Original Paper

A Human Long Non-Coding RNA ALT1 Controls the Cell Cycle of Vascular Endothelial Cells Via ACE2 and Cyclin D1 Pathway

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Key Words

Long non-coding RNA • Endothelial cells • Angiotensin converting enzyme II • Cyclin D1 • Ubiquitination

Abstract

Background/Aims: *ALT1* is a novel long non-coding RNA derived from the alternatively spliced transcript of the deleted in lymphocytic leukemia 2 (*DLEU2*). To date, *ALT1* biological roles in human vascular endothelial cells have not been reported. **Methods:** *ALT1* was knocked down by siRNAs. Cell proliferation was analyzed by cck-8. The existence and sequence of human *ALT1* were identified by 3' rapid amplification of cDNA ends. The interaction between lncRNA and proteins was analyzed by RNA-Protein pull down assay, RNA immunoprecipitation, and mass spectrometry analysis. **Results:** *ALT1* was expressed in human umbilical vein endothelial cells (HUVECs). The expression of *ALT1* was significantly downregulated in contact-inhibited HUVECs and in hypoxia-induced, growth-arrested HUVECs. Knocking down of *ALT1* inhibited the proliferation of HUVECs by G0/G1 cell cycle arrest. We observed that angiotensin converting enzyme II (ACE2) was a direct target gene of *ALT1*. Knocking-down of *ALT1* or its target gene ACE2 could efficiently decrease the expression of cyclin D1 via the enhanced ubiquitination and degradation, in which HIF-1 α and protein von Hippel-Lindau (pVHL) might be involved. **Conclusion:** The results suggested the human long non-coding RNA *ALT1* is a novel regulator for cell cycle of HUVECs via ACE2 and cyclin D1 pathway.

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Published by S. Karger AG, Basel

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Introduction

Long noncoding RNAs (lncRNAs) are defined as noncoding RNAs longer than ~200 nucleotides and lack protein-encoding capacity, which are subgrouped into antisense, intronic, intergenic, sense overlapping and bidirectional transcripts [1, 2]. In contrast to other non-coding RNAs such as microRNAs, studies of lncRNAs in biomedicine are still in their infancy due to much more complex and diverse gene regulation mechanisms [1, 2]. However, increasing evidence has shown clearly that lncRNAs have strong biological functions and may be involved in all biological and disease processes [1-4].

A human long noncoding RNA *ALT1* (GenBank accession no: AF380424) is an alternatively spliced transcript of deleted in lymphocytic leukemia 2 (*DLEU2*), which maps to a critical region at 13q14.3 that is recurrently deleted in malignancy [5]. Although another transcript from *DLEU2* was found to be the critical host gene of miR-15a and miR-16-1 and could play critical role in cell cycle progression and cell proliferation in tumor cells via miR-15a and miR-16-1 [6, 7], the biological roles of *ALT1* has never been studied.

Angiotensin converting enzyme 2 (ACE2) converts angiotensin II (Ang II) into peptides including angiotensin-1-7 (Ang1-7) and angiotensin-1-9 that counteract the effects of Ang II through Mas receptor (MasR) and angiotensin type II receptor (AT2R) respectively [8, 9]. ACE2 activity and expression level were reported to be severely down regulated or absent in proliferating alveolar epithelial cell lines A549 and MLE-12, while its activity and expression level went much higher in quiescent A549 and MLE-12 cells [10]. Loss of ACE2 in mice enhanced the expression of genes that were induced by hypoxia and elevated mice serum Ang II, causing endothelial dysfunction, vasoconstriction and cardiac hypoperfusion [11, 12]. Ang II itself was reported to be a key mediator in the down regulation of ACE2 expression, while HIF-1 α inhibited ACE2 expression indirectly, both of which were depended on HIF-1 α accumulation during hypoxia exposure [13]. Besides, Ang1-7 treatment caused human aortic endothelial cells G0/G1 phase arrest and apoptosis [14]. These results indicate a pivotal role of ACE2 in cell cycle regulation.

In the current study, the existence and sequence of human *ALT1* were identified by 3' rapid amplification of cDNA ends in human umbilical vein endothelial cells (HUVECs). Interestingly, in both contact inhibited and hypoxia-induced growth-arrested HUVEC, the expression of *ALT1* was significantly decreased. Knockdown of *ALT1* could efficiently inhibit the proliferation of HUVECs by G0/G1 cell cycle arrest via its target gene ACE2 and cyclin D1 pathway, in which HIF-1 α and protein von Hippel-Lindau (pVHL) might also be involved.

Materials and Methods

Cell culture

Human specimens and animal tissues acquisition and using were approved by the Institutional Review Board of the First Affiliated Hospital of Sun Yat-sen University. Human umbilical cords were obtained with consent of the donors. HUVECs were identified with CD31 antibody (Fig. 1). Primary HUVECs were cultured

Fig. 1. Primary HUVECs were characterized with antibodies to CD31.

with EGM™-2 Single-Quots® (CC-4176) medium (Lonza) supplemented with 2-5% FBS (Gibco) and cytokines kit that sustained the cell growth and endothelial phenotype. All the cells were incubated at 37°C in an

incubator humidified with 5% CO₂. Hypoxia exposure cells were cultured in 0.2% O₂ in a hypoxia incubator (Galaxy 170 R, New Brunswick). Cells of passages 3 to 8 were used for the experiments in the current study.

3' rapid-amplification of cDNA ends (3'-RACE)

3'RACE assay was performed with SMARTer® RACE 5'/3' Kit(TAKARA, Cat.634858), following the manufacturer's instructions. The primers used in 3'-RACE assay were designed referring to AF380424.1 provided in GeneBank (<https://www.ncbi.nlm.nih.gov>). Primers are listed in Table 1.

AF380424.1(ALT1) and NR_002612 in vitro transcription

AF380424.1(ALT1), NR_002612 and their corresponding antisense sequences were synthesized by bridging pieces of each sequence together through numerous polymerase chain reactions (PCR). AF380424.1, NR_002612 and their antisense were then cloned into pcDNA 3.1 for *in vitro* transcription assays, after the accomplishment of the synthesis of the four sequences. *In vitro* transcription assays were performed with MAXIsript® T7/T3 Transcription Kit(Invitrogen), following the manufacturer's instructions.

Transfection and knockdown

For small interfering RNA oligonucleotides (siRNA) transfection experiments, cells were transfected with Lipofectamine™ RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. siRNAs used in the current study were designed and synthesised (GenePharma, RiBio). siRNA1 (5'-ACC ACC AUU GCC GCC UUC CC dTdT-3'), siRNA2 (5'-GGU GAG AAC UGA CUA AAC U dTdT-3') and siRNA3 (5'-UGU CAG CAA AGA ACU GUA A dTdT-3') were directed against exon 7 and exon 6 of AF380424 (ALT1) respectively. In order to knock down AF380424 with a higher efficiency, a mixture of two of the three siRNAs was applied in the following knock down experiment. siRNA ACE2(5'-GAG GAG ACU AUG AAG UAA A dTdT-3') Scrambled siRNA (5'-UUC UCC GAA CGU GUC ACG U dTdT-3') was used as negative control siRNA (NC siRNA).

Cell viability assay

Cell viability was measured with Cell Counting Kit-8 (Dojindo, Japan) following the instructions. Given different cell types, 3000-5000 cells were seeded in each well of 96 well plates. The absorbance of each well was measured at 450 nm on a spectrophotometer (Thermo) after different small interfering RNA oligonucleotides (siRNAs) transfection, with the absorbance at 650 nm being subtracted to eliminate the background.

Cell cycle and apoptosis analysis

Cells were left untreated as control and transfected with indicated siRNAs. 12-24 hrs after transfection, cells were further incubated for 24 h before harvested. Cells were harvested and fixed overnight in 70% ethanol (V/V) and then centrifuged at 300×g for 3 minutes, resuspended in 300-500 µl PBS containing 0.3% NP-40 (V/V), 20 µg/ml propidium iodide (Sigma) and 100 µg/ml RNase A (Sigma) and incubated 30 min at room temperature before analysis. Cell apoptosis was analyzed using Annexin V-FITC/PI kit (BD Biosciences), following the manufacturer's instructions. 20, 000 cells of each sample were counted and analyzed using FACSCalibur flow cytometer (Becton Dickinson). Both cellular DNA content and cell apoptosis ratio were analyzed using CellQuest software (BD Biosciences).

lncRNAs expression analysis and copies estimation in each cell

The lncRNAs AF380424.1(ALT1) and NR_002612 expression analysis were performed with Hs00863924_m1 and Hs03454364_m1 DLEU2 TaqMan® Gene Expression Assays (Thermo Fisher)

Table 1. Primers applied in 3'-RACE assays (referred to GenBank: AF380424.1)

primers	Sequence 5'-3'
ALT1-F500	TGACATGAGATTAAGGTTTAATAATGAT
ALT1-F763	GATAGATTTGGCTCTAGGCCTACTCTAG
ALT1-F1200	AACTGGGGACTTACAGGAGAATTGAACA
RACE-3RT	5'-AAGCAGTGGTATCAACGCAGAGTAC TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'
Down-long1	5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3'
Down-long2	5'-CTAATACGACTCACTATAGGGC-3'

respectively, GAPDH was detected with Hs00863924_m1 following the instructions. The expression level of other coding genes were performed following the protocols of subgreen real-time fluorescence quantitative PCR (qRT-PCR) as previously described [15]. Primers for subgreen qRT-PCR were listed in Table 2. The copies of ALT1 in each cell was estimated according to the linear correlation between TaqMan Gene Expression Ct values and limit dilution ALT1 expressing pcDNA 3.1 plasmids copies which was calculated with the following formulas: (1) copy number=concentration (ng/ μ L) $\times$ avogadro's constant(6.02×10^{23}) $\times 10^{-9}$ /(660 D/nt $\times$ base number of the ALT1 expressing pcDNA 3.1 plasmid); (2) copy numbers/ μ L=(mass/relative molecular weight) $\times 6.02\times 10^{23}$.

RNA-protein interaction pull down and mass spectrometry analysis

In order to get sufficient lncRNA for RNA-protein interaction pull down assay, lncRNAs DLEU2-004 (AF380424.1, ALT1), DLEU2-004 antisense, DLEU2-010(NR_002612) and DLEU2-010 antisense were all transcribed *in vitro*. Sequences of AF380424, NR_002612 and their antisense sequences were synthesized by a series of PCR reactions and cloned into pcDNA 3.1 plasmid vectors, so as to be templates for transcription reactions *In vitro*. Uracil in the lncRNAs sequences were labeled by biotin during transcription reactions, which were performed with MEGAshortscript™ T7 Transcription Kit (Invitrogen™) following the manufacturer's instructions. Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Scientific™) was applied for RNA-protein interaction pull down assay. HUVECs were lysed with Pierce IP lysis buffer (Thermo Fisher) for protein extraction. Cell lysate was then mixed with DLEU2-004, DLEU2-004AS, DLEU2-010 and DLEU2-010AS respectively. Magnetic beads labeled by streptavidin was added into the mixture and incubated for another one hour at room temperature. After incubation, the whole mixture was placed on a magnetic rack for interacting protein-lncRNA-beads complex purification, and the complex harvested in the last step was boiled with protein loading buffer at 95°C for 10 minutes and analyzed through SDS-PAGE, and then stained with coomassie brilliant blue.

Mass spectrometry analysis was carried out on an Ettan MALDI-TOF mass spectrometer (GE Healthcare) following protocols established and reported elsewhere previously [16]. Matching peptides from potential unknown lncRNA interacting proteins were searched against SwissPort2016 2016 (550960 sequences; 196692942 residues).

RNA immunoprecipitation

Dynabeads® Protein A for Immunoprecipitation (Novex™) was applied in RNA immunoprecipitation assay to analyze the interaction of lncRNAs with target proteins. The RNA immunoprecipitation assay was carried out with Magna RIP RNA-Binding protein Immunoprecipitation Kit (17-700, Millipore) following manufacturer's instructions. The protein A beads that were harvested at the final step of immunoprecipitation were separated equally into two parts: A and B. A, ALT1 from part A beads were extracted with Trizol and quantified by TaqMan probe qRT-PCR. B, proteins in part B beads were analyzed with western blotting.

Table 2. Primers applied in Subgreen qRT-PCR assays

primers	Sequence 5'-3'
GAPDH-F	CGGATTTGGTCGTATTGGG
GAPDH-R	CTGGAAGATGGTGATGGGATT
beta-actin F	CTCCATCCTGGCCTCGCTGT
beta-actin R	GCTGTCACCTTCACCGTTCC
mir-15a-F	GCAGCACATAATGGTTTGTG
mir-15b-F	GCAGCACATCATGGTTTACA
mir-16-1-F	AGCAGCACGTAAATATTGGCG
UNIVERSAL	GTGCAGGGTCCGAGGT
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
KPNA3-F	GCAAATCCAAAATTGCACTGT
KPNA3-R	TGCATTGTCCAGCATCAGGT
RFP2a-F	AAATACATCAGCTTGTAGGAGAC
RFP2a-R	CCGTGGATCATCAAACAGACTACA
C13ORF1-F	GGCAATCCAAAATTGCACTGT
C13ORF1-R	TGCATTGTCCAGCATCAGGT
ALT1-F1	CGCCTTCTCCTTTTCGCAATG
ALT1-R1	TGTGCAGTTTCAGCAAAGCTC
ALT1-F2	TTAGTAGAGGGCAATAAATGCCAC
ALT1-R2	TGAGTCAGCAGAAACAACACTGTTTC
ALT1-F3	TAGACCATGTGGAGAAGGAACTG
ALT1-R3	CCCAGTTACTTACAGTTTCTGCTG
ACE2-F	CATTGGAGCAAGTGTGATCTT
ACE2-R	GAGCTAATGCATGCCATTCTCA
cyclin D1-F	TCCTCTCCAAAATGCCAGAG
cyclin D1-R	GGCGGATTGGAAATGAACTT

Western blotting

Cells were washed with PBS and lysed with ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% TritonX-100, 5 mM EDTA, 0.5% SDS, 0.1 mM PMSF and 1% proteinase inhibitor cocktail). The lysates were then centrifuged (12000 rpm) at 4°C for 15 min. The protein concentrations of each groups were then determined with a BCA Kit(Thermo Fisher). Equal amounts of cell lysates (30-50 µg protein/lane) were loaded into 8% or 10% SDS-polyacrylamide gels. Western Blotting was performed with Bio-Rad system. Proteins were then transferred to PVDF, and then blocked with 5% milk in TBST. The blots were subsequently incubated overnight with specific primary Abs against ACE2 (abcam, cell signaling, proteintech) Cyclin D1(abcam), HIF-1a(proteintech), pVHL(sigma), CUL1 (proteintech), TRIM13 (cell signaling) and β-TUBULIN (proteintech), etc., followed by secondary antibody incubation. The images and bands' gray-scale values were taken and analyzed by Image Quant Las 4000 mini (GE Healthcare).

Statistics

Data are presented preferably as mean±SD. The Student t test and one-way ANOVA test were applied to distinguish the statistically significant differences among groups. The statistically significant level was set at 0.05. All data were processed using GraphPad Prism 5.0 (GraphPad, San Diego, CA), and the statistical analysis was performed with SPSS software, version 16.0 (SPSS Inc, Chicago, IL).

Results

ALT1(AF380424.1), but not NR_002612(DLEU2-010) is expressed in HUVECs

ALT1 is a known lncRNA transcribed from *DLEU2*. However, the entire length and sequence of human *ALT1* have not been identified. By using the 3' rapid amplification of cDNA ends (RACE) assay, we found that the entire length of *ALT1* was 43 nt longer than AF380424.1 in HUVECs. The sequence of the 43 nt is TTT GTG GCC TGC ATG CTT GTA AAT TAA AAT GCA TTG AGT TGT C. The copy number of *ALT1* is approximately 80-100 copies per cell. In contrast, another transcript of *DLEU2* NR_002612 (*DLEU2-010*) could be barely detected in HUVECs (Fig. 2).

ALT1 expression is decreased in HUVECs with contact inhibition or with hypoxia-induced growth arrest

To provide a potential link of *ALT1* and biological function in HUVECs, we determined the expression levels of *ALT1* in HUVECs with contact inhibition, in HUVECs with hypoxia-induced growth arrest, in their control HUVECs. Interestingly, as shown in Fig. 3A, hypoxia exposure or contact inhibition could decrease *ALT1* significantly in HUVECs.

Knocking down of ALT1 declines HUVECs proliferation

ALT1 level in HUVECs was knocked down with 50 nM siRNA1, siRNA2, siRNA3 respectively. Cell viability was analyzed with cck-8 kit. siRNA1 and siRNA3 could decrease *ALT1* levels in HUVECs (Fig. 3B). siRNA1 and siRNA3 declined HUVEC viability significantly (Fig. 3C). siRNA2 was not applied in the following studies, as it's lower efficiency in decreasing *ALT1* level.

Fig. 2. AF380424.1(*ALT1*, ENST00000425586.5, *DLEU2-004*) and NR_002612 (ENST00000621282.4, *DLEU2-010*) levels in HUVECs. The average *ALT1* expression Ct value is 31.11 ± 0.23 in HUVECs, while the *DLEU2-010* expression Ct value is 35.90 ± 0.64 in HUVECs.

Fig. 3. Knocking down of ALT1 declines HUVECs proliferation. A. The effects of siRNA1, siRNA3 and siRNA1+3 on the expression of ALT1 in HUVECs. B. The effect of ALT1 knocking down on HUVECs proliferation. C. ALT1 knockdown declines HUVEC viability. * $P < 0.05$, compared with control.

Fig. 4. Knocking down of ALT1 induces cell cycle arrest of HUVECs in G0/G1 and decreases the cyclin D1 level. A. Y-axis is cell count; X-axis is Propidium Iodide (PI) staining, FL2 channel. a. control, b. NC siRNA, c. siRNA1, d. siRNA3, e. siRNA1+3, f. cell ratio in different cell cycle phase. B. cyclin D1, cyclin E and Pho-cyclin E levels in different groups. Compared with control or NC siRNA, * $P < 0.05$.

Knocking down of ALT1 induces cell cycle arrest of HUVECs in G0/G1

In order to find why the decreasing of ALT1 level decreased HUVECs viability, we performed cell cycle analysis. siRNA1, siRNA3 and siRNA1+3 could significantly induce

Fig. 5. Cell apoptosis was analyzed with flow cytometry, with Annexin V and PI staining. a control, b NC siRNA, c siRNA1, d siRNA3, e siRNA1+3, f cell apoptosis ratio comparison between NC siRNA and siRNA1, siRNA3 and siRNA1+3 respectively.

cell cycle arrest in G0/G1, compared with siRNA control (Fig. 4A). We further explored whether decreasing of *ALT1* level interfered cyclin D1 or cyclin E protein level. Compared with NC siRNA, cyclin D1 level was decreased in HUVECs by siRNA1, siRNA3 and siRNA1+3 transfection, but did not interfere cyclin E and phosphorylation cyclin E levels (Fig. 4B). Cell apoptosis of different groups was analyzed with flow cytometry 36 hours later after siRNA transfection. But we found no significant difference among different groups, which suggested that decreasing *ALT1* level did not activate cell apoptosis directly (Fig. 5). Besides, we also detected the effects of *ALT1* knockdown on the expression of proteins involved in autophagy and apoptosis, at 36 hours after siRNA transfection. We found that the levels of P62, LC3 I / II, total caspase 3 did not change with the knockdown of *ALT1*. Since *ALT1* was characterized to be the antisense of *RFP2* which encodes TRIM 13 [6], we also detected TRIM13 level of each group. The TRIM13 levels were not interfered by the decreasing of *ALT1* level (Fig. 6).

Decreasing of ALT1 does not change the miR-15a, miR-15b and miR-16-1 level, but increases the expression levels of KPNA3, C13ORF1

MiR-15a, miR-15b, miR-16-1, KPNA3, C13ORF1 and *RFP2* are *ALT1* adjacent genes. To determine the effects of *ALT1* on the expression of them, the total RNAs were harvested at 36 hours after siRNA1+3 transfection. *KPNA3, C13ORF1* mRNA expression level was significantly upregulated in siRNA1+3 group ($P < 0.05$), while *RFP2* mRNA expression level was higher in siRNA1+3 group without significance ($P > 0.05$). The levels of miR-15a, miR-15b and miR16-1 were not changed statistically significant by siRNA1+3 transfection ($P > 0.05$) (Fig. 7).

ALT1 interacts with ACE2 and CUL1 in HUVECs

RNA-Protein pull down assays were performed to identify the proteins interacted with *ALT1*. Compared with *ALT1* antisense, NR_002612 sense and NR_002612 antisense, *ALT1*

Fig. 6. TRIM13, p62, LC3I/II, caspase3 levels in control, NC siRNA, siRNA1, siRNA3, siRNA1+3 groups.

Fig. 7. The influences of ALT1 knockdown on ALT1 adjacent genes expression. *P < 0.05, compared with NC siRNA group.

Fig. 8. ALT1 interacts with ACE2 and CUL1 in HUVECs. ALT1, NR_002612 sense and their antisense strands were transcribed in vitro and verified by sequencing. Biotinylated lncRNAs were incubated with cell lysate. Proteins that interacted with lncRNA were pulled down by streptavidin labeled magnetic beads. A. proteins pulled down with lncRNA strands were analyzed by SDS PAGE and stained with coomassie brilliant blue. Differential bands that were pulled down by ALT1 strand were marked with ① and ② respectively. B. Proteins included in the two bands were then identified by mass spectrometer. Proteins with top 10 possibilities were listed.

was found to be pulled down with two different protein bands ① and ② (Fig. 8A). Proteins that included in the two bands were then identified by mass spectrometer, and the top 10 suspected proteins were listed (Fig. 8B).

Fig. 9. RNA immunoprecipitation of ALT1 with ACE2 or CUL1. A. ALT1 retrieved from antibody-labeled protein A beads were extracted with Trizol and quantified by TaqMan probe qRT-PCR. B. Proteins arrested with antibody-labeled protein A beads were analyzed with western blotting.

Fig. 10. Analysis of the interaction between ACE2 and CUL1 by co-immunoprecipitation. ACE2 was precipitated with ACE2 antibody in CO-IP assay, and ACE2 antibody was incubated first followed by CUL1 antibody incubation in WB analysis.

As ACE2 and CUL1 were reported to be involved in cell cycle arrest [17, 18], we further verified the interaction of *ALT1* with ACE2 or CUL1 respectively through RNA immunoprecipitation. *ALT1* could be retrieved about 2-13 folds more with ACE2 antibody and 2-15 folds more with CUL1 antibody, than with isotype control antibody IgG (Fig. 9A and 9B). Besides, *ALT1* could not be retrieved with antibody to TRIM 13 (Fig. 9). We also performed co-immunoprecipitation to verify whether ACE2 could form complexes with CUL1. However, the direct interaction between ACE2 and CUL1 was not found in the current study (Fig. 10).

Knockdown of ALT1 does not alter CUL1 level, but decreases ACE2 level in HUVECs

Knockdown of *ALT1* inhibited cell proliferation, leaving cell confluence at 50-70% in siRNA1, siRNA3 and siRNA1+3 groups. Cell confluence in both control and NC siRNA group were >90%, because cell proliferation in these two groups were not affected. Decreasing of *ALT1* with siRNA did not influence CUL1 level, but the ACE2 levels were significantly higher among siRNA transfection groups, compared with control (Fig. 11A). It is well known that ACE2 expression is cell cycle dependent and is very sensitive to the cell confluence levels [10]. To exclude the influence of cell confluence, we performed the experiment in controlled 50-70% cell confluence. It was clear that the expression of ACE2 was significantly reduced by *ALT1* knockdown in HUVECs (Fig. 11B).

Hypoxia and cell contact inhibition decrease the ACE2 and cyclin D1 mRNA levels in HUVECs

We found that hypoxia and cell contact inhibition could decrease the expression of *ALT1* (Fig. 3) and the ACE2 was a target gene of *ALT1* (Fig. 8, 9 and 11). In this experiment, the effects of hypoxia and cell contact inhibition on the expression of ACE2, as well as the cyclin D1 were determined. Compared with NC siRNA transfection, hypoxia exposure and cell contact inhibition could decrease ACE2 and cyclin D1 mRNA levels (Fig. 12).

siRNA-ACE2 reduced cyclin D1 level, but increased HIF-1α level

Decreased *ALT1* level was found to result in the reduced ACE2 level. We further studied the effects of the decreasing ACE2 with siRNA. Hypoxia exposure increased HIF-1α level despite

Fig. 11. The effects of ALT1 on the expression of CUL1 and ACE2 in HUVECs. A. CUL1 level was not affected by siRNA1, siRNA3 and siRNA1+3 transfection, while the ACE2 levels were significantly lower in siRNA-ALT1 transfection group than that in control and NC siRNA transfection group. B. Cell confluence of each group was controlled within 50-70% by seeding different cell number in different wells of 6 well plates. Compared with both control and NC siRNA, * $P < 0.05$.

the cell confluence. Decreasing *ALT1* and ACE2 with siRNA respectively could significantly increase HIF-1 α level ($P < 0.05$). In addition, the decreasing *ALT1* reduced both cyclin D1 and ACE2 expression levels. ($P < 0.05$). Knocking down of ACE2 with siRNA could also decrease cyclin D1 level ($P < 0.05$) (Fig. 13). Protein von Hippel-Lindau (pVHL) level in each group was intangible during the repetition of the experiment. Therefore, the differences of pVHL levels among different groups were not statistically analyzed temporally. We then controlled cell confluence at $< 70\%$, and focus on the effects of siRNA1+3, siRNA ACE2 and hypoxia on phosphorylation retinoblastoma (phospho-RB) and pVHL levels. Knockdown of *ALT1* and ACE2 significantly reduced phospho-RB levels and increased HIF-1 α and pVHL levels. (Fig. 14).

Fig. 12. The effects of hypoxia and cell contact inhibition on the expression of ACE2 and cyclin D1 in HUVECs. ALT1, ACE2 and cyclin D1 mRNA fold changes in hypoxia and cell contact inhibition. Cell confluence of each group was controlled within 50-70% by seeding different cell number. Compared with NC siRNA, * $P < 0.05$.

Fig. 13. The effects of hypoxia exposure, cell contact inhibition and siRNA-ACE2 transfection on expression levels of HIF-1 α , ACE2 and cyclin D1. HIF-1 α levels in both control and NC siRNA group were compared with the HIF-1 α levels in other groups, * $P < 0.05$. ACE2 levels in both control and NC siRNA groups were compared with the ACE2 levels in other groups, # $P < 0.05$. cyclin D1 levels in both control and NC siRNA group were compared with the cyclin D1 levels in other groups, & $P < 0.05$.

Decreasing ALT1 with siRNA directly induces cyclin D1 ubiquitination

It was reported that cyclin D1 was ubiquitinated and subsequently degraded by pVHL in a cell density dependent manner [19]. Co-immunoprecipitation was performed with cyclin D1 antibody, and found that knockdown of ALT1 or ACE2 with siRNA

respectively could directly lead to cyclin D1 ubiquitination. siRNA1+3 and NC siRNA were transfected into HUVECs of different groups. In NC siRNA group, cell confluence naturally reached to >90%, which resulted in cell contact inhibition and caused cyclin D1 ubiquitination. However, siRNA1+3 transfection significantly induced cyclin D1 ubiquitination and inhibited cell proliferation, leaving cell confluence at about 50-70% (Fig. 15).

Discussion

ALT1 is an alternative splice variant of the DLEU2 Gene [5]. Alt1 is conserved in mouse, but its sequence may be much shorter than ALT1 in human [5, 6]. In this study, the existence and 3' end sequence of human ALT1 were identified by using the 3'-RACE. To date, several homology lncRNAs are found to be transcribed from DLEU2, including NR_002612 which is the longest variant. ALT1 was reported to be regulated by methylation and to be involved in nuclear factor- κ B pathway regulation [20]. However, the functions of ALT1 in cell biology are still unknown.

In the current study, we found that the copy numbers of ALT1 per cell was estimated to be about 80-100 in HUVECs, through TaqMan probe assay. However, NR_002612 copy numbers in HUVECs could merely be detected (Fig. 2). The estimated ALT1 copy numbers in HUVECs was similar to lncRNA-ATB, which is activated by TGF- β in SMMC-7721 hepatoma cells [21].

Knockdown of ALT1 level with siRNA showed clearly that siRNA could significantly decrease ALT1 level in HUVECs, which was verified with TaqMan probe assay (Fig. 16). Decreasing of ALT1 level in HUVECs resulted in significant cell viability reduction. The underlying mechanisms remain unknown, and this was also the reason that we carried on the following experiments in HUVECs. The reduction of HUVECs cell viability was resulted from cyclin D1 degradation after knockdown of ALT1. This was further validated by G0/G1 cell cycle arrest that was detected with flow cytometry after siRNA transfection. Consistent with

Fig. 14. The effects of hypoxia exposure, siRNA 1+3 and siRNA-ACE2 transfection on expression levels of HIF-1 α , phospho-RB, cyclin D1 and pVHL. HIF-1 α levels compared with HIF-1 α levels in both control and NC siRNA group respectively, *P<0.05. Phospho-RB levels compared with Phospho-RB levels in both control and NC siRNA group respectively, #P<0.05. cyclin D1 levels compared with cyclin D1 levels in both control and NC siRNA group respectively, Φ P<0.05. pVHL levels compared with pVHL levels in both control and NC siRNA group respectively, &P<0.05.

Fig. 15. Analysis of cyclin D1 ubiquitination by co-immunoprecipitation. Cyclin D1 was pulled down with cyclin D1 antibody and the ubiquitination of cyclin D1 was detected with ubiquitin antibody. MG 132 (1 μ M, 12 h) was applied to prevent ubiquitinated cyclin D1 from degradation by protease.

this, cyclin D1 level was significantly reduced after knockdown of *ALT1*, while both total cyclin E and phosphorylation cyclin E levels remained unchanged in different siRNA transfection groups. Besides, it was reported that TRIM13 regulates caspase-8 ubiquitination during ER stress and induced cell death [22]. But in the current study, TRIM13, total caspase-3 levels and cell apoptosis rate were not changed significantly by knockdown of *ALT1* (Fig. 5 and 6). Therefore, knockdown of *ALT1* might not directly activate apoptosis or autophagy. All these mentioned above suggested that *ALT1* was directly involved in cyclin D1 degradation.

RNA pull-down assays were then performed to explore RNA-protein interaction. ACE2 and CUL1 were found to be directly interacted with *ALT1*, which was further verified by RNA immunoprecipitation with ACE2 and CUL1 antibodies. In prostate cancer cells, expression of cyclin D1 and cyclin E were decreased after CUL1 knockdown [18]. CUL1 level was not influenced by knockdown of *ALT1* in the current study, the role of interaction between CUL1 and *ALT1* remains obscure. ACE2 is a carboxypeptidase, which was first identified in year 2000 [23, 24]. Ang I and Ang II can be metabolized by ACE2 to Ang-(1-9) and Ang-(1-7) respectively [24, 25]. ACE2/Ang-(1-7)/MAS receptor axis was reported to counteract the ACE/ Ang I /AT1R axis playing vasodilatory, anti-inflammatory and anti-proliferation roles [8, 26, 27]. ACE2 activity and expression level were reported to be severely down regulated or absent in proliferating alveolar epithelial cell lines A549 and MLE-12, while its activity and expression level went much higher in quiescent A549 and MLE-12 cells [10]. However, in the current study, both ACE2 mRNA and protein levels were significantly down regulated by both cell contact inhibition and *ALT1* knockdown in HUVECs.

Indeed, ACE2 could barely be detected when cell confluence was above 90%, and decreasing of *ALT1* level did not elevate ACE2 level but rather reduced ACE2 level and caused cell cycle arrest. This discrepancy might be explained by different ACE2 isoforms translated from different transcripts in different source of cells [28, 29]. In fact, not only ACE2 level was reduced by *ALT1* knock down in the current study, but also the HIF-1 α level was elevated. In accordance with our results, the expression of genes induced by hypoxia was increased in ACE2 knockout mice, causing endothelial dysfunction [12]. Furthermore, ACE2 was reported to be down regulated indirectly by HIF-1 α accumulation in human pulmonary artery smooth muscle cells [13]. Taken together, it is tempting to conclude that knockdown of *ALT1* reduced ACE2 by either direct effect (direct target) and indirect effect via the increased HIF-1 α . Although the underlying mechanism of increasing HIF-1 α level by *ALT1* knockdown remains unknown, *ALT1* level was found to be down regulated by hypoxia, and cell contact inhibition (*in vitro*) in HUVECs. Hypoxia might be caused by imbalance between supply and consumption of oxygen [30]. It was proposed that hypoxia caused by imbalance between oxygen consumption and supply in arterial wall plays key roles in atherosclerosis development [30, 31]. *ALT1* level reduction caused by hypoxia might be one of the mechanisms underlying endothelial dysfunction caused by hypoxia during the early stage of atherosclerosis development. Based on this, hypoxia induced *ALT1* reduction in the current study has shed light on the roles of lncRNAs that are regulated by hypoxia in early stage of atherosclerosis. Cyclin D1 was reported to be substrate of pVHL by expression array analysis between *VHL* defective and intact *VHL* cell lines [32]. In the current study, pVHL levels were elevated in siRNA1+3, siRNA-ACE2 and especially in siRNA-ACE2+hypoxia group. The trend of pVHL expression levels was in line with that of HIF-1 α level. This accordance was supported by a previous study which concluded that pVHL is required for cyclin D1 degradation at a high cell density through HIF in renal carcinoma 786-O cells [19]. Cyclin D1 was also ubiquitinated at a high cell density in the current study, and decreasing of *ALT1* with siRNA1+3 directly induced cyclin D1 ubiquitination as well. A similar conclusion might be made from the current study, because HIF-1 α level was increased after *ALT1* knockdown. Furthermore, cyclin D1 ubiquitination was also induced by knockdown of ACE2 which significantly increased HIF-

Fig. 16. Knockdown of *ALT1* in HUVECs with siRNA. a. NC siRNA and siRNA 1, b. NC siRNA and siRNA3, c. NC siRNA and siRNA1+3.

Fig. 17. Summarized pathway of ALT1 in HUVECs cell cycle regulation. ① whether Skp1-Cdc53/Cul1-F-box complex exists in HUVECs, and what is the substrate? ② How does ACE2 participate in HIF-1 α degradation.

1 α level under normoxia at a low cell density. ACE2 is a carboxypeptidase. ACE2 deficiency caused up-regulation of genes induced by hypoxia [12]. ACE2 knockdown caused increased HIF-1 α level in the current study. Taken together, there is a possibility that ACE2 might hydroxylate the conserved proline residue of HIF-1 α . HIF α could be degraded in an oxygen dependent manner by pVHL when the conserved proline residue was hydroxylated [33, 34]. pVHL loss caused HIF α accumulation and exerted similar effects to ACE2 knockout, including up-regulation of hypoxia-inducible genes [35, 36].

Besides, sequence and structural similarities was found between the identified complex pVHL/elongin C/elongin B/Cul2 and the yeast Skp1-Cdc53/Cul1-F-box complex [37, 38]. This might suggest that the interaction between ALT1 and CUL1 which was found in the current study is involved in the process of cyclin D1 ubiquitination and degradation by pVHL as well.

In summary, knockdown of ALT1 or its target gene ACE2 could efficiently decrease cyclin D1 level via the enhanced ubiquitination and degradation, in which HIF-1 α and protein von Hippel-Lindau (pVHL) was involved. The results suggested the human long non-coding RNA ALT1 is a novel regulator for cell cycle of HUVECs via ACE2 and cyclin D1 pathway (summarized in Fig. 17). However, the role of ACE2 in regulation of HIF-1 α level and the role of interaction between ALT1 and CUL1 remains obscure in ALT1 mediated cyclin D1 ubiquitination. Two issues need to be addressed to reveal the specific mechanisms of ALT1 in regulating cyclin D1 ubiquitination: ① whether Skp1-Cdc53/Cul1-F-box complex exists in HUVECs, and what is the substrate? ② How does ACE2 participate in HIF-1 α degradation?

Acknowledgements

The current study was supported by the National Natural Science Foundation of China to Dr. Wang (81270378; 81070258), Dr. Li (81370368) and Dr Li (81300237). This study was in part supported by the Natural Foundation of Shandong Province to Doctor Li (ZR2016HQ18).

Disclosure Statement

None.

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