Original Paper

Phospholipid Scramblase 1 Functionally Interacts with Angiogenin and Regulates Angiogenin-Enhanced rRNA Transcription

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Key Words

Angiogenin • Phospholipid scramblase 1 • Ribosome RNA transcription • Protein-protein interaction

Abstract

Background: Angiogenin (ANG) can translocate to the target cell nucleus and accumulate in the nucleolus to enhance rRNA transcription, thus promoting cell proliferation. However, the regulation of ANG-enhanced rRNA transcription remains unknown. Previously we identified phospholipid scramblase 1 (PLSCR1) as a potential ANG-interacting protein in yeast two-hybrid screening. **Methods:** The interaction was re-confirmed in yeast cells and further verified by *in vitro* pull down, *in vivo* co-immunoprecipitation (Co-IP), fluorescent resonance energy transfer (FRET) and immunofluorescence analyses. The rRNA transcription level was determined by real-time quantitative PCR and Northern blot. **Results:** PLSCR1 was identified as a novel ANG-interacting protein. Notably, PLSCR1 interacted with ANG in the cell nucleus and regulated rRNA transcription. Furthermore, depletion of cellular ANG expression abolished PLSCR1-enhanced rRNA transcription, which could be rescued by exogenous ANG. **Conclusion:** Our data suggest that PLSCR1 positively regulates rRNA transcription through interacting with ANG, thus deepening our understanding on rRNA transcription regulation.

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Introduction

Angiogenin (ANG) is a 14-kDa protein originally isolated from the conditioned medium of HT-29 human colon adenocarcinoma cells [1]. It exerts its angiogenic function by activating vessel endothelial cells and promoting a series of cellular processes, including cell migration, invasion, proliferation, and formation of tubular structures [2, 3]. Besides its angiogenic function, ANG could directly stimulate cancer cell proliferation [4, 5]. Recently, ANG has been found to be expressed in motor neurons, and its loss of function mutations are associated with amyotrophic lateral sclerosis and Parkinson's disease [6-10], indicating a neuroprotective role of this protein. A number of cell types have so far been reported as ANG's targeting cells, such as vessel endothelial and smooth muscle cells, tumor cells, and motor neurons [3, 5, 7].

Accumulating evidences indicate that the function of ANG on cell growth and survival is related with its nuclear activity in enhancing rRNA transcription [11]. ANG could translocate to the nucleus of its target cells where it accumulates in the nucleolus, binds to the promoter region of ribosomal DNA (rDNA), and stimulates rRNA transcription. It has been reported that ANG-mediated rRNA synthesis is critical for cell proliferation and the maintenance of normal physiological function of motor neurons [11, 12]. However, the molecular mechanisms underlying the ANG-enhanced rRNA transcription and its regulation remain elusive.

Protein-protein interactions play key roles in essential every biological process. To looking for protein partners that may involve in ANG-enhanced rRNA transcription, we performed a yeast two-hybrid screen using ANG as bait, and identified phospholipid scramblase 1 (PLSCR1) as a potential ANG binding protein. In this study, we confirmed the authenticity of the interaction between PLSCR1 and ANG both *in vitro* and *in vivo*. Our further study revealed that the interaction happened in the nucleus of HeLa cells and positively regulated rRNA transcription.

Materials and Methods

Plasmid construction

ANG cloning and pDBLeu-ANG bait generation for yeast two-hybrid analysis were described previously [13]. The obtained prey, pEXPAD502-PLSCR1, was used as the origin of *PLSCR1* gene. The full-length *PLSCR1* was cloned into the *Eco* RI/*Not* I sites of pET-42a-c(+) (Novagen, Darmstadt, Germany) to produce pET-PLSCR1 for expressing GST-fused protein and subsequently used for the GST-pull-down assay. To generate a construct for hemagglutinin (HA)-tagged protein expression, the *PLSCR1* gene was subcloned into the *Sal* I/*Not* I sites of pCI-neo mammalian expression vector (Promega, Madison, WI, USA) which had been pre-inserted a 3×HA tag into the *Eco* RI/*Xba* I sites. For fluorescent resonance energy transfer (FRET) assay, the plasmids pYFP-ANG and pCFP-PLSCR1 were constructed to express yellow fluorescent protein (YFP) fused ANG and cyan fluorescent protein (CFP) tagged PLSCR1 respectively following the same procedure as described in [14]. All the constructs were sequenced to verify their reading frames.

The ANG RNA interference (RNAi) cassette containing plasmid pAng-RNAi, the empty vector pBS/U6 and pBabe-puro were described previously [15]. The siRNA for PLSCR1 interference was synthesized by the GenePharma Co. (Shanghai, China) with a target sequence 5'-AGT CTC CTC AGG AAA TCT G-3' according to the original report [16].

Yeast two-hybrid assay

The PROQUEST™ Two-Hybrid System (Invitrogen, Grand Island, NY, USA) was used to confirm the interaction between ANG and PLSCR1 according to the manufacturer's instruction. Briefly, *S.cerevisiae* strain MaV203 was co-transformed with the bait plasmid pDBLeu-ANG and the prey plasmid pEXPAD502-PLSCR1, and selected on selective drop-out (SD) plates lacking histidine, leucine and tryptophan (SD-His-Leu-Trp). 3-Aminotriazole (50 mM) was added to inhibit *HIS3* leakiness. After incubated at 30 °C for 7 days, the positive colonies were subjected to the X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside)

assay. Yeast control strains (a-e), which represent none interaction (a), weak interaction (b), moderately strong interaction (c), strong interaction (d), and very strong interaction (e) respectively, were applied to the X-gal analysis simultaneously (Fig. 1A). Clones showing blue color within 12h were designated as positive clones.

Western blotting

The protein samples were resolved by 12% SDS-PAGE, and then transferred to a PVDF (Millipore, Billerica, MA, USA) by semi-dry transfer in Towbin's buffer (20% methanol, 25 mM Tris, 192 mM glycine), blocked with 5% skim milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST). All primary and secondary antibodies were diluted in 5% skim milk powder in TBST. The primary antibodies included anti-ANG polyclonal antibody (home-made), anti-PLSCR1 monoclonal antibody IE9, anti-fibrillarin monoclonal antibody C13C3, anti-HA monoclonal antibody 6E2, anti-beta-tubulin polyclonal antibody 9F3 and anti-alpha-tubulin monoclonal antibody 11H10 (Cell Signaling Technology, Danvers, MA, USA). Detected proteins were visualized using enhanced chemiluminescence (Pierce, Rockford, IL, USA).

GST-pull-down assay

The pET-PLSCR1 was transformed into *E. coli* BL21 (DE3) for bacterial expression of the GST chimeric protein. The bacteria were induced with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) for 12 h at 20 °C, harvested and then sonicated in PBS buffer containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) and DTT (5 mM). Lysate containing GST or GST-PLSCR1 fusion protein was pre-cleared by ultra-centrifugation and batch-bound overnight to GSH-agarose (Amersham Biosciences, Pittsburgh, PA, USA). Then the beads containing about 0.1 μ g of GST or GST-PLSCR1 chimeric protein were incubated with 0.5 μ g of ANG in a binding buffer (20 mM Tris, 250 mM NaCl, 1 mM EDTA, 0.5% NP-40) at 4 °C for 6h. The beads were washed, and the bead-bound proteins were subjected to SDS-PAGE and Western blotting.

Cell culture, transfection and nuclear translocation of ANG

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected at 50-60% confluency using the lipofectamineTM 2000 reagent (Invitrogen) in accordance with the manufacturer's protocol. ANG stable knocking-down HeLa cells were generated according to Tsuji's protocol [5], and the interference efficiency was determined in both mRNA and protein levels by using quantitative RT-PCR and immunoblotting approaches respectively. For nuclear translocation of exogenous ANG, HeLa cells were incubated with 0.5 μ g/ml of ANG at 37 °C and monitored by indirect immunofluorescence assay.

Immunofluorescence assay

To visualize the nuclear localizations of ANG and/or PLSCR1, HeLa cells were grown on glass coverslips and fixed with pre-chilled methanol (10 min, -20 °C). After blocking for 1h in 1% BSA, the fixed cells were incubated with 30 μ g/mL of anti-ANG monoclonal antibody 26-2F (provided by Dr. Guo-fu Hu at Tufts University) and/or anti-PLSCR1 polyclonal antibody (Abnova, Taipei, China) with 1:100 dilution for 1h, washed, and incubated with FITC-conjugated goat anti-mouse and TRITC-conjugated goat anti-rabbit secondary antibody at 1:100 dilution for 1h. Anti-fibrillarin antibody was used to stain the nucleolus. Slides were mounted in mounting solution (50% glycerol) and observed under a confocal microscope (Zeiss, Hamburg, Germany).

In vivo co-immunoprecipitation assay

The total cell lysate was extracted with lysis buffer (1% Triton X-100, 100 mM NaCl, 100 mM Tris pH 7.5, 0.5 mM EDTA, 10% glycerol, and protease inhibitor cocktail), and cleared by centrifugation at 16,000 *g* for 30 min at 4 °C. Protein concentration was determined using a modified Bradford protocol.

To obtain the nuclear lysate preparation, the cells were washed twice with ice-cold PBS and resuspended in Chelsky Buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂, 30 mM Sucrose, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) containing complete protease inhibitor mixture (Roche Applied Science). The suspension was incubated on ice for 10 min before adding 10% Triton-X-100 to a final concentration of 0.3% to allow a sufficient breakage of the cytoplasmic membrane. After centrifugation at

1,200 *g* for 5 min at 4 °C, the pellet was obtained and lysed in lysis buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM MgCl₂, 1% Triton-X-100, and 1mM PMSF) containing complete protease inhibitor mixture, then centrifuged again at 16,000 *g* for 30 min at 4 °C.

Equal amounts of the resultant supernatant were incubated overnight with the goat original anti-ANG polyclonal antibody or control IgG (4 °C on rotator), followed by incubation with Protein G-conjugated agarose beads (Millipore). The immune-precipitates were analyzed by Western blotting.

Confocal microscopy and fluorescent resonance energy transfer (FRET) analysis

HeLa cells were co-transfected with pYFP-ANG and pCFP-PLSCR1 plasmids and the fluorescence was examined by confocal microscope with a 40× oil immersion objective. Confocal microscopic images were obtained with a Leica confocal imaging spectrophotometer system (TCS-SP). Photobleaching based FRET assay was performed as previously described [14]. Briefly, the fluorescence images for CFP and YFP were collected repetitively at a 10 seconds interval for five times. After that, YFP was photobleached with the full power of the 514 nm line laser, and the emission intensities of CFP and YFP were monitored for an additional five times. FRET efficiency (FRET_e) was calculated as the percentage of the CFP fluorescence recovery [FRET_e = (CFP_A - CFP_B)/CFP_A × 100%], where CFP_A was the CFP intensity after photobleaching and CFP_B was the CFP intensity before photobleaching of YFP.

Quantitative real-timePCR

Total RNA was extracted using a TRIzol Reagent (Invitrogen). For each reverse transcription, 2 μg of total RNA was converted into cDNA by moloney murine leukemia virus reverse transcriptase (Promega). An equal volume mixture of the products was then quantified by quantitative real-time PCR using an ABI PRISM 7500 Sequence Detector and Power SYBR Green PCR Master Mix (Applied Biosystems, NY, USA). PCR was done with an initial denaturation step of 10 min at 95 °C followed by 40 cycles of amplification consisting of 15 s at 95 °C, 15 s at 59 °C, and 35 s at 72 °C. The primers for 47S pre-rRNA were 5'-CCT GCT GTT CTC TCG CGC GTC CGA G-3' and 5'-GCG TCT CGT CTC GTC TCA CT-3'. The primers for ANG were 5'-CAG GAT AAC TCC AGG TAC-3' and 5'-CGG ACG ACG GAA AAT TG-3'. The primers for PLSCR1 were 5'-AGA ACA GCT TTG GAC AGA GGG TTT ACT TTG C-3' and 5'-CTG AAG GCA GCA GGG ACA ACA ACA G-3'. The primers for the internal control gene GAPDH were 5'-ACG GAT TTG GTC GTA TTG GG-3' and 5'-CGC TCC TGG AAG ATG GTG AT-3'. Each sample was run in triplicate. Average threshold cycle (Ct) values for PLSCR1, ANG or 47S pre-rRNA was normalized against the average Ct values for GAPDH from the same cDNA sample. Fold change between experimental groups (E) and control groups (C) equals $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{PLSCR1/ANG/47S} - Ct_{GAPDH}$ and $\Delta\Delta Ct = \Delta Ct_E - \Delta Ct_C$.

Northern Blot

10 μg total RNA from each groups were separated by electrophoresis through a 1% agarose/2% formaldehyde gel, and transferred overnight in 20 × saline-sodium citrate (SSC) to a positively charged nylon membrane. Northern blots were performed with Digoxin (DIG)-labeled DNA probes specific for 47S pre-rRNA or β-actin mRNA. The probe used for 47s pre-rRNA is 5'-GGT CGC CAG AGG ACA GCG TGT CAG (DIG)-3' that hybridizes with the first 25 nucleotides of the 47s rRNA precursor [15]. The probe used for β-actin is 5'-AGG GAT AGC ACA GCC TGG ATA GCA AC (DIG)-3' which hybridizes with the β-actin mRNA at 484 to 509. Blots were detected using DIG Northern Starter Kit from Roche Applied Science following the manufacturer's instructions.

Statistical analysis

Data were presented as means ± S.D. The differences between groups were determined by Student's t-test (two-tailed). Differences were declared significant at *: *P* < 0.05 and **: *P* < 0.01.

Results

PLSCR1 binds to ANG in vitro and in vivo

Since PLSCR1 was picked as a potential ANG-interacting protein from yeast two-hybrid screening, we first re-confirmed the interaction in the yeast cells. For this purpose, yeast cells

Fig. 1. PLSCR1 interacts with ANG *in vitro* and *in vivo*. **A.** Yeast strain MaV203 cells were co-transformed with pEXP-AD502 or pEXP-AD502-PLSCR1 along with pDBLeu or pDBLeu-ANG as indicated. Transformants and yeast control strains (a-e) were subjected to X-gal analysis. The β -galactosidase-positive colony (blue staining) indicates a positive interaction, while white colonies indicate no interaction. **B.** 0.5 μ g recombinant ANG was incubated with recombinant GST-tagged PLSCR1 in the presence of GSH-agarose beads. The pull-down products were examined by immunoblotting with anti-ANG polyclonal antibody (top panel) and coomassie blue staining (bottom panel). **C.** PLSCR1 was co-immunoprecipitated with ANG in HeLa cells. The cell lysates were incubated with anti-ANG antibody, or normal IgG as control, and the immunoprecipitates were subjected to immunoblot by anti-ANG and anti-PLSCR1 antibodies respectively. The input represents 5% of the lysate used in each binding assay (bottom panel). **D.** HeLa cells were co-transfected with plasmids as indicated and subjected to photobleaching analysis. The bleached cell was marked with a white arrow. The CFP and YFP fluorescence emission intensities in the cells before and after photobleaching were shown in right panel. The FRET efficiency (FRET_e) was calculated as the percentage of the CFP fluorescence recovery. All the data represented the means of three independent experiments with the bars showing standard deviation. Bar scale: 20 μ m.

were co-transformed with pDBLeu or pDBLeu-ANG along with pEXPAD502 or pEXPAD502-PLSCR1. The results showed that the transformants turned blue in X-gal analysis only when ANG and PLSCR1 were simultaneously expressed (Fig. 1A). Comparing to the yeast control strains, the interaction between ANG and PLSCR1 was moderately strong in this system (Fig. 1A).

To further verify the interaction, we performed *in vitro* GST pull-down and *in vivo* co-immunoprecipitation assay. When ANG was co-incubated with bacteria-expressed and bead-bound GST-PLSCR1 or GST, it could be specifically pulled down by GST-PLSCR1 but not by GST (Fig. 1B), indicating a directly physical interaction between ANG and PLSCR1 *in vitro*. We then ectopically expressed HA-tagged PLSCR1 in HeLa cells, and applied 0.5 $\mu\text{g}/\text{ml}$ ANG into the culture medium for 1h. After washing, the cells were harvested, lysed and subjected to co-immunoprecipitation with an anti-ANG antibody. The subsequent immunoblotting analysis revealed that PLSCR1 was precipitated together with ANG (Fig. 1C), indicating that PLSCR1 interacts with ANG in the cellular content.

We then applied FRET technique to test PLSCR1-ANG interaction. After co-transfecting HeLa cells with pCFP-PLSCR1 and pYFP-ANG, the energy acceptor was photobleached by a high-intensity exposure to the YFP excitation light (514 nm laser) to block energy transfer from CFP to YFP, and the energy redistribution of CFP was recorded. If there is a FRET, photobleaching of YFP should increase the emission of CFP at 480 nm. As shown in the Figure 1D, the FRET did occur between PLSCR1 and ANG, further confirming that PLSCR1 interacts with ANG in living cells. The FRET of the two control groups CFP/YFP-ANG and CFP-PLSCR1/YFP were $-10\pm3\%$ and $-9\pm3\%$ respectively, while the FRET of CFP-PLSCR1/YFP-ANG was $47\pm11\%$ (Fig. 1D, Bar graph).

Interestingly, co-expression of CFP-PLSCR1 and YFP-ANG changed both proteins' localization and formed clear dots in the nucleus (Fig. 1D), suggesting that PLSCR1 and ANG interact with each other in cell nucleus.

PLSCR1 binds to ANG in the nuclei of HeLa cells

We then examined the interaction between endogenous ANG and PLSCR1 in HeLa cells. Immunofluorescence results revealed that ANG was predominantly localized in the nucleus while the PLSCR1 was distributed both in nucleus and cytoplasm membrane (Fig. 2A). Merged image showed that endogenous ANG and PLSCR1 were co-localized in the nucleus of HeLa cells, especially in the nucleolus (Fig. 2A), suggesting that the interaction between ANG and PLSCR1 might happen in the nucleolus. Thereafter, we analyzed the localization of ANG and PLSCR1 by subcellular fractionation. The data revealed that ANG was predominantly found in the nuclear fraction while PLSCR1 was present in both nuclear and cytoplasmic fractions (Fig. 2B). To further confirm that ANG interacts with PLSCR1 in the nucleus, we took the nuclear fraction to do nuclear co-immunoprecipitation assay. As expected, we were able to co-immunoprecipitate PLSCR1 with an ANG specific polyclonal antibody, but not the normal IgG (Fig. 2C), indicating that endogenous PLSCR1 interacts with ANG in the nuclei of HeLa cells.

PLSCR1 positively regulates rRNA transcription in HeLa cells

The finding that PLSCR1 interacted with ANG in the nucleus prompted us to examine whether PLSCR1 is involved in the ANG-stimulated rRNA transcription. We first manipulated the PLSCR1 expression in HeLa cells by transfecting a PLSCR1 expressing plasmid or small-interfering RNAs (siRNAs), and then measured the 47S pre-rRNA level by RT-qPCR and Northern blot to evaluate the rRNA transcription activity. The RT-qPCR results showed that over-expression of PLSCR1 led to about 2.5-fold increase of steady-state 47S pre-rRNA level in HeLa cells compared with control (Fig. 3A and B). Knocking down the endogenous PLSCR1 expression by siRNA resulted in about 40% decrease in 47S rRNA compared with its negative control (Fig. 3C and D). The Northern blot also showed similar results (Fig. 3E). These data demonstrate that PLSCR1 is a positive regulator of rRNA transcription in HeLa cells.

Fig. 2. PLSCR1 interacts with ANG in nucleus of HeLa cells. **A.** Subcellular localization of endogenous PLSCR1 and ANG. Non-transfected HeLa cells were subjected to immunofluorescence staining with anti-PLSCR1 and anti-ANG antibodies. Fibrillar was stained to show the nucleolus. The yellow arrow indicates the nucleolus localization of ANG and PLSCR1. Part of the endogenous PLSCR1 also localized in the cell membrane (white arrow). Bar scale: 20 μ m. **B.** PLSCR1 and ANG existed in cell nuclear fraction. Nuclear and cytoplasmic fractions were prepared from the non-transfected HeLa cells. Samples from equivalent numbers of cells were analyzed by immunoblotting with antibodies against PLSCR1, ANG, fibrillar and α -tubulin. Total, whole cell lysate; Cyt, cytoplasmic lysate; Nuc, nuclear lysate. **C.** Co-immunoprecipitation of nuclear PLSCR1 with ANG. Equivalent nuclear extracts prepared in (B) were subjected to co-immunoprecipitation with the anti-ANG polyclonal antibody or IgG, followed by immunoblotting with anti-PLSCR1 and anti-ANG antibodies respectively.

The regulation capacity of PLSCR1 on rRNA transcription depends on the availability of ANG

We next examined if PLSCR1-enhanced rRNA transcription depends on the existence of ANG. The ANG knockdown stable cells and control cells were established by our lab and have been described in previous published report [17]. To visually show the decreased expression of ANG in the cells, we examined the cellular ANG level by immunofluorescent staining. The pictures clearly revealed a much lower cellular ANG protein present in the cytoplasm and no detectable accumulation of this protein in the nucleoli upon ANG knockdown (Fig. 4A, a and b), which could be restored by adding exogenous ANG into the culture medium (Fig. 4A c).

We thus measured the cellular rRNA transcription activity in this ANG-down-regulated cell line with RT-qPCR. In accordance with the original report [5], knocking-down of ANG down-regulated rRNA transcription and addition of exogenous ANG rescued rRNA transcription to control level (Fig. 4B control column). However, over-expression of PLSCR1 did not up-regulate the 47S rRNA level if the endogenous ANG was down-regulated, indicating that the ANG is required for PLSCR1-enhanced rRNA transcription (Fig. 4B PLSCR1 column). In the

Fig. 3. PLSCR1 enhances rRNA transcription. A. Overexpression of PLSCR1 in HeLa cells. HeLa cells were transiently transfected with pCI-3HA vector (control) or pCI-3HA-PLSCR1 plasmid (PLSCR1). Twenty-four hours after transfection, the cell lysate was extracted and the PLSCR1 expression level was determined by western blotting. β -tubulin was served as a loading control. B. PLSCR1 positively regulated pre-rRNA transcription in HeLa cells. The 47S pre-rRNA expression level in either control or PLSCR1 over-expressed cells was determined by RT-qPCR. C. siRNA against PLSCR1 efficiently knocked down the expression of endogenous PLSCR1. The level of endogenous PLSCR1 protein level was detected by immunoblotting. D. Knocking down of PLSCR1 decreased the rRNA transcription level in HeLa cells. Total RNA was extracted and the 47S pre-rRNA transcription level was determined by RT-qPCR. The *GAPDH* level was used as the internal control of RT-qPCR. E/F. Northern blot confirmed the enhancement of PLSCR1 on rRNA transcription. Top panel, Northern blots were detected with DIG-labeled probes specific to the 47S pre-rRNA and β -actin mRNA. Bottom panel, quantification of the 47S pre-rRNA relative to β -actin mRNA by ImageJ analysis.

cking down of PLSCR1 decreased the rRNA transcription level in HeLa cells. Total RNA was extracted and the 47S pre-rRNA transcription level was determined by RT-qPCR. The *GAPDH* level was used as the internal control of RT-qPCR. E/F. Northern blot confirmed the enhancement of PLSCR1 on rRNA transcription. Top panel, Northern blots were detected with DIG-labeled probes specific to the 47S pre-rRNA and β -actin mRNA. Bottom panel, quantification of the 47S pre-rRNA relative to β -actin mRNA by ImageJ analysis.

rescue experiment, introduction of exogenous ANG could recover the PLSCR1-enhanced rRNA transcription, indicating that the regulation of PLSCR1 on rRNA transcription is ANG dependent (Fig. 4B). The results were also confirmed by the Northern blot assay (Fig. 4C). These findings suggest that the interaction between ANG and PLSCR1 plays an important role in regulating rRNA transcription.

Discussion

ANG-stimulated rRNA transcription plays a critical role in vessel endothelial cell proliferation, cancer cell proliferation, and motor neuron survival [11, 12]. However, the mechanism of how this process is regulated remains unknown. In this study, we demonstrated

Fig. 4. The regulation capacity of PLSCR1 on rRNA transcription depends on the availability of ANG. A. HeLa cells were transfected with pAng-RNAi and its control vector pBS/U6. Stable transfectants were selected with 0.5 μ g/ml puromycin. The 0.5 μ g/ml recombinant ANG were added to the medium as a rescued group. ANG was detected with monoclonal antibody 26-2F. The pictures were obtained under the same scanning parameter setting of confocal microscopy. Bar scale: 20 μ m. B. PLSCR1-mediated enhancement of pre-rRNA level was ANG-dependent. The stable trans-

fectants of pBS/U6 and pAng-RNAi were transiently transfected with pCI-3HA vector or pCI-3HA-PLSCR1 plasmid. For the ANG rescue assay, exogenous ANG (0.5 μ g/ml) was added back into the culture medium of the RNAi transfectant. 47S pre-rRNA transcription level was determined by RT-qPCR. The *GAPDH* level was used as the internal control of RT-qPCR. All the data represented the means of three independent experiments, with the bars showing standard deviation. **: $P < 0.01$. C. The 47S pre-rRNA transcription level was analyzed by Northern blot. Left panel, Northern blots were detected with DIG-labeled probes specific to the 47S pre-rRNA and β -actin mRNA. Right panel, quantification of the 47S pre-rRNA relative to β -actin mRNA by ImageJ analysis.

that PLSCR1 interacts with ANG in the nucleus and up-regulates rRNA transcription in an ANG-dependent manner, suggesting that PLSCR1 regulates rRNA transcription through interacting with ANG. Therefore, this study provides evidence on how the ANG-stimulated rRNA transcription is further modulated by an ANG-interacting protein.

PLSCR1 was primarily identified as a multi-palmitoylated, calcium-binding endofacial membrane protein mediating nonselective and bidirectional transport of phospholipids between membrane leaflets [18, 19]. Structural and functional analyses reveal that PLSCR1 has several functionally important domains, including multiple PXXP and PPXY domains, a DNA binding domain, a cysteine palmitoylation motif, a nonclassical nuclear localization signal (NLS), a Ca^{2+} binding EF-hand-like domain, and a single transmembrane domain [20], indicating its potential as a multifunction protein. Further studies do challenge its function

as a major phospholipids translocator and reveal that PLSCR1 mainly acts as a signaling molecule and can translocate to nucleus [20].

Currently, little is known about the function of PLSCR1 in the nucleus. It has been reported that this protein binds to the promoter region (⁻¹⁰¹GTAACCATGTGGA⁻⁸⁹) of the inositol 1,4,5-triphosphate receptor type 1 gene (*IP3R1*) and enhance its expression [21]. Here we reported that PLSCR1 promotes rRNA transcription although it's not clear whether this protein binds to genomic rDNA directly or indirectly. Interestingly, the binding site for PLSCR1 within *IP3R1* promoter contains an E-box core consensus sequence (i.e.CANNTG), and one rRNA gene contains three CATGTG sequences with two in 18s and 28s coding regions and one in intergenic space, and 12 palindromic canonical sequence of CACGTG at various positions, suggesting PLSCR1 might directly bind to rDNA. Furthermore, PLSCR1 hosts a putative transcriptional activation domain [20]. Therefore, it would be worth to determine if PLSCR1 activates rRNA transcription through direct binding to these sequences. On the other hand, ANG has been reported to bind to ABE in the rRNA gene and stimulate rRNA transcription [22, 23]. Therefore, it is also possible that PLSCR1 and ANG form a complex through their nuclear interaction to coordinate the transcriptional regulation capacities of different elements such as E-box and ABE.

To demonstrate that PLSCR1 regulates rRNA transcription through interacting with ANG, a possible method is to disrupt their intermolecular interaction by a competitive peptide mimicking the binding sequence and then observes the change of pre-rRNA transcription. Unfortunately, although we tried to map the binding sites on both proteins by yeast two-hybrid approach, the results revealed that a broad range of peptides involved (data not shown), suggesting a possible conformational binding epitopes. Docking analysis predicted multiple binding regions between these two proteins (Data not shown). Interestingly, Kusano and Eizuru found a very similar binding pattern in PLSCR1 with its interaction partner. They reported that PLSCR1 directly interacts with the amino-terminal region of human T-cell leukemia virus type-1 (HTLV-1) Tax through its 1-100 and 200-250 amino acid regions [24]. Therefore, currently we could not provide direct evidence to prove that PLSCR1 interacts with ANG and then regulates ANG-stimulated rRNA transcription.

PLSCR1 has been implicated in growth factor signaling pathway regulation, cell proliferation, and tumor growth and development [20, 25-28]. However, its underlying mechanism of action remains unclear. Ribosome biogenesis is a fundamental step in cell growth and proliferation, and rRNA synthesis is critical to ribosome assembly. Therefore, our finding that PLSCR1 interacts with ANG in the cell nucleus and thus synergistically regulates rRNA transcription might provide one explanation for the mechanism. Intriguingly, ANG is reported as a permissive factor for proliferation role of growth factor EGF, bFGF, and VEGF [15], and our unpublished data reveal that these factors could increase PLSCR1 expression and nuclear translocation (data not shown), it is possible that the PLSCR1-ANG interaction as well as the up-regulation on rRNA transcription could be one of the down-stream and critical events in response to these growth factors. Taken together, our finding drives us to speculate that PLSCR1 may cooperate with ANG in promoting rRNA transcription, ribosome biogenesis, and cell proliferation.

Overall, the present study identifies nuclear PLSCR1 as an ANG-interacting protein and provides evidence that nuclear PLSCR1 regulates ANG-enhanced rRNA transcription. These data shed light on the nuclear role of PLSCR1 and the mechanism of rRNA transcription regulation.

Acknowledgements

We would like to thank Dr. Guo-fu Hu (Tufts University Medical Center, Boston, MA, U.S.A.) for the gift of ANG RNAi plasmid and the ANG-specific monoclonal antibody 26-2F. The work was supported by grants from National Natural Science Foundation of China (No. 30800173 and 31170721).

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