Biochemical and Biophysical Research Communications 458 (2015) 501–508

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

PRR11 regulates late-S to G2/M phase progression and induces premature chromatin condensation (PCC)

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ARTICLE INFO

Article history:
Received 24 January 2015
Available online 7 February 2015

Keywords:
PRR11
Cell cycle
Late-S phase
G2/M progression
Premature chromatin condensation (PCC)

ABSTRACT

Recently, we have demonstrated that proline-rich protein 11 (PRR11) is a novel tumor-related gene product likely implicated in the regulation of cell cycle progression as well as lung cancer development. However, its precise role in cell cycle progression remains unclear. In the present study, we have further investigated the expression pattern and functional implication of PRR11 during cell cycle in detail in human lung carcinoma-derived H1299 cells. According to our immunofluorescence study, PRR11 was expressed largely in cytoplasm, the amount of PRR11 started to increase in the late S phase, and was retained until just before mitotic telophase. Consistent with those observations, siRNA-mediated knockdown of PRR11 caused a significant cell cycle arrest in the late S phase. Intriguingly, the treatment with dNTPs further augmented PRR11 silencing-mediated S phase arrest. Moreover, knockdown of PRR11 also resulted in a remarkable retardation of G2/M progression, and PRR11-knockdown cells subsequently underwent G2 phase cell cycle arrest accompanied by obvious mitotic defects such as multipolar spindles and multiple nuclei. In addition, forced expression of PRR11 promoted the premature Chromatin condensation (PCC), and then proliferation of PRR11-expressing cells was massively attenuated and induced apoptosis. Taken together, our current observations strongly suggest that PRR11, which is strictly regulated during cell cycle progression, plays a pivotal role in the regulation of accurate cell cycle progression through the late S phase to mitosis.

1. Introduction

Cell cycle progression requires a series of highly coordinated molecular events which ultimately lead to faithful segregation of chromosomes [1–3]. Accumulating evidence strongly suggests that the vast majority of human cancers arise from serious defects in accurate cell cycle regulation, which leads to uncontrolled cell proliferation [3–5]. Numerous of cell cycle regulators such as cyclins, cyclin-dependent protein kinases (CDKs) and cyclin-dependent protein kinase inhibitors (CKIs) have been shown to be deregulated in a variety of cancers, and their aberrant dysregulation has been considered to be tightly linked to the initiation and/or progression of cancers [6–9].

Recently, we have identified a novel gene product, proline-rich protein 11 (PRR11) implicated in the regulation of both cell cycle progression and lung cancer development [10]. Based on our observations, PRR11 appeared to be highly expressed during the cell cycle from G2 to M phases, indicating that the expression of PRR11 is regulated in a cell cycle-dependent manner. Consistent with these observations, siRNA-mediated silencing of PRR11 caused a remarkable growth retardation in both HeLa and lung cancer cells, which might be due to a cell cycle arrest at S phase. Importantly, the expression level of PRR11 in primary lung cancer tissues was higher as compared with that in their corresponding normal ones, and its higher expression level was significantly associated with poor prognosis of patients with lung cancers. In a good agreement with these observations, knockdown of PRR11 in lung cancer cells resulted in an obvious inhibition of cellular proliferation, cell cycle

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http://dx.doi.org/10.1016/j.bbrc.2015.01.139
0006-291X/

progression, cell migration, invasion, colony formation *in vitro*, and reduced tumor growth *in vivo*. These findings strongly suggest that PRR11 is implicated in tumorigenesis through the deregulation of cell cycle progression. However, a precise molecular mechanism(s) behind PRR11-mediated regulation of cell cycle and tumorigenesis remained unclear.

In the present study, we have examined the expression pattern of PRR11 during cell cycle progression in detail, and a functional role of PRR11 in the regulation of cell cycle as well as cellular proliferation in human lung cancer H1299 cells.

2. Materials and methods

2.1. Cell culture and cell synchronization

Human non-small lung carcinoma-derived H1299 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO) and penicillin (100 IU/ml)/streptomycin (100 mg/ml). Cells were maintained at 37 °C in a water-saturated atmosphere of 5% CO^2 in air. Synchronization of the cells was performed by double-thymidine block [11].

2.2. Construction of expression plasmids and transient transfection

The entire coding sequence of human of PRR11 inserted into the mammalian expression plasmid pcDNA3.0 along with N-terminal Flag tag, C-terminal EGFP protein and IRES-EGFP. And then accurate sequence of human PRR11 cDNA were nominated as pcDNA-Flag-PRR11, pcDNA-PRR11-EGFP and pcDNA-PRR11-IRES-EGFP. For transient transfection, cells were seeded at a density of 0.8×10^5 cells/24-well tissue culture plate or 2.5×10^5 cells/6-well tissue culture plate and incubated overnight. Cells were then transiently transfected with the indicated plasmids using Lipofectamine ® 2000 transfection reagent (Invitrogen) following the manufacture protocols.

2.3. siRNA-mediated knockdown

The nucleotide sequences of control siRNA and siRNA against PRR11 were described previously [10]. Prior to transfection, cells were seeded at a density of 5×10^4 cells/24-well tissue culture plate or 2×10^5 cells/6-well tissue culture plate and allowed to attach overnight. The indicated siRNAs were then transiently transfected into cells using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions.

2.4. Real time-PCR (RT-PCR) and Western blotting

The expression of PRR11 was detected as previously described [10,12].

2.5. Indirect Immunofluorescent staining

Cells were fixed and incubated with polyclonal anti-PRR11 (Sigma), monoclonal phospho-Histone H3 (Ser10) (6G3, Cell Signaling Technology), monoclonal anti-cleaved Caspase-3 (Asp175) antibody (9661, Cell Signaling Technology), monoclonal anti-α-tubulin (B-7, Santa Cruz Biotechnology) or with monoclonal anti-BrdU antibody (Roche Applied Science), followed by the incubation with Alexa 488-conjugated anti-rabbit IgG or with Alexa 594-conjugated anti-mouse IgG (Molecular Probes). Cells were then mounted with medium containing 4′6-diamidino-2-phenylindole (DAPI, Sigma), and the preparations were visualized with an Olympus BX51 fluorescence microscope and a Zeiss confocal LSM 768 microscope.

2.6. Flow cytometric analysis

Cell cycle distribution was examined by flow cytometric analysis as previously described [10]. The Annexin V-PE Apoptosis Kit (Biovision) was used according to the manufacturer's instructions to quantify the apoptotic cells. Cell surface expression of phosphatidylserine (PS) was detected with Annexin V during the progression of apoptosis. After transient transfection for 24 h, attached and suspension cells were harvested with a pipette, washed twice with PBS, and resuspended in a 500 μL binding buffer. Annexin V-PE and propidium iodide (PI) (5 μL of each) were added to the cells and incubated at room temperature for 15 min before flow cytometric analysis. Data analysis was performed using the CellQuest software (Becton Dickinson).

2.7. Statistical analyses

Statistical evaluations were performed with GraphPad software (www.graphpad.com), and results were shown as mean ± SD unless otherwise stated. Statistical significance was set at a P value of <0.01, and marked with an asterisk.

3. Results

3.1. PRR11 is highly expressed during late S and G2/M phase of cell cycle

Recently, we have described that the expression level of PRR11 is regulated in a cell cycle-dependent manner, being high in G2/M phase [10], indicating that PRR11 might play a critical role(s) in the modulation of the proper cell cycle progression. To further gain insights into understanding a possible cell cycle regulatory role(s) of PRR11, we sought to examine PRR11 expression pattern during the cell cycle in detail. For this purpose, exponentially growing human lung carcinoma-derived H1299 cells were fixed and stained with anti-PRR11 antibody. As shown in Fig. 1A, PRR11 was largely detectable in cytoplasm under normal condition. Although PRR11 possesses a putative nuclear localization signal (NLS), our indirect immunofluorescence experiments clearly demonstrated that PRR11 is a cytoplasmic protein.

Next, we asked whether cell cycle-dependent oscillation of PRR11 could be observed in H1299 cells. To this end, H1299 cells were incubated in the presence of BrdU, and then cells were subjected to immunofluorescence staining with anti-PRR11 and anti-BrdU antibodies. Representative images were shown in Fig. 1B, and number of BrdU-, PRR11-, and BrdU/PRR11-positive cells were scored (Fig. 1C). Based on our present results, number of double positive cells (9.5 ± 2.7%) was significantly smaller as compared with that of BrdU-positive cells (43 ± 5.2%) (more than 150 and 400 positive-cells were counted, respectively), suggesting that PRR11 is expressed in certain specific stage of S phase. Since our previous immunoblotting analysis revealed a significant upregulation of PRR11 protein 6 h after being released from G1/S boundary by double thymidine block [10], the current results further indicate that PRR11 begins to accumulate at the late S phase.

Finally, we examined the expression of PRR11 during mitosis. Asynchronously growing H1299 cells were fixed and simultaneously stained with anti-PRR11 and anti-phospho-histone H3 (Ser10) antibodies. It has been well-established that phospho-histone H3 at ser10 is a widely used mitotic-specific marker [11–13]. As seen in Fig. 1D, all of the phospho-histone H3-positive mitotic cells expressed PRR11(more than 50 pHH3-positive cells were analyzed). These observations prompted us to determine PRR11 expression during the different mitotic stages. Immunofluorescence-based studies demonstrated that PRR11 is

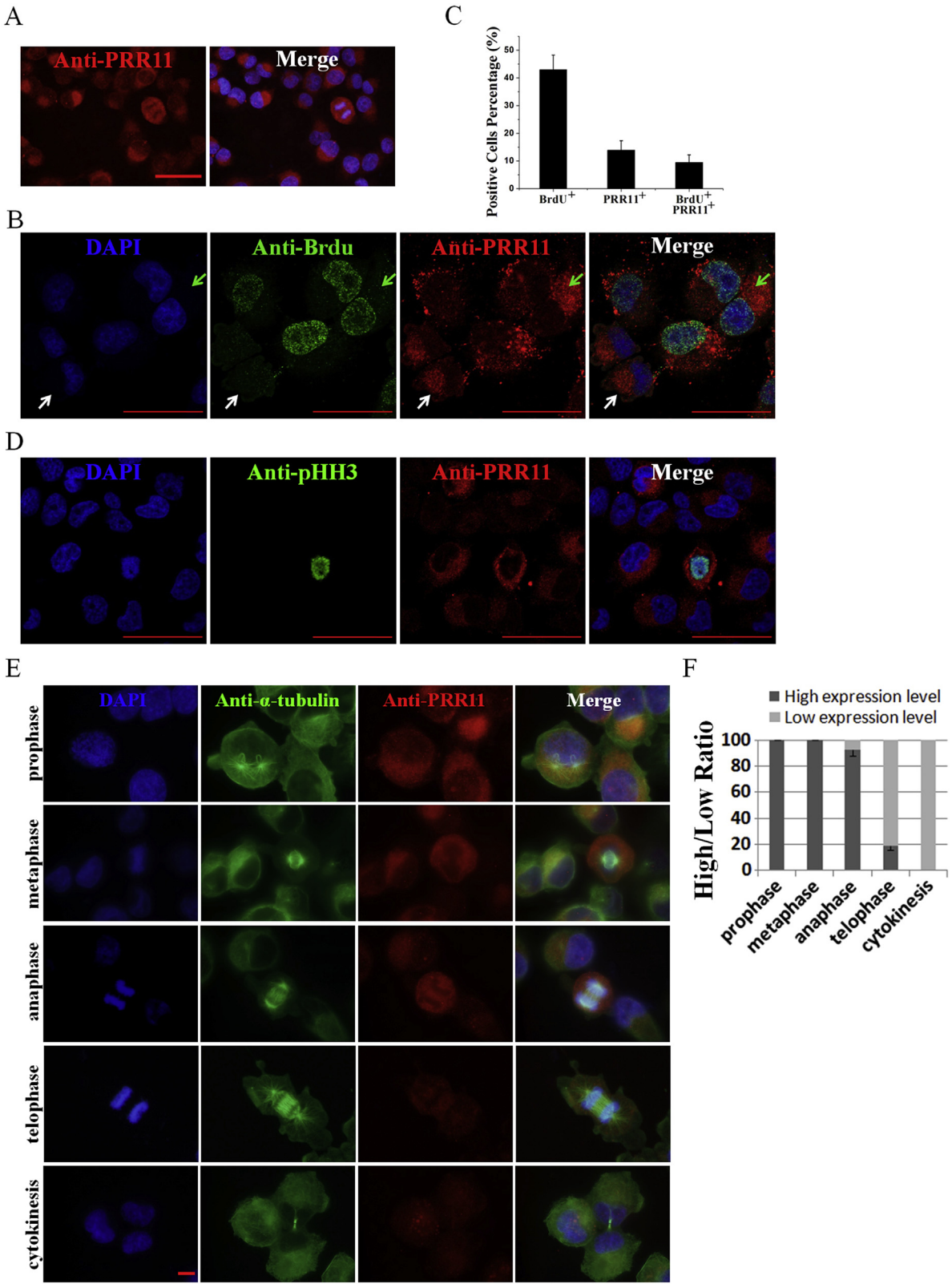

Fig. 1. Immunofluorescence assays of PRR11 expression in H1299 cells. (A) Cytoplasmic localization of PRR11. Exponentially growing H1299 cells were fixed and stained with anti-PRR11 antibody (red). Cell nuclei were stained with DAPI (blue). Scale bar, 50 μm. (B) PRR11 expression in S phase of the cell cycle. Asynchronously growing cells were incubated in the presence of BrdU for 30 min and then stained simultaneously with anti-BrdU and anti-PRR11 antibodies. Cell nuclei were stained with DAPI (blue). White and green arrows indicate the PRR11-positive and PRR11/BrdU-double positive cells, respectively. (C) Relative number of PRR11-, BrdU-, and PRR11/BrdU-positive cells. (D) Expression of PRR11 in M phase of the cell cycle. Exponentially growing cells were fixed and simultaneously stained with anti-PRR11 (red) and anti-phospho-histone H3 (green). Cell nuclei were stained with DAPI (blue). (E) Representative images of each of the mitotic stages such as prophase, metaphase, anaphase, telophase and cytokinesis were shown. Scale bar, 10 μm. (F) Representative histograms of PRR11 expression level High or Low in each phase of mitosis (more than 80 mitotic cells were counted).(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

readily detectable in prophase, metaphase and anaphase cells, whereas its expression level is significantly reduced in telophase cells (Fig. 1E and F). Altogether, these observations further support our previous data of PRR11 expression during cell cycle using quantitative RT-PCR and Immunoblotting methods, and indicate that PRR11 begins to be expressed in the late S phase, its expression level is maintained through mitotic prophase-anaphase, and quickly declines in telophase cells.

3.2. Knockdown of PRR11 leads to cell cycle retardation in late S phase

To address a potential role(s) of PRR11 in the regulation of S phase progression, H1299 cells were transiently transfected with the control siRNA or with siRNA against PRR11. Forty-eight hours after transfection, total RNA and whole cell lysates were prepared and subjected to quantitative real-time PCR and immunoblotting analysis, respectively. As shown in Fig. 2A, the amounts of PRR11 was significantly reduced at both mRNA and protein levels under our experimental conditions. In addition, as shown in Fig. 2B, the cell volume was bigger but thinner in PRR11-knocked down cells than control cells. Next, we checked the cell cycle distribution of control and PRR11-knocked down cells. Consistent with our recent studies [10], silencing of PRR11 caused a significant increase in S phase population (Fig. 2C). Upon close inspection of the flow cytometric data, we have found an obvious increase in the late S phase population in PRR11-depleted cells as compared with control cells (Fig. 2D), suggesting that PRR11 might play a critical role in the regulation of late S phase progression.

Recently, we have found that depletion of PRR11 causes a significant down-regulation of ribonucleotide reductase M1 (RRM1) [10]. RRM1 is a regulatory subunit of ribonucleotide reductase, which catalyzes the rate-limiting step of deoxyribonucleotide formation and is essential for S phase DNA synthesis [14,15]. We then attempted to examine whether PRR11 could collaborate with RRM1 to contribute to DNA synthesis during S phase progression. To this end, H1299 cells were transiently transfected with control siRNA or with siRNA against PRR11, cultured in the presence of 50 mM of dNTPs and then subjected to FACS analysis. As expected, the treatment with dNTPs resulted in an accelerated entry into S phase in control siRNA-transfected cells (Fig. 2C and D). Taken together, these observations suggest that PRR11 is required for proper late S phase progression, which might not be dependent on RRM1-mediated synthesis of deoxyribonucleotides.

3.3. Knockdown of PRR11 caused G2/M progression delay and mitotic defects

To examine a possible functional role(s) of PRR11 in G2/M phase of the cell cycle, H1299 cells were transiently transfected with control siRNA or with siRNA targeting PRR11 and then processed for the indirect immunofluorescence staining with anti-phospho-histone H3 and anti-α-tubulin antibodies. As shown in Fig. 2E and F, knockdown of PRR11 caused a remarkable decrease in number of phospho-histone H3-positive mitotic cells as compared with that of control cells. Thus, it is likely that PRR11 is required for the proper G2/M progression.

Intriguingly, the indirect immunofluorescence analysis also demonstrated that PRR11-depleted cells exhibit the obvious mitotic defects including multipolar spindles and multiple nuclei (Fig. 2G). For example, around 9.8 ± 2.7% of PRR11-knocked down cells displayed the abnormal spindle structures such as multipolar spindles as compared with control cells (1.6 ± 0.4%). Since centrosome is an organelle that serves as the main microtubule organizing center (MTOC) and builds the mitotic spindle [16], we asked whether PRR11 could be a centrosome-associated protein. However, we did not detect the co-localization of PRR11 with the specific centrosomal marker γ-tubulin in mitotic cells (data not shown), indicating that PRR11 might be involved in the mitotic spindle formation through an as yet unknown indirect mechanism.

3.4. Overexpression of PRR11 inhibits cell proliferation and induces premature chromatin condensation (PCC)

We have described that siRNA-mediated knockdown of PRR11 promotes cell cycle arrest and suppresses cellular proliferation in HeLa and lung cancer cells, whereas PRR11 was highly expressed in primary lung cancer tissues as compared with their corresponding normal ones [10], raising a possibility that PRR11 might contribute to cellular proliferation. To address this issue, the expression plasmid for PRR11-EGFP was transiently transfected into H1299 cells. Cells overexpressing PRR11-EGFP were identified based on EGFP signal. However, as clearly seen in Fig. 3A and B, overexpression of PRR11 led to a significant inhibition of BrdU incorporation into cell nuclei. Two weeks after transfection, EGFP-positive cells disappeared which was due to cell death (data not shown). EGFP overexpression transfection had no obvious effects on H1299 cell phenotype or function (Fig. 3C). These observations suggest that the aberrant overexpression of PRR11 inhibits cellular proliferation and causes the subsequent cell death.

Finally, we have asked whether any cell cycle defects could be detectable in PRR11-overexpressing cells. The indirect immunofluorescence analysis demonstrated that 78.2 ± 10.3% of Flag-PRR11-overexpressing cells display the premature chromatin condensation (PCC) (Fig. 3C), whereas no PCC were found in EGFP transfected cells (Fig 3C). Notably, overexpression of PRR11 induced PCC in asynchronous cells, cells synchronized at G1/S boundary and cells synchronized at S phase, indicating that PRR11 induces PCC in a cell cycle-independent manner (Fig. 4A and B). We known that PRR11-overexpression could arrest cell proliferation by inducing the PCC. Then, we employed FACS to analyze the apoptosis for PRR11-EGFP or EGFP positive cells by Annexin V-PE Apoptosis Kit. As shown in Fig. 4C, in contrast to the EGFP positive cells, the ratio of apoptosis and death was remarkably increased in PRR11-EGFP positive cells. However, the ratio of apoptosis was less than PCC by forcing expression of PRR11 (Fig. 4E and F). It suggested that PRR11 overexpression resulted in the apoptosis by inducing the PCC. Taken together, these results suggest that PRR11 can activate the mitotic chromatin condensation, and the uncontrolled PRR11 overexpression impedes cellular proliferation and induces apoptosis through the induction of PCC.

4. Discussion

Mammalian cell growth depends on the cooperative interactions among a number of cellular proteins to form a series of events, which drive the cell cycle from one phase to the next. In the present study, we have found for the first time that PRR11 plays a critical role in the regulation of accurate cell cycle progression through the late S phase to mitosis.

According to our recent observations [10], the expression level of PRR11 in human lung cancer tissues was significantly higher than that in their corresponding normal ones, and its higher expression level was closely associated with the poor prognosis of lung cancer patients. In this study, we have employed human lung carcinoma-derived H1299 cells as a model cell system to investigate a functional implication of PRR11. siRNA-mediated knockdown of PRR11 resulted in a marked delay of the cell cycle progression through S phase as well as from G2 phase to mitosis. Intriguingly, PRR11-

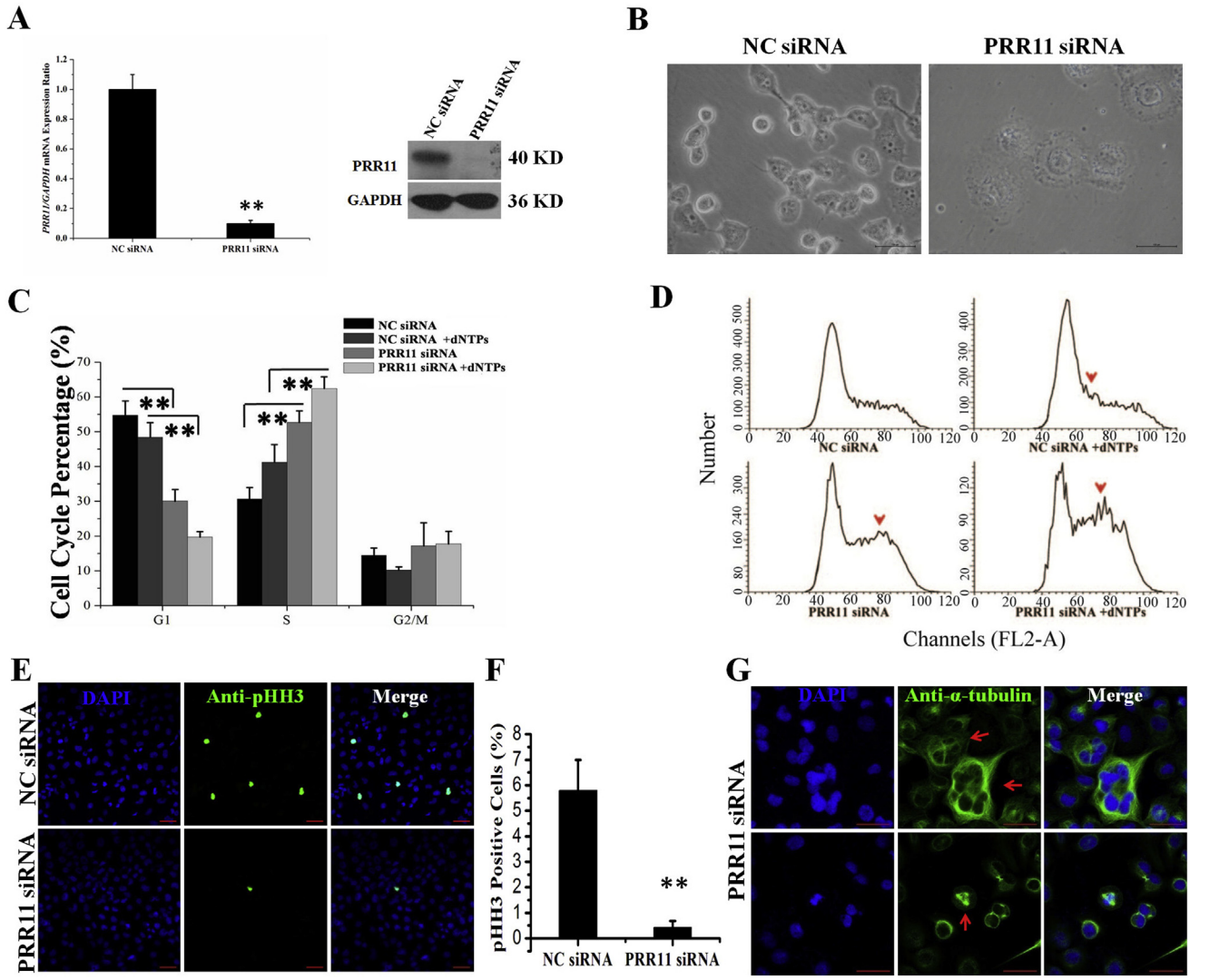

Fig. 2. siRNA-mediated knockdown of PRR11 induces cell cycle arrest in the late S-G2 phase and leads to mitotic defects. (A) siRNA-mediated silencing of PRR11. H1299 cells were transiently transfected with a negative control siRNA (NC siRNA) or with siRNA against PRR11. Forty-eight hours after transfection, total RNA and whole cell lysates were prepared and subjected to RT-PCR (left) and immunoblotting (right), respectively. (B) The effects of PRR11 depletion with the cell shape. H1299 cells were transiently transfected as in (A). Twenty-four hours after transfection, cells were photographed. Scale bar, 100 μm. (C) The effects of PRR11 depletion and/or dNTPs treatment on cell cycle distribution. H1299 cells were transiently transfected as in (A). Four to 6 h after transfection, cells were treated with 50 mM of dNTPs or left untreated. Forty-eight hours post-treatment, cells were harvested, fixed in ethanol, stained with propidium iodide (PI) and subsequently subjected to FACS analysis. Percentage of cells with G1, S or G2/M DNA content was shown. (D) Representative histograms of cell cycle analysis that correspond with data in (C) were shown. Red arrowheads indicated the positions of the early and late S phase. (E) cells were fixed and stained with anti-phospho-histone H3 antibody (green). DAPI was used to stain cell nuclei (blue). Scale bar, 100 μm. (F) Representative histograms of pHH3 positive cells (more than 500 DAPI positive cells were counted, respectively). (G) Depletion of PRR11 induces mitotic defects. cells were fixed and stained with anti-α-tubulin antibody (green). Cell nuclei were stained with DAPI (blue). Representative images of cells with multinuclear structure (Red arrowheads indicated) (upper panels) and multipolar mitotic spindles (Red arrowheads indicated) (bottom panels) were shown. Scale bar, 20 μm.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

knocked down cells exhibited various mitotic defects such as multipolar spindles and multiple nuclei. Since the expression of PRR11 was regulated in a cell cycle-dependent manner with peaks during G2/M phase, it is likely that PRR11 is required for the proper S phase and G2/M phase progression. Notably, exogenous PRR11 overexpression induced prominent PCC and apoptosis. Since PCC can result in genomic instability [17,18] and elevated PCC has been detected in cancers [18,19], it is likely that aberrant PRR11 overexpression might lead to genomic instability and/or aneuploidy through the induction of PCC in cancerous cells. This issue is currently under investigation in our lab.

Together with our recent findings [10], it is possible that PRR11 is regulated at both mRNA and protein level. In this connection, Weinmann et al. described that PRR11 gene locus which contains a putative E2F-1-binding site at 300-bp upstream from its transcriptional initiation site might be regulated by E2F-1 during cell cycle progression [20]. Alternatively, Larance et al. found that PRR11 is rapidly degraded through proteasome, and also indicated that its proteasomal degradation might be mediated by the conserved APC/C and FBXW7-SCF targeting motifs within its COOH-terminal region [21]. Further studies should be required to adequately address this issue.

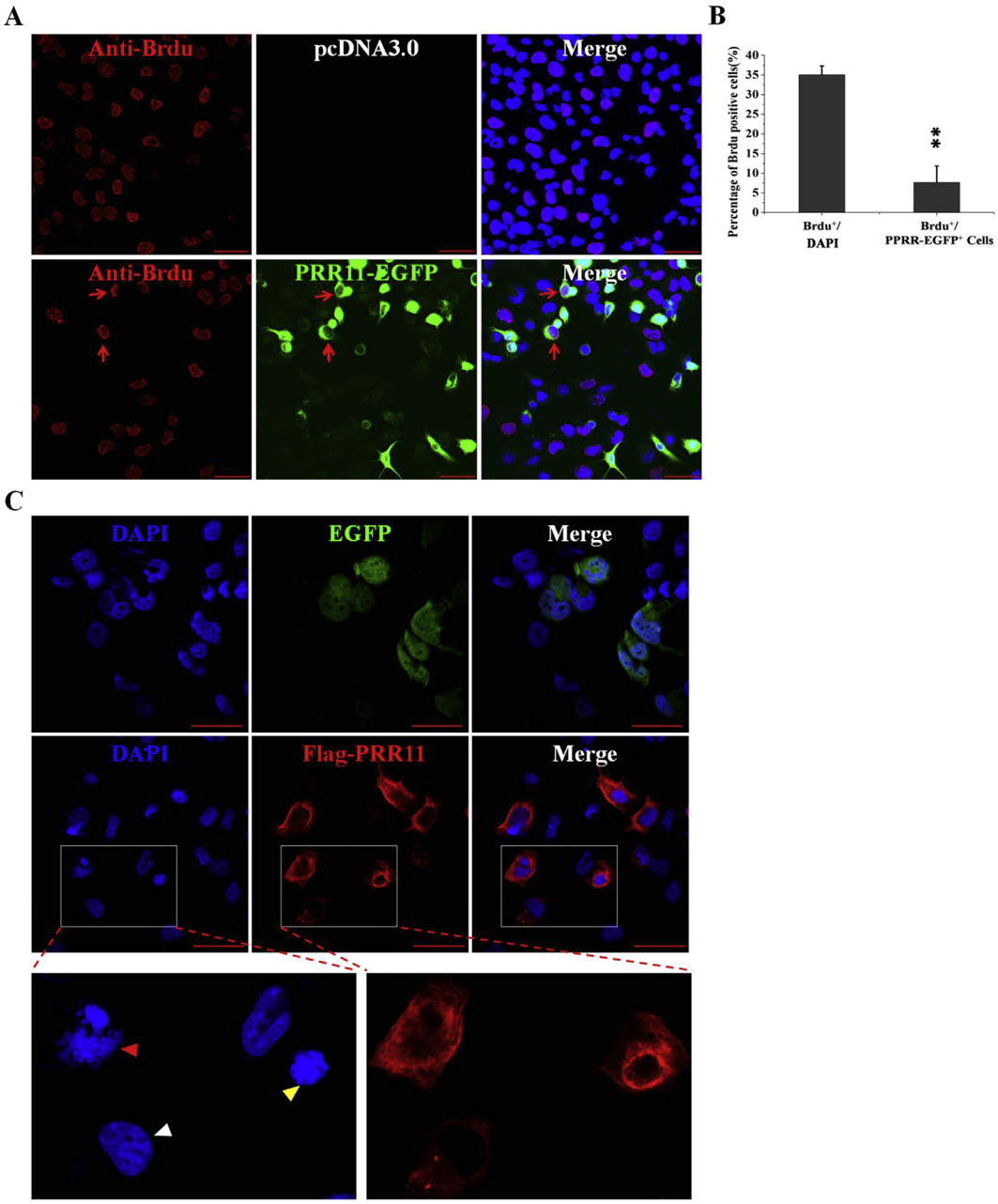

Fig. 3. Forced expression of PRR11 inhibits cellular proliferation accompanied by premature Chromatin condensation (PCC). (A) Overexpression of PRR11 attenuates cellular proliferation. H1299 cells were transiently transfected with the expression plasmid for pcDNA3.0 or PRR11-EGFP (green). Twenty four hours after transfection, cells were incubated in medium containing BrdU for 30 min. Cells were then fixed and stained with anti-BrdU (red). Cell nuclei were stained with DAPI (blue). Red arrowheads indicate both Brdu and PRR11-EGFP positive cells. Scale bar, 50 μm. Under these experimental conditions, number of BrdU-positive cells in all cells (DAPI positive) and only PRR11 overexpressed cells (PRR11-EGFP positive) were scored and calculated, respectively (B). (C) Forced expression of PRR11 induces PCC. H1299 cells were transiently transfected with the expression plasmid for EGFP or Flag-PRR11. Forty-eight hours after transfection, cells were probed with anti-Flag antibody (red). DAPI was used to stain cell nuclei (blue). White arrowheads indicate normal nucleus for PRR11-overexpression cells. Yellow and Red arrowheads indicate G1 or S phase and G2/M phase with PCC for PRR11-overexpression cells, respectively. Scale bar, 50 μm.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To gain insights into understanding the cell cycle regulatory role of PRR11, we have performed microarray analysis and finally identified RRM1 as well as CCNA1 as cellular proteins down-regulated in PRR11-knocked down cells [10]. Since RRM1 has been shown to be essential for the deoxyribonucleotide synthesis during S phase, the down-regulation of RRM1 after silencing of PRR11 suggests that PRR11 might participate in S phase progression through the regulation of deoxyribonucleotide synthesis. Unfortunately, our present results ruled out this possibility. Alternatively, PRR11 contains a proline-rich motif, which might be involved in protein–protein interaction [22,23]. In addition, while we have previously found that high PRR11 levels correlate with tumor progression, the present study revealed that robust exogenous PRR11 overexpression inhibits cell proliferation. This discrepancy also suggests that PRR11 might need to cooperate with other tumor related proteins to exert its tumor-promoting activity. Currently, we have a plan to identify a potential PRR11-binding partner(s) using a yeast-based two-hybrid system.

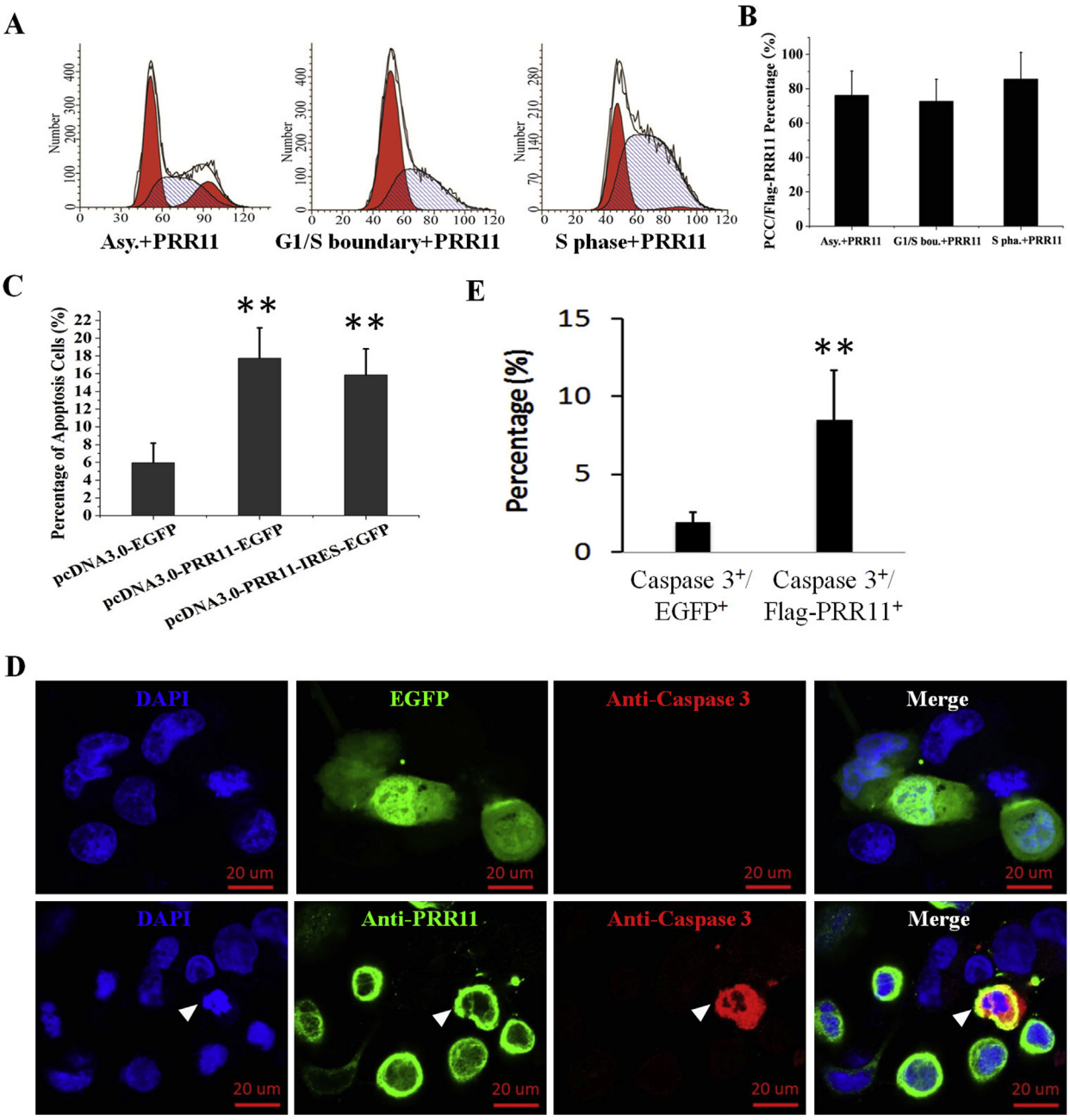

Fig. 4. PRR11-overexpression induces the cell apoptosis by premature Chromatin condensation (PCC). (A) Overexpression of PRR11 in synchronized H1299 cells. Asynchronously growing H1299 cells were synchronized at the G1/S boundary by double thymidine block, and released for 4 h to obtain S phase cells as described in Material and methods. Asynchronous and synchronized G1/S boundary or S phase cells were transiently transfected with the expression plasmid encoding Flag-PRR11, and maintained in normal medium or in medium containing 2.5 mM of thymidine until subsequent analysis, respectively. Twenty-four hours after transfection, cell cycle distribution was assessed by flow cytometric analysis, and number of cells with PCC was scored (B). (C) Representative histograms of cell apoptosis (early apoptosis and late apoptosis) analysis by FACS. H1299 cells were transiently transfected with the expression plasmid for EGFP, PRR11-EGFP and PRR11-IRES-EGFP (PRR11 and EGFP was unconjugated). Twenty-four hours after transfection, attached and suspension cells were harvested, and then the apoptosis of EGFP positive cells were analyzed by FACS. (D) PCC results in Apoptosis. H1299 cells were transiently transfected with the expression plasmid for EGFP or Flag-PRR11. Cells were fixed and stained with anti-Flag (Green) and anti-cleaved Caspase 3 (Red). Cell nuclei were stained with DAPI (blue). White arrowheads indicate apoptosis cells. Scale bar, 20 μm. (E) Representative histograms of Apoptosis analysis (more than 200 Flag-PRR11 or EGFP-positive cells were counted, respectively).(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Conflict of interest

None.

Acknowledgments

This work was supported in part by a grant-in-aid from National Natural Science Foundation of China (30801356 and 81001097 to YB, http://www.nsfc.gov.cn/), Chongqing Natural Science Foundation (cstc2013jcyjA10043 to CZ and cstc2014jcyjA10026 to HZ, http://www.ctin.ac.cn/), and Scientific and Technological Research Program of Chongqing Municipal Education Commission (KJ130326 to HZ, http://www.cqjw.gov.cn/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.139.

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