration, and secretion. Currently, the activation of fibroblasts into myofibroblasts, and subsequent atrial remodeling and fibrosis have now been established as the most important bases for persistent AF. Previous studies revealed that Angiotensin II (Ang II) is the main trigger for fibroblast differentiation and atrial fibrillation. Ang II signaling sparks a cascade of cytokines such as transforming growth factor β1 (TGF-β1) and PDGF-BB, which then constantly activate fibroblasts into myofibroblasts, and finally promote the process of atrial fibrosis. Advanced studies have shown that PDGF-BB stimulates the initiation of integrin-FA signaling in fibroblasts, although the specific mechanisms remain obscure.

Integrins and integrin-related kinases in the field of human fibroblasts have been attracting the attention of many researchers during the last decade. Various extracellular stimuli, mechanical or biochemical, are received by integrins, processed by integrin-related kinases and FA complex in the vicinity of integrins, transmitted by cytoskeleton, and eventually exert numerous effects on cancer cells and fibroblasts. Integrin signaling comes into effect by targeting FA complex to a great extent. Once activated, FA complex recruitment occurs near integrins and integrin-related proteins, followed by proliferation and migration of the cells, although the exact mechanism still needs to be clarified.

CDKs play a fundamental role in mediating the cell cycle, but are not believed to be involved in regulation of any activity outside of the nuclei. However, a recent proteomic and phosphoproteomic analysis by Robertson, et al identified a large number of phosphorylation sites in FA complex proteins as potential CDK1 targets. They reported that CDK1 directly promotes FA complex formation in mouse embryonic fibroblasts (MEFs), which then subsequently may be targeted by integrin signaling to implement various functions. Moreover, the CDK1-specific inhibitor RO-3306 triggers disassembly of FA complex in human foreskin fibroblasts, although the mechanism is also almost unknown. Since CDK1 has been continuously discovered to regulate formation of FA complex in different cells, we aim to determine whether CDK1 is involved in the formation of FA complex in human atrial fi-
broblasts and it has a role in regulating the activation of fibroblasts.

**Methods**

**Antibodies and reagents:** Antibodies were diluted 1:1000 for immunoblotting and 1:100 for immunocytochemistry. Anti-LAR antibody (#73-193) was obtained from Neuro-mab (USA). Antibodies against vimentin (#AF1975) and α-smooth muscle actin (α-SMA) (#AF0318) were purchased from Beyotime (China). Anti-FSP1 (fibroblast-specific protein 1) (#ab124805), -Pyk2 PT402 (#ab4800), -Pyk2 (#ab226798), -GAPDH (#ab8245), -Collagen I (#ab34710), -Collagen III (#ab7778) antibody and recombinant human PDGF-BB protein (#ab155718) were purchased from Abcam (USA). Antibodies against CDK1 (βc-54) was purchased from Santa Cruz Biotechnology (USA). Anti-murine PDGF-BB antibody was purchased from BioVision (USA). DAPI was from Life Technologies (UK). Goat anti-rabbit-IgG HRP-conjugated antibody were purchased from LI-COR Biotechnology (USA). Antibodies against c-Abl (#73-193), FAK PT397 (#8556S), FAK (#3285), paxillin (#2542), cyclin B1 (#4135), Akt PT308 (#9275), Akt (#9272), Src PT416 (#6943S), Src (#2108S), FAK PT925 (#3284S), FAK PT937 (#8556S), FAK (#3285), were purchased from Cell Signaling Technology (USA). Antibodies against c-Abl (#73-193), FAK PT397 (#8556S), FAK (#3285), paxillin (#2542), cyclin B1 (#4135), Akt PT308 (#9275), Akt (#9272), Src PT416 (#6943S), Src (#2108S), FAK PT925 (#3284S), FAK PT937 (#8556S), FAK (#3285), were purchased from Cell Signaling Technology (USA). Antibodies against CDK1 (βc-54) was purchased from Santa Cruz Biotechnology (USA). Anti-murine PDGF-BB antibody was purchased from BioVision (USA). DAPI was from Life Technologies (UK). Goat anti-rabbit-IgG HRP-conjugated antibody and goat anti-mouse-IgG IRDye-conjugated antibody were purchased from LI-COR Biosciences (The Netherlands). Anti-human fibroblast microbeads (#130050601) was obtained from Miltenyi Biotec (Germany). RO-3306 (CDK1 inhibitor, #217699) was purchased from Merck Millipore (UK). Dynabeads protein G (#10009D) was from Thermo Fisher Scientific (USA).

**Masson, HE and sirius red staining:** The study plan was approved by the Institutional Review Board. All work followed the principles of the Declaration of Helsinki. For Masson, HE, and sirius red staining, human atrium tissues were fixed by 4% formaldehyde solution for 24-36 hours, embedded in paraffin, and sectioned serially (6-8 pieces, each with a thickness of 8 um). The staining was conducted according to previously described methods.15

**Magnetic cell sorting and cell culture:** Magnetic cell sorting was utilized to sort human atrial fibroblasts immediately after about 100 mg atrium tissues were taken from patients undergoing cardiac surgeries at the beginning of cardiopulmonary bypass. A total of 87 patients were included in the study. The characteristics of all the patients from whom atrial tissue was obtained are presented in Supplemental Tables I, II, III, IV. Consent was obtained from every patient from whom we took atrial tissue. The sorting process was conducted according to the protocol from Miltenyi Biotec. A single-cell suspension using standard methods was prepared immediately after harvesting the tissue. The cells were then filtered, counted, centrifuged, and incubated with anti-human fibroblast microbeads for 30 minutes at room temperature (19-25°C). Next, the cells were washed and separated using a MACS Separator with an MS column. The resulting cell suspensions were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotic-antimycotic solution. Since fibroblasts would gradually differentiate into myofibroblasts and the majority of fibroblasts would become myofibroblasts within 2 passages, only primary fibroblasts were used for subsequent experiments. For proliferation assay and migration assay, cells were starved for 16 hours in serum-free DMEM before growing to 80% confluence. Recombinant PDGF-BB was applied at a concentration of 20 ng/mL when necessary. Where indicated, fibroblasts were incubated with 10 μM RO-3306 (CDK1 inhibitor, Merck Millipore, Watford, UK) while the carrier DMSO was applied to control ones.

**Immunoprecipitation and immunoblotting:** Lysates of cells were prepared, separated by SDS-PAGE, transferred to nitrocellulose membranes, and then immunoblotted using previously described standards.16 Immunoblots were visualized using fluorescence detection with a Bio-Rad Infrared Imaging System. Immunoprecipitations of cyclin B1 and CDK1, CDK1, and paxillin, as well as paxillin and FAK using Dynabeads protein G (Novex, Life Technologies) were performed according to the manufacturer’s protocol.

**Immunofluorescence:** Fibroblasts were planted on acid-etched glass coverslips after being cultured overnight in serum-free DMEM. Fixed and permeabilized cells were immunostained as previously described.17 Staining was visualized by confocal laser scanning microscopy and analyzed using ImageJ.

**RNA preparation, reverse transcription PCR (RT-PCR), and q-PCR:** Trizol reagent (Invitrogen, USA) was used for the extraction of total RNA. The cDNAs were obtained from 1 μg of total RNA by reverse transcriptase. PCR amplification was carried out for 30 cycles according to standard methods.10 Q-PCR was performed using SYBR Green (Takara, Japan). Relative quantification was calculated normalized to GAPDH. The primers used for RT-PCR and q-PCR were designed and tested by Sangon Biotech (Supplemental Table V).

**RNAi:**

**siRNA** The siRNA duplexes with 21 nucleotides were created by Nippon EGT (Japan). The target sequence based on nucleotides of human CDK1 cDNA 5’-ATCTACACATGCCATGACTAAC-3’ was designed using the software RNAi Designer online (http://sidirect2.irma.jp/). The control (luciferase) siRNA for the target sequence was 5’-AA GCCATTCTATCTCTAGAG-3’, which has already been confirmed to not possess homology to any mammalian gene sequences.

**Plasmids and transfection** The region encoding human paxillin protein was amplified by RT-PCR from human atrium total RNA and inserted into the GFP-expressing mammalian vector pEGFP-C1 (Takara, Japan) based on an existing protocol.19 GFP-tagged paxillin-S106A, paxillin-S230A, and paxillin-S244A constructs were prepared by Pfu-applied PCR using a Quick Change Site-Directed Mutagenesis kit (Stratagene, USA). Plasmid DNAs were transfected into the atrial fibroblasts using Lipofectamine 3000 Reagent (Invitrogen, USA), with pEGFP-C1 (containing GFP) as the control. The transfection efficiency was satisfactory.

**Lentiviruses and transfection (intramyocardial injection)** Lentiviruses in this study were kindly provided by Experi-
Data are presented as the mean ± SD from 3 separate experiments. For all experiments, intergroup comparisons were evaluated by Student’s t-test (group n = 2) or one-way ANOVA with the post hoc Tukey’s test (group n ≥ 3) and are presented as a graph.
24 hours and of FA complex assembly after 30 minutes by PDGF-BB treatment (Figure 2A, B). We also found that PDGF-BB could activate the LAR-Akt-CDK1 pathway after 15 minutes by immunoblotting (Figure 2C). The 3 main integrin-related protein kinases remained highly phosphorylated in both PDGF-BB and DMSO treated cells (Figure 2D). These results not only showed similarities between AFPAFs and PDGF-BB-treated AFPSRs, but also pointed out the possible relationship between CDK1 and FA complex. We subsequently tested if PDGF-BB could activate AFPSRs. Eighteen hours after PDGF-BB was applied to AFPSRs, proliferation of AFPSRs could be observed (Figure 2E), and migration of AFPSRs was also enhanced at 24 hours (Figure 2F). Immunoblotting confirmed increased secretion of collagen I and III (Figure 2F). Immunoblotting confirmed increased secretion of collagen I and III (Figure 2F). Immunoblotting confirmed increased secretion of collagen I and III (Figure 2F).

Figure 1. Different phenotypes of atrial tissue or atrial fibroblasts between patients with AF and with SR. A: Cells were stained for vimentin (green), α-SMA (red), and nuclei (blue). The merged image of all three stains is also shown. More α-SMA positive cells could be observed in AFPAF than AFPSR, which suggested more myofibroblasts in AFPAF. The experiments were repeated 3 times by using fibroblasts from 6 patients. Scale bar: 20 μm. B: Activation of CDK1 pathway was identified in AFPAF. Western blot detected increased expressions of LAR, Akt, PT308, CDK1 PT161, and paxillin PT118, and decreased expression of c-Abl in AFPAF cells, whereas the expressions of Akt, CDK1, and paxillin remained the same between these two kinds of cells. The experiments were repeated 3 times by using fibroblasts from 6 patients. C: FA complex was much larger in the AFPAFs (P < 0.01). Fixed and permeabilized cells were stained using an antibody against paxillin PT118 (grey) to visualize FA complex. Cells were also stained for vimentin (green) and nuclei (blue). The experiments were repeated 3 times by using fibroblasts from 6 patients. Scale bar: 20 μm. D: Western blot analysis showed that high expressions of FAK Tyr925, Src Tyr416, and Pyk2 Tyr402 but not FAK Tyr97 existed in both the cells, which suggested integrin signaling was activated in each group. The experiments were repeated 3 times by using fibroblasts from 6 patients. AF indicates atrial fibrillation; and SR, sinus rhythm.

CDK1 activates cardiac fibroblasts: In order to explore if functional transformation of PDGF-BB-treated AFPSRs and PDGF-BB-caused changes in FA complex are related to the CDK1 pathway, we used a specific CDK1 inhibitor, RO-3306, to test this hypothesis. The expression of CDK1 PT161 and area of paxillin PT118 were both remarkably reduced after RO-3306 had been added (Figure 3A, B). This implied that CDK1 mainly affected the activity of paxillin, but not its expression. Moreover, weakened migration, decreased proliferation, and alleviated secreting function were observed after treatment of RO-3306 to PDGF-BB-treated AFPSRs (Figure 3C-E), without the survival of these cells being significantly affected (Supplemental Figure 2A). These results demonstrated that inactivation of CDK1 could not only cause disassembly of FA complex, but also inhibit activation of AFPSRs. To further confirm these results, we used small interfering RNA (siRNA) to knockdown endogenous CDK1 in AFPSRs (Supplemental Figure 2B). The expressions of CDK1, CDK1 PT161, and paxillin PT118 were significantly suppressed by CDK1 siRNA in PDGF-BB-treated AFPSRs, whereas paxillin remained unaffected (Figure 3A, B). Similar to the effect of RO-3306 observed in PDGF-BB-treated AFPSRs, knockdown of CDK1 decreased proliferation, reduced migration, and alleviated the secreting function of PDGF-BB treated AFPSRs (Figure 3C-E), with the survival of these cells remaining unaffected (Supplemental Figure 2A). A total of 22 atrial tissue donors were included in Parts 2 and 3 (Supplemental Table III). Paxillin at Ser244, a direct target of CDK1, determines assembly of FA complex and differentiation of fibroblasts: Paxillin, originally considered an adaptor protein, is crucial in protein-protein interactions (PPI) in FA complex and possesses many phosphorylation sites. Previous studies have highlighted the critical role of paxillin in FA...
protein recruitment, yet how paxillin was regulated remains unknown.26 Since paxillin is the key molecule that determines assembly and disassembly of FA complex, we explored whether any of its phosphorylation sites are determined by CDK1 and if those phosphorylation sites play critical rules in the FA complex assembly process. We first identified all potential paxillin phosphorylation sites by CDK1 using Group-Based Prediction System (GPS, version 3.0). False positives were minimized with the highest threshold selected. Three phosphorylation sites (paxillin at Ser106, Ser230 and Ser244) were identified as potential targets by CDK1, with Ser244 receiving the highest score (Figure 4A). Since all these phosphorylation sites were predicted based on a perfect sequence match in protein secondary structure, whether they could be phosphorylated in tertiary structure still needs to be clarified. In order to test whether CDK1 directly phosphorylates paxillin and which phosphorylation site it targets in AFPSRs, we performed coimmunoprecipitation assays using PDGF-BB-treated AFPSRs and DMSO-treated AFPSRs. In the past, CDK1 was believed to exert functions only inside the nucleus, while recent studies suggest they also exist in the vicinity of FA complex and may target a number of kinases.25 We identified that paxillin co-precipitated with CDK1 from the lysates of PDGF-BB-treated AFPSRs, which revealed that active CDK1 could phosphorylate paxillin (Figure 4B). Nuclear CDK1 activity requires forming a complex with cyclin B1 during the G2/M period.26 However, no difference in expression of cyclin B1 and CDK1, or association between cyclin B1 and CDK1, was observed between these two kinds of cells (Figure 4C), suggesting that the effect of CDK1 activity on PDGF-BB treated AFPSRs was not a consequence of cyclin availability. Next, to specify whether the 106th, 230th, or 244th site could be phosphorylated by activated CDK1 and if those phosphorylation sites play a role, we used plasmids to express CDK1 with or without kinase domain. We then transfected cells with these constructs and then treated with PDGF-BB (Figure 4D). We found that paxillin-S106A, -S230A, and -S244A tagged to GFP respectively with CDK1 in PDGF-BB-treated AFPSRs. A satisfactory transfection rate was warranted (Figure 4F and Supplementary Figure 2C). We identified that only paxillin-S244A mutant did not coimmunoprecipitate with CDK1, which confirmed that only paxillin at Ser244 could be phosphorylated by activated CDK1 (Figure 4D). We further investigated the effect of paxillin phosphorylation on FA complex (FAK) (a main binding protein of paxillin). Only paxillin-S244A mutant associated more efficiently with PDGF-BB-induced endogenous FAK than did the other 3 paxillin mutants (Figure 4E). This result suggests that phosphorylation at Ser244 affects the interaction of paxillin with Tyr118 with its binding partner FAK, probably leading to the formation of FA complex.

We then applied the non-phosphorylatable mutant paxillin-S106A, -S230A and -S244A tagged to GFP respectively to identify their effects on FA complex size. Only paxillin-S244A could remarkably reduce the size of FA complex (Figure 4F). We then examined the effects of paxillin-S244A on PDGF-BB-treated AFPSRs, and observed reduced proliferation, weakened migration, and al-
siRNA. The experiments with FA and TGF β1 inhibited fibroblasts by Sakata, et al.20) and in our experiment (Figure 5A and Supplemental Figure 2D). First, we transfected the lentivirus into PDGF-BB-treated AFPSRs and observed reduced proliferation, weakened migration, and alleviated secretion of collagen I and III was suppressed by RO-3306 and CDK1 siRNA. The experiments were repeated 3 times by using fibroblasts from the same 12 patients in A.

Discussion

Even though apoptosis of cardiomyocytes is related to atrial fibrillation, fibrosis is still accepted as a hallmark of atrial structural remodeling leading to persistence of AF, while the formation of myofibroblasts forebodes potential fibrosis. However, how fibroblasts are activated to differentiate into myofibroblasts is not fully understood. During the last decade, endothelial-mesenchymal transition (EndMT) has drawn the attention of scientists and researchers. Nevertheless, recent studies suggest that it only has limited effects and that most myofibroblasts derive from resident fibroblasts.

Activation of RAAS is demonstrated to be the most crucial system for fibrosis. Its downstream cytokines, TGF β1 and PDGF-BB, have been proven to possess the broadest effects on the fibrotic process. PDGF-BB is explicit to target integrin-signaling, especially FA complex, to promote the activation of fibroblasts into myofibroblasts. However, how PDGF-BB influences that process remains to be elucidated.

Integrins are dimer membrane receptors that receive stimulations from mechanical force or multiple cytokines and transmit these stimulations to integrin-related kinases. The integrin-related kinases (FAK, Src, Pyk2 and etc.) then phosphorylate FA complex, influence the cytoskeleton, initiate activation of fibroblasts, and finally promote their proliferation, migration, and secretion. Quite a number of studies have demonstrated that FA complex forma-
CDK1 targets Pax at Ser244 to determine assembly of FA complex and differentiation of fibroblasts. A: Three sites (Ser106, Ser230 and Ser244) were identified as potential CDK1 substrates by GPS. B: The immunoprecipitates of CDK1 were immunoblotted with an anti-Pax antibody to detect whether Pax could be phosphorylated by CDK1. Pax could be significantly phosphorylated by CDK1 30 minutes after PDGF-BB was applied. The total protein levels of CDK1 and Pax are also shown. The experiments were repeated 3 times by using fibroblasts from 6 patients. C: No complex was formed between CDK1 and cyclin B1 when Pax was phosphorylated by CDK1, demonstrating the function of CDK1 was not through nuclear activity. The experiments were repeated 3 times by using fibroblasts from the same 6 patients in A, D: Only Pax-S244A mutant did not co-immunoprecipitate with CDK1, confirming that only Pax at Ser244 could be phosphorylated by CDK1. The experiments were repeated 3 times by using fibroblasts from the 12 patients. E: Pax-S244A mutant inhibited its interaction with its binding partners. The experiments were repeated 3 times by using fibroblasts from the same 12 patients in D, F: Immunofluorescence showed that Pax S244A mutant inhibited PDGF-BB-induced FA complex assembly ($P < 0.01$). The experiments were repeated 3 times by using fibroblasts from the 12 patients. Scale bar: 20 μm. G: PDGF-BB-induced proliferation of AFPSRs was inhibited by Pax S244A mutant ($P < 0.01$). The experiments were repeated 3 times by using fibroblasts from the same 12 patients in F. H: Scale bar: 20 μm. H: PDGF-BB-induced migration of AFPSRs was reduced by Pax S244A ($P < 0.01$). The experiments were repeated 3 times by using fibroblasts from the same 12 patients in F. I: Western blotting confirmed PDGF-BB-induced secretion of collagen I and III was suppressed by Pax-S244A. The experiments were repeated 3 times by using fibroblasts from the same 12 patients in F. Pax indicates paxillin, and GPS; Group-Based Prediction System.

Figure 4. CDK1 targets Pax at Ser244 to determine assembly of FA complex and differentiation of fibroblasts. A: Three sites (Ser106, Ser230 and Ser244) were identified as potential CDK1 substrates by GPS. B: The immunoprecipitates of CDK1 were immunoblotted with an anti-Pax antibody to detect whether Pax could be phosphorylated by CDK1. Pax could be significantly phosphorylated by CDK1 30 minutes after PDGF-BB was applied. The total protein levels of CDK1 and Pax are also shown. The experiments were repeated 3 times by using fibroblasts from 6 patients. C: No complex was formed between CDK1 and cyclin B1 when Pax was phosphorylated by CDK1, demonstrating the function of CDK1 was not through nuclear activity. The experiments were repeated 3 times by using fibroblasts from the same 6 patients in A, D: Only Pax-S244A mutant did not co-immunoprecipitate with CDK1, confirming that only Pax at Ser244 could be phosphorylated by CDK1. The experiments were repeated 3 times by using fibroblasts from the 12 patients. E: Pax-S244A mutant inhibited its interaction with its binding partners. The experiments were repeated 3 times by using fibroblasts from the same 12 patients in D, F: Immunofluorescence showed that Pax S244A mutant inhibited PDGF-BB-induced FA complex assembly ($P < 0.01$). The experiments were repeated 3 times by using fibroblasts from the 12 patients. Scale bar: 20 μm. G: PDGF-BB-induced proliferation of AFPSRs was inhibited by Pax S244A mutant ($P < 0.01$). The experiments were repeated 3 times by using fibroblasts from the same 12 patients in F. H: Scale bar: 20 μm. H: PDGF-BB-induced migration of AFPSRs was reduced by Pax S244A ($P < 0.01$). The experiments were repeated 3 times by using fibroblasts from the same 12 patients in F. I: Western blotting confirmed PDGF-BB-induced secretion of collagen I and III was suppressed by Pax-S244A. The experiments were repeated 3 times by using fibroblasts from the same 12 patients in F. Pax indicates paxillin, and GPS; Group-Based Prediction System.

Our study does demonstrate that CDK1 causes FA complex recruitment and has an overall effect on differentiation of human atrial fibroblasts into myofibroblasts. We first verified the remarkable proportion of atrial myofibroblasts in patients with AF. Then we identified that the FA complex recruitment resided in myofibroblasts, and that the CDK1 pathway was responsible for FA complex recruitment and differentiation of fibroblasts into myofibroblasts. The result not only highlights the importance of CDK1 in the progression of AF, but also points out that FA complex is subjected to the CDK1 pathway. We further explored what phosphorylation sites CDK1 directly targets in FA complex. Since paxillin is greatly responsible for maintaining structural integrity and recruitment of FA, we tested whether any of the phosphorylation sites of paxillin could be phosphorylated by CDK1 PT161, the active form of CDK1. Paxillin, with a multi-domain structure and lack of identifiable enzyme motifs, is typically considered only as an adaptor protein. The fact that quite a number of serine/threonine
and tyrosine phosphorylation sites are found dispersed throughout the molecule suggests it may participate in multiple functions. Five leucine-rich regions termed LD motifs that function in protein recognition are within the amino terminus. The carboxy terminus that also mediates PPI is comprised of 4 lin-11, isl-1, and mec-3 (LIM) domains. Plenty of different phosphorylation sites of paxillin were found to be targeted by different protein kinases such as FAK and viculin. Currently, it has become clear that the LD domains adjust proliferation and other activities whereas LIM domains regulate paxillin targeting to FA proteins. However, all these functions are based on constant organization of paxillin to the vicinity of integrins, yet how paxillin initiates that organization remains unknown.

We consequently investigated the potential association of CDK1 and paxillin, and confirmed that activation of CDK1 causes organization of paxillin while the overall paxillin expression remains unchanged. We used Group-based Prediction System (GPS) to identify the possible phosphorylation sites of paxillin that might be CDK1-targeted. Three phosphorylation sites (Ser106, Ser230, and Ser244) were identified as possible CDK1 targets based on their protein secondary sequence. Next, we tested if they could be phosphorylated by activated CDK1 in their tertiary structure and found that CDK1 could bind to paxillin after stimulation of PDGF-BB. This phenomenon is totally independent of cyclin B, which suggested it results from direct phosphorylation of CDK1. We then synthesized non-phosphorylatable paxillin mutant-S106A, -S230A and -S244A, and found that all but S244A could bind with CDK1 and FAK (as paxillin at Tyr118 being an already known phosphorylation site of FAK), which confirmed that only Ser244 is the target of CDK1, and that inactivation of Ser244 results in disassembly of FA complex. We next found that inhibition of CDK1 and paxillin at Ser244 could both well prevent FA recruitment, which proved that CDK1 leads to FA recruitment through phosphorylation of paxillin at Ser244. Eventually, non-phosphorylatable paxillin at Ser244 could inhibit fibroblast activation, demonstrating that CDK1-paxillin at Ser244 pathway was of prime importance in atrial fibroblast activation into myofibroblasts. Moreover, knockdown of paxillin also inhibited fibroblast activation, highlighting the importance of paxillin in the whole process.

Limitations: In this original article, we demonstrated that CDK1 is responsible for FA recruitment, following which integrin-related proteins target FA to perform functions that finally activate AFPSRs, leading to atrial fibrosis. However, limitations still exist. Although MCS could immediately sort AFPSR and AFPAF cells in time and ensure survival of the cells, we have to research the cells...
immediately since in vitro cell culturing itself is a stimulus for activation of fibroblasts. Furthermore, more specific inhibitors against specific phosphorylation sites have not yet been discovered, which increases the difficulty of the research. However, we still established the idea that PDGF-BB-CDK1 signaling regulates FA complex, and integrin signaling targets FA complex to initiate activation of AFSRs.

Disclosures

Conflicts of interest: The authors declare no conflict of interest.

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Supplemental Files

Supplemental Tables I-V,
Supplemental Figures 1 and 2
Please see Supplemental Files;
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