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Research paper

EZH2-mediated α-actin methylation needs lncRNA TUG1, and promotes the cortex cytoskeleton formation in VSMCs

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ABSTRACT

Recent studies have revealed that long non-coding RNAs (lncRNAs) participate in vascular homeostasis and pathophysiological conditions development. But still very few literatures elucidate the regulatory mechanism of non-coding RNAs in this biological process. Here we identified lncRNA taurine up-regulated gene 1 (TUG1) in rat vascular smooth muscle cells (VSMCs), and got 4612 bp nucleotide sequence. The expression level of TUG1 RNA was increased in synthetic VSMCs by real-time PCR analysis. Meanwhile, the expression of enhancer of zeste homolog 2 (EZH2) (TUG1 binding protein) increased in cytoplasm of VSMCs under the same conditions. Immunofluoresce analysis displayed the colocalization of EZH2 with α-actin in cytoplasm and F-actin in cell edge ruffles. This leads us to hypothesize the existence of cytoplasmic TUG1/EZH2/α-actin complex. Using RNA pull down assay, we found that TUG1 interacted with both EZH2 and α-actin. Disruption of TUG1 abolished the interaction of EZH2 with α-actin, and accelerated depolymerization of F-actin in VSMCs. Based on EZH2 methyltransferase activity and the potential methylation sites in α-actin structure, we revealed that α-actin was lysine-methylated. Furthermore, the methylation of α-actin was inhibited by knockdown of TUG1. In conclusion, these findings partly suggested that EZH2-mediated methylation of α-actin may be dependent on TUG1, and thereby promotes cortex F-actin polymerization in synthetic VSMCs.

1. Introduction

Long non-coding RNAs (lncRNAs) are widely involved in the physiological and pathological processes of human body (Esteller, 2011; Kong et al., 2016; Liao et al., 2016). An increasing number of lncRNAs was identified during various biological processes, but the number of lncRNAs with known functions is very small. Vascular smooth muscle cells (VSMCs) can change their phenotype from contractile to synthetic state in response to various environmental stimulus (Gomez and Owens, 2012). Recent studies have revealed that several lncRNAs are involved in cardiovascular development and pathology (Bell et al., 2014; Leung et al., 2013; Thum and Condorelli, 2015). For example, knockdown of smooth muscle and endothelial cell-enriched migration/differentiation-associated long non-coding RNA (SENCR) can decrease the expression of myocardin and numerous smooth muscle contractile genes (Bell et al., 2014). Angiotensin II (Ang II)-regulated lncRNA which is responsible for the production of 2 microRNAs implicated in VSMC proliferation (Leung et al., 2013). But still very few literatures elucidated the roles of non-coding RNAs in VSMC biological processes.

The long non-coding RNA taurine up-regulated gene 1 (TUG1) is first identified in a screen for genes upregulated by taurine in developing retinal cells, and knockdown of TUG1 results in malformed or non-existent outer segments of transfected photoreceptors (Young et al., 2005). Previous studies suggested that TUG1 regulated cell cycle by binding to polycomb repressive complex 2 (PRC2) (Khalil et al., 2009). High expression of TUG1 is associated with carcinogenesis of various cancers, such as bladder urothelial carcinoma (Han et al., 2013), osteosarcoma (Zhang et al., 2013) and colorectal cancer (Sun et al., 2016; Wang et al., 2016). Knockdown of TUG1 inhibits the proliferation, and induces apoptosis in the carcinoma cell lines (Han et al., 2013).

Abbreviations: LncRNAs, long non-coding RNAs; TUG1, taurine up-regulated gene 1; VSMCs, vascular smooth muscle cells; SENCR, smooth muscle and endothelial cell enriched migration/differentiation-associated long non-coding RNA; Ang II, angiotensin II; PRC2, polycomb repressive complex 2; EZH2, enhancer of zeste homolog 2; PDGF, platelet-derived growth factor; qRT-PCR, quantitative real-time PCR; PLMLA, prediction of lysine methylation and lysine acetylation.

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Furthermore, TUG1 promotes cell proliferation partly through epigenetically mechanism (Zhang et al., 2014). However, it is unknown whether and how TUG1 participates in the phenotype switching in VSMCs.

Cell motility requires precisely orchestrated regulation of multiple cellular processes that involve dynamic actin cytoskeletal reorganization. Actin exists in both filamentous form (F-actin) and in soluble monomeric form (G-actin) in all cell types. Smooth muscle (SM) α-actin protein possesses important functions in cytoskeleton dynamics (Lv et al., 2016), such as formation of contractile stress filament and lamellipodia. Enhancer of zeste homolog 2 (EZH2), a component of the PRC2, is one of the key regulators of development in organisms from flies to mice, and exerts its epigenetic function through regulation of histone methylation. Previous studies showed that EZH2 was mainly expressed in nucleus. However, Su et al. demonstrated the existence of cytosolic EZH2 and its essential role in actin polymerization-dependent processes, such as platelet-derived growth factor BB (PDGF-BB) induced dorsal circular ruffle formation in fibroblasts (Su et al., 2005). Khalil et al. found the interaction between EZH2 and TUG1 in various human cells (Khalil et al., 2009). However, the significance of this interaction has not been further elucidated.

In this study, we investigated the expression and potential roles of TUG1 during the phenotypic switching of VSMCs. We first identified rat TUG1 sequence in VSMCs, and found that TUG1 was essential for EZH2 methyltransferase activity in F-actin formation of VSMCs. EZH2 was coupled with α-actin by TUG1, which was required for methylation of α-actin and polymerization of F-actin.

2. Methods

2.1. Ethics statement

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Animal Science and Technology, Hebei medical University and performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (China, 1988).

2.2. Cell culture and treatment

Rat VSMCs were isolated from aortas of 80–100 g male Sprague-Dawley rats anesthetized intraperitoneally with urethane (1.5 g/kg body weight) as described previously (Han et al., 2006). Serum starvation and PDGF-BB treatment were used to prepare contractile and synthetic VSMCs, respectively (Wanjare et al., 2013).

2.3. Rat TUG1 gene cloning and construction of expression vectors

According to sequences of TUG1 from human and mouse, we searched for rat TUG1 by homology analysis and designed primers for gene cloning. PCR primers used in TUG1 gene cloning: TUG1-qRF: TTT CCT TTC AAG AGA AAT GAC TG, TUG1-qRR: TTA AAC AAA AAA GGG AAT TTA TTG G, TUG1-qrf4: CCA TGA ACT CCA GGG TCC CAG A, TUG1-qrf2: TCC AGA CCC TCA GTG CAA AC, TUG1-RF2: TAC GTC CCG TGC CTC CTG AT, TUG1-RR2: AGG GCT GTG CTG AAT CTG GG, TUG1-qrr3: CTG GAC TTC CAT AGA GCG CC, TUG1-qrf3: GTG CAC TGG GTA AAC GTT GG, TUG1-qrf5: GCT GAC AGT TCT TGC AAA GAT GAG C, TUG1-qrr2: AGC AGG CAA ACA ACT TCA GC, TUG1-qrr4: GCA GCT GGT ATT TCT TCC TTG GT. Expression vectors encoding the rat TUG1 gene fragments were constructed into pGEM-T Easy Vector. The full length of TUG1 was 4576 bp, which cloned by primers of TUG1-qRF and TUG1-qRR. TUG1 3588 bp was cloned by primers of TUG1-qRF and TUG1-qrr4 as the template of RNA probe synthesis. TUG1-RF2 and TUG1-RR2 were used for qRT-PCR and the amplification fragment length was 242 bp. Sequence percent identity analysis of TUG1 gene from rat, mouse and human was performed by MegAlign software.

2.4. Immunofluorescence assay

VSMCs were fixed with 4% paraformaldehyde solution for 5 min at room temperature and then washed with PBS, followed by incubation in 10% normal goat serum blocking solution for 20 min in a humidified chamber at room temperature. Cells were incubated in mouse anti-EZH2 antibodies (1:20) mixture, or rabbit anti-α-actin (1:20) and phalloidin (1:20) mixture for 2 h at room temperature. Cells were washed 3 times with PBS and incubated in Fluorescein-conjugated secondary antibodies (anti-rabbit TRITC, red; anti-mouse FITC, green; 1:100) for 60 min at room temperature. The cells were then washed with PBS, mounted with DAPI, and visualized using laser scanning confocal microscope.

2.5. In vitro RNA probe synthesis and RNA pull down assay

The plasmids TUG1-3588 were linearized to serve as templates to generate RNA probes for pull down assay. The RNAs were synthesized using the Biotin RNA Labeling Mix (Roche) and T7/SP6 RNA polymerase (Promega). Briefly, in each 100 μL of reaction mixture, 1 μg of linearized DNA template was transcribed by T7/SP6 polymerase in the presence of 2.5 mM biotin-labeled UTP, CTP, ATP and GTP. After 2 h incubation at 37 °C, the reaction was stopped by adding 2 U of RNase-free DNase I (Promega) for 15 min at 37 °C. The RNA was purified with RNAclean kit (TIANGEN). For the RNA probe pull down assay, 3 μg biotin-labeled sense or antisense RNA was incubated with cell protein extracts (1 mg) respectively, which were then targeted with streptavidin beads and washed. Complexes were resolved using SDS loading buffer, and RNA-binding proteins in the pull down material were analyzed by Western blot analysis using anti-EZH2 and anti-α-actin antibodies.

2.6. SiRNA transfection and Western blot

VSMCs grown to 50–60% confluence were transfected with specific duplex siRNA, TUG1 siRNA (siTUG1) (5′-GCA GAU AUU CUG ACC CAU U-3′, 5′-CCC UUU AUA GUG CAG UCA A-3′ and 5′- CCA AGA AGC AUU CAG CCA UTT-3′), Scrambled siRNA (siCon) (5′-GCU AGA GUA GCG GUG AAU CGTT-3′ and 5′-CGA AUU CAC CGC UAC UCU AGC TT-3′) served as a negative control, using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's protocol. After transfection for 24 h, VSMCs were treated with PDGF (15 ng/mL) for 30 min. RIPA buffer was used to lyse VSMCs (50 mM Tris-Cl, pH 7.5, 1% NP-40, 0.5% Na-deoxycholate, 150 mM NaCl) and mice arteries (50 mM Tris-Cl, pH 7.5, 1% NP-40, 0.5% Na-deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl). The nuclear and cytoplasmic protein separation was performed by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo). The proteins were separated by 10% or 12% SDS-PAGE, and electro-transferred onto a PVDF membrane. Membranes were blocked with 5% non-fat milk in TBST for 2 h at room temperature, and incubated with primary antibodies against SM22α (1:1000, cat. no. ab14106, Abcam), EZH2 (1:500, cat. no. 5246, Cell Signaling), SM α-actin (1:1000, cat. no. 1184-1, Epitomics), β-actin (1:1000, cat. no. sc-47778, Santa Cruz), GAPDH (1:1000, cat. no. sc-137179, Santa Cruz), and Lamin A/C (1:1500, cat. no. ab108595, Abcam) at 4 °C overnight, and then incubated with IRDye800® conjugated secondary antibody (1:20,000, Rockland) for 1 h, following scanning with the Odyssey Infrared Imaging System (LI-COR Biosciences), then the integrated intensity for each detected band was determined with the Odyssey Imager software (v3.0). The experiments were replicated at least three times.

2.7. Actin fractionation

Cells were scraped, washed with PBS, and lysed in buffer A (20 mM Tris/HCl, pH 7.5, 1% Triton X-100, 5 mM EGTA and 1 mM phenylmethylsulfonyl fluoride) on ice for 20 min, and then centrifuged at highest speed and 4 °C for 10 min. The supernatants were harvested.