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Mir-351-5p contributes to the establishment of a pro-inflammatory environment in the H9c2 cell line by repressing PTEN expression

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Abstract The activated renin–angiotensin–aldosterone system modulates several metabolic pathways that contribute to left ventricular hypertrophy and heart failure. In this metabolic system, angiotensin II modulates heart morphophysiological changes triggered by a series of inflammatory and pro-inflammatory responses; however, the fine tuning associated with the control of this biochemical pathway remains unknown. Here, we investigated elements involved in the post-transcriptional regulation of the pro-inflammatory environment in the H9c2 cardiac cell line, focusing on miRNA elements that modulate PTEN expression. A cellular model of investigation was established and the miR-315-5p was identified as a novel element targeting PTEN in this cardiac cell line, thereby controlling the protein level. This interconnected pathway contributes to the control of the pro-inflammatory environment in Ang II-treated cells.

Keywords Angiotensin II · Cell culture · miRNA · Post-transcriptional regulation · PTEN

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Introduction

Cardiovascular diseases are the leading cause of mortality and morbidity worldwide, and heart failure has been implicated as the main cause of such problems over the last 20 years [1]. Cardiac myocytes exposed to chronic stress undergo sequences of phenotypic and metabolic changes that can induce left ventricular hypertrophy and heart failure. A fundamental mechanism leading to both hypertrophy and subsequent heart failure is the activation of the renin–angiotensin–-aldosterone system, which activates protein kinase cascades, the fetal-like genes program, calcium level changes etc. [2]. Moreover, increased production of the main effector angiotensin II (Ang II) co-operates with the inflammatory environment and plays a critical role in left ventricular remodeling and heart failure [3, 4]. It is widely described that Ang II causes a modulatory effect on the pro-inflammatory environment in a dose-dependent manner and modulates the synthesis of autocrine and paracrine elements even in cell culture models [5].

In the pro-inflammatory environment, increased production of cytokines, such as tumor necrosis factor-α (TNF-α) [6, 7], modulates the functional activity of groups of transcription factors such as nuclear factor-κB (NF-κB) [8], which modulates the hypertrophic genes expression reinforcing a close connection between the two molecular pathways [7]. In addition, the pro-inflammatory environment triggers the production of prostanoids and/or eicosanoids via the arachidonic acid (AA) pathway. Prostanoids and eicosanoids are a family of molecules that are involved in many biological mechanisms and tissue homeostasis [9] and the cyclooxygenase-2 (COX-2) enzyme catalyzes the rate-limiting step of the molecular pathway. The role of COX-2 in inflammation and disease has long been appreciated, but it is still not clear how the functional activity of

the enzyme is modulated and how it disrupts cardiac homeostasis. Moreover, many details are lacking concerning the inflammatory and pro-inflammatory processes. Recent studies have correlated COX-2 activity with the phosphatase and tensin homolog deleted on chromosome 10 (PTEN). PTEN was originally characterized as a tumor suppressor molecule [10] that controls the hydrolysis of PI-3,4,5-trisphosphate (PIP3) and the downstream events (i.e., the cell cycle, cellular growth, angiogenesis, and even the inflammatory process) through the activation of the AKT/PKB enzyme [11]. Lee et al. [12] demonstrated using Raw 264.7 macrophages that inflammatory processes can be modulated indirectly by PTEN levels. Additionally, Li et al. [13] demonstrated a negative correlation between COX-2 and PTEN proteins in osteoblasts through the activation of the casein kinase 2 (CK2). This enzyme is able to phosphorylate PTEN at several of its serine and threonine residues, thereby inactivating its phosphatase activity and reinforcing the pro-inflammatory mechanism via AKT modulatory activity. However, the fine tuning that controls the connections between PTEN and the inflammatory/pro-inflammatory mechanisms needs further investigation. In addition, the mechanisms involved with PTEN mRNA processing are currently unknown.

Several elements have been correlated with the post-transcriptional regulation of PTEN. Of these elements, microRNAs (miRNAs) represent interesting molecules that play an important function in the control of gene expression. These small non-coding RNAs (18–25 nt) bind to specific sequences in the messenger RNA, especially in the 3′ of untranslated region (UTR) and decrease protein synthesis by initiating mRNA degradation or translation inhibition [14, 15]. Moreover, growing evidence suggests that miRNAs play critical roles in cardiovascular development and disorders [16–20]. Based on this information, in the present study we investigated miRNA elements that possibly modulate PTEN and contribute to the establishment of the pro-inflammatory environment in a cardiac cell line treated with Ang II. Particularly in this study, we described for the first time in mammals the molecular function of miR-315-5p in cellular mechanism.

Materials and methods

Cell culture and treatment

H9c2 (2-1) cells (American Type Culture Collection, ATCC: CRL-1446) are an embryonic rat ventricular cell line. These cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 100 μg/ml penicillin/streptomycin in 5 % CO_2 atmosphere at 37 °C. All reagents were purchased from Life Technologies™, Brazil. In the experimental cell cultures, 10 μM Ang II (Sigma-Aldrich, USA) was added to the media according to the method described by Smeets et al. [21], and the cells were incubated for up to 48 h. The 0 h time-point represents untreated cells; these cultures served as the controls for our assays.

Cellular viability assays

Cellular viability and proliferation were assessed using 1.5×10^4 cells that were previously grown in 96-well plates. The cultures were grown in the presence or absence of Ang II for up to 48 h. Cellular proliferation and viability were determined using methylthiazolyl diphenyl tetrazolium bromide in the MTT assay at specific time-points [22]. The plates were read at 570 nm using a microplate reader (Packard Instrument Company Inc., Meriden, CT, USA), and the assays were performed in triplicate.

Cellular immunostaining and cell surface area measurement

Enriched suspensions of H9c2 cells were plated at a density of 3.0×10^4 cells/well in 24-well culture plates containing sterile coverslips. The cells were incubated for 24 h in a 5 % CO_2 atmosphere at 37 °C, then incubated with or without 10 μM of Ang II for different time-points up to 48 h. The cells were fixed in 3.8 % paraformaldehyde containing 0.2 % Triton X-100 (Sigma-Aldrich, USA) for 7 min at 37 °C and subjected to immunostaining. To analyze cellular morphological changes after Ang II treatment, the cytoskeleton was labeled in a 1 % bovine serum albumin (BSA) solution containing 100 μg/ml of phalloidin-tetramethylrhodamine B isothiocyanate (TRITC) (Sigma-Aldrich, USA) for 1 h, and the nuclei were counterstained in a solution of 3.33 ng/ml 4′,6 diamino-2-phenylindole (DAPI, Sigma-Aldrich, USA). The coverslips containing cells were subsequently mounted onto slides and subjected to microscopic analysis. Images were obtained with DMLB-100S, Leica™ microscope (Leica Microsystems, Wetzlar, Germany) equipped with an HBO 100 W mercury lamp and the corresponding filter sets, and the surface area of each selected cell was calculated. One hundred randomly selected cells were measured in each of four wells from each treatment group, and the images were analyzed using Image J ® 1.45S, Wayne Hasband software.

TNF-α and PGE2 immunoassays

Cell culture supernatants were used in immunoassays to measure the secretion of tumor necrosis factor alpha (TNF-α) and the prostanoid prostaglandin E2 (PGE2). 50 μl of the cellular supernatants were used in each assay. TNF-α

and PGE2 were measured using specific ELISA kits from Thermo Scientific and Cayman Chemical, respectively, as directed by the manufacturers. The assays were analyzed with a microplate reader (Packard Instrument Company Inc., USA), and the experiments were repeated three times.

Western blot analyses

After incubation of H9c2 cells with the Ang II peptide, whole cell extracts were prepared according to the method of Sambrook et al. [23]. Equal amounts of protein (50 μg) were electrophoresed in 10 % polyacrylamide gels, and then electrotransferred to polyvinylidene fluoride (PVDF) membranes. The membranes were immunoblotted overnight with the murine anti-COX-2 (Cayman Chemical, USA), rabbit anti-PTEN or anti-P-PTEN, anti-AKT or anti-P-AKT, and anti-β actin (Cell Signaling. Inc., USA) polyclonal antibodies, followed by 2 h of incubation with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotech). Immunoreactive bands were visualized with a chemiluminescent detection kit (ECL™, GE Healthcare, USA) and exposed to hyperfilm (GE Healthcare, USA). The bands were quantified with the Quantity One® Software (Biorad). The same procedure was conducted for cells transiently transfected with 10 nM of a custom-designed rno-miR-351-5p inhibitor or a miRNA inhibitor negative control (Cat #4464076, Life Technologies). The transfection procedures were performed with the Lipofectamine 2000 transfection reagent (Life Technologies™) following the instructions of the supplier.

RNA isolation and quantitative PCR

Total RNA was extracted from 1.5×10^6 cells incubated in the presence or absence of Ang II using the TRIZOL® Reagent (Life Technologies™). One microgram of each RNA sample was reverse-transcribed (RT) into first-strand cDNA using Cloned AMV Reverse Transcriptase (Life Technologies™) following the instructions of the supplier. All RT reactions were analyzed using real-time PCR with the SYBR Green Master Mix (Life Technologies™) in an ABI 7300 Sequence Detection System (Applied Biosystems®). The reactions were run in triplicate, and mRNA expression was normalized to the reference gene *β-actin*. The specific sets of primers used in these analyses were as follows: *β-actin*, 5′-TGGTGGGTATGGGTCAGAAG and 5′-CAATGCCGTGTTCAATGG; AKT, 5′-GCTTACTGA GAACCGTGTCC and 5′-GGTCGTGGGTCTGGAATG; *COX-2,* 5′-CAGCCCACCAACTTACAATG and 5′-CATC AGCCACAGGAGGAAG; *PTEN*, 5′-CCAGGACCAG AGGAAACC and 5′-GTCATTATCCGCACGCTC. The results were quantified as C_t values, where C_t is defined as the threshold cycle of PCR at which the amplified product is first detected, and presented as relative gene expression (the ratio of target/control). The $2^{-\Delta\Delta C_t}$ method was applied to calculate the relative quantification.

For miRNA analyses, total RNA was purified from each sample, and cDNA was prepared using Mini Script Reverse Transcription (Qiagen, German), which contains a special stem-loop primer for miRNAs. The analyses of the miRNAs expression miR-190, miR-190b, miR-19-b1*, miR-19-b2*, miR-421, miR-759, miR-351-5p, miR-463, miR-873, miR3597-5p, and miR-9*, whose putative binding sequence were identified in the 3′-UTR sequence of the PTEN RNA sequence from *Rattus norvegicus* (Gene ID: 50557) (using online bioinformatics tools miRBase [24], miRanda [25] and Blast search), were performed using real-time quantitative PCR with the miScript SYBR Green PCR Kit (Qiagen, German) according to the manufacturer's instructions. In addition, the expression level of the rno-miR-26b was investigated considering its molecular connection with COX-2-mRNA. The same ABI 7300 Sequence Detection System (Applied Biosystems®) equipment was used in the analyses and also the $2^{-\Delta\Delta C_t}$ relative quantification method was applied; U6 was used as the internal control. The results were quantified as C_t values and used to calculate relative gene expression.

Plasmid constructs and dual-luciferase reporter assays

Corresponding cDNA sequence of the 3′-UTR of the PTEN (3426–3445 bp, 5′-GAAGAGCACTTTAAGCCTTA) mRNA were cloned into the pGL3-Control vector (Promega) upstream of the firefly luciferase coding sequence via synthetic oligonucleotides ligation. In this assay, H9c2 (2-1) cells were plated at a density of 3×10^4 cells per well in a 24-well plate and transiently transfected with 100 ng of the pGL3-Control vector or 100 ng of pGL3-PTEN-3′-UTR; in addition, the H9c2 (2-1) cells were also transfected with 100 ng of the pGL3-PTEN-3′-UTR along with 30 nM of custom designed rno-miR-351-5p inhibitor or a mirVana™miRNA inhibitor, used as negative control (NC) (Cat #4464076 Life Technologies). The Lipofectamine 2000 transfection reagent (Life Technologies™) was used as the transfection reagent. At 24 h post-transfection, the activities of both luciferases were determined using the Promega dual-luciferase reporter assay (Promega, USA) according to the manufacturer's instructions. The *Renilla* luciferase activity was normalized to the *Firefly* luciferase expression for each sample to account for differences in transfection efficiency. The luciferase activities were measured using a TD20/20 luminometer (Turner Designs).

Graphs and statistical analyses

Values from four independent assays were employed for analysis, and graphs were generated using Graph Pad Prism® 5. Data are presented as the mean ± standard deviation and the differences between the control and treated groups were also measured using one-way analysis of variance (ANOVA), followed by Dunnetts test. Significance was set at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

Ang II stimulates a pro-inflammatory environment in H9c2 cells

Ang II is a peptide that is largely used in vascular smooth muscle cells (VSMC) as a hypertrophic agent and contributes to the development of heart hypertrophy and the establishment of the inflammatory environment in in vivo models [26]. H9c2 cardiac cells were incubated with the peptide at a concentration of 10 μM to investigate the relationship between the pro-inflammatory agent on COX-2 production and its molecular connection with PTEN in our cellular model. MTT analysis demonstrated neither toxic properties of the peptide nor major changes in cellular viability at this chemical concentration (data not shown). Next, the cells were incubated with or without the chemical for different time-points and cellular area were measured. After 12 h of incubation with Ang II, an approximately 170 % increase in the cellular area was observed compared to the control, decreasing thereafter (Fig. 1a). The same cellular area measurements were performed on the untreated cells and no significant changes were observed (data not shown).

Considering the investigated 48 h time-points, TNF-α production by the Ang II-treated cells was measured and the results demonstrated 70 % increased production of this cytokine at 2 h time-point assay. At the succeeding time-points (6, 12 and 24 h) this cytokine maintained an even higher level (110 % increase compared to the control), decreasing thereafter. The results suggest the existence of a pro-inflammatory environment, which is probably controlled by the cells. The same analyses were performed to the production of PGE2. At 12 h time-point, a 70 % increase in PGE2 production was also observed decreasing thereafter (Fig. 1b). Taken together, these results suggested that Ang II induced a pro-inflammatory environment in our cellular model and the activation of prostanoid production and the initial hypertrophic response could be connected to the COX-2 synthesis and activity.

To verified COX-2 production in H9c2-treated cells and its possible connection with PTEN activity in the AKT/PKB molecular pathway, we performed Western blot analysis. It is well known that the negative modulatory effect of PTEN activity positively reinforces the pro-inflammatory environment [11, 27]. Western blot analysis demonstrated an inverse negative cross-regulation between COX-2 and PTEN expression over the 48 h period studied (Fig. 1c). The highest expression level of the COX-2 protein occured between the 2 and 12 h time-points (∼207 % higher on average than the control level); at the same time-points, PTEN presented a reduced expression level (∼74 % reduction on average compared to the control level). Additionally, the phosphorylated form of PTEN reached its highest level (∼240 % increase on average when compared to the control), despite the lower absolute level of the phosphorylated form detected in the assay. In addition, an oscillatory pattern of phosphorylated AKT was observed, at the same time-points where COX-2 protein presented its highest level and PTEN the lowest ones. At 6 and 12 h time-point the phosphorylated AKT protein level increased ∼184 and 147 % on average than the control level, when compared to the control. The unphosphorylated form of AKT did not suffer major changes in its level all along the investigated time-points. These findings reinforce previously results that demonstrated a close connection between the COX-2 and PTEN proteins in the control of inflammatory environment through the AKT/PKB pathway.

Moving forward: miRNAs as post-transcriptional regulatory elements that collaborate with the pro-inflammatory environment

To determine whether post-transcriptional regulatory mechanisms modulate PTEN and the pro-inflammatory environment, mRNAs and miRNAs from Ang II-treated and -untreated H9c2 cells were analyzed in qPCR reactions. The results demonstrated that COX-2 mRNA reached its highest expression level at the 2 h time-point (∼7 times higher than the control); an inverse expression pattern was observed for the rno-miR-26b expression level (Fig. 2a). The results corroborate the previous study of Ji et al. [28], where the authors connected the regulatory function of miR-26b and COX-2 protein expression with carcinoma of nasopharyngeal human cells. Moreover, the qPCR analyses also demonstrated an increasing expression pattern of PTEN mRNA during the first 12 h analyzed and a considerable up-regulation of this mRNA at the 24 h time-point (∼289 % higher than the control level) (Fig. 2a). When compared the mRNA expression pattern to the PTEN protein levels in the assays (Fig. 1c), any correlation between the expression levels of the above molecules is not observed, suggesting that post-transcriptional mechanisms are modulating the protein synthesis of PTEN

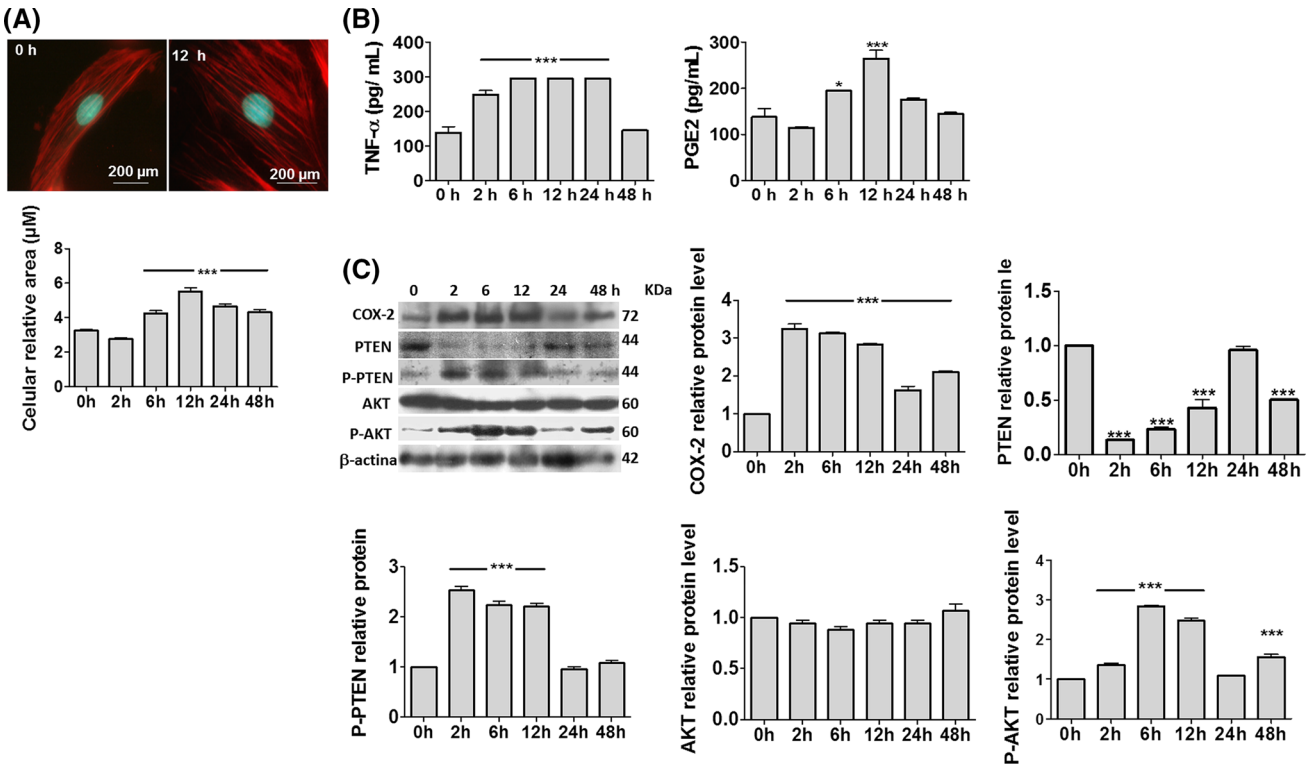

Fig. 1 Angiotensin II modulates the pro-inflammatory environment in cardiac H9c2 cells. **a** Effect of Ang II treatment on H9c2 area. After cellular incubation with or without the peptide for different lengths of time, the cells were fixed, immunostained and the cells sizes were quantified and plotted on graphs. The nuclear material and cytoskeleton were labeled with DAPI and Phalloidin-TRITC. *Bars* = 200 µm. The results were from one representative experiment. **b** The supernatants of treated and untreated cells were used for TNF-α and PGE2 measurement, and the results were plotted in the represented graph. **c** Western blot analyses. In this Figure, the *graphs* present the average values of independent experiments, and the *error bars* represent the standard deviation of the mean. ANOVA analysis showed significant differences between the control and cell samples; the significance level was set at $p < 0.05$ (***)

in Ang II-treated cells. This observation is reinforced by the relative level of phosphorylated form of PTEN protein observed in the assay. Despite the considerable increase in its relative level between the 2 and 12 h time-point of the Ang II cellular induction, that at some degree matches the RNA expression level, the absolute level of the P-PTEN is still present at low absolute level all along the assay. This combined result suggests that the phosphorylated form of PTEN exists in order to contribute to the pro-inflammatory environment that gets set in our cellular model. Despite a lower biological activity, the phosphorylate form of PTEN is more stable [29–31], and in this pro-inflammatory environment, P-PTEN helps to control the PI3K/AKT pathways at its minimal level, which could be appointed as relevant to the cellular homeostasis.

Next, to address how post-transcriptional mechanisms modulate PTEN in the Ang II-treated cells, we performed qPCR analyses to investigate the expression patterns of 11 miRNAs that potentially target the PTEN 3′-UTR. Interestingly, the results suggested that a negative correlation exists between rno-miR-351-5p and PTEN protein expression levels in H9c2 cells treated with Ang II (Fig. 2b). In addition, it was not possible to infer any explanation for the changes in PTEN protein expression patterns based on the analyses of the other miRNAs investigated; moreover 3 of them were not detected in the assays (miR-873, -357-5, and -9*, results not shown). The potential target site for rno-miR-351-5p in the 3′-UTR of PTEN mRNA is also presented in Fig. 2c.

To verify if PTEN 3′UTR is a true target of miR-351-5p, dual-luciferase assays were performed to verify physical interaction between the putative target sequence in the PTEN mRNA from *R. norvegicus* and miRNA-351-5p (Fig. 3a). The pGL3 plasmids (Control and PTEN-3′-UTR) were transfected into H9c2 cells to measure luciferase production in the presence or absence of the miRNA-351-5p inhibitor or miR-inhibitor negative control (NC). The pGL3-Control vector was used to normalize the luciferase production by the cells in the presence of an empty vector. The PTEN-3′-UTR construct reduced the production of luciferase in transfected H9c2 cells by ∼39 % compared to the cells transfected with the pGL3-Control vector. Taken together, the results suggested the presence of molecular mechanisms that were interfering in the PTEN

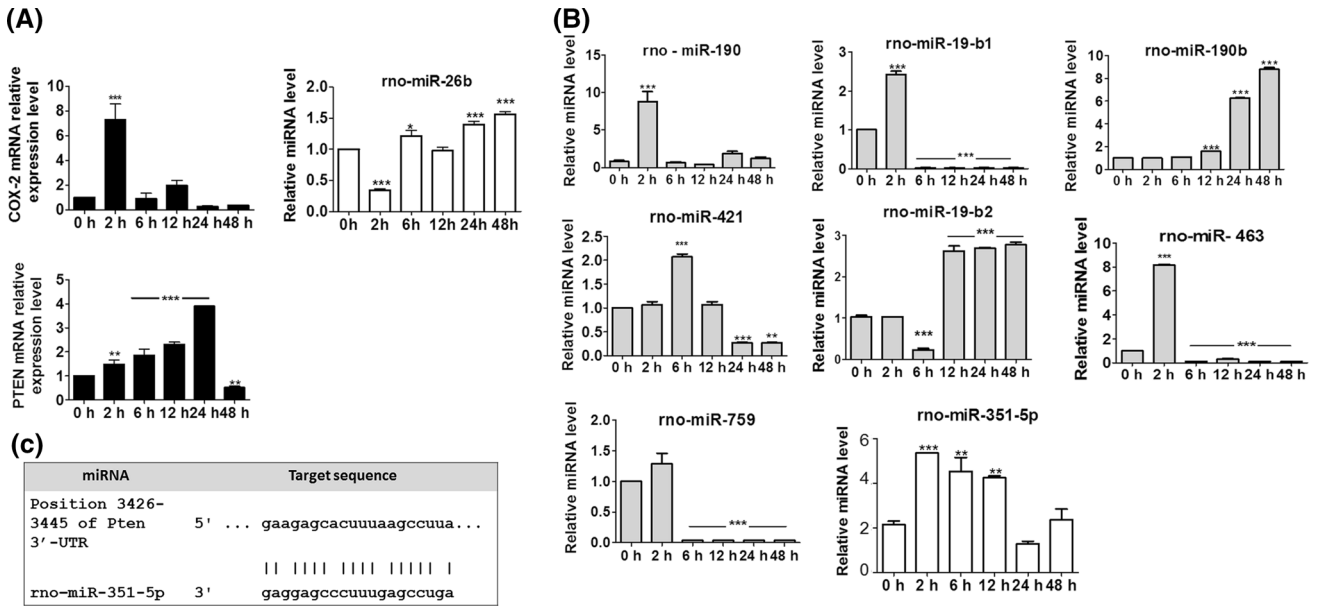

Fig. 2 Transcriptional and post-transcriptional elements that are correlated with the pro-inflammatory environment in Ang II H9c2-treated cells. **a** COX-2 and PTEN mRNA expression levels and miR-26b. **b** Expression pattern of miRNAs that putative bind to the 3′-UTR sequence of the PTEN mRNA in Ang II-treated and untreated cells. **c** Schematic representation of the potential target site for rno-miR-351-5p in the 3′- UTR of PTEN mRNA. The ANOVA analysis showed significant differences between the control and cell samples; the significance level was set at $p < 0.05$ (***)

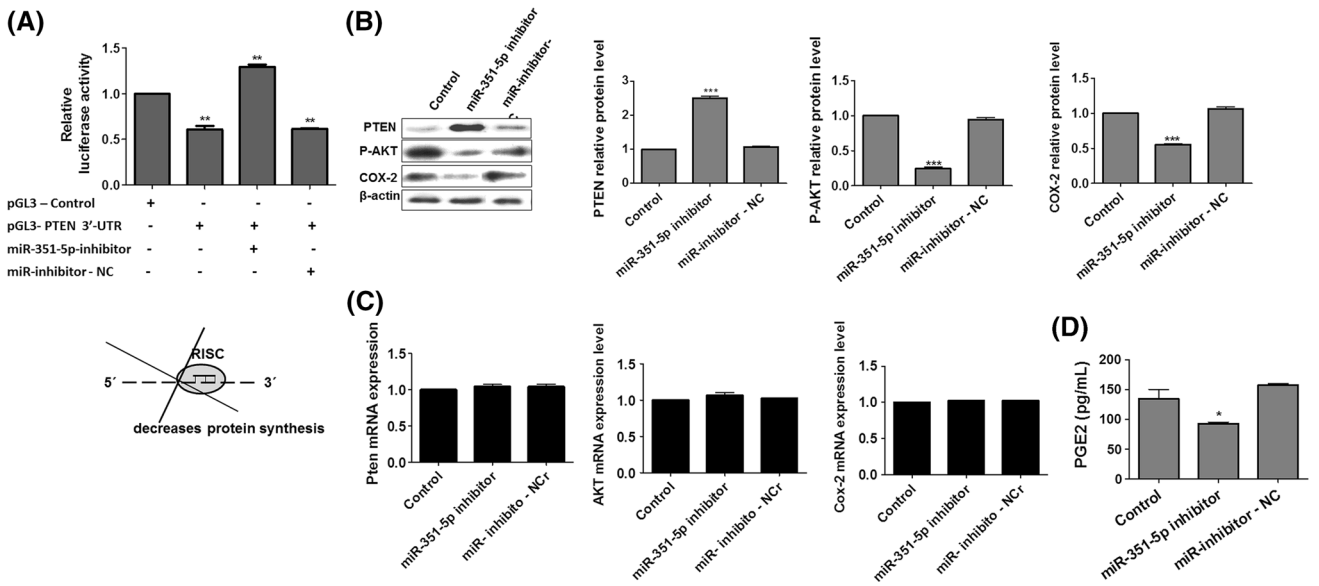

Fig. 3 PTEN is a direct target of miR-351-5p in *Rattus norvegicus*. **a** Relative luciferase activity in cells transfected with pGL3 plasmids (Control and PTEN-3′-UTR) and the miRNA 351-5p-inhibitor and the negative control miR-inhibitor (NC); the results were plotted in representative graphs. **b** Western blot analyses of PTEN, P-AKT, and COX-2 protein expression in H9c2 miR-inhibitor-transfected cells. **c** PTEN, AKT, and COX-2 mRNA expression in H9c2 miR-inhibitor-transfected cells. **d** PGE2 measurements from miR-inhibitor-transfected cells were also analyzed and plotted on graphs. ANOVA analysis showed significant differences between the control and cell samples; the significance level was set at $p < 0.05$ (***)

construct translation, which reduced the luciferase production. Additionally, the presence of the specific miRNA 351-5p inhibitor in H9c2-transfected cells increased the luciferase production by 29.6 %, suggesting that this specific inhibitor blocks the natural miRNA binding to the 3′-UTR of PTEN, facilitating extra rounds of PTEN translation. Moreover, the presence of the negative control miR-inhibitor reduced the luciferase expression of the

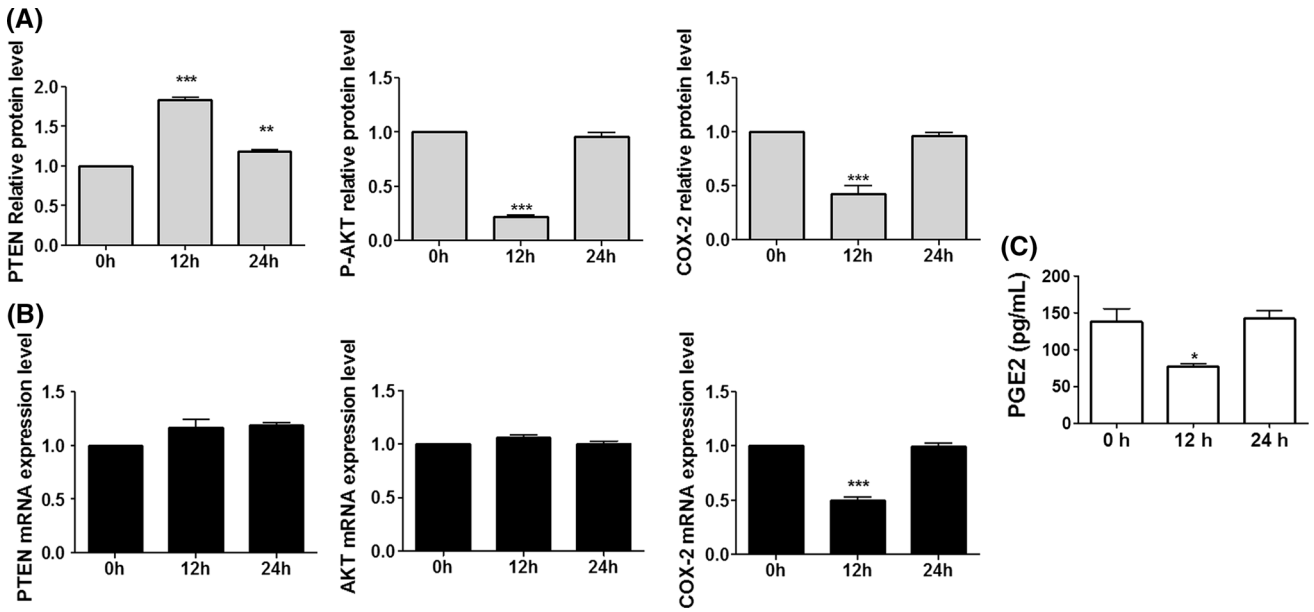

Fig. 4 miR-351-5p contributes to the establishment of the pro-inflammatory environment in H9c2 cells treated with Ang II. **a** Western blot analyses of PTEN, AKT, and COX-2 protein expression and this respective mRNA level after miR-inhibitors transfections and Ang II treatment (**b**). **c** PGE2 measurements from miR-inhibitor-transfected cells and treated with Ang II were also analyzed and plotted on graphs. ANOVA analysis showed significant differences between the control and cell samples; the significance level was set at $p < 0.05$ (***)

H9c2-transfected cell with the pGL3-PTEN-3′-UTR by ~39 % as well as in cells exclusively transfected with the vector, demonstrating any effect in the modulation of miR-315-5p activity on PTEN mRNA translation.

In addition, we investigated PTEN, AKT, and COX-2 expression at transcriptional and translational levels (Fig. 3b, c); also PGE2 production in H9c2 transfected cells was investigated (Fig. 3d) to verify the modulatory effect of the miRNA-351-5p inhibitor on the pro-inflammatory environment. In cells transfected with the miRNA-351-5p inhibitor, PTEN protein levels increased approximately 150 % on average when compared to the control. In contrast, the presence of the NC miR-inhibitor did not significantly alter PTEN expression. At the same time, the presence of the specific miR-inhibitor decreased P-AKT (~75.7 %,) COX-2 synthesis (~50 %) and even PGE2 production (31 % reduction of the prostanoid in the miR-351-5p transfected cells). In addition, no major changes were observed at the transcriptional level of the investigated molecules. The combined data suggest that miR-351-5p helps to control PTEN protein level by direct interfering and controlling in its translational mechanism which contributed to the establishment of the pro-inflammatory environment in the investigated cardiac cell line.

To corroborate such observations, H9c2 cells were transiently transfected with the miR-351-5p inhibitor and, then they were treated with Ang II for up to 24 h. Western blot analyses demonstrated again an inversed correlation between PTEN and COX-2 (Fig. 4a). The phosphorylated form of AKT follows COX-2 protein expression pattern. However, at 12 h time-point, PTEN increased its expression in ~84 %, while COX-2 decreased its level in ~60 %, when compared to the control level, and P-AKT protein decreased in ~78 %. The proteins returned to the near basal level after 24 h transfection, which could be explained by cellular homeostasis return. In Fig. 4b, the mRNA expression levels of PTEN, AKT, and COX-2 are presented. No significant change was observed for PTEN and AKT mRNA expression, however, a decreased expression for COX-2 (~50 % when compared to the control) was observed, as well as PGE2 production was reduced in the assay (Fig. 4c).

Discussion

In this study, the H9c2 cardiac cell line was used to address the mechanism by which PTEN modulates the pro-inflammatory environment and the contribution of post-transcriptional elements (especially miRNAs). In the established model, Ang II increases the cell area, TNF-α and PGE2 production, suggesting the existence of a close connection between pro-inflammatory stimuli and cardiac cell remodeling, as observed in several studies [3, 4]. Loss of PTEN was already appointed as an important element that helps to prevent the development of maladaptive

ventricular remodeling due to the control of angiogenesis and metabolic gene expression in response to pressure overload, but not in response to Ang II. Using our cellular model, we investigated the connections of PTEN with COX-2 in the control of the pro-inflammatory environment. Negative regulatory mechanisms between the molecules were observed in our Western blot results, thereby corroborating the results of previous analyses [13].

Focusing the analyses on transcriptional and post-transcriptional elements to identify miRNAs that could support the close molecular connections between PTEN and COX-2, qPCR analyses were performed. In our assays, the highest expression level of the COX-2 transcripts was observed at 2 h time-point, as a probable consequence of Ang II treatment. The COX-2 mRNA level decreased after the 2 h treatment in kinetic range that was in agreement with its $\sim$1.5 h half-life [32]. In addition, the expression pattern of miR-26b presents an inversed correlation with COX-2 mRNA level suggesting a close connection between the two molecules and reinforcing the previous observations of Ji et al. [26, 28] as previously discussed. However, the mRNA expression pattern did not match the COX-2 protein expression pattern; indeed, COX-2 expression was found at higher levels from the 2–12 h time-points and this finding could be explained based on COX-2 protein half-life. Shao et al. [32] demonstrated that the COX-2 half-life is dependent on the cell line investigated. Despite the considerable mRNA degradation in Ang II-treated cells after the 2 h time-point, COX-2 transcripts were still available for new rounds of transcription, which could contribute to the maintenance of the protein level out to the 12 h time-point. At the same time, PTEN mRNA level was gradually increasing up to 24 h, decreasing thereafter, which could contribute to the homeostatic environment return. In addition, the highest relative expression level of the PTEN transcript (24 h time-point) was in agreement with the relative expression of the PTEN protein. Moreover, the mRNA and protein levels of PTEN were inversely correlated with miR-351-5p expression, which reinforces a positive connection of this miRNA to our investigated molecular connections. Interesting, the increased amount of PTEN mRNA and the miR-351-5p level facilitated the phosphorylation mechanism of PTEN, at some level. The phosphorylated protein presents a reduced biological activity [29–31], which could be considered interesting to the establishment of the pro-inflammatory environment in Ang II-treated cells. Considering that the majority of PTEN mRNA has its translational process blocked by miR-351-5p, probably, a minor fraction of PTEN mRNA still get translated. In a pro-inflammatory environment, the cellular machinery tries to minimize PTEN protein active, by activating kinases and phosphorylating the protein. This mechanism helps to control the circulating level of active PTEN protein and cooperate with the establishment of the pro-inflammatory environment in Ang II-treated cells.

In this study, we demonstrated for the first time that phosphatase and tensin homolog from *Rattus* is a novel target of miR-351-5p, and its binding activity plays a role in the pro-inflammatory processes induced by Ang II in the H9c2 cardiac cell line. The miR-351-5p binding activity collaborates with the control of the PTEN protein level, which reinforces the negative feedback loop that regulates COX-2 production through the indirect modulation of the AKT/PKBt pathway. Till now, evidences correlated the suppression of PTEN activity with its increased phosphorylation at specific residues of Ser/Thr residues (Ser380, Thr382, Thr383, and Ser385) on the PTEN C-terminus by the CK2 protein kinase [13], whose activity is modulated by COX-2 protein activity through the AKT pathway. In our study, we also demonstrated that the miRNA-315-5p inhibitor contributed to increased synthesis of PTEN and decreased levels of the P-AKT and, consequently, COX-2 protein, as well as the down-regulated production of PGE2. This fine tuning helped to amplify the Ang II modulatory effect in the pro-inflammatory environment. While many mechanisms contribute to this effect, our findings increase our understanding of the mechanism controlling the expression of COX-2 and PTEN, which are important modulatory factors in cardiovascular diseases.

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Compliance with ethical standards

Conflict of interest The authors report no conflict of interest.

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