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ORIGINAL ARTICLE

Role of G3BP1 in glucocorticoid receptor-mediated microRNA-15b and microRNA-23a biogenesis in endothelial cells

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Abstract MicroRNAs (miRNAs) are a family of non-coding RNAs that play crucial roles in regulating various normal cellular responses. Recent studies revealed that the canonical miRNA biogenesis pathway is subject to sophisticated regulation. Hormonal control of miRNA biogenesis by androgen and estrogen has been demonstrated, but the direct effects of the glucocorticoid receptor (GR) on miRNA biogenesis are unknown. This study revealed the role of GR in miRNA maturation. We showed that two GR agonists, dexamethasone and ginsenoside-Rg1 rapidly suppressed the expression of mature miR-15b, miR-23a, and miR-214 in human endothelial cells. RNA pulldown coupled with proteomic analysis identified GTPase-activating protein (SH3 domain) binding protein 1 (G3BP1) as one of the RNA-binding proteins mediating GR-regulated miRNA maturation. Activated GR induced phosphorylation of v-AKT Murine Thymoma Viral Oncogene Homologue (AKT) kinase, which in turn phosphorylated and promoted nuclear translocation of G3BP1. The nuclear G3BP1 bound to the G3BP1 consensus sequence located on primary miR-15b ~ 16-2 and miR-23a ~ 27a ~ 24-2 to inhibit their maturation. The findings from this study have advanced our understanding of the non-genomic effects of GR in the vascular system.

Keywords Glucocorticoid · GR · G3BP1 · microRNA · miRNA biogenesis

Introduction

MicroRNAs (miRNAs) are short non-coding RNA molecules of 19–25 nt in length. Over the past decade, accumulating studies have revealed miRNAs have vital role in various physiological processes. The 3′-untranslated regions (3′-UTR) of many human protein-coding genes contain miRNA binding sites and are regulated by miRNAs [1]. The regulation of gene expression by miRNA is mediated through binding to the 3′-UTR of the mRNA in a sequence-specific manner, resulting in translational repression. In the canonical miRNA maturation pathway, mono- or poly-cistronic primary miRNAs (pri-miRNAs) are first transcribed by RNA polymerase II. The pri-miRNAs in the nucleus are recognized by DiGeorge critical region gene 8 (DGCR8) and cleaved by the ribonuclease Drosha to form an intermediate precursor miRNAs (pre-miRNAs), which are then exported into the cytoplasm by nuclear transport factor exportin-5. The cytoplasmic endoribonuclease III, Dicer, cleaves the pre-miRNAs to produce ~22 nt miRNA/miRNA* duplexes. Mature miRNA is then assembled into the effector, the RNA-induced silencing complex (RISC), where it mediates mRNA decay or translational repression [2]. Mounting evidence suggest the processing of pri-miRNA can be affected by diverse signaling pathways, leading to the delicate control

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of the expression of individual miRNAs and the rapid response to extracellular stimuli [3].

Recently, increasing numbers of RNA-binding proteins (RBPs) involved in the miRNA maturation process have been identified [4]. GTPase-activating protein (SH3 domain)-binding protein 1 (G3BP1) is a ubiquitously expressed protein that has diverse functions, including RNA metabolism, signal transduction, cell cycle regulation, and even disease progression [5]. Structurally, the C-terminus of G3BP1 comprises two RNA recognition motifs (RRM) and a conserved arginine–glycine-rich box, which indicates G3BP1 is predominantly a RNA-binding protein. An earlier study showed G3BP1 specifically cleaves c-myc mRNA between CA dinucleotides, and a G3BP1 consensus binding sequence (ACCCMYMSGCMS) was also identified [6]. On the other hand, the NTF2-like domain of G3BP1 suggests that it could shuttle between the cytoplasm and nucleus through a nuclear pore complex [7]. Initial studies demonstrated G3BP1 as a signaling protein involved in downstream signaling of the Ras pathway. Subsequent studies showed that phosphorylation of G3BP1 at Ser-149 affected a number of its functions, including nuclear translocation [8], endoribonuclease activity [6], and stress granule recruitment [9]. All of these data indicate that G3BP1 may play a role in coupling signaling transduction and RNA metabolism. A recent study suggested that G3BP1 is directly involved in miRNA processing [10]. However, the regulatory mechanism of G3BP1 in miRNA biogenesis remains unclear.

Although regulation of miRNA biogenesis by hormones such as estrogen [11] and androgen [12] had been previously demonstrated, the non-genomic role of glucocorticoid and its receptor in miRNA biogenesis machinery is not yet fully understood. Ligand binding of glucocorticoid receptor (GR) can lead to a conformational change resulting in nuclear translocation and exert its effects through genomic actions (transactivation and transrepression). Also, the ligand-bound GR can interact with many effector proteins such as phosphoinositide 3-kinase (PI3K) or Raf-1 proto-oncogene (RAF) to induce downstream phosphorylation signaling pathways such as v-AKT Murine Thymoma Viral Oncogene Homologue (AKT) or Mitogen-Activated Protein Kinase (MAPK) pathways [13]. Apart from these well-studied GR-mediated phosphorylation pathways, recent evidences demonstrated that glucocorticoids affected the expression of specific mature and precursor miRNAs, but these studies did not report on the mechanism involved in their processing [14–16]. In our previous study, we showed that ginsenoside-Rg_1 (Rg_1), a natural GR agonist [17], could stimulate angiogenesis in both in vitro and in vivo models [18]. Subsequently, using miRNA microarray, we found Rg_1 could regulate the expression of subset of miRNAs [19]. Furthermore, we demonstrated several Rg_1-down-regulated miRNAs, including miR-15b, miR-23a, and miR-214 could functionally enhance the expressions of vasculoprotective proteins such as vascular endothelial growth factor receptor-2 (VEGFR-2) [20], hepatocyte growth factor receptor (MET) [21], and endothelial nitric oxide (eNOS) [19], respectively. These down-regulated miRNAs and up-regulated proteins together contributed to the angiogenesis-stimulating effect of Rg_1. However, the mechanism by which the activated GR regulates the expression of these miRNAs remains elusive.

The anti-inflammatory effects of GR have been well-studied; but the short-term cardiovascular protective functions are less recognized, and the molecular mechanism remains elusive. In this study, we identified G3BP1 as a mediator of GR-regulated miRNAs. We found GR-activated by Rg_1 or dexamethasone (Dex) could rapidly induce phosphorylation of AKT followed by G3BP1, the phosphorylated G3BP1 then translocated from the cytoplasm to the nucleus. Nuclear G3BP1 then bound to Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 containing the G3BP1 consensus binding sequence and then inhibiting their microprocessing, leading to decreased expressions of their mature miRNAs. These findings suggest that G3BP1 is a novel regulator of miRNA microprocessing, mediating the rapid effect of GR agonists on the expression of miRNAs.

Methods

Cell culture and treatments

Human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Walkersville, MD, USA) were cultured as described previously [20]. All experiments were performed on the same lot of HUVECs which were used between passages 2–8. Cells with or without transfection were treated with glucocorticoid receptor agonists, dexamethasone (Dex) (10 nM) or ginsenoside-Rg_1 [22] (Rg_1) (150 nM), prepared in DMSO.

RNA extraction and quantitation of miRNA expression

Total RNA was extracted from HUVECs using TRIzol (Invitrogen) and then treated with DNase I (Invitrogen). For primary miRNAs, RNA was converted to cDNA by the SuperScript III First-Strand Synthesis System (Invitrogen) using specific reverse primers [23]. The expression levels were then determined by real-time PCR using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Woburn, MA, USA), with specific primer sequences (primers 1–6) as reported elsewhere [23]. Quantitation of mature miRNAs

was performed by TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) [24]. All real-time PCR reactions were determined on a StepOnePlus real-time PCR system (Applied Biosystems). The relative expressions of target miRNAs were normalized to the level of U6B small nuclear RNA in the same sample.

Small interfering RNA (siRNA) transfection

HUVECs at 70% confluence were transiently transfected with siRNA (50 nM; Applied Biosystems). The cells were incubated with RNA molecules complexed to Lipofectamine RNAiMAX transfection reagent (Invitrogen) in Opti-MEM for 24 h. After transfection, cells were rinsed with Opti-MEM followed by the GR agonists treatment as appropriate. Non-targeting siRNA (Applied Biosystems) was used in parallel with the gene-specific siRNA.

Biotinylated RNA pulldown assay

RNA-binding proteins involved in the biogenesis of specific miRNAs were identified by biotinylated RNA pulldown assay [25]. Nuclear lysate (60 μg) was incubated with purified 3′-biotinylated oligonucleotide (1 μg) complementary to the specific 5′-flanking sequence of the target primary miRNAs (primers 7–9) in binding buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 0.2 U RNaseOUT, and 0.05% protease inhibitor) at 4 °C. After 30 min incubation at room temperature, NeutrAvidin Agarose Resin (20 μl) (ThermoScientific) was added and allowed to bind at 4 °C overnight under constant shaking. After washing the reaction mixture with binding buffer, the pulldown materials were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane for immunoblot analysis.

iTRAQ labeling and mass spectrometric analysis of miRNA-binding proteins

Protein samples obtained from the RNA pulldown assay were used for protein extraction and peptide preparation for iTRAQ labeling and mass spectrometric analysis according to our previous study [26].

Protein extraction and immunoblot analysis

Immunoblot analysis was performed on protein extracts as described previously [19]. Whole cell lysate was extracted with lysis buffer (Novagen, Madison, WI, USA) containing protease (0.5%, v/v) and phosphatase inhibitor cocktails (0.5%, v/v; Calbiochem, San Diego, CA, USA). The nuclear extract was prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocol. The protein concentration of the cell lysates was determined by detergent-compatible DC protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated by 8–12% SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was soaked in blocking buffer (1% non-fat milk in TBS-T) and incubated with the primary antibody at 4 °C overnight. The washed membrane was then incubated with horseradish peroxidase-conjugated goat-anti-rabbit or mouse IgG secondary antibody (Invitrogen), and immunoreactive bands were visualized using SuperSignal West Pico Kit (Thermo Scientific). Anti-G3BP1 (1:1000, v/v; BD Transduction Laboratories, Franklin Lakes, NJ, USA), anti-phospho-G3BP1 (1:500, v/v; pSer-149; Sigma-Aldrich), anti-hnRNPA1 (1:1000, v/v; R196; Cell Signaling), anti-hnRNPK (1:1000, v/v; R332; Cell Signaling), and anti-Lamin A/C (1:2000, v/v; H-110; Santa Cruz) were used in the analysis.

Immunofluorescence microscopy

Immunofluorescence microscopy was carried out as described previously [27]. Briefly, HUVECs (1×10^4) were plated onto a glass coverslip in a 24-well plate overnight. After treatment for the indicated time, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature, respectively. The fixed cells were incubated with primary anti-G3BP1 antibody (H-10, 1:200, v/v; Santa Cruz) at 4 °C overnight. The coverslip was washed with PBS and incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:250, v/v; Invitrogen) at room temperature for 2 h. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 0.5 μg/ml). The coverslip was washed and mounted on a slide using DAKO fluorescence mounting medium (Carpinteria, CA, USA). Fluorescence images were acquired with an Olympus Fluoview FV1000 (Olympus) confocal laser-scanning microscope equipped with a 60×/1.35-NA oil immersion objective lens (Tokyo, Japan). The xy frame was set to 512 × 512 pixels and laser intensity was set to 10% power.

RNA immunoprecipitation (RIP)

Cell lysate (400 μg) was extracted for immunoprecipitation assay using a Pierce Co-immunoprecipitation Kit (Thermo Scientific) according to manufacturer's protocol. Anti-G3BP1 antibody (2 μg, H-10; Santa Cruz) was used to capture G3BP1 with mouse IgG used as the control. RNA was recovered from the immunoprecipitate by phenol–chloroform extraction, and subjected to reverse

transcription and real-time PCR. Protein was extracted from the immunoprecipitate was subjected to immunoblot analysis.

RNA electrophoretic mobility shift assay (RNA EMSA)

The DNA template for in vitro transcription was amplified by PCR from genomic DNA of HUVECs using a primer containing a T7 promoter sequence on the 5′-region (primers 10–11). *In vitro* transcription of primary miRNA substrates were synthesized using MEGAscript T7 Transcription Kit (Ambion) and then biotin-labeled by RNA End Biotinylation Kit (Thermo Scientific). The labeled pri-miRNAs (1 μg) were incubated with recombinant G3BP1 (Abcam, Cambridge, MD, USA) in binding buffer (10 mM HEPES, 20 mM KCl, 1 mM $MgCl_2$, 1 mM DTT; pH 7.3) at room temperature for 30 min. The samples were loaded onto a 12% native polyacrylamide gel for electrophoresis and transferred onto Hybond-N^+ positively charged nylon membrane (GE Healthcare Life Sciences, Piscataway, NJ, USA) followed by UV crosslinking of RNA on the membrane. The membrane was blocked with blocking reagent (Roche) and incubated with streptavidin-horseradish peroxidase conjugate (1:100, v/v) at room temperature for 20 min. After a thorough washing with PBS, the chemiluminescent signal was visualized using a SuperSignal West Pico Kit (Thermo Scientific).

In silico prediction of putative G3BP1 on Pri-miR-15b~16-2 and Pri-miR-23a~27a~24-2

The RNA sequences of Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 were aligned with the G3BP1 consensus binding sequence (ACCCMYMSGCMS), using Global Alignment of BLAST which employed the Needleman–Wunsch alignment. The secondary structure of the pri-miRNAs was predicted by Mfold using default conditions [28] (Supplementary figure 2).

In vivo pri-miRNA processing assay

The activity of primary miRNA processing was monitored by a dual-luciferase reporter system [29]. Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 were amplified from genomic DNA (primers 12–15). The PCR products were digested with *Sac*I and *Hin*dIII and then inserted into the 3′-UTR of the firefly luciferase in the pMir-Report Luciferase vector (Ambion). The stability and the amount of firefly luciferase will be inversely proportional to the intracellular Drosha activity. A mutation vector containing mutated Pri-miR-15b ~ 16-2 or Pri-miR-23a ~ 27a ~ 24-2 sequences was generated using a QuickChange Lighting Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with specific mutagenic primers (primers 16 and 17). The sequences of the insert were validated by Sanger sequencing (Supplementary figure 1). The recombinant vector and the Renilla luciferase vector pRL-TK (Promega) were co-transfected into COS-7 cells. After transfection for 24 h, the cells were incubated with GR agonists for another 24 h. After the cells were lysed with a passive reporter lysis buffer, luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) using an Infinite F200 (Tecan, Männedorf, Switzerland) microplate luminometer. The relative Drosha activity was calculated by the formula: relative microprocessing activity = $1/[(\text{firefly luciferase activity/Renilla luciferase activity})_{\text{treatment}}/(\text{firefly luciferase activity/Renilla luciferase activity})_{\text{control}}]$.

In vitro angiogenesis assays

Cell proliferation, cell migration, and tube-like structure formation assays were performed on HUVECs as described previously [30].

Statistical analysis

All results were expressed as the mean ± standard derivation (SD) of at least three independent experiments. Student's *t* test was used to compare two means. One-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test was performed for multiple comparisons. A p value <0.05 was considered to be significant.

Results

GR agonists rapidly inhibited the expression of mature miR-15b, miR-23a, and miR-214

To investigate the non-genomic effect of GR agonists on the expression of those miRNAs which have been functionally validated in our previous studies, the expression of primary and mature miR-15b, miR-23a, and miR-214 from HUVECs (human umbilical vein endothelial cells) treated with GR agonists Rg_1 or Dex were examined by real-time RT-PCR (Fig. 1a). Treatment with Rg_1 or Dex for 1 h reduced the expressions of mature miR-15b, miR-23a, and miR-214 to between 40 and 60%, but no significant changes were found in the expression of their primary forms. The non-genomic actions of GR can occur in minutes, while the genomic actions arise after few hours. To determine the mode of action of the GR agonists on those miRNAs, we have examined the expressions of the primary

and mature form of the miR-15b, miR-23a, and miR-214 at 15 min, 30 min, 1 h, and 24 h. The GR agonists had no significant effects on the primary miRNAs, but caused the expression levels of mature miRNAs to be rapidly down-regulated in a time-dependent manner (Fig. 1b). To confirm that the decrease of miRNAs was dependent on the activation of GRα, we knocked down the expression of GRα by transfection with a GR-specific siRNA. The suppressive effects of GR agonists on mature miRNAs were completely abolished by GR-specific siRNA (Fig. 1c). These data strongly suggest that GR activation regulates a subset of miRNAs in a posttranscriptional manner.

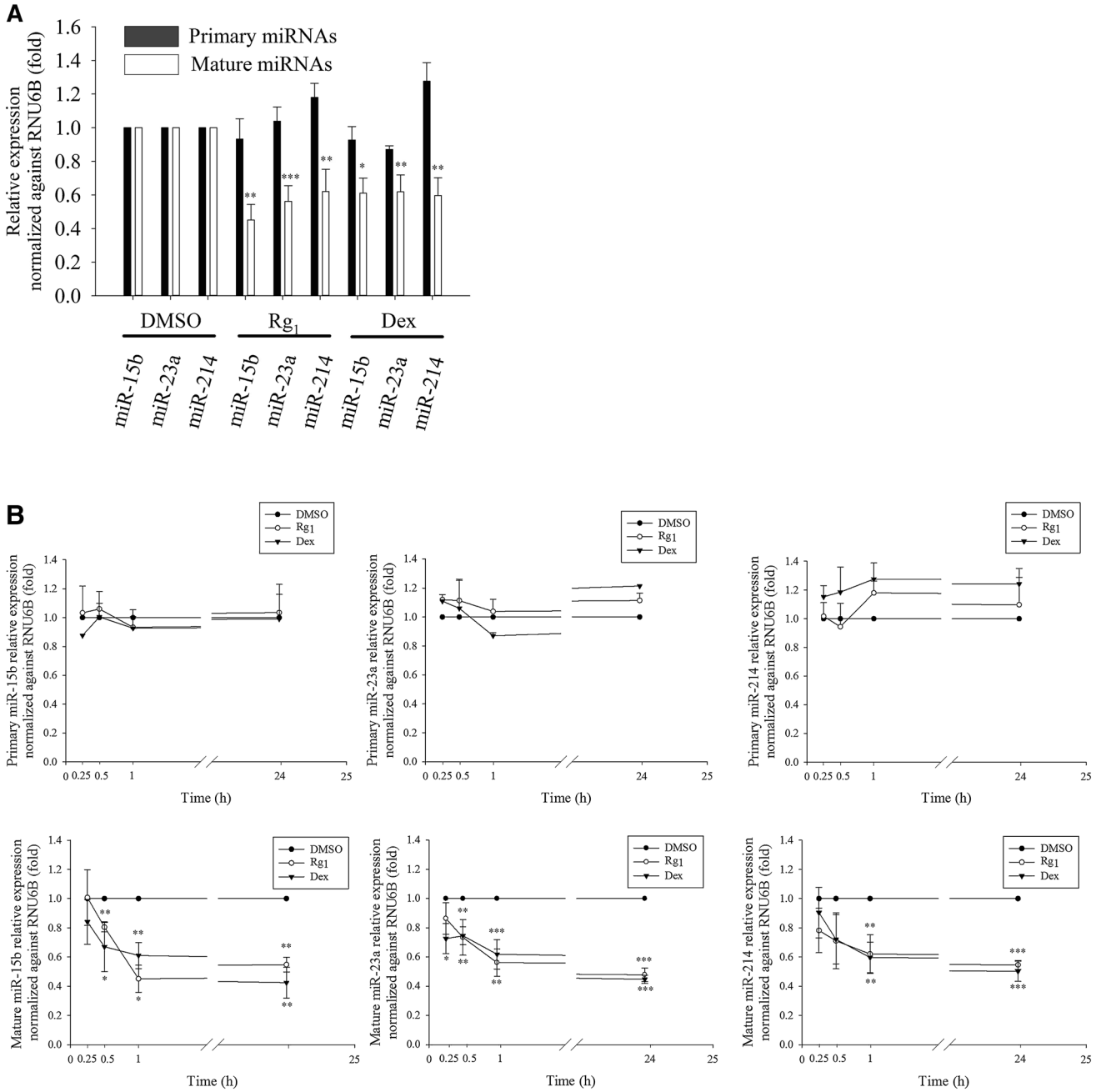


Fig. 1 GR agonists rapidly inhibited the expression of mature miR-15b, miR-23a, and miR-214. **a** HUVECs (passage 2–7) were treated with Rg_1 (150 nM) or Dex (10 nM) for 1 h. **b** HUVECs (passage 2–7) were treated with Rg_1 (150 nM) or Dex (10 nM) for the indicated time (15 min to 24 h). **c** HUVECs (passage 2–7) transfected with control siRNA or GR-specific siRNA were treated with Rg_1 (150 nM) or Dex (10 nM) for 1 h. The expressions of primary and mature miRNAs were detected by SYBR qRT-PCR and TaqMan microRNA assay, respectively. Knockdown efficiency of GRα in HUVECs was determined by immunoblotting (upper left panel). Values are presented as the mean ± SD of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. DMSO vehicle control, $n = 6$

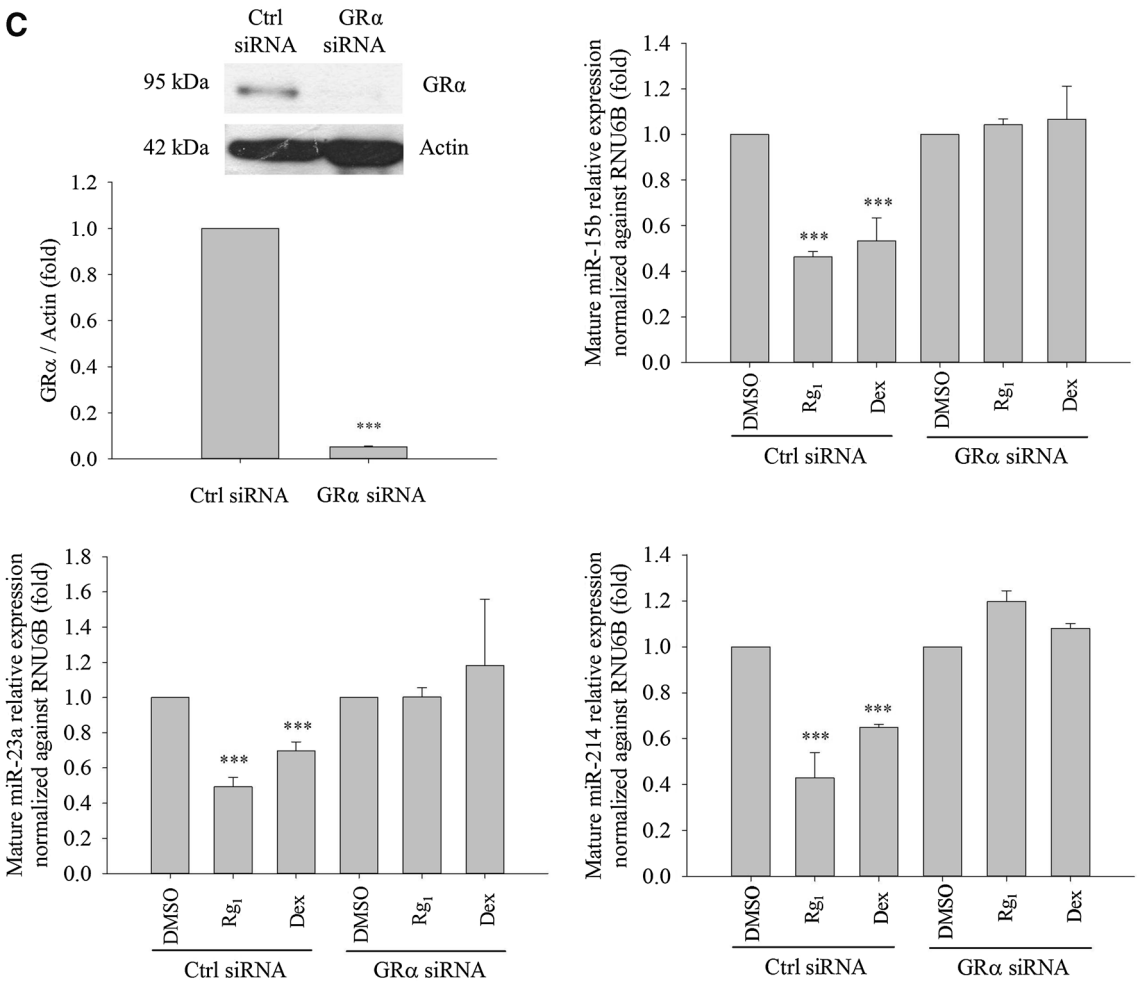


Fig. 1 continued

GR agonists suppressed the expression of a subset of miRNAs by recruiting RNA-binding proteins

To identify the proteins which mediate the effects of GR on miRNA biogenesis, we carried out the RNA pulldown assay with biotinylated Pri-miR-23a∼27a∼24-2. The putative RBPs on the captured Pri-miR-23a∼27a∼24-2 cluster complex were further examined by iTRAQ-based quantitative proteomic analysis. Table 1 shows the proteins that were found to be increased in association with the Pri-miR-23a∼27a∼24-2 cluster. We identified four proteins namely: hnRNPA1, hnRNPK, and PCBP2 (hnRNPE2) that belong to the heterogeneous nuclear ribonucleoprotein (hnRNP) family, and G3BP1, a heterogeneous nuclear RNA-binding protein. To confirm the results of the proteomic analysis, immunoblotting coupled with a RNA

Table 1 Identification of regulatory RNA-binding proteins that mediate GR-inhibited miRNA expressions

Gene name	Gene ID	Protein name	Rg_1/Ctrl (fold)	Dex/Ctrl (fold)
HNRNPA1	3178	Heterogeneous nuclear ribonucleoprotein A1	2.045	2.879
HNRNPK	3190	Heterogeneous nuclear ribonucleoprotein K	7.353	5.718
PCB2 (HNRNPE2)	5094	Poly(rC) binding protein 2	4.809	2.838
G3BP1	10146	GTPase-activating protein (SH3 domain) binding protein 1	6.498	7.129

HUVECs (passage 3) were treated with Rg_1 (150 nM) or Dex (10 nM) for 1 h. RNA pulldown assays were carried out on 60 μg of nuclear lysate with 1 μg of biotinylated RNA oligonucleotide complementary to the 5′-flanking sequence of the primary miRNAs. Bound proteins in the pulldown materials were analyzed by iTRAQ labeling and mass spectrometry. Fold changes were determined by comparing the treatment group with the DMSO vehicle control

pulldown assay was performed on Pri-miR-15b∼16-2, Pri-miR-23a∼27a∼24-2, and pri-miR-214 (Fig. 2a). Similar to the proteomic analysis, the immunoblot analysis showed Pri-miR-15b∼16-2 and Pri-miR-23a∼27a∼24-2 had increased associations with hnRNPA1, hnRNPK, PCBP2, and G3BP1. However, no increased binding was seen between pri-miR-214 and these proteins, which indicated the biogenesis of miR-214 might be regulated by a distinct mechanism, so subsequently we focused on only miR-15b and miR-23a. The hnRNPs are RNA binding proteins that can directly associate with RNA and influence their metabolism. The effects of hnRNPs, especially hnRNPA1, on miRNA regulation have been reported previously [31]. However, the regulatory mechanism of G3BP1 on miRNA processing is not clear. To verify that the binding of G3BP1 to the Pri-miR15b∼16-2 and Pri-miR-23a∼27a∼24-2 clusters was mediated via activation of GR, cells were pre-treated with a GR antagonist RU486 before addition of the GR agonists. The RNA pulldown assay confirmed the association of G3BP1 with the target miRNAs was dependent on GR activation (Fig. 2b). Knockdown of G3BP1 by specific siRNAs partially reversed the reduced expression of mature miR-15b, and completely reversed downregulated miR-23a that resulted from the action of the GR agonists (Fig. 2c). This confirmed G3BP1 had a role in decreasing the expression of mature miR-15b and miR-23a. As we have previously demonstrated, Rg_1 increased VEGFR2 and MET protein expressions by suppressing miR-15b and miR-23a, respectively. The knockdown of G3BP1 could also abolish the increased VEGFR2 and

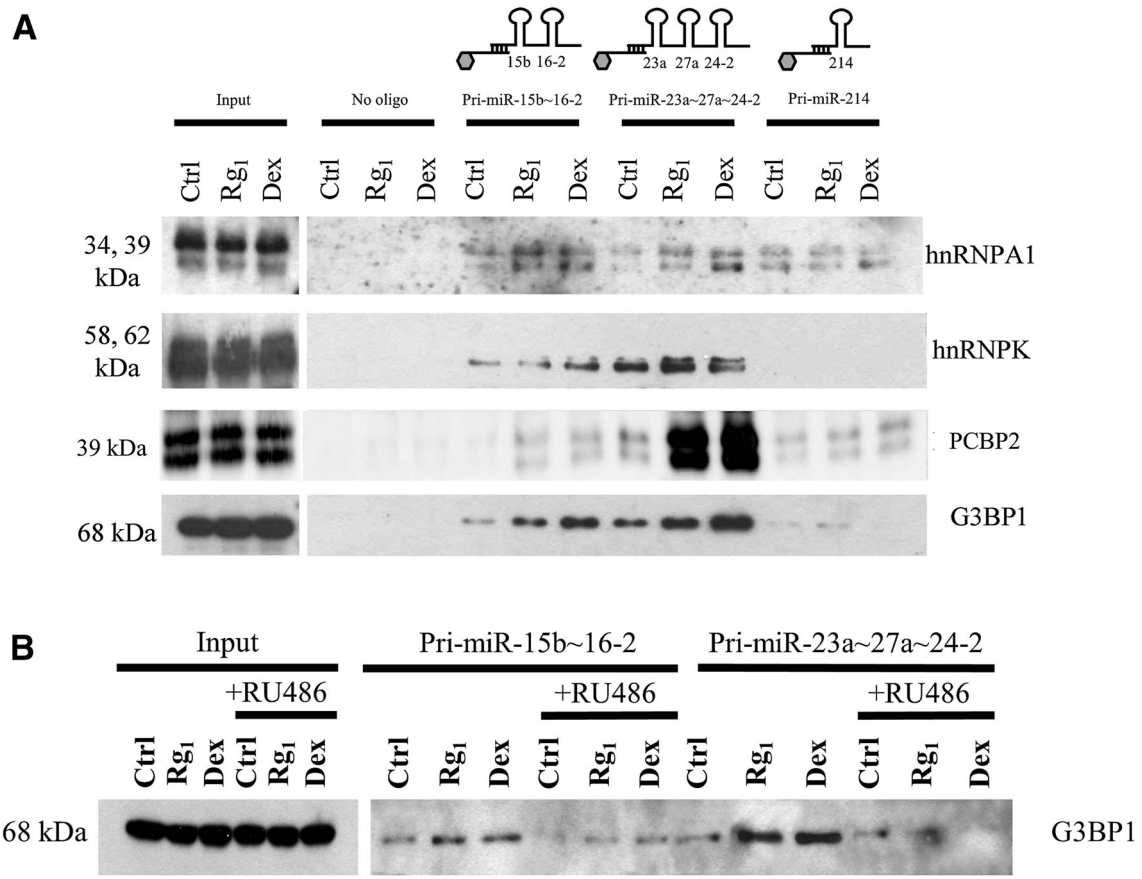


Fig. 2 Identification of regulatory RNA-binding proteins that mediated GR-inhibited miRNA expressions. **a** HUVECs (passage 5–8) were treated with Rg_1 (150 nM) or Dex (10 nM) for 1 h. RNA pulldown assays were carried out on 60 μg of nuclear lysate with 1 μg of biotinylated RNA oligonucleotide complementary to the 5′-flanking sequence of the primary miRNAs. Total bound proteins in the pulldown materials were analyzed by immunoblotting with specific antibodies. Nuclear lysate (60 μg) without RNA pulldown was used as input for loading control, $n = 4$. **b** HUVECs (passage 5–7) were pre-treated with a GR antagonist RU486 (10 μM), the GR antagonist, for 1 h before Rg_1 (150 nM) or Dex (10 nM) treatment. RNA pulldown assays were performed as described above, $n = 3$. **c** HUVECs (passage 5–7) transfected with control siRNA or G3BP1-specific siRNA were treated with Rg_1 (150 nM) or Dex (10 nM) for 1 h. The expressions of mature miRNAs were detected by TaqMan microRNA assay. Knockdown efficiency of G3BP1 in HUVECs was determined by immunoblotting (*upper left panel*), $n = 3$. **d** The protein expressions of MET and VEGFR-2 in HUVECs (passage 6–8) after knockdown of G3BP1 were determined by immunoblotting. The image shown in the *upper panel* is representative of three independent experiments. Semi-quantitative analysis of three independent experiments is shown in the *lower panel*. Values are presented as the mean ± SD of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. DMSO vehicle control, $n = 3$

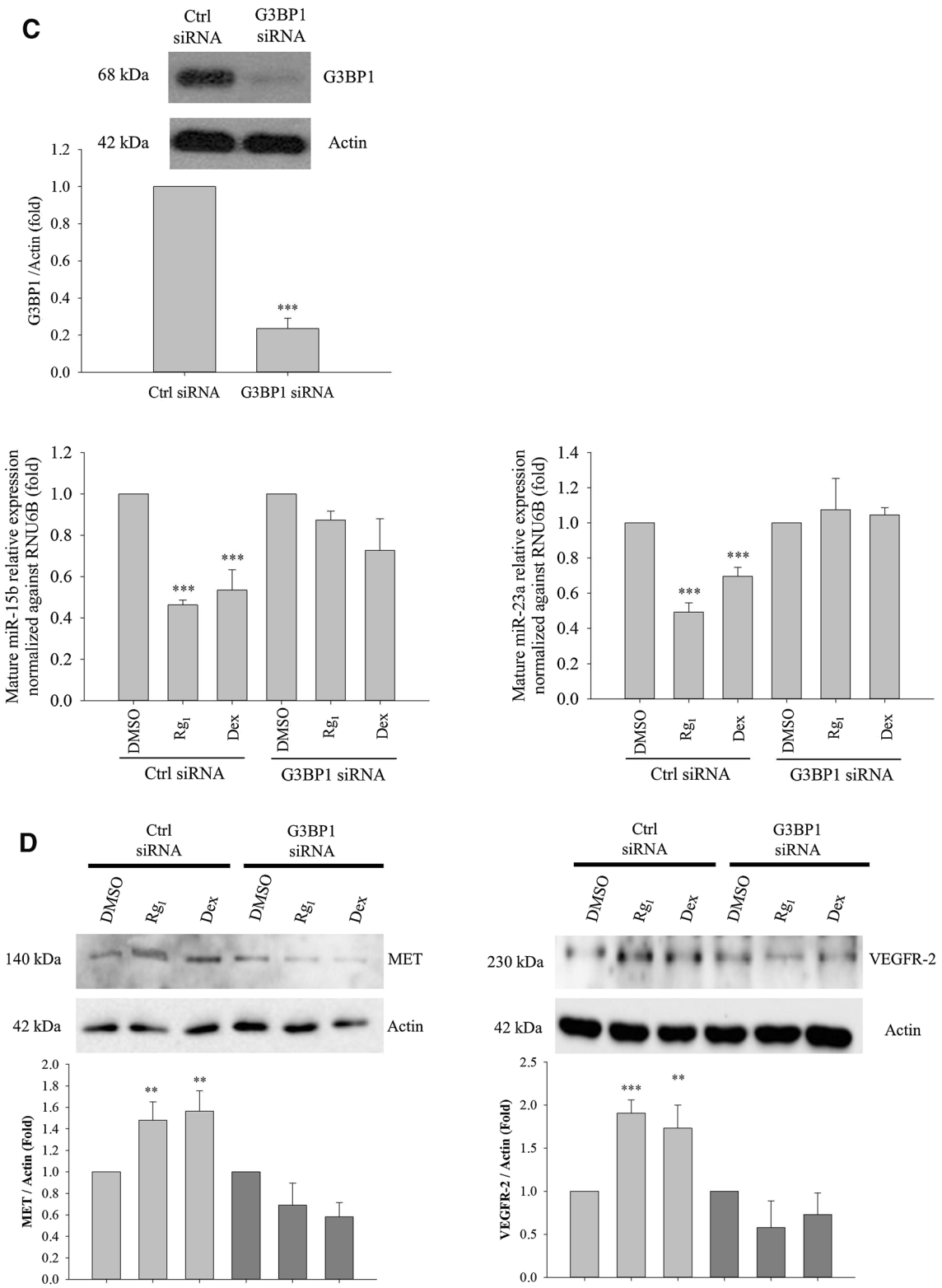


Fig. 2 continued

MET protein expressions induced by the GR agonists (Fig. 2d).

G3BP1 phosphorylation is induced by GR agonists, and its nuclear translocation is AKT-dependent

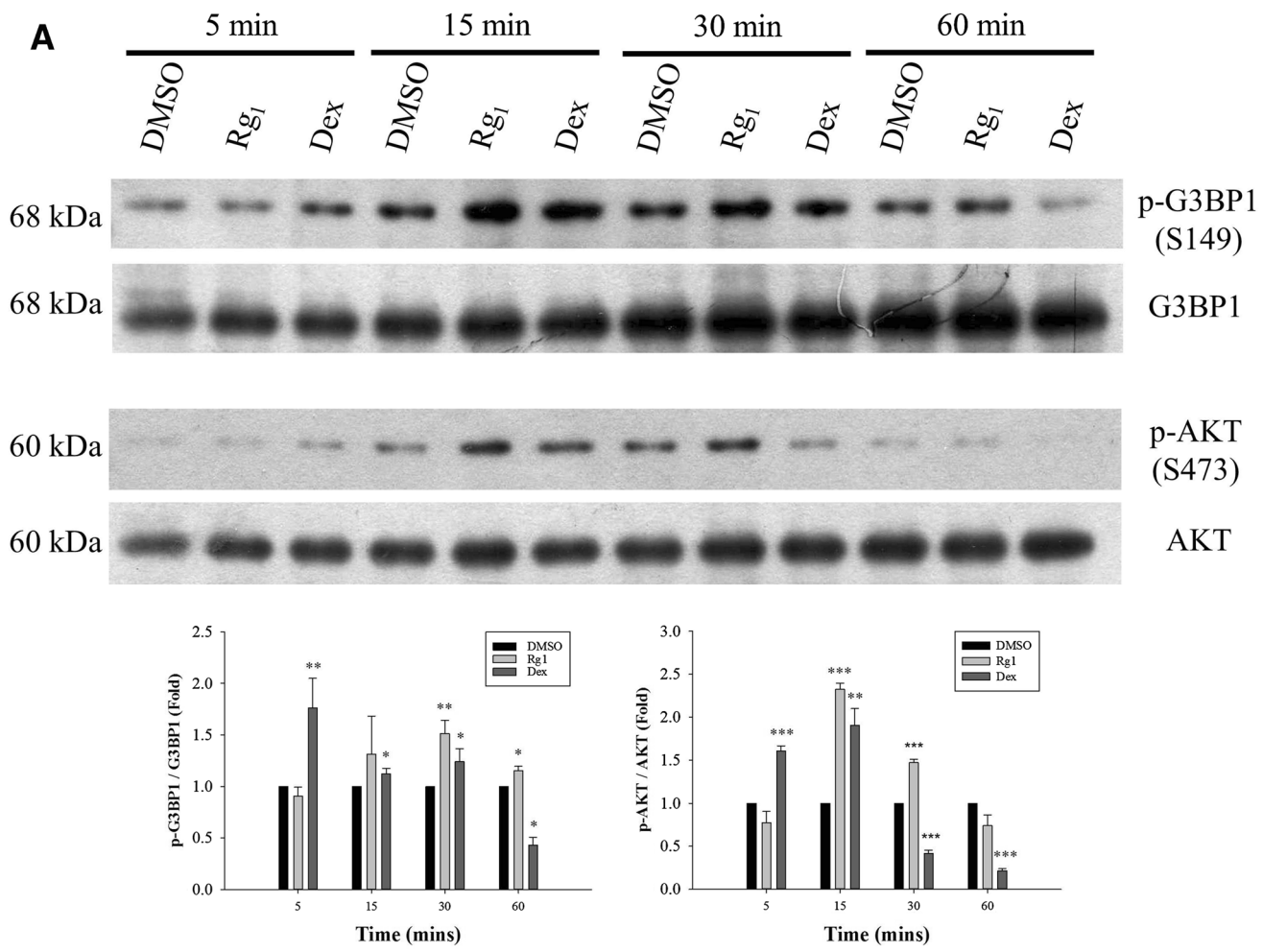
We investigated how GR agonists induced G3BP1 translocation from the cytoplasm to the nucleus. Under ambient conditions, G3BP1 is primarily present in the cytoplasm, but can translocate to the nucleus when specifically induced. It has been demonstrated that nuclear translocation of G3BP1 was dependent on phosphorylation at Ser-149 [8], but the upstream pathway which mediates its nuclear translocation is unknown. The immunoblot analysis demonstrated that GR agonists rapidly induced phosphorylation of G3BP1 at Ser-149 within 5–30 min (Fig. 3a). We also found concomitant rapid phosphorylation of AKT. Subcellular fractionation of total protein extracts showed that phosphorylation of G3BP1 is increased after treatment with the GR agonists (Fig. 3b). Confocal imaging further demonstrated that the GR agonists rapidly increased G3BP1 (green fluorescence) accumulation in the nucleus (Fig. 3c). We previously demonstrated the angiogenesis-stimulating effects of Rg_1 were mediated through the activation of the AKT pathway [17]. To test whether G3BP1 Ser-149 phosphorylation was also mediated through the AKT pathway, cells were pre-treated with an AKT-specific inhibitor FPA124 before addition of the GR agonists. Pre-treatment with FPA124 fully prevented phosphorylation of G3BP1 by GR agonists (Fig. 3d).

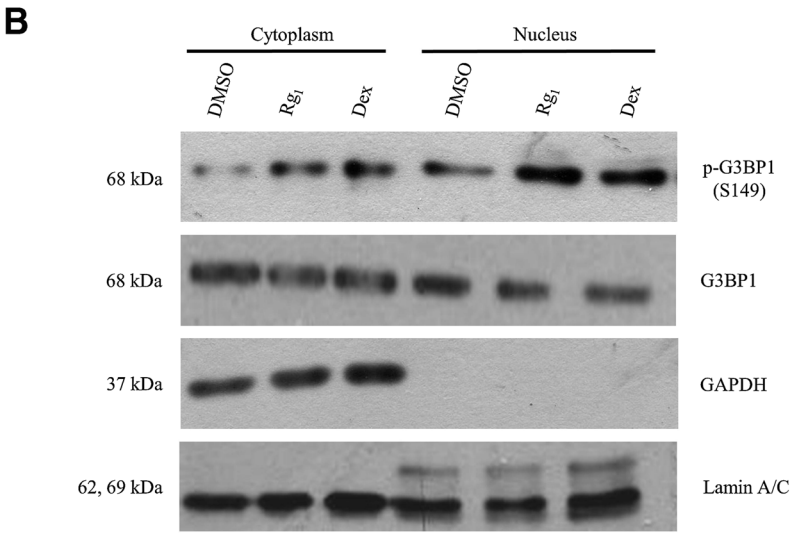
GR agonist-induced binding of G3BP1 to Pri-miR-15b~16-2 and Pri-miR-23a~27a~24-2

It has been shown that G3BP1 can interact with the specific RNA consensus sequence (ACCCMYMSGCMS) and exclusively cleaves between CA dinucleotides [6]. To investigate if G3BP1 binds onto primary miRNAs containing this consensus sequence, the G3BP1 consensus binding sequence was aligned with the sequence of Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 clusters (Fig. 4a). In the Pri-miR-15b ~ 16-2 cluster, one putative binding site was found at the beginning of pre-miR-16-2. The folded RNA structures are shown in Fig. 4b. Two putative binding sites were found on Pri-miR-23a ~ 27a ~ 24-2, one was located just at the beginning of pre-miR-23a, and the other was located at the end of pre-miR-27a. This demonstrated the binding region of G3BP1 and the recognition site of the microprocessor were proximal. To investigate the association of G3BP1 with Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 after treatment with GR agonists, we immunoprecipitated the RNA–protein complex by G3BP1-specific antibody and analyzed the bound RNAs by RT-PCR. We found increased amounts of Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 were associated with G3BP1 in the GR agonist-treated cells (Fig. 4c). No significant associations were found with pri-miR-214, which confirmed that pri-miR-214 might be regulated by a distinct mechanism. We further examined the direct binding of G3BP1 to pri-miR15b and Pri-miR-23a ~ 27a ~ 24-2 by RNA EMSA using full-length recombinant G3BP1 (rec G3BP1) (Fig. 4d). Consistent with the RIP results, rec G3BP1 was found to be associated with in vitro transcribed Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2. Taken together, these results demonstrated G3BP1 physically interacts with Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2.

GR agonists inhibited microprocessing of Pri-miR-15b~16-2 and Pri-miR-23a~27a~24-2 via G3BP1

Binding of RBPs to primary miRNAs can exert either an inhibitory or stimulatory effect on the microprocessing. To determine the effects of G3BP1 on Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 microprocessing, an in vivo pri-miRNA processing assay was performed using a reporter vector [29] with wild-type or mutated Pri-miR-15b ~ 16-2 or Pri-miR-23a ~ 27a ~ 24-2 constructs (Fig. 5a). GR agonists transiently inhibited the microprocessing of both Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 after 0.25–1 h, which recovered after 24 h (Fig. 5b, upper panel). Mutation of the putative G3BP1 binding regions on both Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 abolished the inhibitory effects of GR agonists on the microprocessing (Fig. 5b, lower panel). To further confirm the microprocessing activity is not affected by the altered secondary structure of the mutated primary miRNAs, we compared the microprocessing activity and the level of mature miRNAs expressed from wild-type or mutant construct (Supplementary figure 3). No significant differences are found between wild-type or mutant constructs, which indicated that the Drosha-DGCR8 activity is not compromised. Knockdown of GRα and G3BP1 prevented the inhibitory effects resulting from the addition of GR agonists (Fig. 5c). These findings indicated that activated GR could rapidly regulate the microprocessing of Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 via G3BP1, and the inhibition was dependent on the specific RNA sequence in the pri-miRs.


A
5 min
15 min
30 min
60 min
DMSO
Rg1
Dex
68 kDa
p-G3BP1 (S149)
G3BP1
60 kDa
p-AKT (S473)
AKT
p-G3BP1 / G3BP1 (Fold)
p-AKT / AKT (Fold)
Time (mins)


B
Cytoplasm
Nucleus
DMSO
Rg1
Dex
68 kDa
p-G3BP1 (S149)
G3BP1
37 kDa
GAPDH
62, 69 kDa
Lamin A/C

◀**Fig. 3** GR agonists induced phosphorylation and nuclear translocation of G3BP1 in an AKT-dependent manner. **a** HUVECs (passage 4–6) were treated with Rg_1 (150 nM) or Dex (10 nM) for the indicated time. Phosphorylation and total G3BP1 or AKT were detected by immunoblotting with specific antibodies. The image shown in the *upper panel* is representative of three independent experiments. Semi-quantitative analysis of three independent experiments is shown in the *lower panel*. **b** HUVECs (passage 4–6) were treated with Rg_1 (150 nM) or Dex (10 nM) for 1 h. Cytoplasmic and nuclear extracts were prepared as described in the "Methods". Phosphorylation of G3BP1 (Ser-149) and total G3BP1 were detected by immunoblotting with specific antibodies. GAPDH and Lamin A/C were used as the cytoplasmic and nuclear markers, respectively. **c** HUVECs (passage 5–7) were immunofluorescence stained with anti-G3BP1 antibody (*green*) after Rg_1 (150 nM) or Dex (10 nM) treatment for 5, 15 or 30 min. DAPI (*blue*) was used to indicate the nucleus. **d** HUVECs (passage 4–6) were pre-treated with a GR antagonist RU486 (10 μM) or an AKT inhibitor FPA124 (1 μM) for 1 h before Rg_1 (150 nM) or Dex (10 nM) treatment for 1 h. Phosphorylation and total G3BP1 or AKT were detected by immunoblotting with specific antibodies. The image shown is representative of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. DMSO vehicle control, $n = 3$

Discussion

We previously studied the effects of the GR agonist Rg_1 [17] on miRNAs expressions and their respective angiogenesis-stimulating protein targets [19–21]. However, the mechanism by which activated GR regulates miRNA expression was not elucidated. In this study, we first confirmed that GR agonists Rg_1 and Dex could rapidly downregulate the expressions of miR-15b, miR-23a, and miR-214 in endothelial cells. Next, the potential RBPs involved in the biogenesis of the target miRNAs were identified by miRNA pulldown assay and iTRAQ-based mass spectrometric analysis. We then investigate the role of G3BP1 with these identified RBPs on the miRNA maturation process. Furthermore, we showed that G3BP1 could bind onto specific regions of primary miRNAs to inhibit their microprocessing, resulting in decreased expressions of the mature miRNAs.

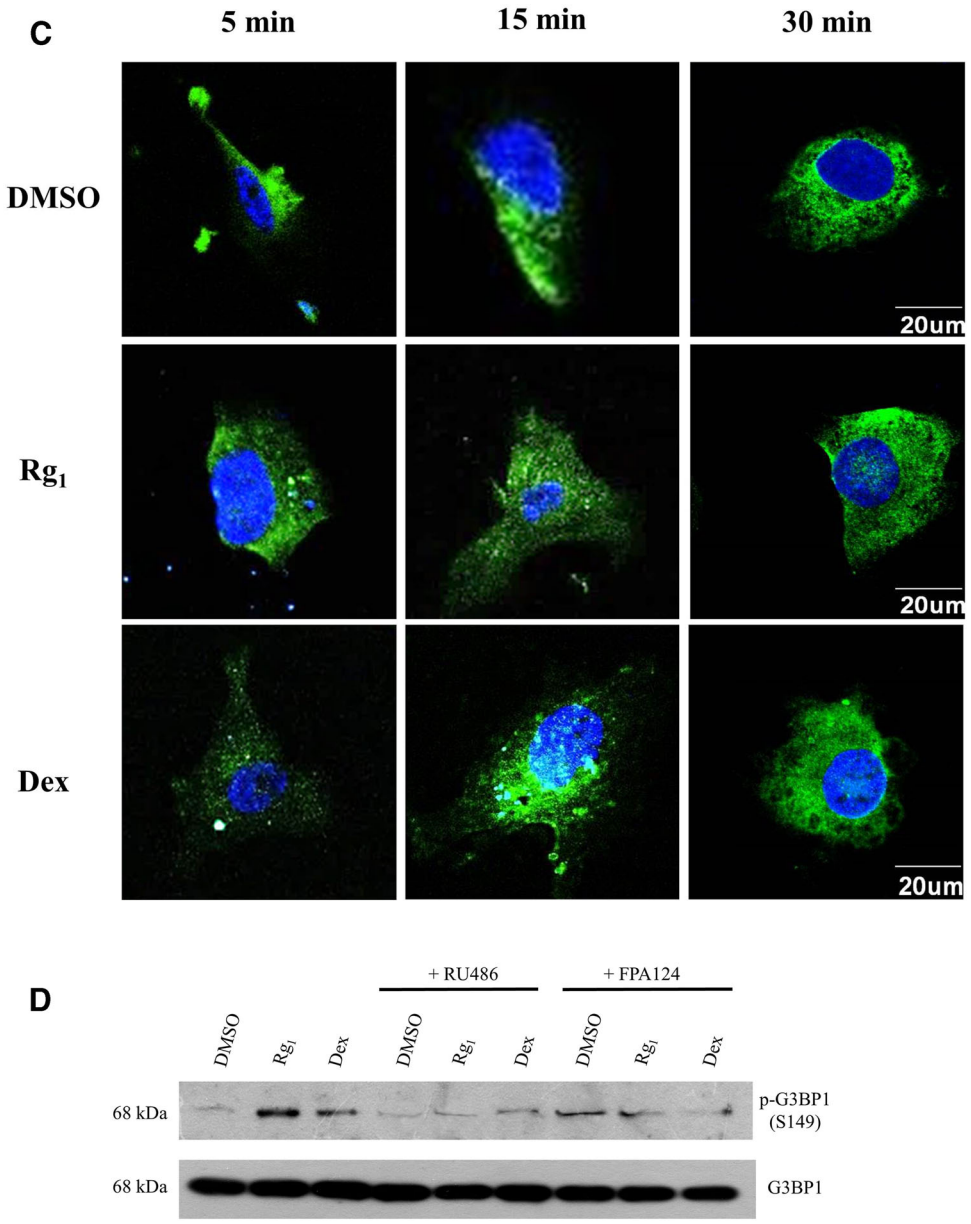
The canonical miRNA maturation pathway is dependent on diverse regulatory mechanisms. Although studies have indicated GR has effects on the expressions of different miRNAs [14, 16], few studies addressed the direct regulatory mechanism of GR on miRNA biogenesis. The rapid effects of GR agonists on the target mature miRNAs suggest a possible posttranscriptional mechanism for the role of GR in miRNA maturation. As GR has been suggested to function as an RBP [32, 33], it is plausible to speculate that the activated GR may bind directly onto primary transcripts of target miRNAs. However, in our pilot RIP experiment, we found no direct association between GR and miR-15b, miR-23a, or miR-214. Using proteomic analysis, we then further explored the putative intermediate players involved in GR-regulated miRNA biogenesis. Several RBPs were found to associate with the target primary miRNAs including, hnRNPA1, hnRNPK, PCBP2, and G3BP1. Notably, hnRNPA1 could bind to the conserved terminal loop of Pri-let-7a-1 and inhibited its processing by Drosha [34]. It was also demonstrated that hnRNPA1 can influence the processing of various primary miRNAs in different ways according to the RNA sequences and structural variations [31]. The role of hnRNPK on miRNA biogenesis was less clear. A study showed that it could inhibit miR-16 binding to the 3′-UTR of COX-2 mRNA [35]. Meanwhile, PCBP2 could interact with Dicer and promoted the processing of miRNA precursor [36]. Intriguingly, knockdown of G3BP1 could completely reverse the expression of GR-suppressed miRNAs, which implies that the inhibition of these RBPs on primary miRNAs may mutually dependent. However, more studies are required to dissect its interaction with different RBPs and the combined effects on primary miRNAs processing.

The multi-functional roles of G3BP1 include transducing diverse extracellular stimulations into specific gene expressions. Recently, G3BP1 was reported to play a key role in the formation of stress granules [9, 37]. Theoretically, the formation of stress granules under cellular stress can preserve energy and lead to translational repression. However, the findings from this study show an opposite scenario has been shown in this study. We found nuclear G3BP1 inhibited miRNA processing, which subsequently relieved the translational inhibition of particular miRNAs to allow some specific proteins to be translated. This suggests the function of G3BP1 is dependent on its subcellular localization. We demonstrated that GR-activated AKT could induce phosphorylation of G3BP1 on Ser-149, resulting in its nuclear translocation. Indeed, the secondary structure of the primary miRNAs is critical for the processing of the primary miRNAs by various RBPs. In the Pri-miR-23a∼27a∼24-2 cluster, the G3BP1 binding site overlaps with the recognition region of the DiGeorge critical region gene 8 (DGCR8) on both miR-23a and miR-27a, DGCR8 is an essential microprocessing protein. The G3BP1 may compete with the Drosha complex for the same binding region on the primary miRNAs, resulting in a decreased microprocessing. In the Pri-miR-15b∼6-2 cluster, the G3BP1 binding site was located near the DGCR8 recognition region of miR-16-2 rather than on miR-15b. However, the secondary structure revealed that the DGCR8 binding sites of miR-15b and miR-16-2 were close together. The binding of G3BP1 on miR-16-2 may prevent interaction with the microprocessing complex. Although physical interactions of G3BP1 with miR-15b and miR-23a have been shown, we did not detect transient accumulation of Pri-miR-15b∼16-2 and Pri-miR-23a∼27a∼24-2 upon GR agonists treatment. This could

be explained by the endonuclease activity of G3BP1 reported in the previous study [37]. Indeed, the function of G3BP1 during miRNA biogenesis was unknown until very recently. He et al. demonstrated that inhibition of endogenous G3bp1 resulted in an increased levels of mature miR-1 and restricted endothelin1-induced cardiomyocyte hypertrophy [10]. In their study, although the consensus sequence of G3BP1 was also found on pre-miR-1, pre-miR-1-2 could not be co-precipitated with G3bp1 due to technical problems. Nevertheless, our findings strongly support the hypothesis that G3BP1 is a negative regulator of miRNA biogenesis. Although our data showed that the binding of G3BP1 and its subsequent effects on miRNA processing was sequence-specific, how the

Fig. 4 GR agonists induced binding of G3BP1 to Pri-miR-15b ~ 16-2 ▶ and Pri-miR-23a ~ 27a ~ 24-2. **a** G3BP1 consensus binding sequence. Putative binding sites of G3BP1 on the Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 are *underlined* and sequences of the precursor miRNAs are *highlighted*, the possible G3BP1 cleavage sites are indicated by *red arrows*. **b** The secondary structures of Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 clusters. *Red boxes* indicate the putative binding sites of G3BP1 and *arrows* indicate the Drosha cleavage points. Computed secondary structures of Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2 were predicted by Mfold as described in "Methods". **c** HUVECs (passage 3–5) were treated with Rg_1 (150 nM) or Dex (10 nM) for 1 h. RIP was performed with G3BP1 or non-specific IgG antibody, followed by SYBR qRT-PCR for Pri-miR-15b ~ 16-2 and Pri-miR-23a ~ 27a ~ 24-2. Values are presented as the mean ± SD of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. DMSO vehicle control, $n = 3$. **d** RNA EMSA was performed with increasing amount of recG3BP1 (0–20 ng) with biotin-labeled Pri-miR-15b ~ 16-2 or Pri-miR-23a ~ 27a ~ 24-2 (1 μg), $n = 3$

Fig. 3 continued



A **G3BP1 Consensus Binding Sequence: ACCCMYMSGCMS**

Pri-miR-15b~16-2

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  1 GAUUUUUAAU UUUACAGGUA AGUUUAUUAA AGACUUCAAA GAUUCUCUUA UUCUUGUUAC
 61 UUUUUUUUCU AUAAAGCUAG GUUGGAUGAA UCCUACAUUU UUGAGGCCUU AAAGUACUGU   Pre-miR-15b
121 AGCAGCACAU CAUGGUUUAC AUGCUACAGU CAAGAUGCGA AUCAUUAUUU GCUGCUCUAG
181 AAAUUUAAGG AAAUUCAUUC AAAACUAUGU UUUCAUCAUC AGAUGUUCGU UUUAUGUUUG
241 GAUGAACUGA CAUACUUGUU CCACUCUAGC AGCACGUAAA UAUUGGCGUA GUGAAAUAUA   Pre-miR-16-2
301 UAUUAAACAC CAAUAUUACU GUGCUGCUUU AGUGUGACAG GGAUACAGCA ACUAUUUUAU
361 CAAUUGUUUG UAUUUCCCUU UAAGGUAACA UUUUAAAUGA AAUGUAUUAU AUUUUAAUCU
421 AUCCUUUUCC UUUGUUUU
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Pri-miR-23a~27a~24-2

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  1 GUGUCCCCAA AUCUCAUUAC CUCCUUUGCU CUCUCUCUCU UUCUCCCCUC CAGGUGCCAG
 61 CCUCUGGCCC CGCCCGGUGC CCCCCUCACC CCUGUGCCAC GGCCGCCUGG GGUUCCUGGG   Pre-miR-23a
121 GAUGGGAUUU GCUUCCUGUC ACAAAUCACA UUGCCAGGGA UUUCCAACCG ACCCUGAGCU
181 CUGCCACCGA GGAUGCUGCC CGGGGACGGG GUGGCAGAGA GGCCCCGAAG CCUGUGCCUG
241 GCCUGAGGAG CAGGGCUUAG CUGCUUGUGA GCAGGGUCCA CACCAAGUCG UGUUCACAGU   Pre-miR-27a
301 GGCUAAGUUC CGCCCCCCAG GCCCUCACCU CCUCUGGCCU UGCCGCCUGU CCCCUGCUGC
361 CGCCUGUCUG CCUGCCAUCC UGCUGCCUGG CCUCCCUGGG CUCUGCCUCC CGUGCCUACU   Pre-miR-24-2
421 GAGCUGAAAC ACAGUUGGUU UGUGUACACU GGCUCAGUUC AGCAGGAACA GGGGUCAAGC
481 CCCCUUGGAG CCUGCAGCCC CUGCCUUCCC UGGGUGGGCU GAUGCUUGGA GCAGAGAUGA
541 GGACUCAGAA UCAGACCUGU GUCUGGAGGA
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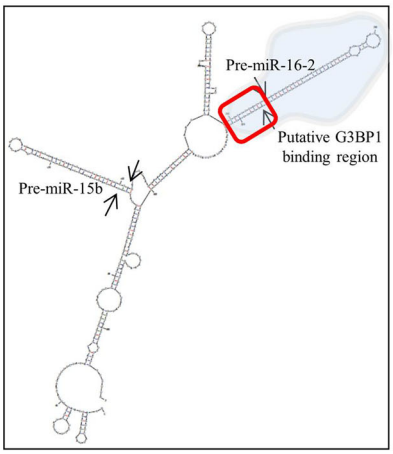
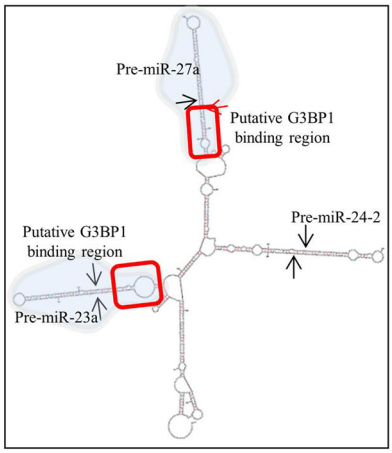
B

Pri-miR-15b~16-2



Pri-miR-23a~27a~24-2



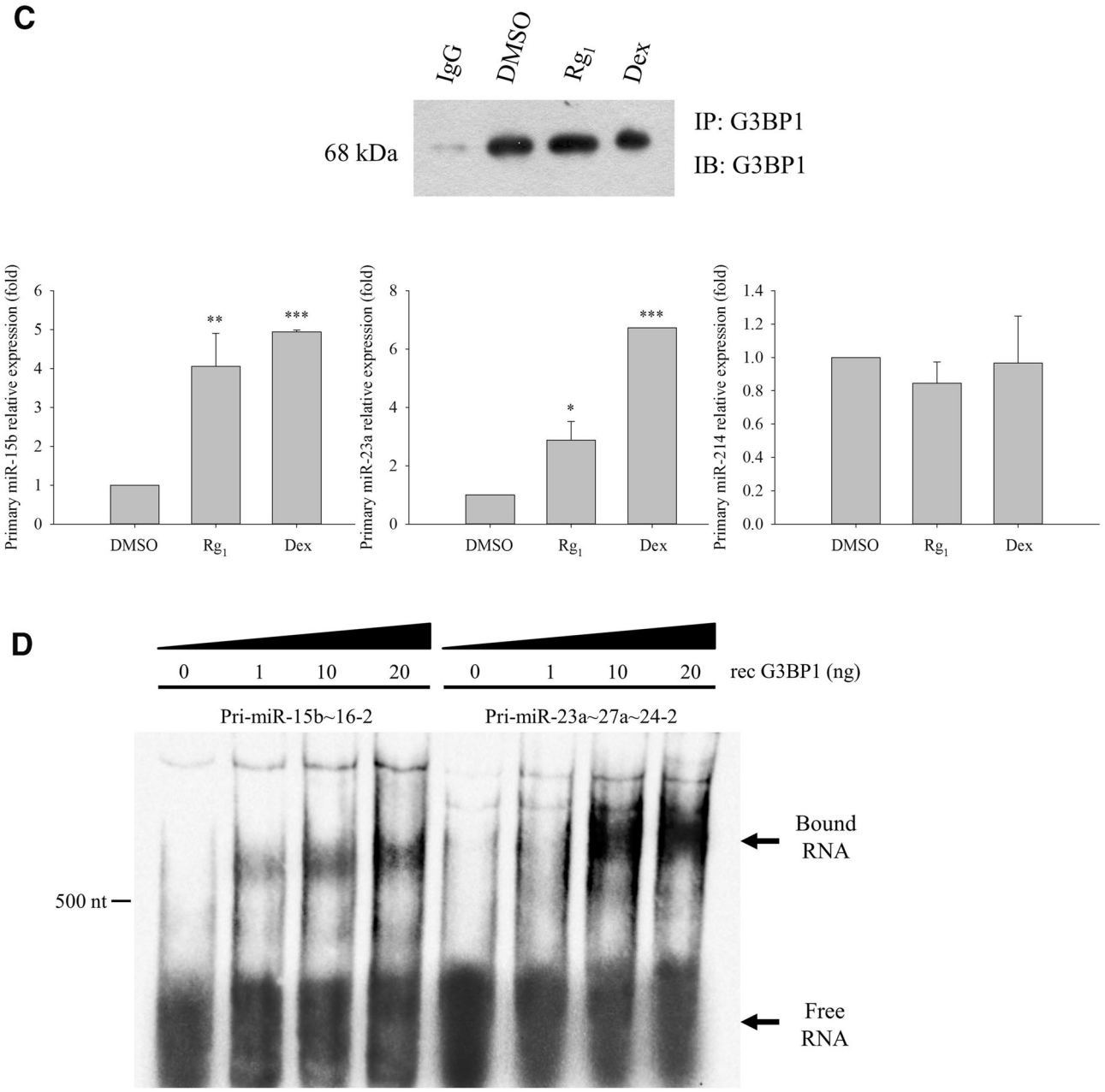


Fig. 4 continued

phosphorylation-dependent endoribonuclease activity of G3BP1 affects the microprocessing of primary miRNAs warrants further study. Though the microprocessing activities have been down-regulated by GR agonist, the levels of the three primary miRNAs remain unchanged. Possible explanation is that primary miRNAs are subject to various posttranscriptional regulations such as RNA editing or alternative splicing. It has been suggested that the partially double-stranded RNA structure of primary miRNA may be a target of adenosine deaminases acting on RNA (ADARs) [38]. In our case, the unprocessed primary miRNAs may be further regulated by such unexplored posttranscriptional mechanisms.

Glucocorticoids are potent anti-inflammatory agents used in standard treatments for dermatitis, allergies, asthma, or other autoimmune diseases. Many studies have also reported on the rapid cardiovascular protective effects of glucocorticoids [39–41]. However, concerns

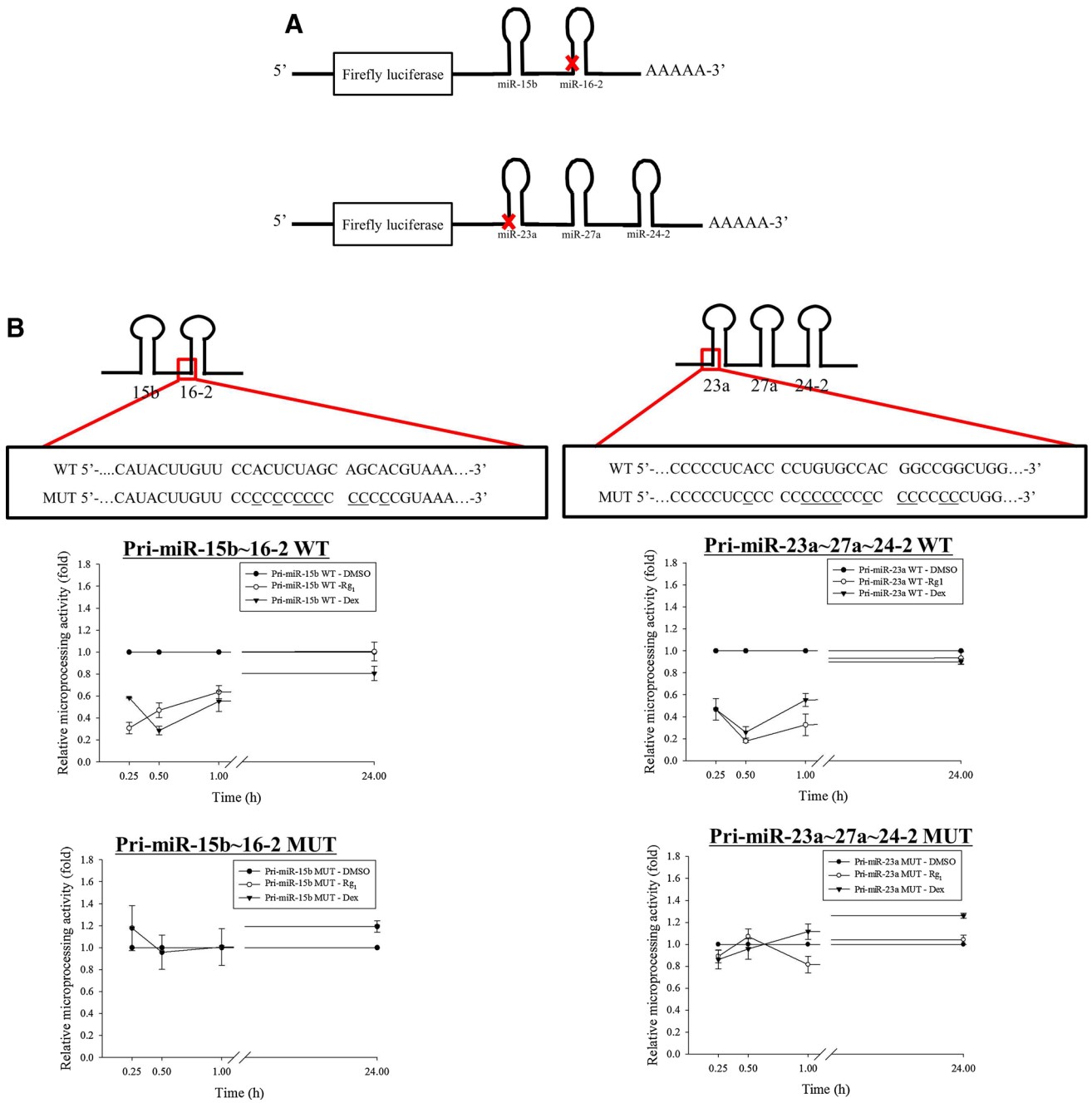


Fig. 5 GR agonists inhibited the microprocessing of Pri-miR-15b∼16-2 and Pri-miR-23a∼27a∼24-2. **a** Pri-miR-15b∼16-2 or Pri-miR-23a∼27a∼24-2 was cloned into the 3′-UTR of the luciferase reporter vector. The *red crosses* indicate the sites of mutation on the vector. **b** The microprocessing activity was determined by an in vivo pri-miRNA processing assay. COS-7 cells were transfected with wild-type (*upper panel*) or mutated (*lower panel*) luciferase vector harboring sequences of the Pri-miR-15b∼16-2 or Pri-miR-23a∼27a∼24-2 clusters. After transfection for 24 h, cells were treated with Rg_1 (150 nM) or Dex (10 nM) for another 24 h, $n = 3$. **c** COS-7 cells were co-transfected with control siRNA, GR-specific siRNA or G3BP1-specific siRNA. After transfection for 24 h, the cells were treated with Rg_1 (150 nM) or Dex (10 nM) for another 24 h. Relative microprocessing activity was determined as described in the "Methods". Knockdown efficiency of GRα and G3BP1 in COS-7 cells were determined by immunoblotting (*upper panel*). Values are presented as the mean ± SD of three independent experiments. ***$p < 0.001$ vs. DMSO vehicle control, $n = 3$

have been raised about the propensity of chronic administration of glucocorticoids for hyperglycemia, hypertension, and dyslipidemia [42]. It is generally recognized that the genomic action of classic glucocorticoids contributes to both beneficial clinical effects and side effects. Nevertheless, interest in the development of novel glucocorticoids that selectively activate the non-genomic mechanism of GR [41] is

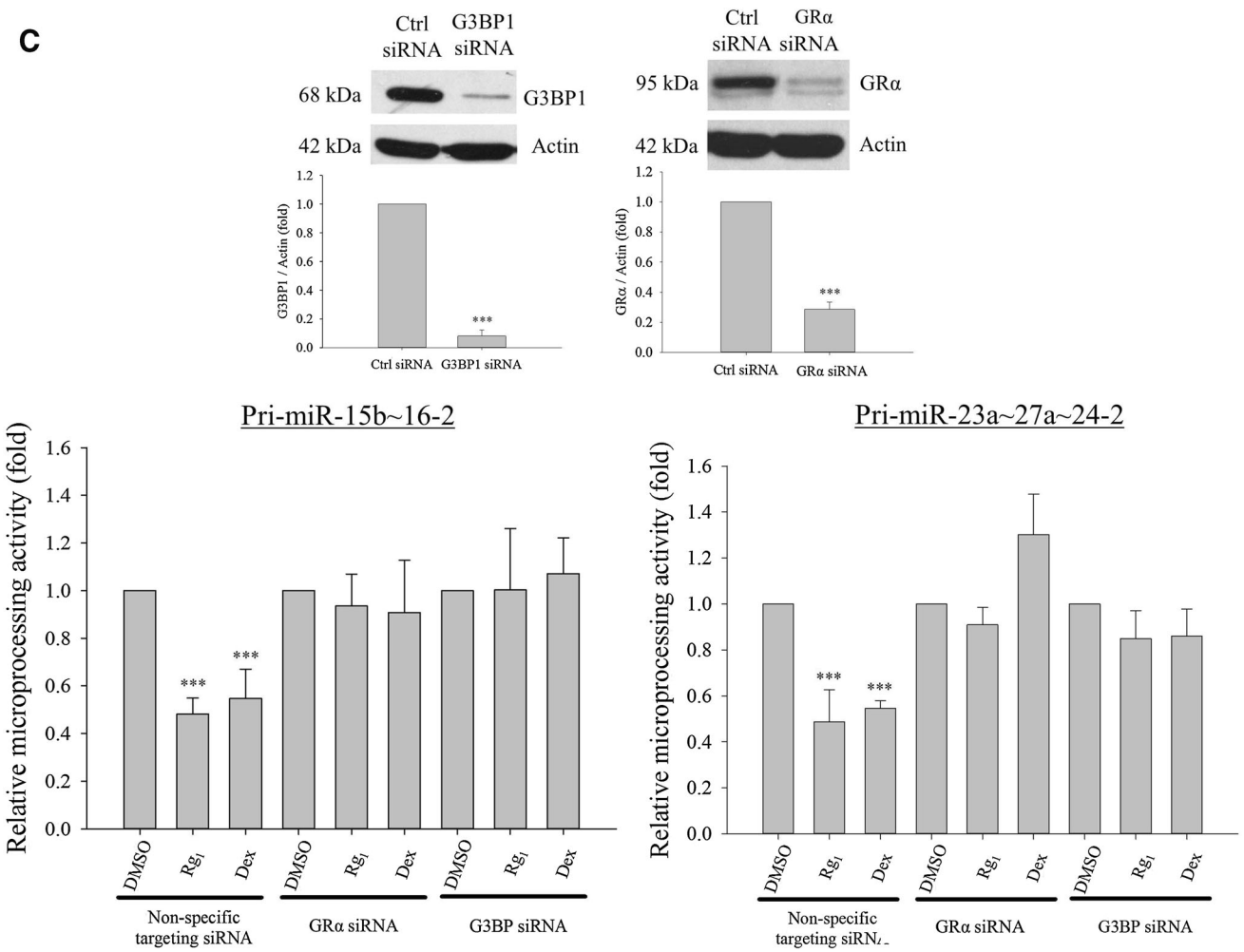


Fig. 5 continued

growing. Such novel glucocorticoids should produce similar therapeutic effects, but have reduced systemic adverse effects. In short term, glucocorticoids inhibited leucocyte–endothelial interactions in the vasculature through rapid non-genomic pathways in the cardiovascular system [43]. Glucocorticoids also protected the myocardium from experimental ischaemic injury [44]. In endothelial cells, treatment with Dex stimulated eNOS activity via a non-transcriptional pathway [40]. In this study, we have revealed another layer of regulatory mechanism on target genes by activated GR, through its rapid action on miRNAs biogenesis. The rapid effects of GR agonists on miRNAs and protein could support the use of corticosteroids for acute cardiovascular protective effects.

In conclusion, the present study revealed that GR has rapid non-genomic effects on miRNA biogenesis through the action of G3BP1. This study highlighted a previously uncharacterized mechanism for GR regulation of the miRNA biogenesis machinery and the rapid modulation of

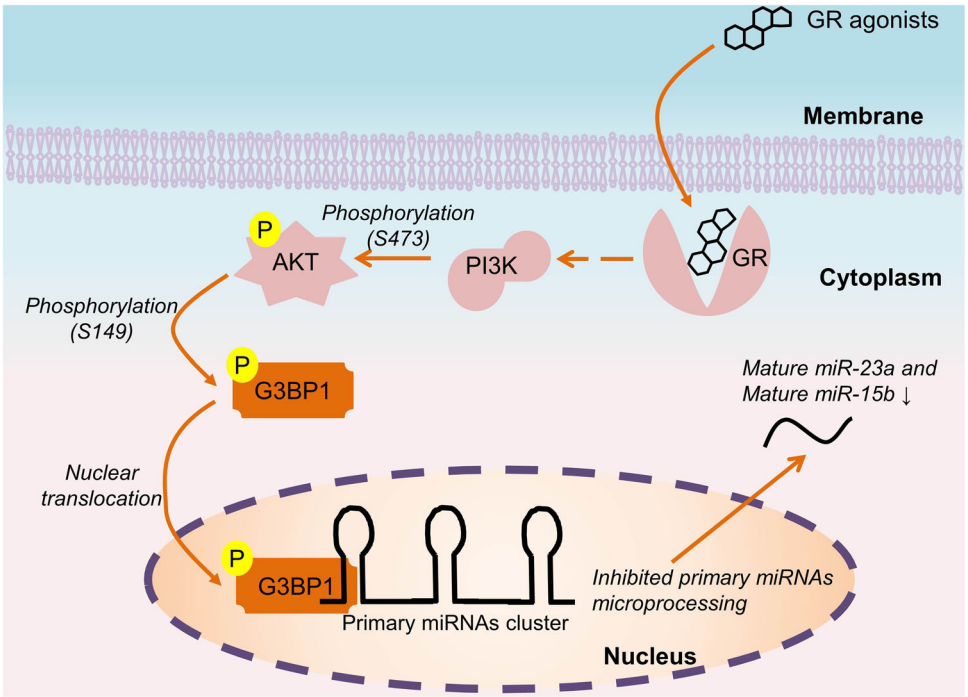


Fig. 6 Proposed mechanism of action for G3BP1-regulated miRNAs biogenesis. Ligand-activated GR induced phosphorylation of AKT and subsequently G3BP1 at serine sites. The phosphorylated G3BP1 translocated from the cytoplasm to nucleus and bound to primary miRNAs harboring the G3BP1 consensus sequence. The binding of G3BP1 inhibited the microprocessing of primary miRNAs and reduced the expressions of their mature forms

the expression of vasculoprotective proteins (Fig. 6). Further study on the interaction of different RBPs is needed to further advance our understanding of the mechanism of GR-regulated miRNA biogenesis.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no competing interests.

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