·Basic Research·

Msx2 plays a critical role in lens epithelium cell cycle control

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Abstract

• **AIM: To investigate the effects of *Msx2* on lens epithelium cell cycle, and evaluate the changes of the proliferation, apoptosis of lens epithelium cells.**

• **METHODS: Mice lens epithelium cells were cultured and transfected with *pEGFP-Msx2* and control. *Msx2*-deficient mice (*Msx2*$^{-/-}$) lens tissue were isolated. Lens tissue and transfected cells were prepared for mRNA extraction using Trizol reagent. *CyclinD1* and *Prox1* expression were evaluated by real-time RT-PCR. BrdU incorporation and apoptosis rate were investigated by immunofluorescence and flow cytometry analysis.**

• **RESULTS: After transfected with *pEGFP-Msx2*, lens epithelium cells failed to incorporate BrdU and anti-phospho-histone-3 immunofluorescence failed to detect cell nuclei which GFP were positive. *Msx2* over expression resulted in increasing apoptosis rate in lens epithelium cells. *CyclinD1* and *Prox1* expression increased significantly in *Msx2* knockout mice by real-time RT-PCR quantization and *CyclinD1* expression decreased significantly in *Msx2* overexpressed cell.**

• **CONCLUSION: *Msx2* has the effect of inhibiting proliferation and differentiation, triggering apoptosis on mice lens epithelium cells.**

• **KEYWORDS:** *Msx2*; lens epithelium cell; cell cycle

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INTRODUCTION

Lens provides an essential function of transmitting and focusing light onto the retina. The lens contains a monolayer of epithelial cells at the anterior surface and a mass of elongated fiber cells that extend from the anterior to the posterior sutures [1]. Epithelial cells proliferate, migrate to the equator, and differentiate to produce new fiber cells, leading to radially symmetric lens growth[2,3].

Many factors influence the lens development, including *Msx* homeobox gene [4-10]. *Msx* genes are important regulators of eye developmental processes. Developmental anomalies associated with gain- and loss-of-function mutations in *Msx1* and *Msx2* clearly demonstrate the importance of these homeodomain transcriptional factors in controlling the development of the skull, hair follicles, teeth, heart and the brain [11-17]. Consistent with their role in controlling eye development is our previous finding that loss of *Msx2* function down-regulated the *FoxE3* expression and resulted in anterior segment dysgenesis resembling peters anomaly[10]. These phenotypes could be consistent with *Msx2*'s role in regulating cell proliferation and differentiation processes. Temporal control of cell fate decisions is critically important, which ensure that differentiation events are executed in the correct sequence. In this study, we attempted to clarify the role of *Msx2* in lens epithelium cell proliferation and differentiation.

MATERIALS AND METHODS

Cell culture and DNA transfection Alpha-TN4 mice lens epithelium cells were seeded into 6-well plates and maintained in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) in the presence of 10% fetal bovine serum and 20μmol/L BrdU. When the cells attained 60%-80% confluence, transfections were performed using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to existing transfection protocol [18]. *pEGFP-Msx2* and *pEGFP-C1* plasmid (a gift from professor Yi-Hsin Liu, Southern California University, USA) were used and each transfection was carried out in triplicate.

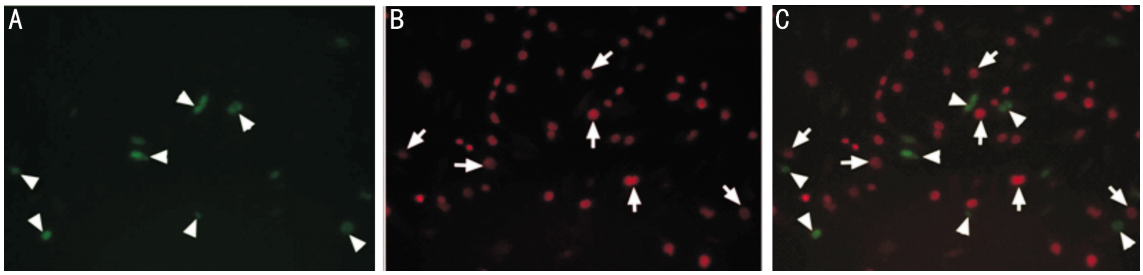

Figure 1 *Msx2*-GFP positive cells failed to incorporate BrdU A: *Msx2*GFP transfected cells (arrow head); B: BrdU positive cells (arrow); C: Merged image showed that GFP positive cells failed to incorporate BrdU.

Experimental mice breeding and genotyping All animal experiments followed the guidelines of the Association for Research in Vision and Ophthalmology Statement for the 'Use of Animals in Ophthalmic and Vision Research'. Experimental mice are the off springs of homozygous *Msx2*-deficient mice strains (*Msx2*$^{-/-}$)[19]. Mice genomic DNA from the embryonic tail tissue was extracted using the hot sodium hydroxide and Tris (HotSHOT) method.

Immunofluorescence Twenty-four hours after transfection, Alpha-TN4 cells were fixed in 4% PFA for 10 minutes and washed with PBS. Anti-BrdU and anti-phospho-histone3 antibody (Sigma-Aldrich, St. Louis, MO, USA) were added into the well and incubated for 2 hours at room temperature. To probe for the primary antibodies, a goat anti-rabbit or a rabbit anti-mouse secondary antibody conjugated to rhodamine red (Invitrogen, Carlsbad, CA, USA) was added and incubated for 30 minutes. Fluorescence images were acquired using an Olympus inverted fluorescent microscope equipped with a SPOT CCD camera.

Quantitative teal –time reverse transcription PCR Twenty-four hours after transfection, total RNA was isolated from transfected Alpha-TN4 cells. And the pregnant females were sacrificed at E19 days and lens tissue of the embryo were isolated in ice-cold PBS. Lens tissue and transfected cells were prepared for mRNA extraction using Trizol reagent (Invitrogen) according to the manufacturer's recommendation. cDNA were synthesized using the SuperScript III First-Strand Synthesis kit (Invitrogen).

Quantitative real-time PCR was carried out using SYBR Green fluorescence dye (TaKaRa China) on a Realtime-PCR system (Applied BioSystems, CA). PCR primers used in this study were listed in Table 1. All samples were run in triplicate, and the relative levels of each of mRNA were normalized to those of beta-actin.

Flow cytometry analysis The effects of *Msx2* on the apoptosis of Alpha-TN4 cells were determined by flow cytometry using the Annexin V: propidium iodide (PI) Apoptosis Detection Kit (KAIJI Biotech, Nanjing, China) according to the recommended protocol. Ten thousand cells were counted, per sample. The data were analyzed with cell quest software.

Table 1 PCR primer sequences used in material and method

Primer	Sequence (5'-3')
CyclinD1	GAGGAGCAGAAGTGCGAAGA
	GAGGGTGGGTTGGAAATGA
Prox1	TTGATGTGGATCGCTTATGT
	CTTCTGCTTGCGTTTGTTT
Beta-actin	CATCCGTAAAGACCTCTATGCCAAC
	ATGGAGCCACCGATCCACA

Statistical Analysis For the statistical analysis of two groups, the unpaired *t*-test was used. Statistical tests were performed using commercial software (SPSS, version 13.0). Error bars are ±SEM.

RESULTS

***Msx2* inhibited lens epithelium cell proliferation** In over expressed *Msx2* alpha-TN4 cell line, transfected cells were then labeled with BrdU to ascertain the status of cell proliferation. As shown in Figure 1, *Msx2*-GFP positive cells failed to incorporate BrdU. We found anti-phospho-histone-3 immunofluorescence failed to detect GFP-positive cell nuclei which confirm that cells over-expressed *Msx2*GFP were selectively prevented from entering mitosis (Figure 2).

***Msx2* induced lens epithelium cell apoptosis** To further characterize the putative properties of *Msx2*, we examined the effect of *Msx2* on alpha-TN4 cells by using flow cytometry analysis. As shown in Figure 3, percentage of apoptotic cells was significantly increased in alpha-TN4 cells transfected with *Msx2*, as compared to those with control (P<0.05).

***Msx2* inhibited *CyclinD1* and *Prox1* expression in *Msx2* knockout mice lens tissue** Of the cell cycle regulatory genes examined, we found *Prox1* and *CyclinD1* expression increased significantly in *Msx2* knockout mice lens comparing with their wildtype littermates (9.81 fold and 6.46 fold) (Figure 4A, B). And, we found that the most prominent effect of *Msx2* over-expression was a robust decrease in *CyclinD1* expression (5.13 fold) whereas over-expression of the *control plasmid* had no significant effect by real time RT-PCR (Figure 4C).

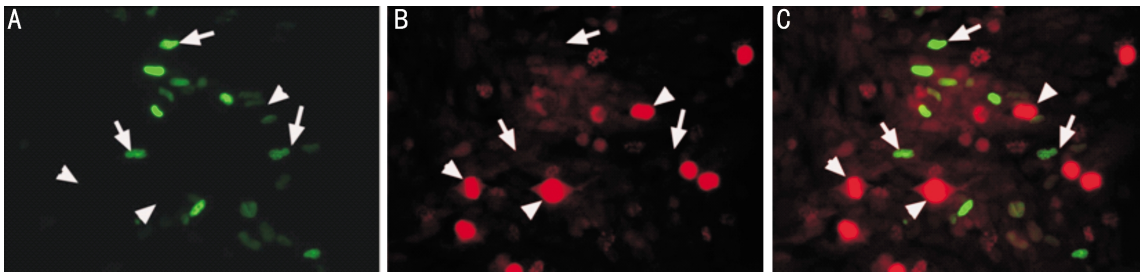

Figure 2 Anti-phospho-histone-3 immunofluorescence failed to detect cell nuclei that were GFP positive A: *Msx2*-GFP transfected cells (arrow); B: Anti-phospho-histone-3 positive cells (arrow head); C: Merged image showed that anti-phospho-histone-3 immunofluorescence failed to detect cell nuclei that were GFP positive.

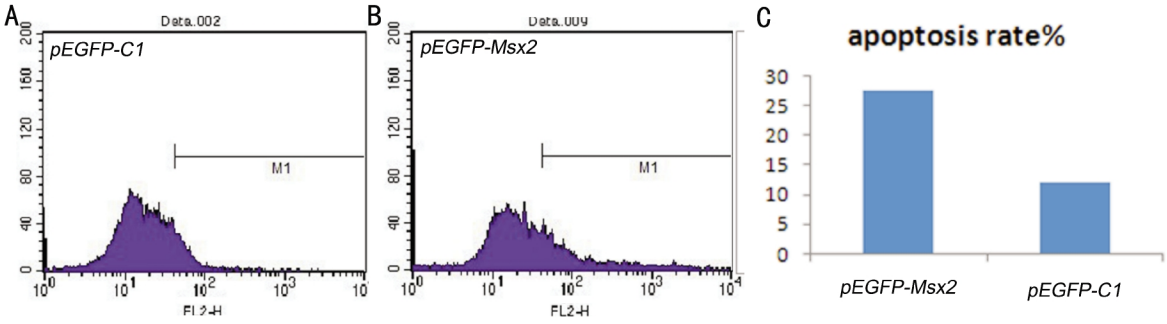

Figure 3 Over expressed *Msx2* induced lens epithelium cell apoptosis A: pEGFP-C1 transfected cells, apoptosis cell rate was 11.19%; B: pEGFP-*Msx2* transfected cells, apoptosis cell rate was 26.52%; C: Bar diagram of apoptosis cell rate of both group (P<0.05).

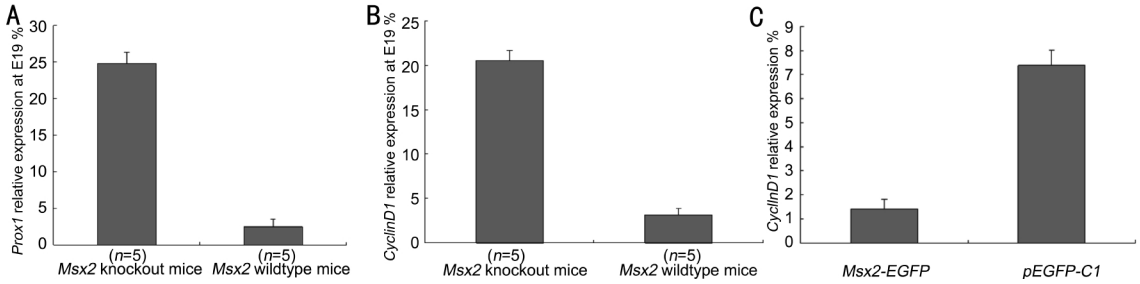

Figure 4 *Prox1* and *CyclinD1* expression in *Msx2* knockout mice lens and lens epithelium cells A: *Prox1* expression level in *Msx2* knockout mice lens (24.82±1.53) and their wildtype littermates (2.53±1.02) (P<0.05); B: *CyclinD1* expression in *Msx2* knockout mice lens (20.54±1.14) and their wildtype littermates (3.18±0.73) (P<0.05); C: *CyclinD1* expression in *Msx2* overexpressed lens epithelium cells (1.43±0.38) and control (5.38±0.64) (P<0.05).

DISCUSSION

Homeobox genes are a large family of developmental regulatory genes that have diverse activities during embryogenesis. The biological functions of many homeobox genes have been elucidated through loss- and gain-of-function analyses, which have demonstrated their essential roles in controlling cellular proliferation and differentiation during development. Previously, over-expression of the *Msx2* gene in transgenic animals resulted in microphthalmia. These phenotypes could be consistent with *Msx2*'s role in regulating cell proliferation and differentiation processes. We have demonstrated that *Msx2* was a significant contributor to eye development. Loss-of-function of *Msx2* led to a range of eye defects with a minuscule lens vesicle and persistent lens stalk as being the most severe phenotypes [10]. So we overexpressed *Msx2* in lens epithelium cell in order to observe the effect on cell cycle.

Our results showed that over-expressed *Msx2* inhibited lens epithelium cell incorporate BrdU, and anti- phospho-histone-3 immunofluorescence was negative which meant that cells proliferation process were disturbed. And overexpressed *Msx2* induced apoptosis by flow cytometry analysis, which signified *Msx2's* important role in controlling the cell cycle of the lens epithelium cell. Therefore, *Msx2* inhibited lens epithelium cell proliferation and induced apoptosis in the cell cycle. Previous study proved that over expression of *Msx2* transiently suppressed the expression of *CyclinD1* and blocked cell proliferation in the retina and delayed the expression of RGC-specific differentiation markers (Math5 and Brn3b), which showed that *Msx2* could affect the timing of RGCs fate commitment and differentiation by delaying the timing of cell cycle exit of retinal progenitors. So we detected the *CyclinD1* expression and found that over expression *Msx2* in lens epithelium cell also suppressed the expression of *CyclinD1* and affected the

lens epithelium cell cycle. For *in vivo* quantization test in *Msx2* null mutant mice, we proved that *CyclinD1* expression increased in the lens of *Msx2*$^{-/-}$ mice.

Prox1, which affected the terminal differentiation and elongation of lens fiber cell through regulating the cell cycle controlled gene like *Cdkn1c* [20] was a very important gene. Ectopic expression of *Prox1* in the mouse retina forced retinal progenitor cells to exit the cell cycle [21]. Furthermore, results from several other studies provided supporting evidence for *Prox1* 's role in promoting neuronal differentiation and cell cycle withdraw. This pro-differentiation function of *Prox1* appears to be evolutionarily conserved among fish, chick and mouse [22, 23]. Thus, it is reasonable to hypothesize that high level of *Prox1* expression found in the *Msx2*$^{-/-}$ lens may lead to premature cell cycle withdrawal and thus promote cell differentiation. Previous study proved that *Prox1* expression was upregulated in the *Msx2*$^{-/-}$ lens vesicles due to the reduced *FoxE3* expression[10], but there are no quantitative results by now. *Prox1* expression was hard to detect in alpha-TN4 lens epithelium cell line even after transfected *Msx2* gene, so we used *Msx2* null mutant mice and quantitatively proved that *Prox1* expression increased in developing lens.

In summary, our study provided the direct molecular evidence for *Msx2* regulating lens epithelium cell proliferation, apoptosis and differentiation, affecting the cell cycle. The observed changes in the expression of *CyclinD1* and *Prox1* further support the importance of *Msx2* in controlling transcription of target genes critical for lens development. Further investigation into these complex interactions among *Msx2* and various transcriptional regulators and signaling molecules in directing growth and morphogenetic events in the developing eye should help clarify the function of *Msx2*

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