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The Role of Herpud1 in Metanephric Mesenchymal Cells and Its Mechanism: Postprint

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Abstract

To preliminarily investigate the biological role of Herpud1 in kidney development, vectors for Herpud1 overexpression and knockdown were constructed and transfected into metanephric mesenchymal cells. The expression changes of epithelial-mesenchymal transition (EMT) marker proteins E-cadherin, Vimentin, and Snail, as well as endoplasmic reticulum stress marker proteins glucose-regulated protein 78 (GRP78) and eukaryotic initiation factor 2α (eIF2α), were detected by RT-PCR and Western blotting. The effect of Herpud1 on cell proliferation was assessed by EDU cell proliferation assay, and cell migration ability was evaluated by wound healing assay. The results showed that, compared with the blank control group, the Herpud1 overexpression group exhibited decreased E-cadherin expression at both mRNA and protein levels, while Snail and Vimentin expression was increased, accompanied by reduced cell proliferation activity and enhanced cell migration ability, along with elevated endoplasmic reticulum stress-related proteins GRP78 and eIF2α. In the Herpud1 knockdown group, E-cadherin expression was increased at both mRNA and protein levels, while Snail and Vimentin expression decreased, cell migration ability was reduced and cell proliferation activity was increased, and endoplasmic reticulum stress-related proteins GRP78 and eIF2α were also decreased. These results demonstrate that Herpud1 can promote the EMT process in MK3 cells, enhance cell migration ability, inhibit cell proliferation activity, and its mechanism may be associated with cellular endoplasmic reticulum stress.

Full Text

Preamble

The Effects of Herpud1 on Metanephric Mesenchymal Cells and Its Mechanism

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Abstract

To investigate the biological role of Herpud1 in kidney development, we constructed overexpression and knockdown vectors for Herpud1 and transfected them into metanephric mesenchymal cells (MK3). Using RT-PCR and Western blot, we examined expression changes in epithelial-mesenchymal transition (EMT) markers (E-cadherin, Vimentin, and Snail) and endoplasmic reticulum (ER) stress markers (GRP78 and eIF2α). Cell proliferation was assessed by EDU assay, and migration capacity was evaluated by wound healing assay. Compared with the control group, Herpud1 overexpression significantly decreased E-cadherin expression at both mRNA and protein levels while increasing Snail and Vimentin expression, accompanied by reduced cell proliferation and enhanced migration. Concurrently, ER stress-related proteins GRP78 and eIF2α were elevated. Conversely, Herpud1 knockdown increased E-cadherin expression and decreased Snail and Vimentin levels, resulting in reduced migration but enhanced proliferation, with corresponding decreases in GRP78 and eIF2α. These findings demonstrate that Herpud1 promotes EMT, enhances migration, and inhibits proliferation in MK3 cells, potentially through modulation of ER stress.

Keywords: Herpud1; EMT; cell proliferation; ER stress

Introduction

The kidney is a vital organ responsible for waste excretion, electrolyte homeostasis, and hormone secretion. During vertebrate gastrulation, kidneys develop from the intermediate mesoderm located between paraxial and lateral mesoderm, exhibiting spatial and temporal continuity. At embryonic day 10.5 in mice, the kidney primordium consists primarily of the ureteric bud (UB) and metanephric mesenchyme (MM). The MM promotes branching morphogenesis of the UB, while MM cells surrounding the UB undergo mesenchymal-to-epithelial transition (MET) to induce nephron differentiation. Thus, metanephric mesenchymal cells are crucial for kidney development, and regulatory factors affecting these cells significantly impact nephrogenesis.

Herpud1 is a homocysteine-induced, ubiquitin-like ER membrane protein whose expression correlates with elevated ER stress. It participates in ER-associated degradation (ERAD), facilitating the clearance of misfolded or unfolded proteins. As an important protein expressed in various tissues and cell types, Herpud1 has been detected in human embryonic kidney 293T cells, which have been used to study its promoter activity. However, the role of Herpud1 in embryonic kidney development remains largely unexplored. This study investigates how Herpud1 influences EMT, migration, and proliferation in MK3 metanephric mesenchymal cells. Given that Herpud1 is inducible by ER stress, we hypothesized that its functions may be mediated through ER stress pathways.

Materials and Methods

1.1 Cells and Reagents

MK3 cells (early metanephric mesenchymal cells) were kindly provided by Professor S. Steven Potter from the Children's Medical Center. Dulbecco's Modified Eagle Medium (DMEM) and 0.25% trypsin were purchased from Gibco (USA). Fetal bovine serum (FBS) was obtained from Biological Industries (BioInd, Israel). TRIzol reagent and Lipofectamine 2000 transfection reagent were from Invitrogen. The reverse transcription kit was purchased from Sigma, RT-PCR primers were synthesized by Sangon Biotech (Shanghai), and Ultra SYBR Mixture was from CWBio (Jiangsu). Primary antibodies against Herpud1, E-cadherin, Snail, Vimentin, GRP78, eIF2α, and β-actin were from Proteintech (USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were from Sangon Biotech (Shanghai). ECL reagent was purchased from Millipore.

1.2 Cell Culture

MK3 cells, an adherent cell line, were cultured in DMEM high-glucose medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a 5% CO_2 incubator. Cells were passaged at 90% confluence using 0.25% trypsin.

1.3 Vector Construction

The coding sequence (CDS) of mouse Herpud1 was amplified from MK3 cells by PCR and cloned into the pCDH-CMV-MCS-EF1-copGFP vector to generate the Herpud1 overexpression plasmid. shRNA sequences targeting the mouse Herpud1 CDS were inserted into the pLKO.1 vector to construct the knockdown plasmid. Primers were: Herpud1-OV-F: 5' -AGACGCCAAGTGTCGTTGTG-3'; R: 5'-TCAGTTGGCTAGGGCTGGT-3'. shHerpud1: 5'-CCGGCGTTATTCTGAAGAGCTTTAACTCGAGTTAAAGCTCTTCAGAATAACGTTTTT 3'and 5'-AATTCAAAAACGTTATTCTGAAGAGCTTTAACTCGAGTTAAAGCTCTTCAGAATAACG-3' .

1.4 Cell Transfection

MK3 cells were seeded in 12-well or 6-well plates. At 40% confluence, cells were transfected with pCDH-CMV-MCS-EF1-copGFP-Herpud1 (pCDH-Herpud1) or pLKO.1-shHerpud1 vectors using Lipofectamine 2000 according to the manufacturer' s protocol.

1.5 Real-Time Quantitative PCR (RT-PCR)

After 48 hours (overexpression) or 72 hours (knockdown) post-transfection, cells were harvested for RNA extraction. Two micrograms of RNA were reverse-transcribed into cDNA, diluted 10-fold, and 4 µl was used as template for RT-PCR to detect EMT marker expression. The PCR program consisted of: 95°C for 10 min; 40 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s; followed by melting curve analysis from 65°C to 95°C. Data were analyzed using Bio-Rad software. Primers were: Herpud1-F: 5' -GTTGGATTGGACCTATTCCG-3' , R: 5' -CTCTGTCTGAACGGAAACCA-3' ; E-cadherin-F: 5' -CAGGTCTCCTCATGGCTTTGC-3' , R: 5' -CTTCCGAAAAGAAGGCTGTCC-3'; Vimentin-F: 5'-GGATCAGCTCACCAACGACA-3', R: 5'-AAGGTCAAGACGTGCCAGAG-3'; Snail-F: 5'-AGCCCAACTATAGCGAGCTG-3' , R: 5' -CCAGGAGAGAGTCCCAGATG-3' .

1.6 Western Blot

Treated cells were harvested and total protein was extracted. Protein concentration was determined by BCA assay. Twenty micrograms of protein were loaded onto 10% SDS-PAGE gels, separated at 100 V, and transferred to PVDF membranes (Millipore) at 300 mA for 2 hours. Membranes were blocked with 5% skim milk for 2 hours, incubated with primary antibodies (1:1000 dilution) overnight at 4°C, washed three times for 5 minutes each, then incubated with goat anti-mouse or anti-rabbit IgG (1:5000 dilution) for 2 hours at 37°C. After three 10-minute washes, bands were visualized using ECL reagent on a Bio-Rad chemiluminescence imaging system.

1.7 Cell Proliferation Assay

At 48 hours (pCDH-Herpud1) or 72 hours (shHerpud1) post-transfection, cells were washed three times with PBS and processed using the EDU Cell Proliferation Kit (RiboBio) according to the manufacturer' s instructions. Proliferating cells were observed and counted under a fluorescence microscope (Nikon).

1.8 Wound Healing Assay

When transfected cells in 6-well plates reached 80-90% confluence, wounds were created using a yellow pipette tip. After washing three times with PBS, cells were cultured in medium containing 1% FBS. Initial images were captured under an inverted microscope at marked positions. After 12 hours of incubation,

images were captured at the same locations. Migration rate was quantified using ImageJ as: (wound width at 0 h - wound width at 12 h) ÷ wound width at 0 h × 100%.

All experiments were performed independently three times. Data are presented as mean ± standard deviation and analyzed using GraphPad Prism 5. Statistical significance was defined as $P < 0.05$, $\boldsymbol{P < 0.01}$, $P < 0.001$.

Results

2.1 Efficiency of Herpud1 Overexpression and Knockdown Vectors in MK3 Cells

Following transfection with pCDH-Herpud1 or pLKO.1-shHerpud1 vectors, efficiency was verified by RT-PCR and Western blot. Herpud1 expression was significantly elevated at both mRNA and protein levels in the overexpression group [Figure 1: see original paper]A, B. In cells transfected with pLKO.1-shHerpud1, Herpud1 expression was markedly reduced compared to controls [Figure 1: see original paper]C, D. These results confirm successful and efficient transfection of both vectors for subsequent experiments.

2.2 Herpud1 Overexpression Promotes EMT in MK3 Cells

After Herpud1 overexpression or knockdown in MK3 cells, we examined EMT markers by RT-PCR and Western blot. Herpud1 overexpression significantly decreased the epithelial marker E-cadherin while increasing mesenchymal markers Vimentin and Snail at both mRNA and protein levels [Figure 2: see original paper]A, B, indicating promotion of EMT. Conversely, Herpud1 knockdown increased E-cadherin expression and decreased Vimentin and Snail levels [Figure 2: see original paper]C, D, further confirming that Herpud1 promotes EMT in MK3 cells.

2.3 Herpud1 Overexpression Inhibits MK3 Cell Proliferation

While Herpud1 has been reported to promote apoptosis, its effect on proliferation remained unclear. Using the EDU assay, we found that Herpud1 overexpression reduced cell proliferation by 54% compared to controls ($P < 0.05$) [Figure 3: see original paper]. Knockdown of Herpud1 significantly increased proliferation [Figure 3: see original paper]. These data demonstrate that Herpud1 upregulation inhibits MK3 cell proliferation.

2.4 Herpud1 Enhances MK3 Cell Migration

Wound healing assays revealed that Herpud1 overexpression markedly increased cell migration rate at 12 hours post-wounding [Figure 4: see original paper]A, B.

In contrast, Herpud1 knockdown reduced migration compared to controls [Figure 4: see original paper]C, D. These findings establish that Herpud1 promotes MK3 cell migration.

2.5 Herpud1 Overexpression Activates ER Stress

To investigate the mechanism underlying Herpud1-mediated EMT and proliferation changes, we examined ER stress markers by Western blot. Herpud1 overexpression significantly increased GRP78 and eIF2α protein levels by 1.6-fold and 1.4-fold, respectively [Figure 5: see original paper]A, B, demonstrating that Herpud1 activates ER stress in MK3 cells.

Discussion

The kidney is a vital organ whose developmental study is crucial for understanding both normal function and disease. Since Starkiewiczowa' s 1967 report on morphological and functional changes during kidney development, molecular mechanisms have been extensively investigated. Genes including Pax2/8, Six1/2/4, and Eya1 are known to be expressed in metanephric mesenchyme and influence nephrogenesis. Our study identifies Herpud1 as a novel regulator in MK3 cells.

Herpud1 is an ER membrane protein induced by ER stress and involved in ERAD-mediated clearance of misfolded proteins. While Herpud1 and ATF4 accumulation has been implicated in DDK syndrome embryonic lethality, its role in kidney development was unknown. We demonstrate that Herpud1 overexpression in MK3 cells promotes EMT, as evidenced by decreased E-cadherin and increased Snail1 and Vimentin expression. This EMT promotion likely underlies the enhanced migration we observed in wound healing assays. Additionally, Herpud1 overexpression inhibited proliferation, ruling out proliferative effects as a confounding factor in migration studies.

Andreas Kispert' s work showed that metanephric mesenchyme undergoes MET to form renal vesicle epithelia and subsequently individual nephrons, highlighting MET as a critical step in kidney development. Our finding that Herpud1 promotes EMT—the reverse of MET—suggests Herpud1 may influence nephrogenesis by regulating mesenchymal plasticity.

The mechanism of Herpud1 action appears to involve ER stress induction. Previous studies reported that Herpud1 can trigger ER stress, which is both necessary for and can induce EMT. We found that Herpud1 overexpression elevated ER stress markers GRP78 and eIF2α, suggesting that Herpud1 promotes EMT and migration in MK3 cells through enhanced ER stress signaling.

In summary, this study demonstrates that Herpud1 promotes EMT, migration, and apoptosis in metanephric mesenchymal MK3 cells, potentially through ER stress induction. These findings illuminate Herpud1' s important role in kidney

development and provide new insights for future research into the molecular mechanisms governing nephrogenesis.

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