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Knockdown of *Trnau1ap* inhibits the proliferation and migration of NIH3T3, JEG-3 and Bewo cells via the PI3K/Akt signaling pathway

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The tRNA selenocysteine 1 associated protein 1 (Trnau1ap, initially named SECp43) is involved in Selenocysteine (Sec) biosynthesis and incorporation into selenoproteins, which play a key role in biological processes, such as embryonic development. We previously reported that downregulation of Trnau1ap inhibited proliferation of cardiomyocyte-like H9c2 cells. However, the effects of Trnau1ap on cell proliferation and migration of embryonic development are not known, and the mechanisms remain elusive. Herein, lentiviral shRNA vectors were transfected in NIH3T3, JEG-3 and Bewo cells (embryonic, trophoblast and placental cells). We found that knockdown of Trnau1ap resulted in reduced expression levels of selenoproteins. The data of Cell Count Kit-8 (CCK-8) assay and wound scratch assay revealed the proliferation and migration rates were reduced in the Trnau1ap-shRNA groups. Furthermore, western blot analysis showed that the phosphorylation level of Akt in the phosphatidylinositol 3-kinase (PI3K)/Akt pathway was attenuated. These results indicate that Trnau1ap plays an important role in regulation of cell proliferation and migration through the PI3K/Akt signaling pathway, as well as being essential for embryonic development by regulating the expression of selenoproteins.

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1. Introduction

Selenium is recognized as an essential trace element in mammals [1]. The main active form of selenium in selenoproteins is Sec, which is identified as the 21_{st} amino acid to incorporate into selenoproteins [2,3]. The process of Sec biosynthesis requires Sec tRNA $^{[Ser]\ Sec}$ and Sec insertion sequence (SECIS) to redifine the UGA codon from a stop codon to the Sec codon [4,5]. And the process of Sec incorporation needs several cis- and trans-acting factors, such as SECIS, Translation initiation factor (eIF4A3), Trnau1ap, soluble liver antigen (SLA), SECIS binding protein 2 (SBP2), Ribosomal Protein (RPL30), Sec Elongation Factor (EFsec), Selenophosphate Synthetase 1 (SPS1) and Selenophosphate Synthetase 2 (SPS2) [6]. The SECIS element with a stem-loop structure locates at the 3′-untranslated region of selenoprotein mRNAs. SBP2 binds to the SECIS element, and associates with other factors, then translocate to the ribosome [7]. RPL30 on the ribosome is recognized as the second binding protein of the SECIS element [8]. SPS2 is a selenoenzyme as well as a selenium donor that can promote the biosynthesis of selenoproteins, but knockdown of SPS1 could not influence the expression of selenoprotein, it was supposed to impact the recycling of Sec [9].

Another factor is Trnau1ap, which is identified as a globular and nuclear protein with 43 kDa, regulates the biosynthesis of selenoproteins with promoting the synthesis of Um34 on tRNA $^{[Ser]\ Sec}$ and the nuclear translocation of SLA in mammals [10,11]. Trnau1ap interacts with SLA, eIF4A3, EFsec, SBP2 and SPS1 to form a multiprotein complex. Meanwhile, it associates with Sec tRNA $^{[Ser]\ Sec}$ to promote its methylation [12]. Prevently, one research reported that constitutive deletion of exons 7 + 8 of Trnau1ap was embryonic lethal, but there was no effect of Trnau1ap mutations on selenoprotein expression [13].

At least 25 selenoproteins in human and 24 selenoproteins in rodents have been identified [14]. Among these selenoproteins such as Glutathione Peroxidases (GPxs), Thioredoxin Reductases (TrxRs) and Iodothyronine Deiodinases (DIOs), selenoprotein N (SelN) and selenoprotein K (SelK) are associated with redox

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reactions [15]. GPxs are performed to reduce cellular hydroperoxides [16]. And GPx4 is essential for spermatogenesis and embryogenesis [17]. TrxRs catalyze the reduction of oxidized thioredoxin by the oxidation of NADPH [18]. TrxR1 and TrxR2 are also essential for embryogenesis [19]. DIOs are identified as regulators in the metabolism of thyroid hormones [20]. SelN is identified to protect against cellular oxidative stress and play a role in redox-related calcium homeostasis [21]. Overexpression of SelK may improve the antioxidant activity of the heart [22].

It has been well known that the PI3K/Akt signaling pathway could be involved in cell proliferation, migration and cell fusion [23–25]. When PI3K is activated, the downstream factor Akt will be phosphorylated [26]. The phosphorylation sites include Threonine 308 and Serine 473 [27]. But the pathway with knockdown of Trnau1ap in cells is unknown. And several studies have reported the structure and location of Trnau1ap [10,11]. However, studies about the effects of Trnau1ap on cell proliferation and migration are seldom. Thus we constructed the lentiviral shRNA vectors to knock down the Trnau1ap. And our purpose is to find out cellular mechanisms about the Trnau1ap in NIH3T3, JEG-3 and Bewo cells. Finally, we found that knockdown of Trnau1ap inhibited cell proliferation and migration through attenuating the PI3K/Akt signaling pathway.

2. Materials and methods

2.1. Cell culture

The mouse embryonic fibroblast cell line (NIH3T3), the human trophoblast choriocarcinoma cell line (JEG-3) and the placental choriocarcinoma cell line (Bewo) were purchased from ATCC. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, USA) plus 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (Biological Industries). And cells were cultured in an incubator with 5% CO_2 at 37 °C.

2.2. Transfection of lentiviral Trnau1ap-shRNA vectors

The lentiviral Trnau1ap-shRNA vector (Trnau1ap-shRNA group) and lentiviral negative control-shRNA vector (NC-shRNA group) were purchased from Obio Technology (Shanghai, China). The target sequence of mouse Trnau1ap shRNA was 5′-CGTGGACGATGGCATGCTGTA- 3′, and the target sequence of human Trnau1ap shRNA was 5′-CTGAGAAGTGTTTGCATAA-3’. Exponential growth cells were seeded into 24-well plates with 2×10^4 cells per well. And cells were transfected with lentiviral shRNA vectors. The multiplicities of infection (MOIs) were 40 in NIH3T3 cells, 30 in JEG-3 and Bewo cells. Meanwhile, polybrene (6 μg/ml) was added to improve transfective efficiency [28]. After 5 days, puromycin (2 μg/ml) was added for screenning positive cells.

2.3. Western blot analysis

The total protein of the Trnau1ap-shRNA group and NC-shRNA group was lysed by RIPA buffer, phosphatase inhibitor and protease inhibitor (100:1:1), and the concentration was measured by BCA assay. The loading quantity of protein was 50 μg. The total protein was separated by 12% SDS/PAGE with 120 V, followed by transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Madison, WI, USA) with 200 mA for 1 h and 30 min. The membranes were incubated with primary antibodies at 4 °C overnight. Primary antibodies include SelK (1:1000), GPx1 (1:1000), GPx4 (1:1000), DIO (1:1000), Trnau1ap (1:1000), TrxR1 (1:1000), SelN (1:1000) (Proteintech, Wuhan, China), Akt and p-Akt (1:1000) (Thr308, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (1:1000) (Boster), then membranes were incubated with secondary antibodies (Boster) for 2 h at room temperature. Finally the bands were detected with ECL reagents (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using BioSpectrum Imaging System (UVP, LLC Upland, CA, USA) and were analyzed by image J software.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted by TRIzol reagent (Invitrogen, USA) according to manufacturer's instructions, the concentration was measured by NanoDrop (2000) (Thermo Scientific, USA). RNA samples were transcripted to cDNA with PrimeScript™ RT reagent Kit (TAKARA, Dalian, China). Quantitative Real Time-PCR was performed to measure the expression level of Trnau1ap mRNA using 7500 ABI (Applied Biosystems, Life Technology, USA) with SYBR Green (Roche, Shanghai, China). The qRT-PCR primers for the Trnau1ap and β-actin mRNA were purchased from GENEWIZ. The primer sets are shown in Table 1. The qRT-PCR process was at 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min, melting curve stage was at 95 °C for 15 s, 60 °C for 1 min, and at 95 °C for 30 s.

2.5. Detectation of malondialdehyde assay

The way to detect the cellular oxidative stress level was using Malondial-dehyde (MDA) assay (Beyotime Biotechnology, Shanghai, China). Cells were collected with 0.25% trypsin (Gibco, USA), broken by ultrasonic wave in the extracting solution, then added MDA reagent to the suspension, followed by a waterbath at 95 °C for 40 min, finally measured at OD 532 nm with microplate Molecular Devices (SpectraMax M3, USA).

2.6. Migration assay

The wound scratch assay was performed to detect the cell migration. The Trnau1ap-shRNA cells and NC-shRNA cells were seeded into 6-well plates, scratched by 10 μl plastic pipette tips, and continued cultured in the serum-free DMEM. Cells were imaged at 0 h, 24 h with a fluorescence microscope (Olympus, Tokyo, Japan). The wound healing rates of cells were perfomed with the ratio (width of the healed wound/width of the original wound).

2.7. Cell proliferation assay

The CCK-8 (Beyotime Biotechnology, Shanghai, China) was performed to detect cell proliferation. The exponential growing Trnau1ap-shRNA cells and NC-shRNA cells were seeded into 96-

Table 1
Primer sets used in quantitative Real-Time PCR.

Gene name	Forward primer	Reverse primer
Mouse *β-actin*	CCGTAAAGACCTCTATGCCAACA	CGGACTCATCGTACTCCTGCT
Mouse *Trnau1ap*	ATGGGAGACCTGGAACCCTA	TCAGGGCTATTGTCTGGCTG
Human *β-actin*	GCAAAGACCTGTACGCCAAC	GATCTTCATTGTGCTGGGTGC
Human *Trnau1ap*	CCAGAACACAGGCAGCTACA	GCAACCTGGCTTGTCCTTTG

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