Biomedicine & Pharmacotherapy 93 (2017) 444–450

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Original article

Carnitine/organic cation transporter 2 (OCTN2) contributes to rat epididymal epithelial cell growth and proliferation

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ARTICLE INFO

Article history:
Received 1 March 2017
Received in revised form 12 June 2017
Accepted 19 June 2017

Keywords:
Apoptosis
Cell proliferation
Epididymal epithelial cells
OCTN2
Spermatogenesis

ABSTRACT

Carnitine/organic cation transporter 2 (OCTN2) is localized at the basolateral membrane of epididymal epithelial cells, and mainly serves to reabsorb carnitine as an essential factor for sperm maturation; however, its functional features in epididymal epithelial cells have remained unclear. We isolated primary epididymal epithelial cells from rat epididymides and verified their phenotype by detecting the presence of cytokeratin-19 (CK-19, an epithelial cell marker) and the absence vimentin (an interstitial cell marker). We found that cultured epididymal epithelial cells isolated from rat epididymides expressed high levels of CK-19 but barely expressed vimentin. Gain-of-function assays, which included the CCK-8 assay and EdU flow cytometry assay, indicated that overexpression of OCTN2 significantly promoted epididymal epithelial cell growth and proliferation. Moreover, forced expression of OCTN2 inhibited the cell apoptosis process, and at the same time increased expression of the pro-apoptosis factor BAX, and decreased expression of the anti-apoptosis factors BCL-2 and Survivin. Furthermore, we also found that OCTN2 overexpression dramatically increased the levels of biomarkers associated with spermatogenesis, including azoospermia-like (DAZL), phosphoglycerate kinase 2 (PGK2), and protamine 2 (PRM2). These results demonstrate that OCTN2 plays a positive role in epididymal epithelial cells, and might be useful in the clinical treatment of male infertility by serving as a key regulatory factor.

1. Introduction

Infertility, defined as the inability of a sexually active individual to conceive a child, is a major problem that affects ~20% of couples, and 20%–55% of cases can be attributed to male factors [1,2]. A male's spermatogenesis index and number of viable sperm significantly impact his fertility potential [3,4]. Spermatozoa released from the germinal epithelium of the testes usually have little motility and fertilizing ability, and are regarded as non-functional gametes [5]. Those spermatozoa can only acquire progressive motility and the ability to fertilize ova by migrating through the length of the epididymis to the vas deferens [6]. During epididymal transport, spermatozoa undergo a series of morphological, biochemical, and physiological changes, and become mature germ cells [7]. Therefore, gaining a better understanding of the biological functions of the epididymis may be important for improving sperm maturation and mammalian fertilization.

The mammalian epididymis is derived from the Wolffian duct, and exists as a long and single tubule located downstream of the testis. It is lined by a continuous layer of epithelial cells that maintain an optimal luminal environment for sperm maturation, protection, transport, and storage [8,9], and also form a blood-epididymis barrier that protects maturing spermatozoa by controlling the luminal fluid environment [10,11]. Additionally, the epididymal epithelium actively transports certain chemical substances, including lipids, steroids, and glycoproteins, into the epididymal lumen, which contains high levels of carnitine [12]. Polyspecific organic cation transporter 2 (OCTN2) is a high affinity carnitine transporter protein encoded by solute carrier family 22

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http://dx.doi.org/10.1016/j.biopha.2017.06.057
0753-3322/

member 5 (*Slc22a5*), and is localized at the basolateral membrane of epididymal epithelial cells [13–15]. OCNT2 is primarily responsible for carnitine-reabsorption. Interestingly, a recent study by Wang et al. [16] showed that knockdown of OCTN2 inhibited L-carnitine intake, and also resulted in lipid droplet accumulation and suppression of cell proliferation in breast cancer, suggesting OCTN2 as a critical regulator of both lipid metabolism and cell growth [16]. However, no information is currently available regarding how OCTN2 contributes to the proliferation of epididymal epithelial cells.

This study was designed to investigate the functional features of OCTN2 in epididymal epithelial cells by use of gain-of-function assays. Furthermore, we also examined the mechanism underlying the growth inhibition induced by OCTN2 overexpression.

2. Materials and methods

2.1. Isolation and culture of rat epididymal epithelial cells

Immature Sprague–Dawley (SD) male rats were purchased from the Shanghai Experimental Animal Center (Shanghai, China), and their epididymal epithelial cells were isolated via a method adapted from a previous study [17]. Briefly, epididymides dissected from the immature rats were minced into 1–2-mm^3 fragments and transferred into 10 mL of EDTA solution containing 0.25% trypsin (GIBCO, Grand Island, NY, USA). After incubation with shaking (60 cycles per min) for 30 min at 32 °C, the tissue fragments were centrifuged at 800 g for 5 min, and then further digested with 1 mg of collagenase type II (Sigma Aldrich, St. Louis, MO, USA) in PBS for 30 min. The dispersed cells were pelleted by centrifugation (800 g for 5 min) and the supernatant was removed. Next, the resultant cell pellets were resuspended in Dulbecco's Modified Eagle Medium (DMDM, GIBCO, USA) and filtered through filter gauze. After being washed twice with PBS, the isolated epididymal epithelial cells were resuspended in DMDM medium and incubated at 32 °C for 10 h. After incubation, the cells were suspended as single cells using 0.25% trypsin and seeded onto new culture plates, which were incubated for 21 consecutive days.

2.2. Epididymal epithelial cell transfection

For OCTN2 overexpression, the rat *OCTN2* gene coding sequence was cloned into pcDNA3.0/OCTN2 or an empty vector (pcDNA3.0) (GenePharma Co. Ltd, Shanghai, China). Transfection was performed using Lipofectamine™ 2000 in accordance with the manufacturer's instructions. Stable epididymal epithelial cells over-expressing OCTN2 and the empty-plasmid transfected epididymal epithelial cells were designated as OCTN2 cells and negative control (NC) cells, respectively, and their expression of OCTN2 was verified by immunocytochemistry, qRT-PCR, and western blotting. Non-transfected cells served as a control group.

2.3. Immunofluorescence

The common marker for epithelial-like cells is cytokeratin (CK)-positive, while vimentin, fibronectin, and N-cadherin are the common markers for interstitial-like cells. Thus, we used one type of cytokeratin (CK19) to confirm that we had successfully isolated primary epididymal epithelial cells from rat epididymides, and used vimentin as a negative control to exclude the presence of mixed interstitial cells (e.g. fibroblasts) when culturing samples of primary tissue. Epididymal epithelial cells were identified and their OCTN2 expression was detected with the use of immunofluorescence staining kits manufactured by Cytelligen (San Diego, CA, USA). Briefly, the cells were fixed in 4% paraformaldehyde in PBS; after which, they were washed with PBS, and their endogenous peroxidase activity was blocked by incubation in 2% hydrogen peroxide for 10 min. The cells were then incubated with primary antibodies which included anti-cytokeratin 19 (CK19), anti-vimentin, and anti-OCTN2 (Abcam, Burlingame, CA, USA) for 1 h at room temperature. After incubation, the cells were thoroughly washing in PBS, and then incubated with the corresponding secondary antibodies for 1 h at room temperature. DAPI (4′, 6-diamidino-2-phenylindole) was used for nuclear counterstaining. The stained cells were observed and photographed using a Zeiss LSM700 confocal microscope (Carl Zeiss, Germany).

2.4. RNA isolation and qRT-PCR

Total RNA was isolated using a TRIzol kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcriptase enzyme was used to synthesize cDNA from the extracted RNA. The qRT-PCR was performed in a Stratagene MX3000P Detection System (Agilent) using the following primers: O*ctn2* forward: 5′-TGGGGCTCTCTGGTGGTT-3′, *Octn2* reverse: 5′-GTGCGACTGAGGCTTCTTG-3′; *Dazl* forward: 5′-TTCATCAGCAAC-CACCAGTC-3′, *Dazl* reverse: 5′-ACAAATCCATAGCCCTTCGA-3′; *Pgk2* forward: 5′-GAGAAAGCCTGTGCCAACC-3′, *Pgk2* reverse: 5′-AACTGTGAGCCCGATGTGC-3′; *Prm2* forward: 5′-AATGAGGAGCCC-CAGTGAG-3′, *Prm2* reverse: 5′-TGTGGCGGCAGGAGTGT-3′. The thermo cycler parameters were as follows: initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation for 20 s at 58 °C, and 40 s of annealing extension at 72 °C. Gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method, and normalized with a housekeeping gene (*GAPDH*).

2.5. Protein extraction and western blotting

Transfected cells were washed twice with PBS and then lysed in RIPA lysis buffer (Beyotime, Jiangsu Province, China). After centrifugation at 12,000 rpm for 10 min, the total cellular proteins were extracted according to the protocol provided with a nuclear protein extraction kit manufactured by KeyGen Bio TECH (Nanjing, China). The total protein concentrations were determined using a BCA protein assay kit. Aliquots of extracted protein were separated on SDS-PAGE gels; after which, the separated protein bands were transferred onto PVDF membranes (Millipore Co., Billerica, MA, USA). Next, the membranes were blocked with 5% non-fat milk and 0.1% Tween-20 at room temperature, and then incubated overnight at 40 °C with primary antibodies against OCTN2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA 1:2000), DAZL (Cell Signaling Technology, Danvers, MA, USA, 1:1000), PGK2 (Abcam, 1:1000), PRM2 (Santa Cruz Biotechnology, 1:3000), and GAPDH (Abcam, 1:10000). Following incubation with the primary antibodies, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Antibody staining was detected with an ECL chemiluminescence system (Santa Cruz Biotechnology). GAPDH served as an internal control.

2.6. CCK-8 assay

Cell growth rates were determined using a Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol. In brief, the NC or OCTN2 transfected cells were seeded into 96-well plates at a density of 2000 cells per well. Next, the cells were first incubated at 37 °C for periods of 24, 48 or 72 h, and then incubated with CCK-8 solution (DOJINDO, Kumamoto, Japan). The optical density (OD) of each well at 450 nm was detected using a microplate reader (Bio-Rad, Hercules, CA, USA).