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ORIGINAL ARTICLE

Cx43 and AKAP95 regulate G1/S conversion by competitively binding to cyclin E1/E2 in lung cancer cells

Renzhen Chen[1], Yu Chen[2], Yangyang Yuan[3], Xuan Zou[1], Qian Sun[1], Hongyan Lin[1], Xiaoyi Chen[1], Mingda Liu[1], Zifeng Deng[1], Youliang Yao[1], Dongbei Guo[1] & Yongxing Zhang[1]

1 State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiamen, China
2 School of Medicine, Xiamen University, Xiamen, China
3 Henan provincial Clinical Research Center for Perinatal Medicine, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Keywords
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Correspondence
Dongbei Guo, State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiamen, China.
Tel: +86 158 5923 0680
Fax: +86 059 2181578
Email: guodb@xmu.edu.cn

Yongxing Zhang, State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiamen, China.
Tel: +86 189 5924 4508
Fax: +86 0592 2181578
Email: z63y94x@xmu.edu.cn

Co-first authors: Renzhen Chen and Yu Chen

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Abstract

AbstractBackground: This study aimed to overexpress or silence connexin 43 (Cx43) and A-kinase anchoring protein 95 (AKAP95) in human A549 cells to explore their effects on cyclins and on G1/S conversion when the interrelationship of Cx43, AKAP95, and cyclin E1/E2 changes.

Methods: The study mainly used Western blot analysis and Co-immuno precipitation to detect the target protein in Cx43/AKAP95 over expressed human A549 cells, and the relationship of proteins Cx43, AKAP95 and Cyclin E during G1-S phase was explored with qualitative and quantitative analysis.

Results: The overexpression of Cx43 inhibited the expression of cyclin D1 and E1 by accelerating their degradation and reduced the Cdk2 activity that blocked the DNA transcription activity. However, the overexpression of AKAP95 increased the expression of cyclin D1 and E1 and inhibited their degradation, and enhanced the Cdk2 activity that promoted the DNA transcription activity. Cx43 and AKAP95 competitively bound to cyclin E1/E2, and the competitive binding affected the Cdk2 activity, Rb phosphorylation, DNA transcription activity, and G1/S conversion.

Conclusions: This study showed that the expression of ERK1/2, PKA, and PKB increased when BEAS-2B cells were treated with PDGF-BB, suggesting that ERK1/2, PKA, and PKB might be involved in the binding of AKAP95 with cyclin E, or the separation of AKAP95 from Cx43 from cyclin E1/E2. The specific mechanism underlying this process still needs further exploration.

Introduction

Previous studies have shown that connexin 43 (Cx43) and A-kinase anchoring protein 95 (AKAP95) present a regular periodic expression, and combine and separate in a whole cell cycle. The Cx43 protein can be loaded into the nucleus in the late G1 phase and G1/S conversion by combining with AKAP95.[1] Cx43 is considered to be the second class of anticancer genes.[2,3] It has an important role in the cell gap junctional intercellular communication (GJIC), regulating cell proliferation and differentiation and inhibiting malignant cancerization of cells.[4–6] AKAP95 is a member of the AKAPs family, and a typical nucleoprotein.[7] It acts as both PKA anchoring and scaffold proteins that participate in the regulation of multiple cell signaling pathways by forming different complexes with various biomacromolecules, such as gene expression regulation, DNA transcription, cell cycle regulation, etc.[8–10] Research has shown that AKAP95 participate in cell cycle regulation by binding to cyclin D and cyclin E,[11] which play vital roles in cell cycle, together with CDK2 and CDK4, which

participate in the conversion of G1/S phase,[1] and the combination can promote cell cycle progression.[12]

This study has further explored how interbinding of Cx43 and AKAP95 influenced G1 phase and G1/S conversion in A549 cells and BEAS-2B cells.

Results

Overexpression of Cx43 inhibited conversion of G1/S by degrading cyclin D1 and cyclin E1

Our previous study indicated that Cx43 and AKAP95 combined with each other in the interphase of cells.[1] AKAP95 could promote the G1/S phase conversion by combining with cyclin E1 and E2, indicating a tumor promotion effect,[12] whereas Cx43 had an antitumor-promoting effect.[2,3] To explore how the combination of Cx43 and AKAP95 affected G1/S conversion in cell cycle further, Cx43 was overexpressed and silenced in A549 cells and the expression levels of cyclin D1, cyclin E1, Cdk2, and Cdk4 were detected. The results showed that the expression levels of cyclin D1, cyclin E1, and Cdk4 declined when Cx43 was overexpressed (Fig 1a), whereas they increased when Cx43 was silenced. However, no significant change was observed in the expression of Cdk2 in both situations. The phosphorylation of cyclin D1 at threonine 286 increased when Cx43 was overexpressed in A549 cells, whereas no obvious change was observed in the degree of cyclin D1 phosphorylation when Cx43 was silenced (Fig 1bi and 1bii, columns 1–3). The phosphorylation of cyclin D1 at threonine 286 induced transfer of cyclin D1 from the nucleus to the cytoplasm, making it a target for ubiquitination, and was finally degraded by proteasome.[13–15] The overexpression of Cx43 was found to reduce the expression of cyclin D1 by increasing the phosphorylation of cyclin D1 at threonine 286 and degrading cyclin D1. No significant change in the phosphorylation of cyclin D1 at threonine 286 was observed when AKAP95 was overexpressed or silenced in A549 cells (Fig 1bi and 1bii columns 4–6), suggesting no effect of AKAP95 on the degradation of cyclin D1. In addition, the expression levels of F-box and WD repeat domain-containing 7 (FBXW7) were detected. FBXW7 is a broad antitumor gene and a key factor in skp1-cul1-f-box ubiquitin ligase E3 complex that can recognize substrates specifically and target the degradation of cyclin E, c-myc, c-jun, or other cyclins.[16,17] The results showed that the expression of FBXW7 increased when Cx43 was overexpressed (Fig 1bii, column 2), but decreased when AKAP95 was overexpressed (Fig 1bii, column 3), indicating that Cx43 degraded cyclin E1 and E2 by increasing the expression of FBXW7. Moreover, AKAP95 inhibited the degradation of cyclin E1 and E2 by inhibiting the expression of FBXW7.

The change in Cdk2 activity was detected by means of a radioisotope labeling experiment, and the results showed that the activity of Cdk2 improved when AKAP95 was overexpressed, but deteriorated when AKAP95 was silenced (Fig 1c, columns 2–3). However, the activity of Cdk2 tended to deteriorate when Cx43 was overexpressed, but improved when Cx43 was silenced in A549 cells (Fig 1c, columns 4–5). It seems that the expression of Cdk2 did not change after manipulation of Cx43, whereas Cdk2 activity fluctuated with expression of Cx43. In fact, the activity of Cdk2 was mainly aroused in the mid-late middle G1-S phase, and we concluded that Cx43 might affect cell-cycle related protein by both expression and activity, or either of these. It prompted us to detect Rb phosphorylation in the following experiments to uncover when and how Cx43 influenced G1-S conversion.

Cyclin D1-Cdk4 and cyclin E1-Cdk2 are essential to the phosphorylation of Rb in G1/S conversion.[18] The expression of cyclin D1 and Cdk4/6 occurs prior to that of cyclin E1 and Cdk2 in the G1 phase; both Cdk4/6 and Cdk2 are important to the phosphorylation of Rb,[19,20] and the phosphorylation degree of Rb directly affects the release of transcription factor E2F in HDAC-Rb-E2F.[21] Serine 795 of Rb is the preferred phosphorylation site of cyclin D1-Cdk4 in the early G1 phase. The phosphorylation of Rb at serine 780 promotes the phosphorylation state of Rb,[22] and the phosphorylation of Rb at serine 567 finally inhibits the combination of AB pocket of Rb and E2F and switch on E2F.[23] These three serine sites of Rb represent three different stages of Rb phosphorylation. Therefore, the effect of Cx43 and AKAP95 overexpression on the phosphorylation of Rb at serine 795, 780 and 567 was detected, and the results are shown in Fig 1d. Compared with the control group (Fig 1d, column 1), the phosphorylation of Rb at serine 795, 780 and 567 significantly decreased when Cx43 was overexpressed (Fig 1d, rows 1–3, column 4), but increased when Cx43 was silenced (Fig 1d, rows 1–3, column 5). The phosphorylation of Rb at serine 795, 780 and 567 increased significantly when AKAP95 was overexpressed (Fig 1d, row 1–2, column 2), whereas no obvious change was observed when AKAP95 was silenced (Fig 1d, rows 1–2, column 3). The phosphorylation of Rb at serine 567 was similar to that in the control group when AKAP95 was overexpressed. The results suggested that Cx43 inhibited the phosphorylation of Rb in the whole G1 phase, whereas AKAP95 promoted the primary phosphorylation and super-phosphorylation of Rb, but could not eventually promote the phosphorylation of Rb at serine 567 that inhibited the combination of Rb and E2F.

The effects of overexpression and silencing of AKAP95 on cyclin E1, cyclin E2, Cdk2, and Cdk4 in A549 cells have been reported in detail in a previous study[12]: The overexpression of AKAP95 promoted high expression of cyclin E1 and cyclin E2 in A549 cells, whereas silencing of AKAP95 reduced the expression of cyclin E1 and cyclin E2, but had no effect on the expression of Cdk2/4.

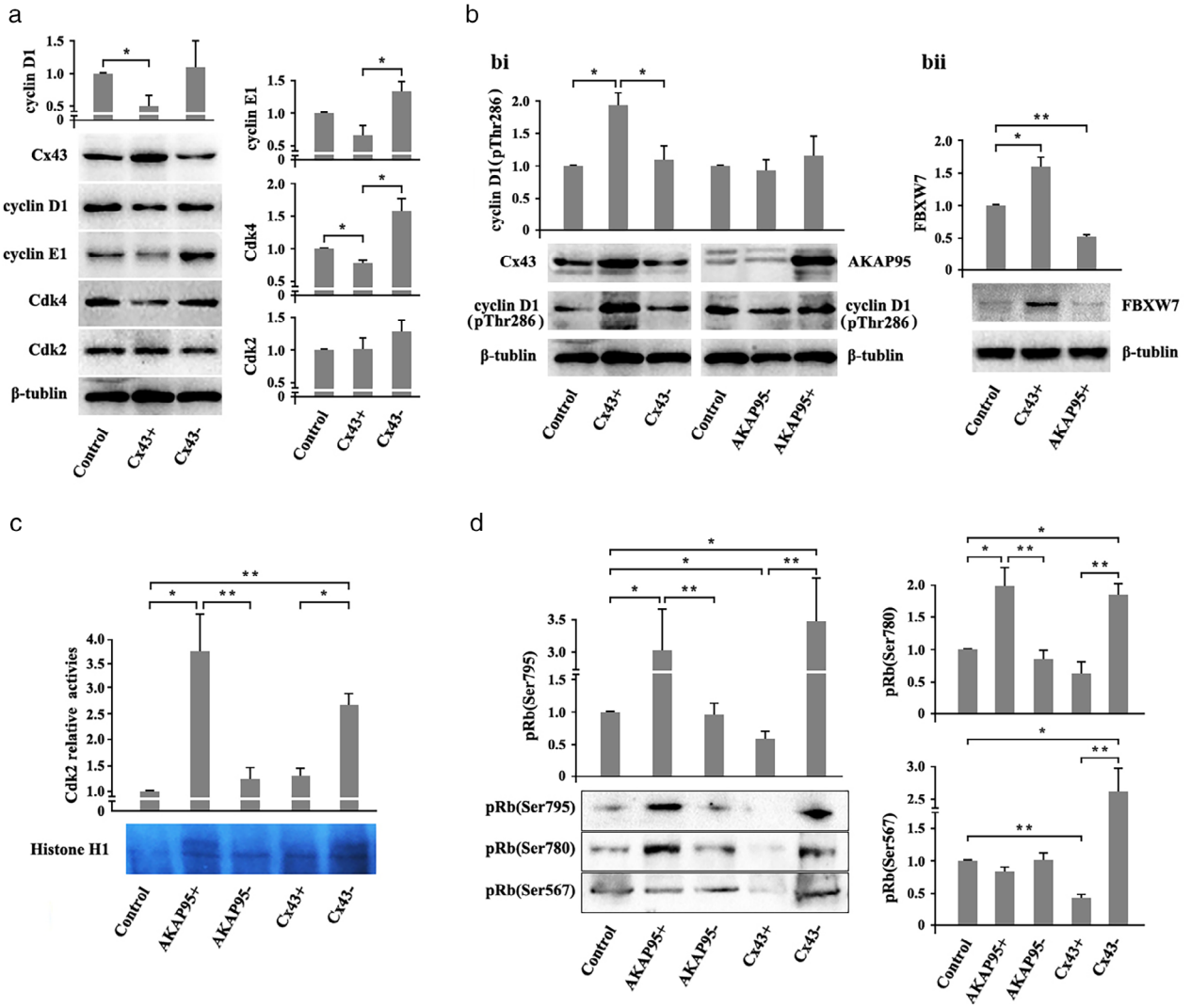

Figure 1 Effect of overexpression of Cx43 and AKAP95 on cyclins. (**a**) Cx43-overexpressed plasmid (Cx43+) and Cx43-silenced plasmid (Cx43-) were transfected in A549 cells for 24 hours. The total cell protein was extracted for western blot analysis. The control group cells were cultured under normal conditions synchronously. $*P < 0.05$; $**P < 0.01$. (**bi**) Cx43/AKAP95- overexpressed plasmids (Cx43+, AKAP95+) and Cx43/AKAP95-silenced plasmids (Cx43-, AKAP95-) were transfected in A549 cells for 24 hours. The total cell protein was extracted for western blot analysis to detect the expression of cyclin D1-T286. $*P < 0.05$; $**P < 0.01$. (**bii**) Cx43/AKAP95-overexpressed plasmids (Cx43+, AKAP95+) and Cx43/AKAP95-silenced plasmids (Cx43-, AKAP95-) were transfected in A549 cells for 24 hours. The total cell protein was extracted for western blot analysis to detect the expression of FBXW7. $*P < 0.05$; $**P < 0.01$. (**c**) Cx43/AKAP95-overexpressed plasmids (Cx43+, AKAP95+) and Cx43/AKAP95- silenced plasmids (Cx43-, AKAP95-) were transfected in A549 cells for 24 hours. The total cell protein was extracted to detect Cdk2 activity using radioassay. Each gray value of the band corresponding to histone H1 (1:500) reflected Cdk2 activity. $*P < 0.05$; $**P < 0.01$. (**d**) Cx43/AKAP95-overexpressed plasmids (Cx43+, AKAP95+) and Cx43/AKAP95-silenced plasmids (Cx43-, AKAP95-) were transfected in A549 cells for 24 hours. The total cell protein was extracted for western blot analysis. The expression of c pRb-Ser795, pRb-Ser780, and pRb-Ser567 was detected. $*P < 0.05$; $**P < 0.01$.

These results indicated that Cx43 decreased the expression levels of cyclin D and E by accelerating their degradation. It deteriorated the activity of Cdk2 and Cdk4, further inhibiting the primary phosphorylation (at serine 795 site), superphosphorylation (at serine 780 site), and final phosphorylation of Rb (at serine 567 site), and finally antimotivated G1/S conversion and DNA transcription. However, AKAP95 increased the expression levels of cyclin E1 and cyclin E2 by reducing their degradation, and improved Cdk2 activity, which increased the phosphorylation of Rb at serine 795 and 780 sites, eventually accelerating the cell cycle process. Therefore, the theory that Cx43 and AKAP95 are just like a pair of regulatory switches that influence the activity of cyclin D1-Cdk4 and cyclin E1-Cdk2 was proposed.

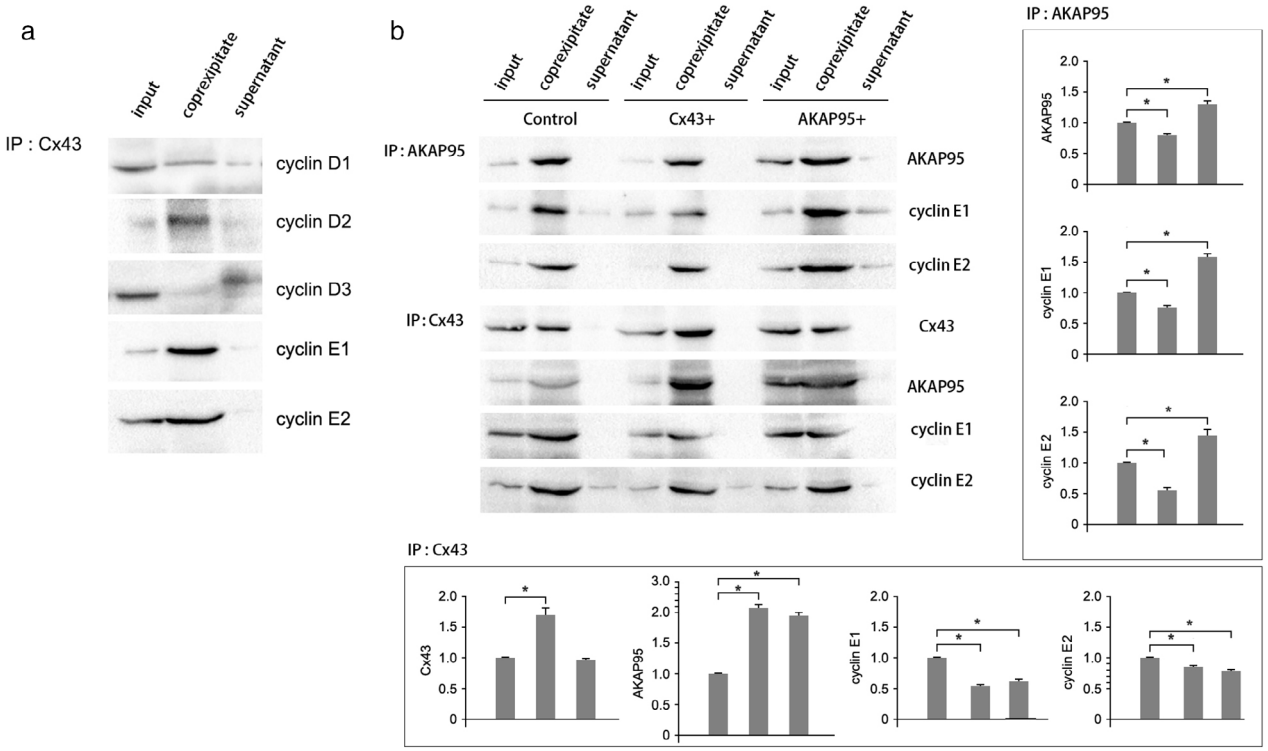

Figure 2 Effect of overexpression of Cx43 and AKAP95 on their combination with cyclin E1/E2. (**a**) Total cell protein was extracted from A549 cells. Mouse anti-Cx43 antibody was used for Co-IP. Co-IP products were detected using western blot analysis with cyclin D1 (rabbit), cyclin D2 (mouse), cyclin D3 (mouse), cyclin E1 (rabbit), and cyclin E2 (rabbit). (**b**) Total protein of 500 μg extracted from A549 cells was taken. Mouse anti-AKAP95 antibody 4 μg and mouse anti-Cx43 antibody 4 μg were used for Co-IP. An equivalent buffer for Co-IP product resuspension was taken. Rabbit antibodies AKAP95, cyclin E1, and cyclin E2 were applied to detect the Co-IP product of AKAP95 antibody (row 1–3). Rabbit antibodies Cx43, AKAP95, cyclin E1, and cyclin E2 were used to detect the Co-IP product of Cx43 antibody by western blot analysis (row 4–7). The ratio of Cx43+ group and AKAP95+ group to control group was taken as a statistical variable for comparison. The single-sample *t*-test was used to analyze the comparison of gray values between the experimental and control groups. A single-factor ANOVA method was used to compare the gray values among different groups. $*P < 0.05$.

Cx43 and AKAP95 regulated G1/S conversion by competitively binding to cyclin E1/E2

AKAP95 can bind to cyclin D1–D3 proteins during the G1 phase[24] and combine with Cx43 and cyclin E1/E2 during G1/S conversion.[1,12] Coimmunoprecipitation (Co-IP) was employed to determine the interaction of Cx43 and cyclin D1-D3, Cx43, and cyclin E1/E2. The results showed an interaction between Cx43 and cyclin D1/D2 (Fig 2a, rows 1–2, column 2) and between Cx43 and cyclin E1/E2 (Fig 2a, rows 4–5, column 2), but no interaction was found between Cx43 and cyclin D3 (Fig 2a, row 3, column 2). This suggested that Cx43 played a role from the early to the late G1 phase. The proliferation of vascular smooth muscle cells (VSMCs) depended on the combination of Cx43 C-terminal with cyclin E, and the platelet-derived growth factor-BB (PDGF-BB) promoted the combination to induced cell proliferation.[25] Cyclin D and E are two key proteins that regulate G1 and S phases in the cell cycle. Cyclin D is essential in the early and middle G1 phases[26] whereas cyclin E is essential in the middle and late G1 phases and in G1/S conversion.[27] The overexpression of Cx43 inhibited the cell cycle process by promoting or accelerating the degradation of cyclin D1 and E1/E2, whereas the overexpression of AKAP95 promoted the cell cycle process by increasing the expression of cyclin E1/E2, inhibiting the degradation of cyclin E1/E2, and promoting G1/S conversion. Therefore, how the combination of Cx43-AKAP95, Cx43-cyclin E1/E2, and AKAP95-cyclin E1/E2 changed when the overexpression of Cx43 with G1/S conversion was blocked, or the overexpression of AKAP95 with G1/S conversion was induced is discussed below.

The Co-IP results are shown in Fig 2b. Compared with the control group (Co-IP product; Fig 2b, column 2), the combination of AKAP95 and cyclin E1/E2 (Co-IP product; Fig 2b, rows 2–3, column 5) and that of Cx43 and cyclin E1/E2 (Fig 2b, rows 6–7, column 5) decreased when Cx43 was overexpressed, whereas the combination of Cx43 and

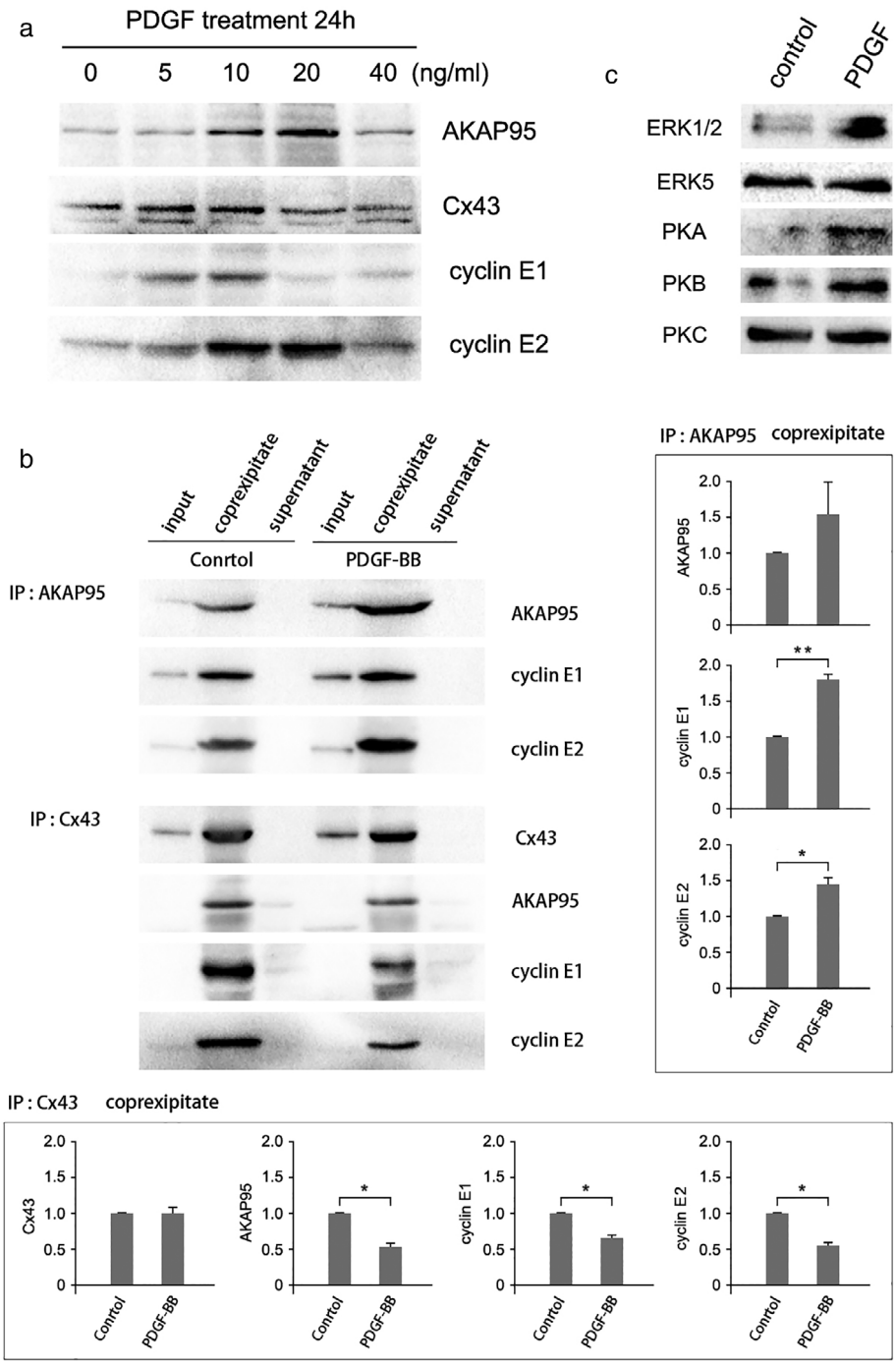

Figure 3 Effects of PDGF-BB treatment on the combination of Cx43, AKAP95, and cyclin E1/E2. (**a**) Starvation treatment with serum- and -antibiotic-free medium for 24 hours was employed when the cell density reached 50%–60%. The cells were then treated with a medium containing series concentration gradient of PDGF-BB of 5, 10, 20, and 40 ng/mL for 24 hours. The control group cells were cultured under normal conditions synchronously. The total protein was extracted to detect the expression of AKAP95, Cx43, cyclin E1, and cyclin E2 by western blot analysis. (**b**) Starvation treatment with serum- and -antibiotic-free medium for 24 hours was employed when the cell density reached 50%–60%. The cells were then treated with a medium containing 15 ng/mL PDGF-BB for 24 hours. Total protein of 500 μg extracted from the experimental and control groups was taken. The operation and process to detect Cx43, AKAP95, cyclin E1 and cyclin E2 were similar to that in Fig 2b. The ratio of PDGF-BB treatment group to control group was taken as a statistical variable for comparison. $*P < 0.05$; $**P < 0.01$. (**c**) Starvation treatment with serum- and antibiotic-free medium for 24 hours was employed when the cell density reached 50%–60%. The cells were then treated with a medium containing 15 ng/mL PDGF-BB for 24 hours. Total protein of 500 μg extracted from the experimental and control groups was taken to detect the expression of kinases with antibodies ERK1/2, ERK5, PKA, PKB and PKC using western blot analysis. $*P < 0.05$; $**P < 0.01$.

AKAP95 significantly increased (Fig 2b, row 5, column 5). However, the combination of AKAP95 and cyclin E1/E2 (Co-IP product, Fig 2b, rows 2–3, column 8) and the combination of Cx43 and AKAP95 (Fig 2b, row 5, column 8) increased when AKAP95 was overexpressed. Simultaneously, the combination of Cx43 and cyclin E1/E2 decreased (Fig 2b, rows 6–7, column 8) and the combination of cyclin E1/E2 and AKAP95 increased. The overexpression of Cx43 not only accelerated the degradation of cyclin E1/E2, but also inhibited the combination of AKAP95 and cyclin E1/E2 by binding to AKAP95 itself, leaving G1/S conversion blocked. However, the overexpression of AKAP95 inhibited the degradation of cyclin E1 and E2 (Fig 1bi) and restricted or inhibited the

combination of Cx43 and cyclin E1 and E2 by binding to both of them. Therefore, this study suggested that Cx43 and AKAP95 might affect Cdk2 activity by competitively binding to cyclin E1/E2 and control G1/S conversion in A549 cells. This view was confirmed by subsequent experiments.

PDGF-BB induces the proliferation and differentiation of various tissue cells, such as fibroblasts, endothelial cells, and smooth muscle cells.[28] In this study, PDGF-BB was used to treat BEAS-2B cells and the Co-IP products were detected to further confirm the interaction of Cx43, AKAP95, and cyclin E1/E2. The range of medication concentration was set at 0–20 ng/mL, and the expression levels of AKAP95 and cyclin E2 gradually increased when the concentration of medication was increased (Fig 3a, rows 1–4). The expression of AKAP95 and cyclin E2 decreased when the concentration reached 40 ng/mL. The expression of cyclin E1 gradually increased by 0–10 ng/mL and decreased by 20 ng/mL. No obvious change was observed in the expression of Cx43 during the whole concentration range. The BEAS-2B cells were treated with a concentration of 15 ng/mL for 24 hours, and the interrelationship among Cx43, AKAP95, and cyclin E1/E2 was determined. Compared with the control group (Fig 3b, column 2), the combination of AKAP95 and cyclin E1/E2 significantly increased (Fig 3b, rows 2–3, column 5), but the combination of AKAP95 and Cx43 decreased when the expression of AKAP95 increased (Fig 3b, row 5, column 5). Moreover, the combination of Cx43 and cyclin E1/E2 decreased significantly when the expression of cyclin E1/E2 increased (Fig 3b, rows 6–7, column 5). The results verified that Cx43 and AKAP95 controlled G1/S conversion by competitively binding to cyclin E1/E2 proteins and regulated the activity of Cdk2.

Moreover, the expression levels of kinases, such as ERK1/2, ERK5, PKA, PKB, and protein kinase C (PKC), changed with the treatment of PDGF-BB. The results showed that the expression of ERK1/2, PKA, and PKB increased (Fig 3c), suggesting their involvement in the combination or separation of AKAP95, Cx43, and cyclin E.

Discussion

A previous study has shown that Cx43 and AKAP95 present a regular combination and separation, and that they combine with each other in the interphase and separate from each other in the cell division phase (M phase) in the cell cycle.[1] In this study, the effect of the combination of Cx43 and AKAP95 on G1/S conversion in A549 and BEAS-2B cells was demonstrated, and the intercombination mediated the inhibition of G1/S conversion. In the early and middle G1 phases, Cx43 bound to cyclin D1 and D2 and decelerated the cell cycle process by promoting the degradation of cyclin D1. In the middle and late G1 phases, Cx43, AKAP95, cyclin E1, and cyclin E2 combined with each other, and Cx43 inhibited the Cdk2 activity by accelerating the degradation of cyclin E1/E2 and inhibiting the phosphorylation of Rb that eventually blocked the DNA transcription process. Cx43 inhibited or decelerated the cell cycle process in the early, middle, and late G1 phases, whereas AKAP95 had a lateral effect in the middle and late G1 phases. It inhibited the degradation of cyclin E1/E2, thereby promoting the Cdk2 activity and Rb phosphorylation, and eventually motivating the DNA transcriptional activity. Moreover, Cx43 and AKAP95 competitively combined with cyclin E1/E2, which is important in the regulation of G1/S conversion in cells.

Therefore, it was speculated that a positive regulation mechanism could be motivated when the cells were stimulated by a proliferation factor; AKAP95 and Cx43 could competitively bind to cyclin E1/E2, leaving the bonding of AKAP95 and cyclin E1/E2 increased but the bonding of AKAP95 and Cx43, and Cx43 and cyclin E1/E2 decreased, thereby blocking the inhibitory activity of Cdk2. On the contrary, the negative regulatory mechanism could be motivated and the expression level of Cx43 increased when the cells were stimulated by an antiproliferation factor, making Cx43 competitively bind to cyclin E1/E2 against AKAP95. The combination of Cx43 and AKAP95 and that of Cx43 and cyclin E1/E2 also increased, whereas the combination of AKAP95 and cyclin E1/E2 decreased and the proliferation of cells was blocked. The complex of Cx43-AKAP95 acted as a switch that regulated the activity of cyclin E1/E2-Cdk2, affecting the G1/S transition of cells by regulating the degradation of D-type cyclin and E-type cyclins (Figure 4).

Johnstone *et al.*[25] showed that the hyperplasia of VSMCs depended on the combination of Cx43 and cyclin E, and the combination could be promoted by PDGF-BB. Also, the phosphorylation of Cx43 mediated by mitogen-activated protein kinase (MAPK) could promote cell growth. However, our study showed that although PDGF-BB promoted cell proliferation by increasing the expression of AKAP95, cyclin E1, and cyclin E2, the combination of Cx43 and cyclin E1/E2 decreased whereas the combination of AKAP95 and cyclin E1/E2 increased on treatment with PDGF-BB.

As a PKA anchoring protein, AKAP95 is closely related to PKA. It has been shown that the activity of PKA could affect the expression and localization of Cx43 and enhance the GJIC function by phosphorylating Cx43 directly.[29] Moreover, MAPK and PKC also affected the phosphorylation of Cx43.[30] The MAPK family mainly includes four signal transduction pathways: ERK1/2, JNK, p38, and ERK5.[31] This study showed that the expression of ERK1/2, PKA, and PKB increased when BEAS-2B cells were treated with

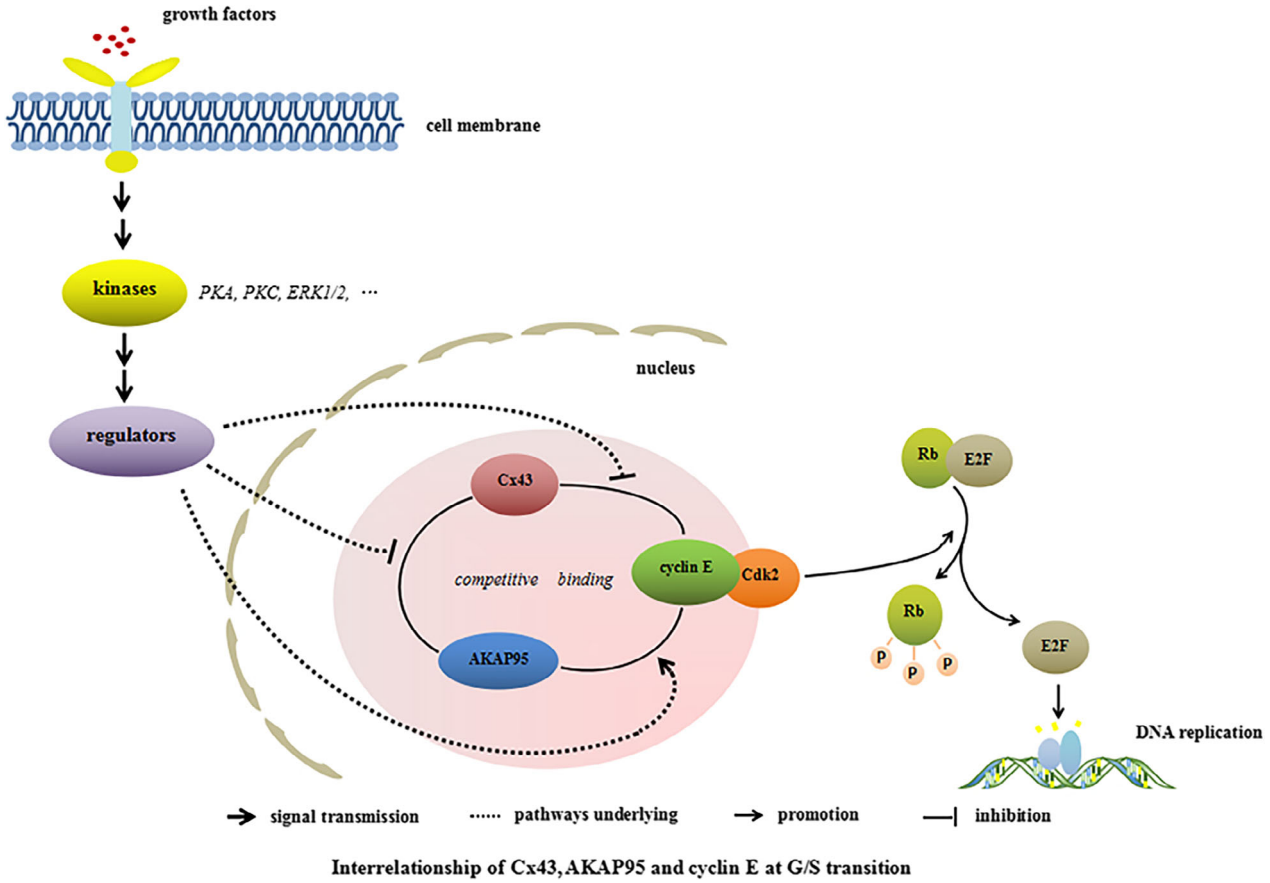

Figure 4 Illustration of Cx43 and AKAP95 competitively binds with cyclin E.

PDGF-BB, suggesting that ERK1/2, PKA, and PKB might be involved in the binding of AKAP95 with cyclin E, or the separation of AKAP95 from Cx43 from cyclin E1/E2. The specific mechanism underlying this process still needs further exploration.

Methods

Monoclonal antibodies

Mouse anti-AKAP95 (sc-100 643), anti-Cx43 (sc-13 558), anti-cyclin D2 (sc-53 637), anti-cyclin D3 (sc-6283), and anti-Cdk2 (sc-70 829) antibodies were purchased from Santa Cruz Biotechnology (TX, USA). Rabbit anti-cyclin D1 (ab134175), anti-PKB (ab8805), anti-PKC (ab32376), anti-ERK1/2 (ab17942), anti-ERK5 (ab40809), anti-AKAP95 (ab140628), anti-cyclin E1 (ab33911), and anti-cyclin E2 (ab40890) antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-cyclin D1-T286 (3300S) antibody was purchased from Cell Signaling Technology (MA, USA). Mouse anti-GAPDH (D190090-O100) was purchased from BBI Life Sciences (Shanghai, China).

Polyclonal antibodies

Rabbit anti-Cx43 (sc-9059), anti-pRb-Ser567 (sc-32 824), anti-pRb-Ser780 (sc-24 550) antibodies, and goat anti-pRb-Ser795 (sc-21 875) antibody were purchased from Santa Cruz Biotechnology. Rabbit anti-β-tubulin (ab6046) antibody was purchased from Abcam. Cdk4 (AF4034) antibody was purchased from Affinity Biosciences (OH, USA). Rabbit anti-Histone H1 (PA5-30055) antibody was purchased from Thermo Scientific (MA, USA). Rabbit anti-Cx43 (15386-1-AP) antibody was purchased from Proteintech (IL, USA); rabbit anti-FBXW7 (D222081-0025) and PKA-C-beta (D151962-0025) antibodies were purchased from BBI Life Sciences Corporation. Goat anti-rabbit lgG (H + L) (GAR007) and goat anti-mouse lgG (H + L) (GAM007) antibodies were purchased from Multisciences Biotech (Hangzhou, Zhe Jiang, China).

Reagents and plasmids

Protein A/G Plus-Agarose (sc-2003) was purchased from Santa Cruz Biotechnology. Human PDGF-BB (100-14B-2UG) was purchased from PeproTech (NJ, USA). DMEM/

high glucose (SH30243.01B) and fetal bovine serum (SH300 84.03HI) were purchased from HyClone (UT, USA). BCA Protein Assay Kit (NCI1059CH) and western blot stripping buffer (21059) were purchased from Thermo Scientific. RIPA (P0013D). Western stripper was purchased from Beijing Appligen Technologies (Beijing, China). WesternLab Peroxidase (E1050) was purchased from Beijing Lablead Biotech (Beijing, China). PMSF (ST505) and Mini Plasmid Kit (DP103-03) were purchased from Beyotime Biotech (Haimen, Jiang Su, China).

The AKAP95-overexpressed plasmid pcDNA3.1-AKAP95 (NCBI, NM_005858.3), Cx43-overexpressed plasmid pcDNA3.1 (+)-Cx43(NCBI, NM_000165.3), AKAP95-silenced plasmid pcDNATM6.2-GW/EmGFP-miR-RNAi-AKAP95 (NCBI, NM_005858.3), and Cx43-silenced plasmid pcDNATM6.2-GW/EmGFP-miR-RNAi- Cx43 (NCBI, NM_000165.3) were the four kinds of plasmids constructed and preserved in the laboratory.

Cell culture and total protein extraction

The human lung cancer cell line A549 and human bronchial epithelial cell-line BEAS-2B are stored and permanently available in our own laboratory and were cultivated under conventional conditions: DMEM medium, 10% fetal bovine serum, 5% CO_2, 90% humidity, and incubation at 37°C. The cells were immediately transfected, and total cell protein was extracted after transfection for 24 hours. The protein concentration was detected using the bicinchoninic acid (BCA) method.

Coimmunoprecipitation

The following steps were adopted to quantitatively compare the changes in the interaction between the experimental and control groups. A total cell protein sample of 500 μg was taken, and 4 μg of specific antibody added. The mixture was incubated gently in a vertical shaker at 4°C for 8 hours, which was continued for 6 hours after the addition of 40 μL of protein A/G plus-agarose beads. The mixture was then centrifuged at 3000 rpm for 5 minutes at 4°C. The supernatant was discarded, and the precipitate was washed three times with PBS. Then, 40 μL buffer was added to resuspend the Co-IP products of each group. Finally, thermal denaturation was performed for 5 minutes at 100°C for western blot analysis. The incubation concentration of rabbit antibodies cyclin D1, cyclin E1, cyclin E2, AKAP95, and Cx43 were 1:4000, and mouse antibodies cyclin D2, cyclin D3 were 1:1000.

Western blot analysis

A protein sample (50 μL) was taken, and a constant voltage of 80 V was applied for electrophoresis and 300 mA constant current for conversion. The cellulose nitrate membrane was incubated with skimmed milk powder for 1 hour, then incubated with the first antibody at 4°C for 8 hours and then the second antibody at room temperature for 1 hour. Images were taken using the Chemi DOC XRS+ Imaging System (BioRad, CA, USA), and the gray scale value of protein bands was scanned using the Image Lab5.0 (Biorad). The incubation concentration of several main antibodies were 1:6000 (cyclin D1, cyclin E1), 1:1000 (Cdk4, FBXW7), 1:2000 (Cdk2), and 1:4000 (pRb-Ser-795, pRb-Ser780, pRb-Ser567, ERK1/2, ERK5, PKA, PKB, PKC).

Radioactive isotope detection

Histone H1 was added to the Co-IP product. A 30 μL reaction system consisting of Tris-HCl (pH 7.5), $MgCl_2$, ATP, and [γ-32P]-ATP was formulated. A buffer was then added and the mixture was heated at 70°C. The supernatant was collected after centrifugation, and the SDS-PAGE electrophoresis was performed. Moisture was drained under negative pressure at 70°C, and bands were detected by autoradiography.

Statistical analysis

In statistical analysis, α means a level of test, and normally we set α no more than 0.05, which means type I error, namely the probability that we reject the original hypothesis when it is true. α is a given premise of statistical analysis while P value is the result of statistical analysis under α, we can only get P value and draw conclusions after the statistical analysis, and it's rudiments of statistics. The gray value of the bands were analyzed using SPSS 13.0 (SPSS Inc., IL, USA). The control group value was set to one. The ratio of the experimental to control group was taken as a statistical variable for comparison between groups. The single-sample *t*-test was used to compare the gray values between the experimental and control groups, and the single-factor analysis of variance method was used to compare the gray values among different groups ($\alpha < 0.05$).

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Disclosure

The authors report no conflict of interest in the study.

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