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Original Article

AGE/RAGE/Akt pathway contributes to prostate cancer cell proliferation by promoting Rb phosphorylation and degradation

Ji-Ming Bao[1,2*], Min-Yi He[3*], Ya-Wei Liu[4], Yong-Jie Lu[5], Ying-Qia Hong[6], Hai-Hua Luo[2], Zhong-Lu Ren[7], Shan-Chao Zhao[1], Yong Jiang[2]

[1]Department of Urology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China; [2]Key Laboratory of Proteomics of Guangdong Province and Key Laboratory of Transcriptomics and Proteomics of Human Diseases Supported by The Ministry of Education of China, Southern Medical University, Guangzhou 510515, China; [3]Department of Organ Transplantation, Zhujiang Hospital, Southern Medical University, Guangzhou 510515, China; [4]Department of Neurosurgery, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China; [5]Molecular Oncology, Barts Cancer Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK; [6]Department of General Surgery, Affiliated Chaozhou Central Hospital of Southern Medical University, Chaozhou 521021, China; [7]Department of Bioinformatics, School of Basic Medical Sciences, Southern Medical University, Guangzhou510515, China. [*]Equal contributors.

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Abstract: Metabolomic research has revealed that metabolites play an important role in prostate cancer development and progression. Previous studies have suggested that prostate cancer cell proliferation is induced by advanced glycation end products (AGEs) exposure, but the mechanism of this induction remains unknown. This study investigated the molecular mechanisms underlying the proliferative response of prostate cancer cell to the interaction of AGEs and the receptor for advanced glycation end products (RAGE). To investigate this mechanism, we used Western blotting to evaluate the responses of the retinoblastoma (Rb), p-Rb and PI3K/Akt pathway to AGEs stimulation. We also examined the effect of knocking down Rb and blocking the PI3K/Akt pathway on AGEs induced PC-3 cell proliferation. Our results indicated that AGE-RAGE interaction enhanced Rb phosphorylation and subsequently decreased total Rb levels. Bioinformatics analysis further indicated a negative correlation between *RAGE* and *RB1* expression in prostate cancer tissue. Furthermore, we observed that AGEs stimulation activated the PI3K/Akt signaling pathway and that blocking PI3K/Akt signaling abrogated AGEs-induced cell proliferation. We report, for the first time, that AGE-RAGE interaction enhances prostate cancer cell proliferation by phosphorylation of Rb *via* the PI3K/Akt signaling pathway.

Keywords: AGEs, RAGE, prostate cancer, proliferation, retinoblastoma, Akt

Introduction

Recent metabolomics studies have reported that human metabolites play important roles in prostate cancer development and progression. Advanced glycation end products (AGEs) are non-enzymatic protein modifications that are produced during the normal aging process [1]. The major AGEs receptor, the receptor for advanced glycation end products (RAGE, also called AGER) [2], is overexpressed in a variety of tumor types including prostate cancer [3, 4]. It has been suggested that AGE-RAGE interaction is involved in the development, growth and metastasis of a number of tumor types [5-7], including prostate cancer [3]. However, the AGE-RAGE related molecular mechanisms regulating these effects in prostate cancer cell remain unclear.

Previous studies have suggested that AGEs stimulation significantly increases the number of cells in the S phase of the cell cycle and decreases the percentage of cells in G1 phase [5]. Furthermore, it has been demonstrated that RAGE knockdown induces cell cycle arrest in the G1 phase [8, 9]. Therefore, it is likely that AGE-RAGE interaction affects cell cycle genes controlling the G1/S phase transition.

The retinoblastoma (Rb)/E2F pathway plays a key role in cell cycle progression and proliferation [10]. Rb is an important regulator of the G1

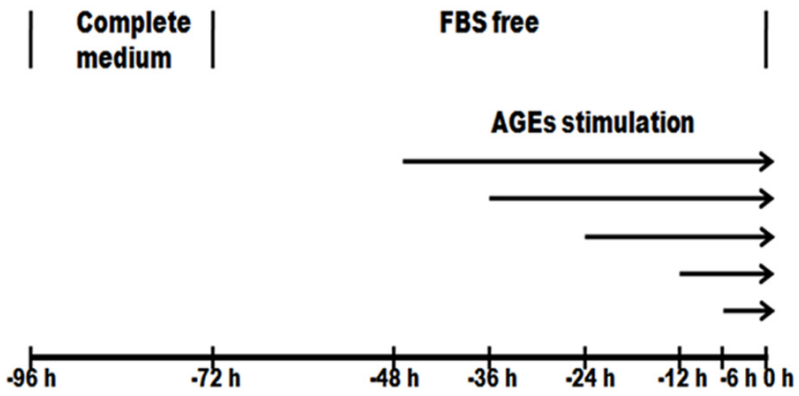

Figure 1. AGEs stimulation methods. PC-3 cells were plated in petri dishes in complete medium for 24 h, and then the medium was changed to FBS free medium for 24 h before stimulation. For concentration gradient stimulation, cells were treated with 0 μg/ml, 1 μg/ml, 10 μg/ml, 100 μg/ml, 200 μg/ml or 400 μg/ml for 48 h. For the time course treatment, cells were treated with 200 μg/ml for 48 h, 36 h, 24 h, 12 h, 6 h and 0 h.

to S-phase transition in the cell cycle [11]. Through interaction with the E2F family of cell-cycle transcription factors, Rb represses the transcription of genes required for the G1 to S-phase transition and suppresses cell proliferation [11]. Additionally, certain carcinogens promote the G1 to S-phase transition by activating the phosphatidylinositol 3'-kinase/Akt (PI3K/Akt) signaling pathway which controls cell proliferation and induces the phosphorylation of Rb [12].

In studies reporting the functional inactivation of Rb in prostate cancer, it has been suggested that *RB1* mutations [13-15] and reduced Rb expression [16, 17] facilitate prostate cancer development. Furthermore, PTEN/PI3K/Akt alterations have been frequently reported in prostate cancer, including the loss of PTEN [18-20] and the aberrant activation of the PI3K/Akt signaling pathway [21, 22].

We hypothesized that AGEs enhance prostate cancer cell proliferation by regulating Rb function and the Akt pathway. Therefore, in the present study, we explored the mechanisms of AGE/RAGE regulation of Rb and the effects of this regulation on prostate cancer cell proliferation.

Materials and methods

Cell culture and treatment

PC-3 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and phenol red (GIBCO, Life Technologies, Grand Island, NY, USA), containing L-glutamine (2 mM) (Invitrogen, Life Technologies) and penicillin sodium (100 U/ml)/streptomycin sulfate (100 mg/ml) (Invitrogen) in a humidified incubator at 37°C with 5% CO_2.

PC-3 cells were cultured in 60-mm dishes or 96 well plates for 24 h. After 24 h, the medium was changed to FBS-free MEM, and then cells were incubated for another 24 h before stimulation with AGEs (BioVision, San Francisco, CA, USA). Cells were treated for 48 h with a concentration gradient of AGEs (0 μg/ml, 1 μg/ml, 10 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml); or with 200 μg/ml for various durations (48 h, 36 h, 24 h, 12 h, 6 h and 0 h; **Figure 1**). Total RNA or cell lysates were extracted and analyzed in each experiment.

CCK-8 cell proliferation assay

PC-3 proliferation was evaluated using the CCK-8 assay according to the manufacturer's instructions (Dojindo, Kumamoto, Japan). Cells were cultured in FBS medium as described above at 3×10^3 cells per well (n = 5) in 96-multiwell plates, and then 10 μl CCK-8 (5 mg/ml) was added to each well. After 4 h incubation at 37°C, the optical density (OD) of each well was measured using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm. Each experiment was repeated three times.

Bioinformatics analysis of RAGE and RB1 expression in prostate cancer

RAGE and *RB1* expression in prostate cancer was analyzed by bioinformatics. All data were downloaded from The Cancer Genome Atlas (TCGA) database (http://cancergenome.nih.gov/), and all data used were TCGA data level 3 (Segmented or Interpreted Data). Three-hundred and eighty-three of the prostate cancer samples in the data set were prostate adenocarcinoma (PRAD). All gene quantification was done by RNA-seq on an Illumina HiSeq_RNASeqV2 platform (Illumina, San Diego, CA, USA) and RSEM normalization (http://dewey-

Table 1. Primers and siRNA Sequences

Name	Sequence	Usage
β-actin	5'-TGGCACCACACCTTCTACAATG-3' (forward)	For qRT-PCR
	5'-TCTCAAACATGATCTGGGTCATCT-3' (reverse)	
RAGE	5'-AAACATCACAGCCCGGATTG-3' (forward)	For qRT-PCR
	5'-TCCGGCCTGTGTTCAGTTTCT-3' (reverse)	
RB	5'-GCAGTATGCTTCCACCAGGC-3' (forward)	For qRT-PCR
	5'-AAGGGCTTCGAGGAATGTGAG-3' (reverse)	
siRAGE	5'-CCACCUUCUCCUGUAGCUUTT-3'	For RNAi
	5'-AAGCUACAGGAGAAGGUGGTT-3'	
siRB	5'-GGCCACAUAUAGCAGAAGUTT-3'	For RNAi
	5'-ACUUCUGCUAUAUGUGGCCTT-3'	
siNC	5'-UUCUCCGAACGUGUCACGUTT-3'	For RNAi
	5'-ACGUGACACGUUCGGAGAATT-3'	

lab.biostat.wisc.edu/rsem). Normalized readings represented the gene expression level.

Data analysis was done in the R language environment. The correlation between *RB1* and *RAGE* was obtained by Pearson correlation and the significance of correlation result was confirmed with a correlation test. Finally, data was visualized using a scatter plot in which the horizontal axis represents *RB1* quantification, the vertical axis represents *RAGE* quantification, and a blue line represents the linear regression line.

RAGE and RB1 knockdown using RNA interference (RNAi)

The target small interfering RNA (siRNA) for *RAGE* (siRAGE), *RB1* (siRB) and negative-control siRNA (siNC) were purchased from GenePharma (Shanghai, China). siNC consisted of an irrelevant sequence. **Table 1** lists the siRNA sequences used. Exponentially growing cells were plated in 6 cm or 96-well plates at 30 to 50% confluence, and then incubated for 24 h. After incubation, cells were transfected with small RNAs in serum free medium OPTI-MEM-I (Invitrogen) according to the manufacturer's protocol. Gene knockdown efficacy was evaluated using Western blot and Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis.

qRT-PCR analysis for gene expression

RAGE, *RB1* and β-actin primers were purchased from Invitrogen. Total cell RNA was extracted using Trizol (Invitrogen) following the manufacturer's instructions. Mature mRNA quantification was performed using the Quantitect SYBR Green PCR Kit (Stratagene, La Jolla, CA, USA) and the MX3005P multiplex quantitative PCR system (Stratagene) according to the manufacturers' recommendations. β-actin mRNA was chosen as a housekeeping gene. Relative mRNA expression was calculated using the comparative CT (ΔΔCT) method as previously described [23, 24]. Fold-changes were calculated by the equation $2^{-\Delta\Delta Ct}$. All primers used are listed in **Table 1**.

Protein extraction and Western blotting

Protein expression levels were assessed by Western blotting analysis. In brief, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 1 × PBS, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate and protease inhibitors. Protein concentration was quantified and an equal amount of protein was loaded in each well of an SDS-PAGE gel. Next, select targets were detected using the following antibodies: RAGE, primary anti-RAGE antibody (CST 4679, 1:2000; Cell Signaling Technology (CST), Danvers, MA, USA); Rb, anti-Rb antibody (CST 9309, 1:2000; CST); phospho(p)-Rb (Ser807/811), anti-p-Rb antibody (CST 8516, 1:1000; CST); Akt, primary anti-Akt antibody (CST 4691, 1:1000; CST); phospho(p)-Akt (Ser473), primary anti-p-Akt (Ser473) antibody (CST 9309, 1:2000; CST); phospho(p)-Akt (Thr308), primary anti-p-Akt (Thr308) antibody (CST 4060, 1:1000; CST); β-actin, Rabbit anti-β-actin (1:500; Santa Cruz, CA) antibody. β-actin served as an internal control.

Ly294002 blockade of PI3K/Akt signaling pathway

Ly294002 (CST 9901, CST), a highly selective PI3K inhibitor, was used to block PI3K-dependent Akt phosphorylation and, thus, the PI3K/Akt signaling pathway. Prostate cancer cells were treated with 20 nmol/ml Ly294002 for 24 h, then p-Akt (Ser473), p-Akt (Thr308) and total Akt were measured using Western blotting to confirm blockage of the PI3K/Akt signaling pathway.

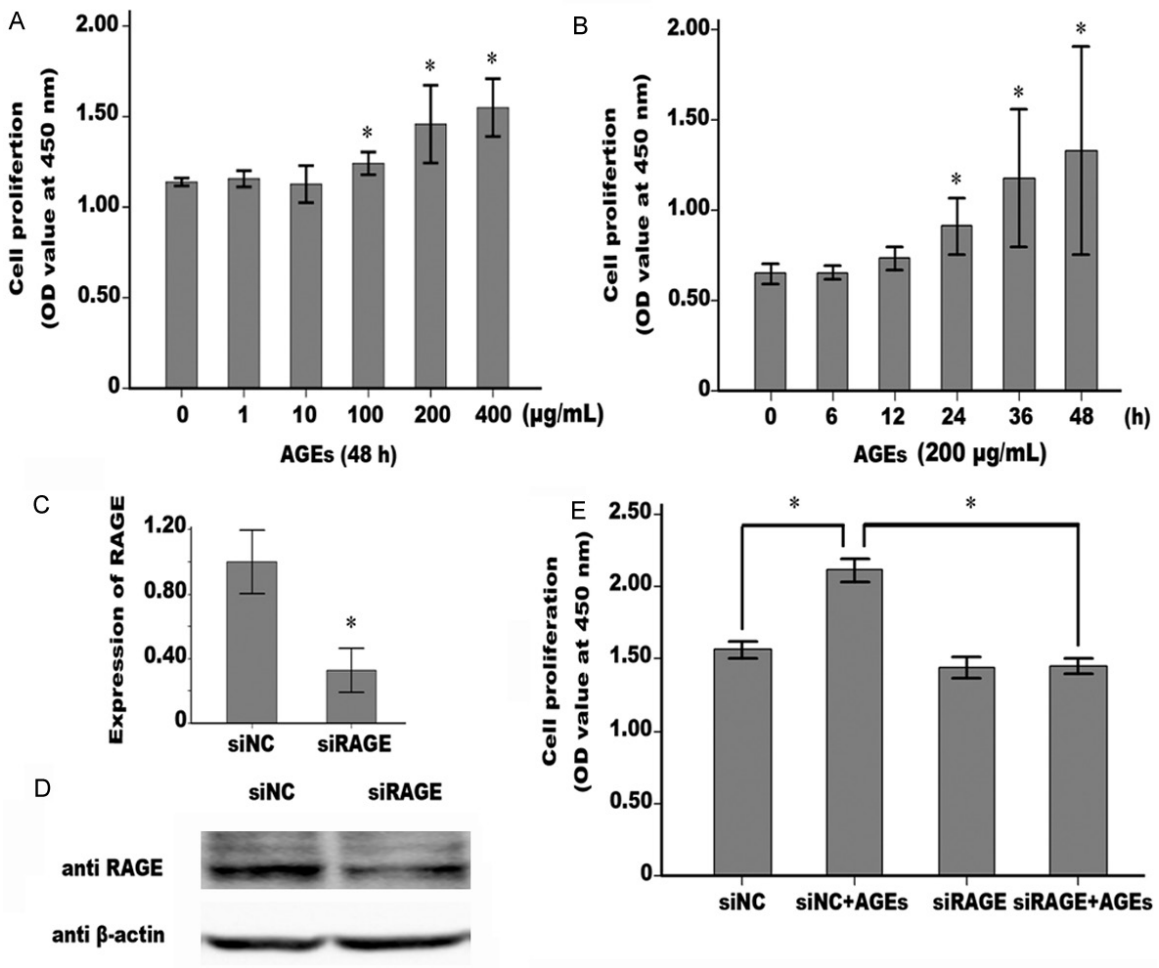

Figure 2. The effects of AGE-RAGE interaction on PC-3 cell proliferation. (A) PC-3 cells were stimulated with AGEs in a gradient concentration for 48 h, and then cell proliferation was evaluated by the CCK-8. AGEs (≥ 100 μg/ml) stimulation promoted cell proliferation when compared with the no treatment (0 μg/ml) group (*$p < 0.05$). (B) PC-3 cells were treated with a time course of 200 μg/ml AGEs, and then cell proliferation was evaluated by the CCK-8. More than twenty-four h 200 μg/ml AGEs stimulation promoted cell proliferation when compared with the no treatment (0 h) group (*$p < 0.05$). (C) RAGE expression was detected by qRT-PCR analysis after the cells were transfected with siRNA. siRAGE significantly knocked the expression of RAGE down when compared with siNC (*$p < 0.01$). (D) RAGE expression was detected by Western blot after the cells were transfected with siRNA. siRAGE significantly knocked the expression of RAGE down when compared with siNC. (E) PC-3 cells were treated with or without AGEs after siRAGE or siNC transfection, and then cell proliferation was determined by CCK-8 assay. The OD value of the siNC + AGEs group was higher than the siNC group (*$p < 0.01$). The OD value of the siRAGE + AGEs group was lower than the siNC + AGEs group (*$p < 0.01$).

Statistical analysis

All data are presented as means ± SD. When the variance was homogeneous, one-way ANOVA was applied to analyze the differences between groups and least significant difference (LSD) tests were used to compare the means of two groups. When the variance was heterogeneous, Welch's t test was applied to analyze the differences between each groups and Dunnett's T3 test was used to compare the means of two groups. $p < 0.05$ was considered as statistically significant (two-tailed).

Results

AGEs induce prostate cancer cell proliferation via AGE-RAGE interaction

PC-3 cells were stimulated for 48 h with AGEs in a gradient concentration. Cell viability improved significantly with increasing AGEs concentrations (**Figure 2A**). When the cells were stimulated with increasing durations of 200 μg/ml AGEs, cell proliferation increased along with the increasing duration of AGEs exposure (**Figure 2B**).

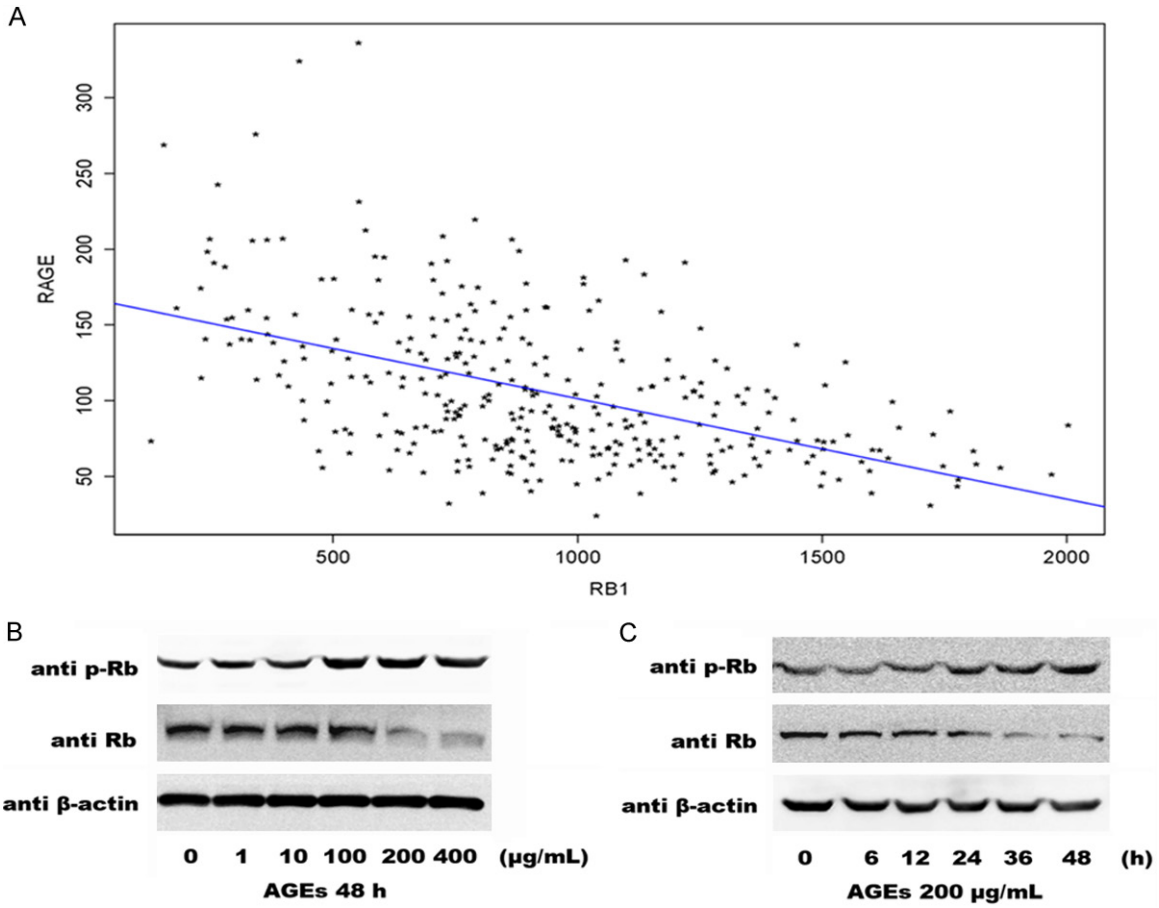

Figure 3. The correlation between RAGE and Rb. (A) *RAGE* and *RB1* expression in prostate cancer tissues. Data from 383 prostate cancer samples from the TCGA data portal were included in our bioinformatics analysis. The horizontal axis represents the quantification of the *RB1* gene and the vertical axis represents the quantification of the *RAGE* gene (RPKM value). Each black star represents one prostate cancer sample. The blue line is a linear regression line. The correlation between the two genes in these cancer samples is -0.50 (p < 2.2e-16). (B) The effect of AGEs gradient stimulation on Rb and p-Rb. After 48 h AGEs gradient treatment, Rb and p-Rb were detected using Western blot. (C) The effect of increasing AGEs stimulation duration on Rb and p-Rb. Cells were subjected to increasing durations of AGEs stimulation, and then Western blotting was used to detect Rb and p-Rb.

To investigate whether AGEs exposure required RAGE to enhance PC-3 proliferation, an RNAi knockdown of RAGE expression was performed. After RAGE siRNA transfection, Western blotting and qRT-PCR were used to evaluate gene silencing efficiency. After examining both RAGE mRNA and protein levels, we observed that the RAGE siRNA knockdown was successful (**Figure 2C** and **2D**, p < 0.01). Next, PC-3 cells, with or without RAGE knockdown, were stimulated with 200 μg/ml AGEs for 48 h. CCK-8 assays demonstrated that RAGE siRNA pretreatment abrogated AGE-induced proliferation (**Figure 2E**, p < 0.01). These results suggested that the proliferation-promoting activity of AGEs was mediated by AGE-RAGE interaction.

RAGE and RB1 gene expression in prostate cancer samples

Data from 383 prostate cancer tissue samples were collected for bioinformatics analysis. Correlation tests indicated a highly significant negative correlation between *RAGE* and *RB1* expression (p < 2.2e^{-16}). A similar correlation was also seen between *RAGE* and *RB1* in prostate cancer samples by linear regression analysis (p < 2e^{-16}; **Figure 3A**).

Continued AGEs stimulation promoted phosphorylation and decreased levels of Rb

To confirm the correlation between AGE-RAGE and Rb, we detected total and phosphorylated

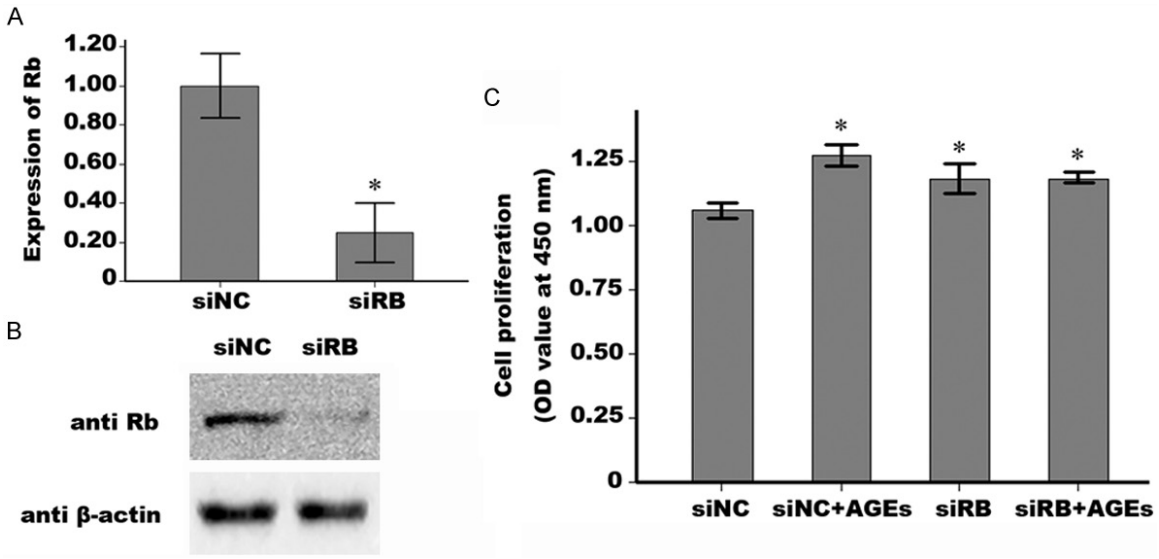

Figure 4. The effect of Rb expression on AGEs induced PC-3 cell proliferation. (A) Rb expression was detected in PC-3 cells with qRT-PCR after siRNA transfection. siRB significantly reduced Rb expression when compared with siNC (*p < 0.05). (B) Rb expression was detected in PC-3 cells with Western blotting after siRNA transfection. siRB significantly reduced Rb expression when compared with siNC. (C) PC-3 cells were treated with or without AGEs after siRB or siNC transfection, and then cell proliferation was evaluated by CCK-8 assay. The OD value of the siNC + AGEs, siRB and siRB + AGEs groups were higher than the siNC group (*p < 0.05).

Rb levels after AGEs stimulation by Western blot. The results showed increased p-Rb, but decreased total Rb levels. This response occurred in a time and AGEs concentration dependent manner (**Figure 3B** and **3C**).

AGE-RAGE interaction enhanced prostate cancer cell proliferation by regulating Rb

We next confirmed that AGE-RAGE regulation of Rb promoted prostate cancer cell proliferation. Rb expression was inhibited using RNAi, and then cell proliferation was measured using the CCK-8 cell viability assay. Western blotting and qRT-PCR analysis indicated that Rb mRNA and protein levels were both significantly decreased by Rb RNAi treatment (**Figure 4A** and **4B**). CCK-8 assays demonstrated that the PC-3 cell proliferation rates of the siNC + AGEs, siRB and siRB + AGEs treated cells were significantly higher than the proliferation rate of siNC treated cells (p < 0.05). However, no significant proliferation differences were observed between siNC + AGEs, siRB or siRB + AGEs treated PC-3 cells (p > 0.05; **Figure 4C**). Both AGEs stimulation and Rb silencing induced PC-3 cell proliferation, but cell proliferation was not further elevated by AGE-induced stimulation after Rb silencing. Thus, these results indicated that AGE-RAGE induces cancer cell proliferation by regulating Rb.

PI3K/Akt signaling pathway activation was required for AGEs induced PC-3 proliferation

To analyze the role of the Akt pathway in AGEs/Rb mediated PC-3 cell proliferation, we examined Akt phosphorylation status. The results showed increased Akt (Ser473) phosphorylation in response to AGEs treatment in a time and concentration dependent manner. However, the phosphorylation of Akt (Thr308) showed no significant response to treatment (**Figure 5A** and **5B**). These results indicated that the PI3K/Akt signaling pathway was activated by AGEs treatment.

To confirm PI3K/Akt signaling pathway involvement in AGEs/Rb mediated PC-3 cell proliferation, Ly294002 was used to block Akt phosphorylation and the PI3K/Akt signaling pathway. Twenty-four h after Ly294002 treatment, p-Akt (Ser473), p-Akt (Thr308) and total Akt were detected using Western blotting. Both p-Akt (Ser473) and p-Akt (Thr308) decreased dramatically, indicating the successful blockade of the PI3K/Akt signaling pathway (**Figure 5C**). A subsequent CCK-8 assay showed that 48 h of AGEs stimulation could not promote PC-3 cell proliferation when the PI3K/Akt signaling pathway was blocked by Ly294002 pretreatment (**Figure 5D**).

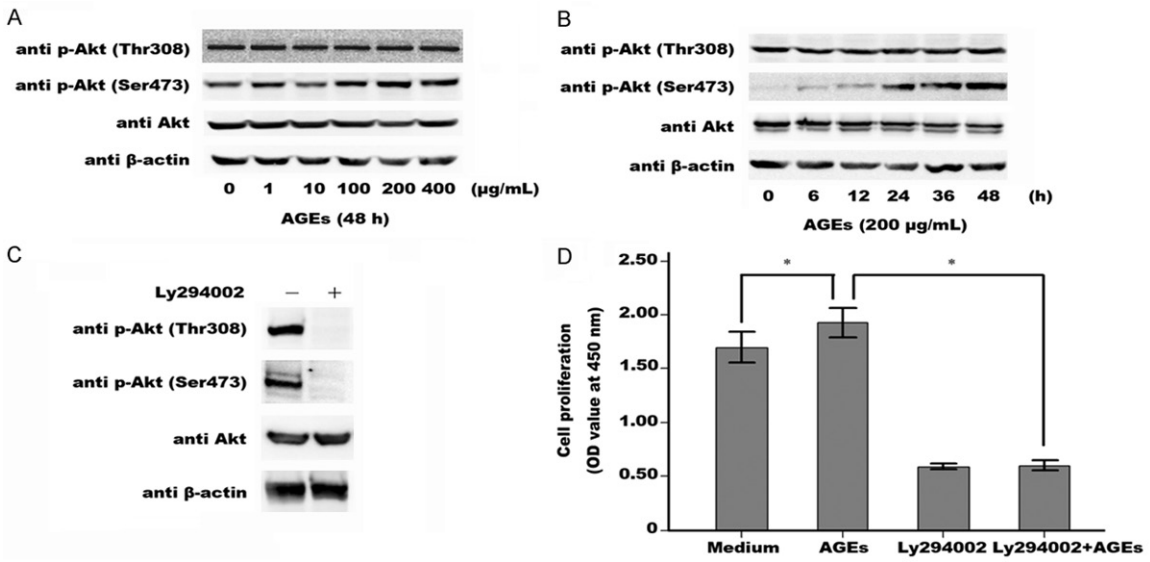

Figure 5. The effect of the PI3K/Akt signaling pathway on AGEs induced PC-3 proliferation. (A) The phosphorylation status of Akt after stimulation with an AGEs concentration gradient. PC-3 cells were treated with an AGEs concentration gradient for 48 h, p-Akt (Ser473), p-Akt (Thr308) and total Akt were detected by Western blot. (B) The phosphorylation status of Akt after increasing durations of AGEs stimulation. PC-3 cells were treat with 200 μg/ml AGEs for increasing periods of time, and then Western blotting was used to detect p-Akt (Ser473), p-Akt (Thr308) and total Akt. (C) Ly294002 blocked p-Akt (Ser473) and p-Akt (Thr308) phosphorylation. Cells were treated with Ly294002 for 24 h, and then p-Akt (Ser473), p-Akt (Thr308) and total Akt were detected with Western blotting. (D) The proliferation of PC-3 cells was significantly inhibited after Ly294002 pretreatment to block the PI3K/Akt pathway. Cells were treated with or without AGEs after Ly294002 pretreatment or without Ly294002 pretreatment, and then cell proliferation was evaluated by CCK-8 assay. The OD value of the AGEs treatment group was higher than the no AGEs control group (*$p < 0.05$). The OD value of the AGEs treatment (without Ly294002) group was higher than the Ly294002 pretreatment + AGEs treatment group (*$p < 0.01$).

Discussion

Epidemiologic studies have suggested that diabetes is associated with an increased risk of prostate cancer recurrence [25, 26] and cancer-related mortality [27, 28]. The production and accumulation of AGEs are accelerated in diabetes [29], indicating the possibility of a link between diabetes, increased prostate cancer risk and AGEs accumulation. In our earlier work, we observed that RAGE expression was associated with prostate cancer progression and poor patient outcome [30]. The study we report here confirmed that AGEs treatment stimulated prostate cancer cell proliferation in a dose- and time-dependent manner. Furthermore, this observation is supported by other studies [3, 5, 31]. However, the mechanism remained unknown. Here, we suggest a role for the Akt/Rb pathway in AGEs induction of prostate cancer cell proliferation.

We demonstrated that AGE-RAGE interaction promoted prostate cancer cell proliferation by inducing the phosphorylation and subsequent degradation of Rb. This result established, for the first time, an association between AGE-RAGE and Rb. During prostate cancer progression, tumor cells undergo a variety of molecular alterations that lead to the acquisition of uncontrolled growth properties. Rb is a key cell cycle inhibitor and tumor suppressor. The unphosphorylated, active form of Rb interacts with E2F1 and represses its transcription activity, leading to cell cycle arrest [32]. Hyperphosphorylation of RB by the cyclin-dependent kinases 4 and 6 (CDK4/6) leads to the disassociation of the Rb-E2F complex and proteasome degradation of Rb via a ubiquitin-dependent pathway [32]. The release of E2F from the Rb-E2F complex triggers the activation of a number of genes required for G1/S transition and tumorigenesis [33]. Therefore, it is highly possible that AGE-RAGE interaction in prostate cancer cell induces Rb phosphorylation, the resultant dissociation of the Rb-E2F complex, the subsequent degradation of Rb through the ubiquitin-proteasome complex and, finally, the

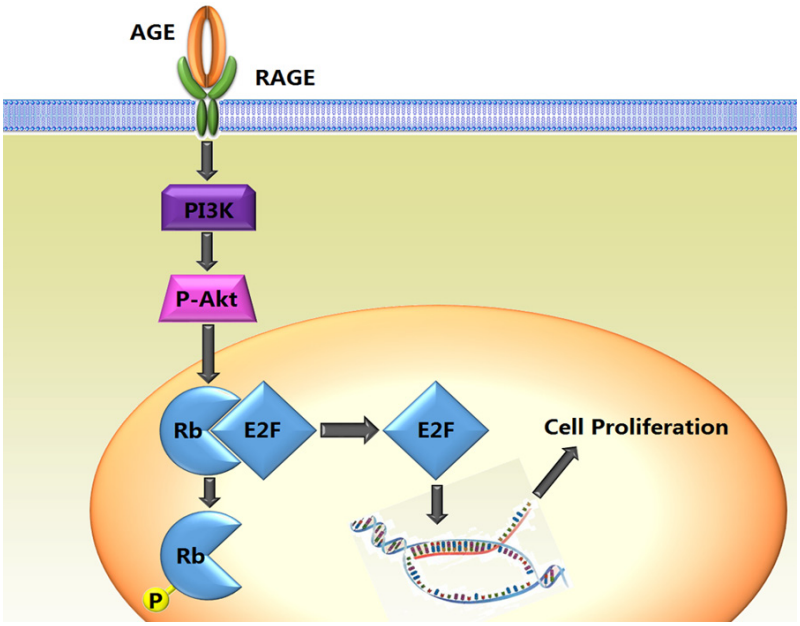

Figure 6. AGE-RAGE interaction activates the PI3K/Akt pathway and enhances Rb phosphorylation and degradation. E2F is released from the E2F/Rb complex and promotes prostate cancer cell proliferation.

promotion of cancer cell proliferation by allowing cell cycle progression from the G1 to the S-phase. Additionally, bioinformatics analysis of a large number of prostate cancer samples from the TCGA database also indicated a significant negative correlation between the expression quantification of *RAGE* and *RB1* genes.

To investigate the mechanisms underlying AGE-RAGE regulation of Rb, we analyzed related signaling pathways. Many previous studies in various cell types have explored the signaling pathways mediated by RAGE, including the MAPK [34], JAK/STAT [35] and NF-κB [36] pathways; however, the involvement of RAGE related signaling pathways has not been frequently reported in prostate cancer. Here, we observed that AGE-RAGE interaction activated the PI3K/Akt signaling pathway by increasing Akt (Ser473) phosphorylation. This observation is consistent with previous studies reported that activation of the PI3K/Akt pathway could be mediated by the interactions between RAGE and its ligands [5, 37-39]. The PI3K/Akt signaling pathway has been shown to be essential for the survival of a number of cell types and some forms of human cancer [40, 41]. Furthermore, evidence indicates that PI3K/Akt signaling may be critical for prostate cancer cell survival and proliferation [22, 42, 43]. The PI3K/Akt pathway is commonly activated in prostate cancer through Akt mutations [44, 45] or PTEN inactivation [42, 46, 47], and phosphorylation of Akt (Ser473) has been considered an excellent predictor of poor clinical outcome in prostate cancer patients [48]. Here, we demonstrated that ability of AGEs stimulation to promote cell proliferation was lost when the PI3K/Akt pathway was blocked. This suggested that the PI3K/Akt signaling pathway might play a critical role in AGE-RAGE induced cell proliferation. Previous studies have shown that Rb was a target protein of PI3K/Akt signaling pathway [12, 49], and it is possible that AGE-RAGE interaction regulates Rb through the PI3K/Akt signaling pathway. This evidence suggests that Rb degradation by the PI3K/Akt/Rb signaling pathway may play a key role in AGEs induced prostate cancer development.

Conclusion

This study indicates that the interaction of AGEs with RAGE enhances prostate cancer cell proliferation by inducing Rb phosphorylation *via* the PI3K/Akt signaling pathway (**Figure 6**). Therefore, inhibiting the formation of AGEs or RAGE downstream signaling is a promising and novel therapeutic strategy for the prevention and treatment of prostate cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Shan-Chao Zhao, Department of Urology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China. E-mail: zhaoshanchaosmu@sina.cn; Yong Jiang, Key Laboratory of Proteomics of Guangdong Province and Key Laboratory of Transcriptomics and Proteomics of Human Diseases Supported by The Ministry of Education of China, Southern Medical University, Guangzhou 510515, China. E-mail: jiang48231@163.com

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