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Original Article

Are Sertoli cells a kind of mesenchymal stem cells?

Daoyuan Gong[1*], Chunfu Zhang[2*], Tao Li[3], Jiahui Zhang[3], Nannan Zhang[3], Zehua Tao[3], Wei Zhu[3], Xiaochun Sun[3]

[1]School of Stomatology, Foshan University, 5# Hebing Road, Foshan 528000, Guangdong, P. R. China; [2]The Second People's Hospital of Kunshan, 42 Zhongshan Road, Kunshan, Jiangsu 215300, P. R. China; [3]School of Medicine, Jiangsu University, 301# Xuefu Road, Zhenjiang 212001, Jiangsu, P. R. China. [*]Equal contributors.

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Abstract: Objective: Sertoli cells (SCs) are a major component of testis which secrete a variety of cytokines and immunosuppressive factors, providing nutritional support and immune protection for sperm growth and development. The purpose of this study was to investigate the relationship between SCs and bone marrow mesenchymal stem cells (BMSCs) in order to provide a theoretical basis for better application of SCs. Methods: We used the adherence method to isolate Sprague-Dawley rat SCs and BMSCs. Cells surface markers were detected by flow cytometry. The capacity of cells to differentiate was determined by osteogenic and adipogenic induction. Assessment of cell proliferation was performed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-H-tetrazolium bromide] assay. Changes in the nucleus were analyzed by Hoechst nuclear staining. Cell aging was observed with β-galactosidase, which is a biological marker of senescence. RT-PCR was employed to detect the expression of cytokines. Results: From the aforementioned experiments, we found that the surface markers of SCs and BMSCs were almost exactly the same. Proliferation of SCs, as well as osteogenic and adipogenic differentiation, were weaker than in BMSCs. Compared with BMSCs, Hoechst nuclear staining showed that the chromatin of SCs began to aggregate and was slightly larger. β-galactosidase staining showed that SCs were in a slightly aging state. The secretion of cytokines from SCs was slightly less than the secretion from BMSCs. Conclusion: SCs are a kind of mesenchymal stem cells which have begun the process of differentiation.

Keywords: Mesenchymal stem cells, Sertoli cells

Introduction

Sertoli cells (SCs) are non-germ cells which localized in seminiferous epithelium. SCs can secrete a variety of immune protection factors, growth factors and nutritional factors, which play a role in the support and nutrition of spermatogenic cells, particularly, they can synthesize and secrete androgen-binding protein [1]. SCs also secrete a variety of cytokines and provide nutritional support for islet cells and nerve cells both *in vivo* and *in vitro*. Greene et al. originally observed immune privilege in germ cells [2], and Selawry et al. confirmed immune privilege in SCs [3]. The cultures of SCs have recently been utilized in a wide range of applications. For example, culture of SCs with neurons and primordial germ cells can promote the growth of the co-cultured cells. SCs can exert immune privilege after transplantation of islet cells [4, 5].

Friedenstein and other researchers, when carrying out bone marrow cell culture *in vitro*, accidentally discovered a fibroblast-like cell which, in the presence of inducing agents, could differentiate into bone cells, fat cells and muscle cells [6-9]. These pluripotent cells subsequently displayed clonal growth of fibroblast-like cells, defined by Caplan as bone marrow mesenchymal stem cells (BMSCs) [10]. BMSCs secrete a large number of hematopoiesis-related cytokines to promote the growth and development of hematopoietic stem cells [11]. In addition, BMSCs can spontaneously produce cell adhesion molecules and chemokines to facilitate interaction with different cell types and attract hematopoietic stem cells [12]. Many studies [13-16] have shown that mesenchymal stem cells (MSCs) have a strong effect on immune regulation, but the specific mechanism has not been fully clarified. Currently, it is thought that MSCs act by inhibiting immune cell activation

Table 1. Specific primers for control and target genes

ID	Gene	Primers sequence, 5' to 3'	Size, bp	Annealing, °C
1	TPO-F	ATTGCTCCTCGTGGTCAT	220	56.0
	TPO-R	CTCCTCCATCTGGGTTTT		
2	SCF-F	TGGATAAGCGAGATGGTA	189	54.0
	SCF-R	TTCTGGGCTCTTGAATGA		
3	VEGF-F	CCTTGCTCTACCTCCAC	280	61.0
	VEGF-R	ATCTGCATCCTGTTGGA		
4	GAPDH-F	GAGTCTACTGGCGTCTTCAC	272	58.0
	GAPDH-R	GTCTTCTGAGTGGCAGTGAT		

and inducing tolerance in immune cells including natural killer (NK) cells, B lymphocytes, T lymphocytes and dendritic cells [13-20]. In the present study, we found that the morphology and some biological functions of SCs are similar to those of MSCs. We therefore, speculate that SCs are a type of MSCs which represent the early stage of differentiation.

Material and methods

Isolation and culture of SCs and BMSCs

SCs and BMSCs were isolated and cultured according to standard protocols [21]. Cells from 80 g male Sprague-Dawley rats were collected by flushing testis tissue and femurs with phosphate-buffered saline (PBS). Cells were cultured in low-glucose Dulbecco's modified Eagle's medium (L-DMEM; Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 U/ml streptomycin under a humidified atmosphere at 37°C with 5% CO_2. To remove non-adherent cells, the medium was changed every 5 days after initial plating. Subsequently, all culture medium was replaced at 3 day intervals. When adherent cells reached 80%-90% confluence, the cells were trypsinized with 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) and subcultured in new flasks for further expansion. Cells at passages 3-5 were used for experiments. The experimental protocol was approved by the Institutional Animal Care Committee of Jiangsu University, Zhenjiang, China.

Flow cytometry

After the third passage, SCs and BMSCs were trypsinized with 0.25% trypsin-EDTA, washed twice with PBS and stained on ice. The phenotype of SCs and BMSCs was analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Cells were immunostained with FITC-conjugated monoclonal antibodies against CD29, CD44 and CD45 (Becton-Dickinson) for 30 min at 4°C. FITC-IgG1 isotypic immunoglobulins were used as isotype controls.

Multi-differentiation capacity

Adipogenesis: SCs and BMSCs were plated in 24-well plates in L-DMEM with 10% FBS. When the cells reached 60% confluence, the culture medium was changed to adipogenic induction medium (medium with 10 μg/ml insulin, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine and 200 μM indomethacin). Three weeks later, adipogenic differentiation was detected by the presence of intracellular lipid vesicles stained with Oil Red O and visualized using an inverted microscope (TE300; Nikon Corporation, Tokyo, Japan).

Osteogenesis: SCs and BMSCs were seeded at 1×10^4 cells/cm^2 in 6-well plates in L-DMEM with 10% FBS. After overnight incubation at 37°C with 5% CO_2, the cells were treated with osteogenic induction medium (0.1 μM dexamethasone, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 4 μg/ml basic fibroblast growth factor). Two weeks later, osteogenic differentiation was assessed by alkaline phosphatase (ALP) staining following the manufacturer's instructions (SHANGHAI SUN Biotech, Shanghai, China).

MTT assay

Assessment of the proliferative ability of SCs and BMSCs was performed using MTT assays. The cells were seeded in 96-well plates at a density of 3000 cells per well and allowed to attach overnight. After test culture periods of 0, 24, 48 and 72 h, MTT (20 μl) was added to each well for the last 4 h. The reactions were terminated by removing all culture medium and adding 150 μl dimethylsulfoxide (Sigma, St. Louis, MO, USA) to each well. Following uniform oscillation for 10 min to ensure complete solubilization of the purple formazan crystals, the absorbance values were determined at 490 nm with an enzymelinked immunosorbent plate

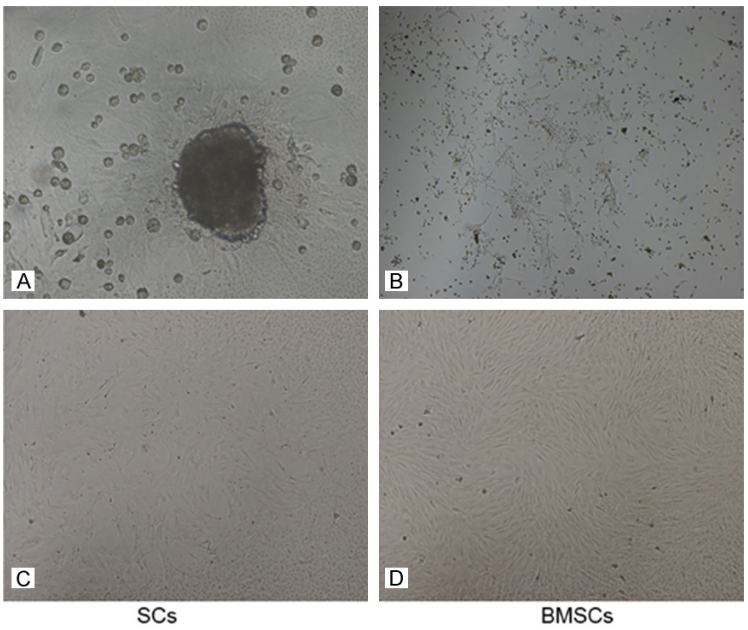

Figure 1. Morphology of SCs and BMSCs. A. SCs were observed extending from testis tissue. SCs presented as polygonal, spindly and fibroblast-like after 8 days in primary culture. B. BMSCs were long, spindle-shaped and fibroblastic in appearance after 7 days in primary culture. C. The second generation of SCs showed a long spindle shape. D. The second generation of BMSCs showed a long spindle shape and were fibroblast-like.

reader (FLX800, BioTek instruments, Vermont, USA).

Hoechst staining

SCs and BMSCs were seeded into 24-well plates, 2×10^4 cells per well. After culture for 48 h, the medium was removed and cells were washed with PBS three times. After incubation with 4% paraformaldehyde for 20 minutes, the fixative was removed and cells were again washed with PBS. Hoechst staining solution (5 μm/ml solubilized in PBS) was added and cells were incubated in the dark at room temperature for 5 min. The staining solution was removed and cells were washed with PBS three times, 5 min per wash, then observed directly under a fluorescence microscope.

Senescence-associated β-galactosidase staining

Expression of senescence-associated β-galactosidase (SA-β-gal) was analyzed in SCs and BMSCs using a SA-β-gal staining kit (Beyotime Institute of Biotechnology, Shanghai, China). In short, cells were washed with PBS, fixed for 15 min with 4% formaldehyde and washed with PBS. Subsequently, the cells were incubated overnight at 37°C in a CO_2-free chamber with freshly prepared SA-β-gal stain solution. The SA-β-gal-positive cells exhibited blue. The number of positive cells was counted in a total of 200 cells using light microscopy.

Reverse transcription-polymerase chain reaction (RT-PCR)

The cycling conditions for PCR were as follows: Initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58-61°C for 30 s and extension for 30 s at 72°C, and a final extension at 72°C for 10 min. The PCR products were checked by electrophoresis on a 1.5% agarose gel with ethidium bromide staining, visualized under UV light and analyzed using the Gel Image Analysis System (Tanon 2500R, Tanon Science and Technology, Shanghai, China). GAPDH was used as internal control. The cDNA samples were subjected to PCR with specific primers synthesized by Invitrogen Life Technologies (**Table 1**).

Statistical analysis

All data were expressed as means ± standard deviation (SD). Differences between more than two groups were analyzed by one-way analysis of variance with the Newman-Keuls multiple comparison test using the GraphPad Prism V5.0 software program (GraphPad, San Diego, CA, USA). *P* values <0.05 were considered statistically significant.

Results

Characterization of SCs and BMSCs

In testicular tissue primary cultures grown for up to 8 days, long spindle cells were observed extending from the testis tissue (**Figure 1A**, **1C**). In bone marrow primary cultures grown for 7 days, fibroblast-like spindle cells were observed which grew in colonies (**Figure 1B**, **1D**). According to flow cytometric analysis, SCs and BMSCs were positive for CD29 and CD44, but

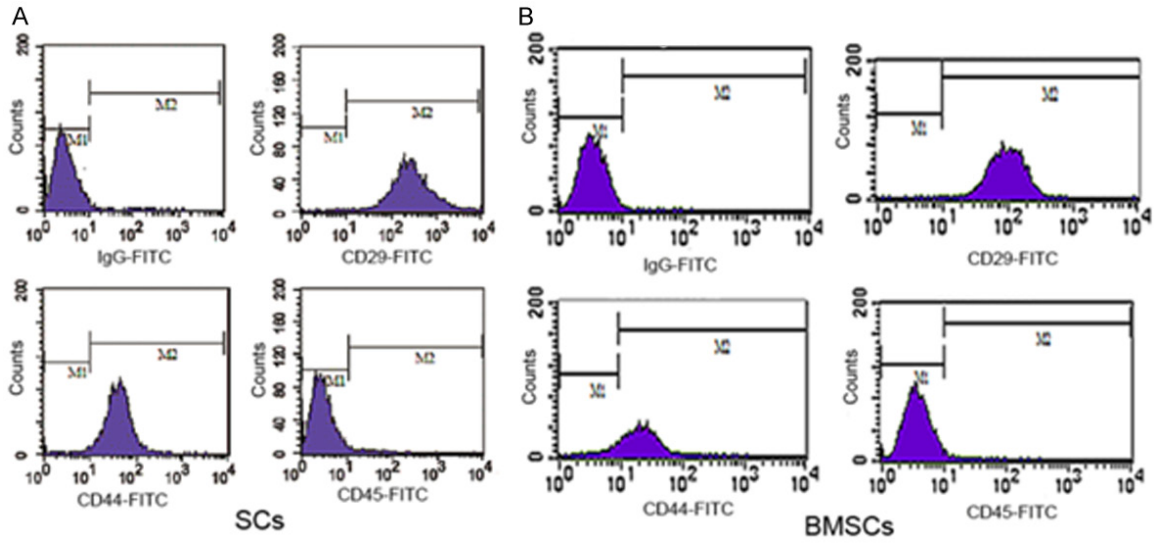

Figure 2. Surface markers identified by FACS analysis. A. IgG indicates isotypes. SCs are positive for CD29 and CD44, but negative for CD45. B. IgG indicates isotypes. BMSCs are positive for CD29 and CD44, but negative for CD45.

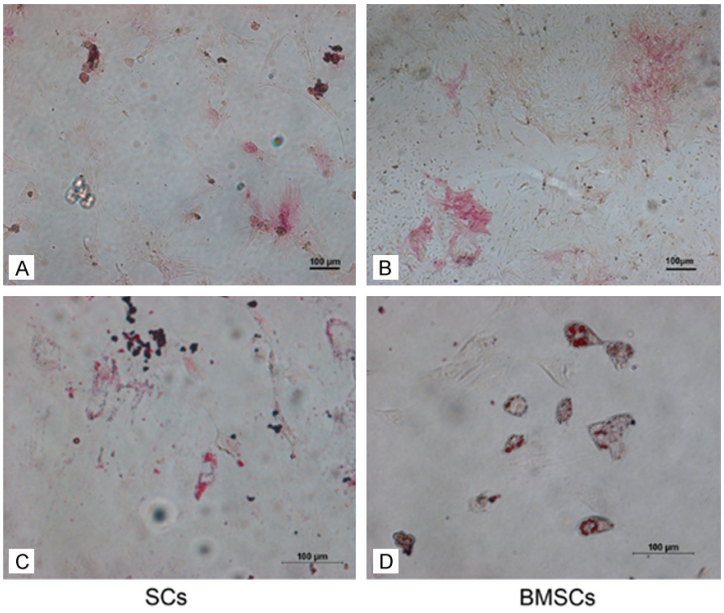

Figure 3. Differentiation potential of SCs and BMSCs. A, B. ALP detection in cell cultures grown for 2 weeks in osteogenic medium. A portion of SCs and MSCs became ALP positive. C, D. Oil Red O staining after three weeks in adipogenic medium. A number of the cells contained numerous Oil Red O-positive lipid droplets.

negative for CD45 (**Figure 2**). The phenotypes of these two cell types were consistent with characteristic MSC surface markers.

Multi-differentiation capacity

In order to investigate the differentiation potential of SCs and BMSCs, the cells were induced to undergo adipogenic or osteogenic differentiation. After induction of differentiation, SCs and BMSCs stained with Oil Red O or ALP. The percentage of Oil Red O-positive or ALP-positive cells did not show significant difference between the SC and BMSC group (**Figure 3**).

Cell viability MTT assay

After 24 h, when MTT assays showed both types of cells entering the logarithmic growth phase, the proliferative activity of SCs was slightly lower than that of BMSCs (**Figure 4**).

Nuclear staining

Hoechst staining showed that, compared with the BMSCs, SCs nuclei were slightly larger, and the nuclear chromatin was not homogeneous, but there did not appear to be any chromatin cohesion and aggregation, irregular shapes and apoptotic bodies (**Figure 5**).

Senescence-associated β-galactosidase staining

Senescence-associated β-galactosidase staining showed that, compared with the BMSC

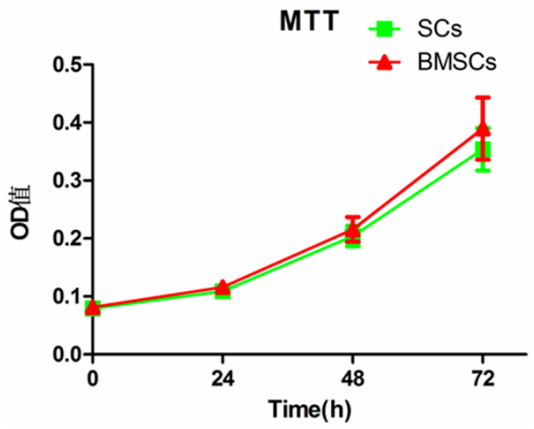

Figure 4. MTT assay. SCs and BMSCs were incubated for 24-72 h and cell viability was determined by MTT assay. MTT, 3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyl tetrazolium bromide. Date were listed as mean ± SD of three samples.

group, SCs had more senescent cells, suggesting that SCs have begun to age (**Figure 6**).

Expression of genes associated with hematopoiesis

RT-PCR was used to characterize SCs and BMSCs for expression of cytokines which participate in the process of hematopoiesis. As shown in **Figure 7**, there was no significant difference in the expression of TPO, SCF and VEGF compared to the BMSC group.

Discussion

Basic and clinical research regarding MSCs is an important frontier in biomedical research. The plasticity of MSCs has enabled their therapeutic application in the treatment of many diseases. Use of MSCs for cell therapy and gene therapy of seed cells has been widely used in clinical treatment [21-25].

The multiple differentiation pathways of BMSCs make them attractive targets for cell transplantation in the treatment of Parkinson's disease, cerebral infarction, brain injury, spinal cord injury, nervous system diseases, bone tissue engineering, cardiovascular disease, liver repair and pulmonary fibrosis therapy. The potential for BMSCs to reduce bone marrow transplantation rejection offers great application possibilities for transplantation [26-29]. BMSCs also exert an inhibitory immune regulatory effect which helps to maximize immune tolerance after transplantation, but their effect alone is not sufficient to completely suppress the rejection reaction, and with further differentiation the inhibitory effect of BMSCs on immune function gradually declines [30-32].

For many years, SCs were thought to be a scaffold for germ cells [2]. We now know that SCs are involved in germ cell differentiation and development of the micro-environment, not just in the seminiferous epithelium, and play a central role in nutrition, phagocytosis, secretion, immune privilege and other functions [33, 34]. SCs inhibit the immune response, and graft transplantation significantly prolongs allograft and plant survival *in vivo* and reduces transplant immune rejection [35, 36]. SCs secrete a variety of substances that promote cell growth, proliferation and differentiation [37]. SCs can produce high levels of FasL, which has been shown to induce T lymphocyte apoptosis following Fas activation, which may be one of the mechanisms underlying testicular tissue immune privilege [38].

Because of their unique cell structure and morphology and their function to secrete various neurotrophic factors, SCs are essential for understanding male sterility disease pathogenesis, and their ability to promote cell growth may have important applications. SCs also present opportunities to design new male contraceptive drugs, especially in the field of tissue engineering and organ transplantation, which will be important research topics in the future. The ethical limitations of SCs transplantation can be solved by the many ways in which SCs can be induced by stem cells and xenogeneic transplantation. Studies have also shown that SCs have a beneficial effect on xenografts [39-41].

By contrasting SCs and BMSCs, our experimental results show that these two types of cells are fusiform, with cell surface marker consistent with MSC surface markers, and similar biological functions. Previous research has shown that SCs and BMSCs secrete a large number of cytokines and can reduce the immune response [18, 19, 40, 41]. Given their very similar biological functions, we believe that SCs are a type of MSCs, but to what extent SCs fully share the characteristics of MSCs remains to be determined.

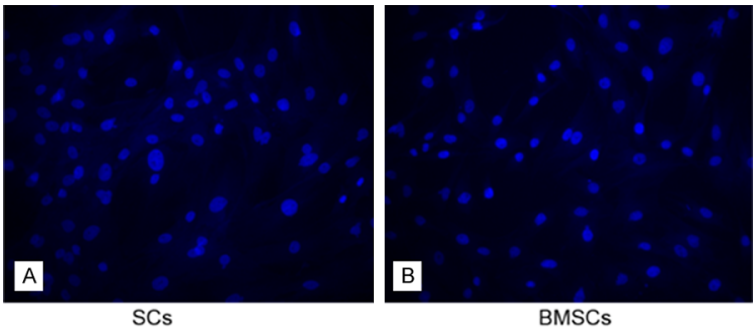

Figure 5. Hoechst staining. A. Nuclear analysis of SCs by Hoechst staining. B. Nuclear analysis of BMSCs by Hoechst staining. SCs nuclei were slightly larger compared with the BMSCs.

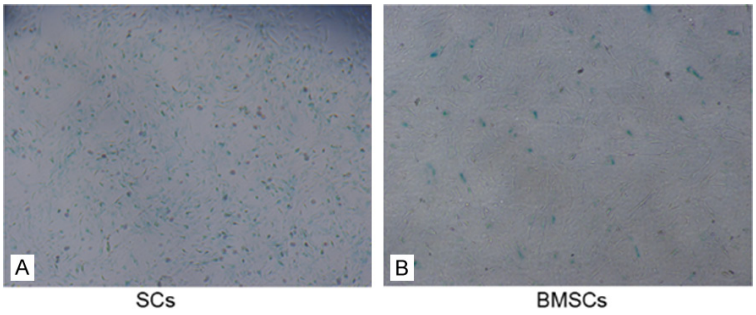

Figure 6. Senescence-associated β-galactosidase staining. A. Senescent SCs detected by SA-β-gal staining. B. Senescent BMSCs detected by SA-β-gal staining. SA-β-gal, senescence-associated β-galactosidase. SCs had more senescent cells compared with the BMSC group.

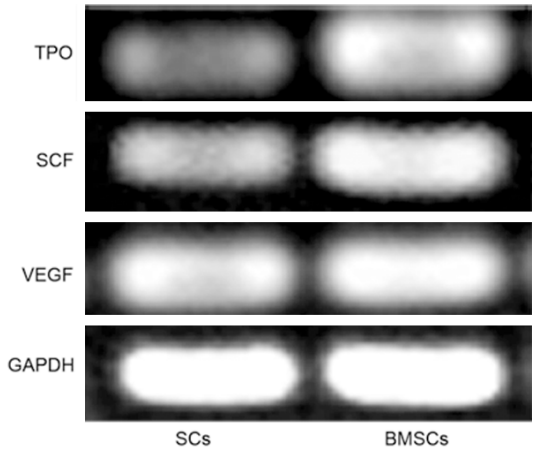

Figure 7. Expression of genes associated with hematopoiesis. mRNA expression of TPO, SCF and VEGF was determined by PCR in SCs and BMSCs. The expression of TPO, SCF and VEGF was not significantly compared to the BMSC group.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaochun Sun, School of Medicine, Jiangsu University, 301# Xuefu Road, Zhenjiang 212001, Jiangsu, P. R. China. E-mail: xiaochun@ujs.edu.cn

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