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Down-regulation of lncRNA MEG3 promotes endothelial differentiation of bone marrow derived mesenchymal stem cells in repairing erectile dysfunction

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ABSTRACT

Aims: In the treatment of diabetes mellitus associated erectile dysfunction (DMED), the intracavernous and periprostatic implantations of bone marrow derived mesenchymal stem cells (BM-MSCs) represent the new therapeutic approaches with great applied prospect. However, the specific mechanisms of BM-MSCs protecting erectile function remain largely unknown.

Materials and methods: The DMED rats were induced and the erectile function was assessed in the models with or without BM-MSCs implantation using intracavernous pressure (ICP)/mean arterial pressure (MAP) ratio. The differentiation of BM-MSCs toward endothelial cells (ECs) was induced by exogenous vascular endothelial growth factor (VEGF) in vitro. RNA pull-down and RIP assays were performed to explore the interaction between MEG3 and FOXM1 protein.

Key findings: Intracavernous implantation of BM-MSCs effectively improved the erectile function of DMED rats, which was accompanied by a significant decrease in the expression of MEG3 in the corpus cavernosum tissues. Also, our study revealed that MEG3 expression was significantly down-regulated during the endothelial differentiation of BM-MSCs in vitro. The down-regulation of MEG3 was further confirmed to be conducive to the differentiation of BM-MSCs toward ECs. More importantly, MEG3 promoted the degradation of FOXM1 protein via facilitating FOXM1 ubiquitination, thereby decreasing VEGF expression, which ultimately regulated the endothelial differentiation of BM-MSCs.

Significance: Taken together, our findings presented the vital role of MEG3 in the repairing processes of BM-MSCs for erectile function and provided new mechanistic insights into the BM-MSCs-mediated DMED repairing.

1. Introduction

Erectile dysfunction (ED) is one of the common diseases that threaten the health of middle-aged and elderly men [1,2]. Clinical studies have shown that diabetes mellitus (DM) associated with ED, and 50%–75% of the patients with DM suffer from ED to some degree [3]. Epidemiological investigation revealed that ED was 1.9–4 times more common in DM patients than in non-diabetic patients [4]. DM-induced ED (DMED) is a complicated pathophysiologic process which is related to multiple factors such as endothelial dysfunction, nerves, endocrine disorder and psychology. Phosphodiesterase 5 inhibitor (PDE5i) is the first choice in the clinical treatment of ED [5]. Unfortunately, the therapeutic effect of PDE5i for DMED patients was relatively poor than that for common ED patients [2]. Developing new therapeutic approaches for DMED has an important clinical significance and social meanings.

At present, stem cell transplantation is one of the most promising methods for the treatment of DMED. Among them, bone marrow derived mesenchymal stem cells (BM-MSCs) with multi-lineage differentiation properties have been confirmed to be used as an ideal source of cell transplantation for recovering DMED [6,7]. In addition to differentiation into various lineages of tissue cells, BM-MSCs were also reported as regulating tissue cell proliferation, apoptosis, and immunomodulation through synthesizing and secreting cytokines and nutrition factors [8,9]. Importantly, BM-MSCs was identified to have the potential to differentiate toward cavernous smooth muscle cells (SMCs) and endothelial cells (ECs) in the cavernosum of the Sprague-Dawley rats, which indicated that BM-MSCs potentially be used as a good candidate for restoring erectile function [10]. Studies have reported that intracavernous and periprostatic implantations of BM-MSCs

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effectively improved erectile function in cavernous nerve injury (CNI) models by increasing cavernous ECs and SMCs content [11,12]. However, the specific mechanisms of BM-MSCs protecting erectile function remain to be fully elucidated.

Increasing studies reported the involvement of long noncoding RNAs (lncRNAs) in the biological functions of BM-MSCs, including lncRNA maternally expressed gene 3 (MEG3). MEG3 is a myeloid-related lncRNA that found to be dysregulated in human diseases, especially in tumorigenesis [13]. Moreover, MEG3 was reported as regulating the osteogenic differentiation of BM-MSCs that involved in postmenopausal osteoporosis progression [14]. It was also recently demonstrated that MEG3 could repress the proliferation and migration of ECs [15]. However, whether MEG3 controls BM-MSCs differentiating toward ECs has yet to be explored.

The bioinformatics analyses showed that MEG3 probably interacted with FOXM1 protein. FOXM1, belonging to Forkhead box (Fox) transcription factor family, was found to physically interacted with the vascular endothelial growth factor (VEGF) promoter to transcriptionally activate VEGF expression [16]. Additionally, VEGF possessed the efficiency to induce BM-MSCs to undergo to EC differentiation [17,18]. We hypothesized that MEG3 involved in the biological behavior of BM-MSCs through regulating FOXM1/VEGF. Based on this, we explored whether MEG3 controls BM-MSCs differentiating toward ECs and the underlying mechanisms in recovering DMED.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats aged 10 weeks were purchased from the Animal Center of Military Medical Science Academy, Beijing, China. All rat experiments were supported by the Ethics Committee of the First Affiliated Hospital of Nanchang University and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals released by Ministry of Science and Technology, China.

2.2. BM-MSCs culture

BM-MSCs were isolated from the bone marrow of SD rats as described in previous study [7]. The rat was sacrificed by cervical dislocation and immersed in 75% ethanol for systemic disinfection. Under sterile conditions, the femora and tibia were detached and the metaphysis was dissected. Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco) was used to elute the bone marrow from cavities. The eluate was cultured in DMEM containing 10% FBS, 1% penicillin-streptomycin (HyClone) at 37 °C with 5% CO_2. After 48 h, the medium was replaced with fresh medium to remove non-adherent cells. BM-MSCs were sub-cultured by trypsin (0.25%, Sigma-Aldrich) digestion, and the 3rd generation cells were used for the subsequent experiments.

2.3. Rat DMED model and BM-MSCs transplantation

The DM rat models (n = 30) were induced by intraperitoneal injection of streptozotocin (60 mg/kg, Sigma-Aldrich), and were perceived as successful if the blood sugar level was above 16.7 mmol/L 72 h later. The ED was also confirmed in DM rat models by apomorphine (APO) test as described in previous study [19]. With subcutaneous injection of APO (100 μg/kg, Sigma Chemical Co., Ltd.), 21 out of 30 DM rats successfully developed diabetic ED were used for further experiments. The pathological features of DMED rat were glans penis hyperemia, increase of penis volume and erection of the terminal phallosome. BM-MSCs (5×10^6 cells in 100 μL of PBS) were injected into the corpora cavernosum of DMED rats (n = 7). The rats (n = 7) receiving 100 μL of PBS were used as the controls.

2.4. Determination of ICP/MAP

After anaesthesia, the rat was supinely fixed to monitor mean arterial pressure (MAP) by the carotid artery. A ventral midline incision was made to expose the main pelvic ganglion and cavernous nerve under an operative microscopy. The cavernous nerve was entangled by the electrode. The needle was inserted into the bottom of corpora cavernosum to detect the intra-cavernous pressure (ICP). The electrical stimulation was at 5.0 mV and a frequency of 20 Hz with a pulse width of 5 ms for 60 s. The ratio of ICP/MAP was calculated.

2.5. Quantitative real-time PCR analysis

Penile tissues or BM-MSCs were harvested for RNA extraction using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The purity and quantification of isolated RNA were determined by the A260 and A280 using a NanoDrop 2000C (Thermo Scientific). And the RNA was subjected to DNase digestion (Roche, Mannheim, Germany) at room temperature for 10 min and terminated by the incubating EDTA (2.3 mM) for 15 min at 70 °C. The cDNA was generated from 1 μg of RNA with a 20 μL reaction using iSCRIPT cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed with cDNA product and gene-specific primers using SYBR Premix EX Taq TM (TaKaRa) according to manufacturer's instructions. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as control. The gene-specific primers were synthesized as the following sequences: MEG3 forward, 5′-AGACAACAGGCC GTCCAGGAG-3′ and reverse, 5′-GAAGAGCGAGTCAGGAAGCAGTG-3′; vWF forward, 5′-CTCTGGCTGTGACGACTTCAA-3′ and reverse, 5′-CAA ACAGGGGCCGTATCTCC-3′; VE-cadherin forward, 5′-GCCACGCCACT GTCTTGTACC-3′ and reverse, 5′-TCTTGTGCTTCCACCACGATCTTG-3′; eNOS forward, 5′-CACAGGCATCACCAGGAAGAAGAC-3′ and reverse, 5′-TTCACACGCTTCGCCATCACC-3′.

2.6. RNA pull-down assay

RNA pull-down assay was performed to explore the interaction between MEG3 and FOXM1 protein using RNA-Protein Pull-Down Kit (Thermo Scientific) according to the manufacturer's instructions. MEG3 was biotinlabeled with the Biotin RNA Labeling Mix (Roche) according to the manufacturer's instructions. Biotinylated RNAs were then incubated with streptavidin magnetic beads (Invitrogen) and the cell lysates for 1 h. The RNA-protein complexes were used for Western blot analysis of FOXM1 level. The input sample was used as a control to verify that the Western blot is properly functioning.

2.7. RNA-binding protein immunoprecipitation assay

RNA-binding protein immunoprecipitation (RIP) assay was further performed with anti-FOXM1 antibody to determine whether MEG3 could be enriched in the complex immunoprecipitated by anti-FOXM1 antibody. And anti-IgG antibody was used as the negative control. According to the manufacturer's protocol of the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore), BM-MSCs (5.0×10^6 cells) were lysed with RIP lysis buffer and the cell lysates were incubated with the magnetic beads coated with anti-FOXM1/IgG antibody in RIP buffer for 8 h at 4 °C. The complexes eluted by wash buffer were then incubated with Proteinase K to remove the protein. And the immunoprecipitated RNA was used for qRT-PCR analysis of MEG3 level as previously described.

2.8. Ubiquitination assays

HEK293T cells were co-transfected with MEG3, His-ubiquitin (Ub) and myc-FOXM1 expression vectors and treated by MG132 for the last 4 h. After 24 h of transfection, the cells were lysed by urea buffer. The