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Gulo regulates the proliferation, apoptosis and mesenchymal-to-epithelial transformation of metanephric mesenchyme cells via inhibiting Six2

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ABSTRACT

During kidney development, the balance between self-renewal and differentiation of metanephric mesenchyme (MM) cells, mainly regulated by Sine oculis-related homeobox 2 (Six2), is critical for forming mature kidney. L-gulono-γ-lactone oxidase (Gulo), a crucial enzyme for vitamin C synthesis, reveals a different expression at various stages during kidney development, but its function in the early renal development remains unknown. In this work, we aim to study the role of Gulo in MM cells at two differentiation stages. We found that Gulo expression in undifferentiated MM (mK3) cells was lower than in differentiated MM (mK4) cells. Over-expression of Gulo can promote mesenchymal-to-epithelial transformation (MET) and apoptosis and inhibit the proliferation in mK3 cells. Knock-down of Gulo in mK4 cells made its epithelial character cells unstabilized, facilitated the proliferation and restrained the apoptosis. Furthermore, we found that Six2 was negatively regulated by Gulo, and over-expression or knock-down of Six2 was able to rescue partially the MET, proliferation and apoptosis of MM cells caused by Gulo. In conclusion, these findings reveal that Gulo promotes the MET and apoptosis, and inhibits proliferation in MM cells by down-regulating Six2.

1. Introduction

The formation of mature kidney originates from the mutual inductive interactions of two adjacent structures, ureteric bud (UB) and metanephric mesenchyme (MM). The UB is induced by MM cells to grow and branch repeatedly, and ultimately forms the collecting duct system of the kidney. Reciprocally, the MM cells surrounding the tips of the branching UB are induced by UB and condensed into cap mesenchyme (CM) cells, which subsequently form pre-tubular aggregates (PTA) and renal vesicles (RV) and finally differentiate into various segments of the nephron epithelium: the podocyte, proximal tubule, loop of Henle and distal tubule [1].

During renal development, the balance between self-renewal and differentiation of MM cells is critical for forming mature kidney with normal morphology and function [2]. The MM is directed by the inductive interactions from UB cells secreting Wnt9b to activate a canonical Wnt-β-catenin signaling pathway in the MM cells [3]. Sine oculis-related homeobox 2 (Six2) highly expressed in undifferentiated MM cells combines with Lef/Tcf complexes to prevent β-catenin from activating these genes which are essential for nephron progenitor commitment [4,5]. Then these $Six2^{+}$ undifferentiated cells sustain their own proliferation (self-renewal) potential, repress consumption (apoptosis) and restrain their premature differentiation through mesenchymal-to-epithelial transformation (MET) [5,6]. On the other hand, a portion of the MM cells lowering Six2 level, takes to expressing Fgf8 and Wnt4 which trigger these menenchymal cells differentiation (MET) into epithelium [7]. All in all, the Six2 is essential to keep the balance of self-renewal and differentiation of cap mesenchyme. But it is still not clarified completely that how the Six2 level is regulated and

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what its upstream regulators are in the process.

Vitamin C mainly functions as an antioxidant and a cofactor of some enzymes implicated in biological reactions in animals [8–10]. L-gulono-γ-lactone oxidase (Gulo) is necessary for catalyzing the last step of biosynthesis of vitamin C [11]. In the case of human being, guinea pigs and some primates, lacking functional GULO, need a dietary intake of vitamin C [12]. In some mammals, Gulo is expressed in liver and kidney both [13]. Vitamin C insufficiency could lead to an abnormal behavioral phenotype, aggravated thioacetamide-induced liver fibrosis and more susceptible to toxic carcinogenesis in Gulo-knockout mice [14,15]. And vitamin C co-operates with TET1 to regulate MET of induced pluripotent stem cells during somatic cell reprogramming and inhibits the migration and epithelial-to-mesenchymal transition (EMT) of epithelial cells [16,17]. However, the physiological effects of Gulo or vitamin C on kidney development have so far not been investigated.

In this study, we aimed to study whether Gulo was an up-stream regulator of Six2 and was involved in MM cells' self-renewal and differentiation.

2. Materials and methods

2.1. Cell culture and transient transfection

mK3 and mK4 cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY, USA), incubated at 37 °C, 5% CO2. Lipofectamine 2000 (Invitrogen, Grand Island, NY) was used for plasmids (pLKO.1-Six2-shRNA/pLKO.1-Control-shRNA and pCDN3.1(+)-Six2/pCDN3.1(+)).

2.2. Plasmid construction

The coding sequence (CDS) of m.Gulo was amplified via polymerase chain reaction (PCR) from the cDNA of mK3 cells and cloned at the site of *Bam*HI and *Eco*RI to generate CMVHIS-GFP-Gulo by ligation-independent cloning (LIC) [18]. The CDS of m.Six2 was amplified via PCR from mK3 cells and cloned into the *Hin*dIII/*Bam*HI site of pCDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA) by LIC. The m.Gulo shRNA and m.Six2 shRNA sequences were acquired from the SIGMA ALORICH with m.Gulo target: 5′-CCCGGCAGCAGAA-CAAGAAAGTGAA-3'; m.Six2 target: 5′-CCTCCACAAGAATGAAAG CGT-3'.

2.3. Lentivirus packaging and infection of cells

The lentivirus production is performed under the guidance of predecessor. Virus packaging was performed by transient transfection of HEK 293 T cells with a transfer pLKO.1-Control-shRNA/pLKO.1-Gulo-shRNA/CMVHIS-GFP-Gulo/CMVHIS-GFP-Control plasmid and two packaging vectors: pMD2.G and psPAX2. Forty-eight hours after the transfection, the lentiviral particles were collected and filtered. Then, parental mK3 and mK4 cells grown in the growth medium were infected with the packaged lentiviral particles with polybrene (8 μg/ml) for 24 h. Subsequently, stable cell lines was screened by puromycin (invivogen) at 10 μg/ml. The established stable cell lines were used for experiments.

2.4. Scratch wound healing assay

mK3 and mK4 stable cell lines were cultured in 6-well plates in complete medium. When the cells aggregation reached approximately 90%, a wound track was scraped across the monolayer cells by a sterile 200 μl pipette tip and cell debris was washed away by 1 × PBS. Then continue to culture the cells in serum-free DMEM. The fluorescence microscope (Nikon, Tokyo, Japan) and SPOT Diagnostic (Sterling Heights, Michigan) CCD camera were together used to acquire time-lapse photographs at 0, 12 and 24 h after scratching. All images were collected from 6 separate areas of each set of samples and the relative migration velocity of the edge of the wound was measured by NIH Image J software (National Institutes of Health, Bethesda, MD, USA).

2.5. 5-Ethynyl-2′-deoxyuridine (EdU) assays

mK3 and mK4 stable cells over-expressing or knocked-down Gulo were incubated in 96-well plate (2000 cells per well) for 24 h, then transfected with plasmids. After 48 h of transfection, the proliferation of mK3 cells were determined in vitro via the EdU DNA Proliferation in Detection kit (RiboBio, Guangzhou, China).

2.6. Flow cytometry assay

The apoptosis of mK3 and mK4 cells was assayed by flow cytometry with the Annexin V-FITC/7-AAD or PI staining (KeyGEN BioTECH, Nanjing, China). For mK3 and mK4 stable cells the cells were cultured in 6-well plates for 48 h, then harvested with trypsin. For transient transfection of mK3 and mK4 stable cell lines, the cells cultured in 6-well plates were transfected with corresponding plasmids when the cells grew to 70% confluence. Then the cells were collected 48 h later with trypsin. The flow cytometry assay were operated by the Flow Cytometry Room, College of Life Sciences, Chongqing Medical University.

2.7. Western blot

The total proteins of mK3 and mK4 cells were dissolved in RIPA lysis buffer (Beyotime Biotechnology, China) complemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Darmstadt, Germany). Then Double Bicamonic Acid (BCA) Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) was used to determine the protein concentration. The protein (15 μg each sample) was resolved by 10% SDS-PAGE. The separated proteins in the gel were electrically transferred to nitrocellulose membrane (Merck, Billerica, MA, USA) after electrophoresis. The nitrocellulose membrane was blocked with 5% non-fat milk diluted in Tris-buffered saline plus 0.05% Tween-20 (TBST). After incubation overnight at 4 °C with primary antibody (anti-E-cadherin (Bioworld, Nanjing, China), anti-Vimentin (Cell Signaling Technology, Danvers, MA, USA), anti-Six2 (Proteintech, Wuhan, China), or anti-Gapdh (CWBIO, Guangzhou, China)), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG (CWBIO, Guangzhou, China) were applied. The positive immune reactive signal was visualized by using enhanced chemiluminescenc detection system with Immolbilon Western HRP Substrate (Merck, Billerica, MA, USA). The expression level of target proteins was normalized to Gapdh.

2.8. Collection of embryo kidney

The morning of the discovery of the vaginal plug was considered as E0.5. We waited until E11.5, E12.5, E14.5, E15.5, E19.5 and P1, collected the kidneys of these embryos for RNA extraction, or stored at −80 °C.

2.9. Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA of embryo kidneys and cultured mK3 and mK4 cells were extracted with TRIzol (Invitrogen) and the RNA was reverse-

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