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Microcystin-LR activates the oncogenes via the LncRNA-THOR/RAS signaling pathway in mouse testicular sertoli cells

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Abstract: Microcystin-LR (MC-LR) is a type of cyanobacterial toxin and a possible carcinogen that can activate proto-oncogenes in testicular cells, but the mechanism is not clear. Sertoli cells are important functional cells in the testis and play a key role in maintaining the internal environment of spermatogenesis. Therefore, the present study using the mouse testicular sertoli cells (TM4) to investigate the possible mechanism of proto-oncogene activation by MC-LR. The results showed that with the increase of MC-LR concentration, the activity of TM4 cells, the activity of TM4 cells declined and the expression of proto-oncogenes (*c-fos*, *c-jun* and *c-myc*). Further studies revealed that MC-LR increased the expression of *Lnc-THOR* and up-regulated the expression of its downstream cancer-related genes *IGF2BP1*, *RAS*, *MAPK* and *ERK* in TM4 cells with a dose-dependent manner. In short, our results indicated that the LncRNA-THOR/RAS signaling pathway had an essential regulatory role in the activation of proto-oncogenes in TM4 cells. This study is the first to explore the mechanism of MC-LR-activated proto-oncogene in the testicular sertoli cells, and provides new insights for further studies on the carcinogenic effects of MC-LR on the male reproductive system.
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1. Introduction

With the development of economy and the acceleration of social industrialization, a large number of nitrogen and phosphorus pollutants are discharged into the water body, which makes the water body more susceptible to eutrophication. Eutrophication of water body will lead to the growth of algae and release a large number of algal toxins into the water and posing a serious threat to human health. Microcystins (MCs) are the most widely distributed pollutants produced by cyanobacteria bloom, and are the most threatening pollutants to the ecological environment and human health [1, 2].

MCs have a cyclic heptapeptide structure. More than 200 kinds of MCs homologues have been found [3]. Microcystin-LR (MC-LR) is the most abundant and toxic isomer among them [4]. MC-LR is chemically stable and can be accumulated in a variety of animals, and then ingested by human body through the food chain. At present, many methods have been developed to remove MCs from water, but there is no reliable method to remove MCs completely [5]. In order to reduce the risk of MCs to human health, the World Health Organization (WHO) and Standards for drinking water quality of China (GB5749-2006) stipulate that the concentration of MC-LR in drinking water should not exceed 1 μg/L [6]. MC-LR is a potential carcinogen for animals and humans. The International Agency for Research on Cancer (IARC) has listed MC-LR as a suspected human carcinogen (category 2B) [7].

MC-LR has multiple organ toxicity, and gonad is the second largest target organ of MC-LR [8]. Testis is the main organ of male reproductive system, and a variety of cells work together to complete the process of spermatogenesis. Sertoli cells play an important role in the development and cycle of Spermatogenic cells in the whole life cycle. Sertoli cells can provide morphological and nutritional support for spermatogenesis, and their dysfunction is usually the cause of spermatogenesis failure. Several studies have shown that exposure to MC-LR increases the mRNA expression levels of *c-fos* and *c-jun* (two important proto-oncogenes) in sertoli cells and testes, suggesting that MC-LR may have a cancer-promoting effect on the male reproductive system [9].

Long non-coding RNAs (LncRNAs) are non-coding RNAs with the length of more than 200 nucleotides, which are involved in the growth, apoptosis, invasion, proliferation, autophagy and other

processes of cells. In recent years, it has been found that LncRNA, as an active regulatory molecule and biomarker, has been involved in the development and progress of various cancers such as liver cancer, lung cancer, ovarian cancer, testicular cancer and so on. The recent study found that more than 30 LncRNAs were significantly altered in human normal liver cells (LO2) after exposure to MC-LR, indicating that LncRNAs were involved in MC-LR-induced hepatotoxicity. More than 300 LncRNAs were found to have significant changes after long-term exposure to MC-LR, suggesting that LncRNA plays an important role in male reproductive toxicity induced by MC-LR. LncRNA-THOR is a cancer-related long non-coding RNA specifically expressed in testis. It can activate the downstream Ras pathway and induce the activation of proto-oncogenes by binding with insulin-like growth factor-2 mRNA-binding proteins 1 (IGF2BP1) [10]. However, it is not clear whether MC-LR can activate LncRNA-THOR and then activate proto-oncogenes through Ras pathway to exert reproductive toxicity.

In this study, we used mouse sertoli cells (TM4) to investigate the effects of MC-LR on proto-oncogenes, LncRNA-THOR and downstream RAS pathway-related genes, aiming to clarify the role of the LncRNA-THOR/RAS pathway in MC-LR-induced proto-oncogenes activation in male germ cells.

2. Material and Methods

2.1 Reagents

MC-LR (Beijing Express Technology Co, Beijing, China); Trizol reagent (Ambion, Beijing, China); RevertAid First Strand cDNA Synthesis Kit (Thermo, China); RT-qPCR kit (TaKaRa Bio Inc., Japan).

2.2 Cell culture and treatment

Sertoli cells (TM4) were grown in DMEM/high-glucose enriched with 10% FBS, 4.0 mM of L-glutamine, 4,500 mg/L of glucose, and 100 U/mL of penicillin/streptomycin. Cells were cultured in a humidified CO_2 chamber at 37°C under normal cell culture conditions. MC-LR was dissolved in PBS to generate 1 mg/mL of stock solution and further diluted with culture medium to the desired concentrations, prior to incubation with TM4 cells for 24 h.

2.3 RT-qPCR assay

Total RNA was isolated from TM4 cells exposed to various concentrations of MC-LR (0, 5, 10, 20 μg/mL) by Trizol reagent. RevertAid first Strand cDNA Synthesis kit was used to synthesize cDNA in 20 μL reaction system following manufacturer's instructions. SYBR premix Ex Taq was used to prepare 10 μl system, and QuantStudio 7 Flex real time PCR system (Life Technologies, USA) was used to perform RT-PCR. All samples were assayed in triplicate and the expression levels were normalized to the gene of *β-actin*. The PCR primer sequences were presented in Table 1.

Table 1. Sequences of the primers used for real-time quantitative PCR

genes	Forward primers (5'-3')	Reverse primers (5'-3')
c-jun	*TCCAAGTGCCGAAAAAGGAAG*	*CGAGTTCTGAGCTTTCAAGGT*
c-fos	*GGTGAAGACCGTGTCAGGAG*	*CCGCTTGGAGTGTATCTGTCA*
c-myc	*GGTGTCTGTGGAGAAGAGGCAAAC*	*GGCGTAGTTGTGCTGGTGAGTG*
Lnc-THOR	*CAAGGTGCTTCTCTCTGGATTT*	*GCCAAAGTCATTTGTTGGGTAT*
Lnc-GAS5	*AGGAGGATGAAGGCTTACGAGGAC*	*AGTCACTGCATGTCCACTTGTCAC*
Lnc-TUG1	*CAGAAGGAAGGTCATTGGCAGGTC*	*GAGACACGACTCACCAAGCACTG*
Lnc-smad7	*CACCTGCGAACTGGCTCCAATC*	*TACCACCAGACGTGCATCAACAAC*
RAS	*CCATCAACAACACCAAGTCCTTC*	*GTGGGTTCAGTTTCCGCAATT*
MAPK	*GGAGAACTGAAGGATGACGACT*	*CGCTGTAGAACGCACCATAGA*
ERK	*TGACCTCAAGCCTTCCAACCT*	*GCCAGAGCCTGTTCTACTTCAA*
β-actin	*TCAAGATCATTGCTCCTCCTGAG*	*ACATCTGCTGGAAGGTGGACA*

2.5 Statistical analysis

Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyze the significant differences between groups, Student-Newman-Keuls test (SNK) was used for multiple comparisons in

variances with homogeneity, and Dunnett T3 test was used for variances without homogeneity. $P<0.05$ was considered statistically significant. All statistical analyses were carried out using SPSS 21.0 (Armonk, NY, USA, 2012).

3. Results

3.1 MC-LR decreased the activity of TM4 cells.

After TM4 cells were exposed to different concentrations of MC-LR for 24 hours, the effects of MC-LR on TM4 cell activity were analyzed by CCK8 kit combined with microplate reader. With the increase of MC-LR (1-60 μg/mL), cells activity decreased gradually (Figure 1). The half-inhibitory concentration (IC_{50}) of MC-LR in TM4 cells was 19.54 μg/mL. Therefore, MC-LR exposure doses of 5, 10 and 20 μg/mL were used for subsequent experiments.

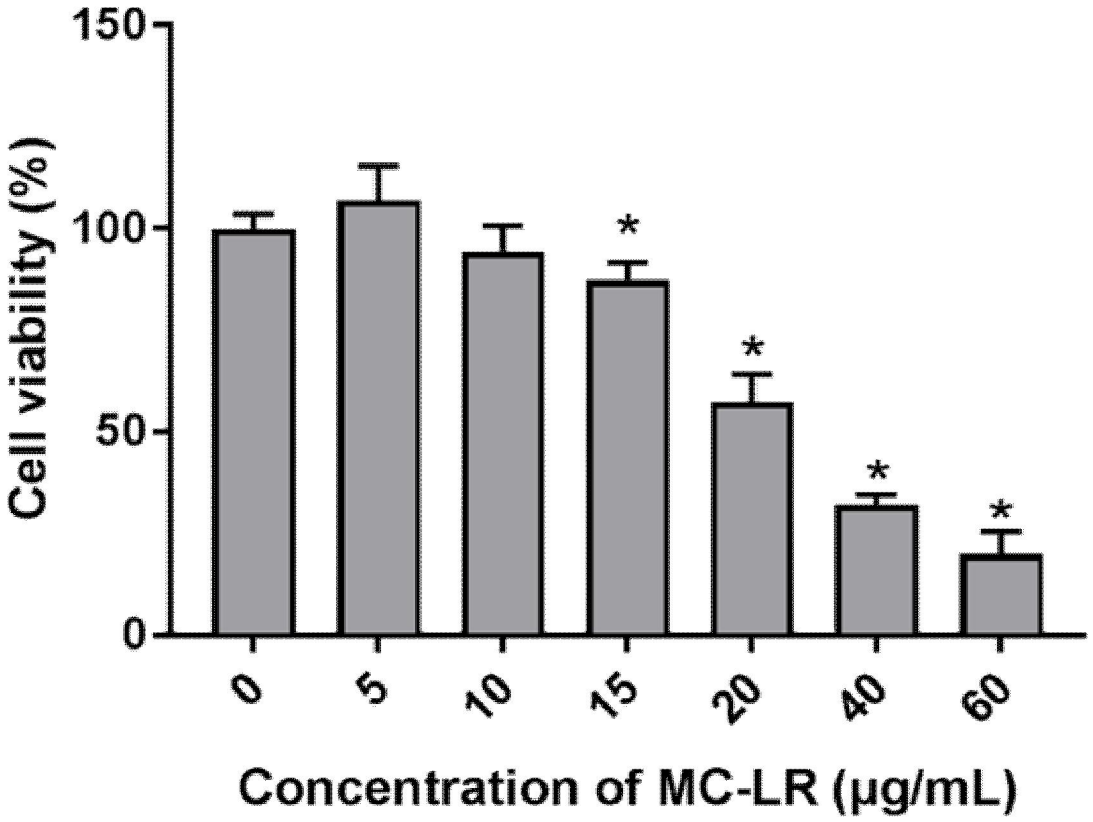

Figure 1. Effect of MC-LR (0-60 μg/mL) exposure for 24 hours on TM4 cells viability
Note: *$P<0.05$ vs. the control group

3.2 MC-LR raised the expression of proto-oncogenes in TM4 cells.

The results showed that as the concentration of MC-LR increased, three proto-oncogenes (*c-fos*, *c-jun* and *c-myc*) in TM4 cells gradually increased, suggesting that MC-LR could increase the expression of proto-oncogenes in TM4 cells. (Figure 2).

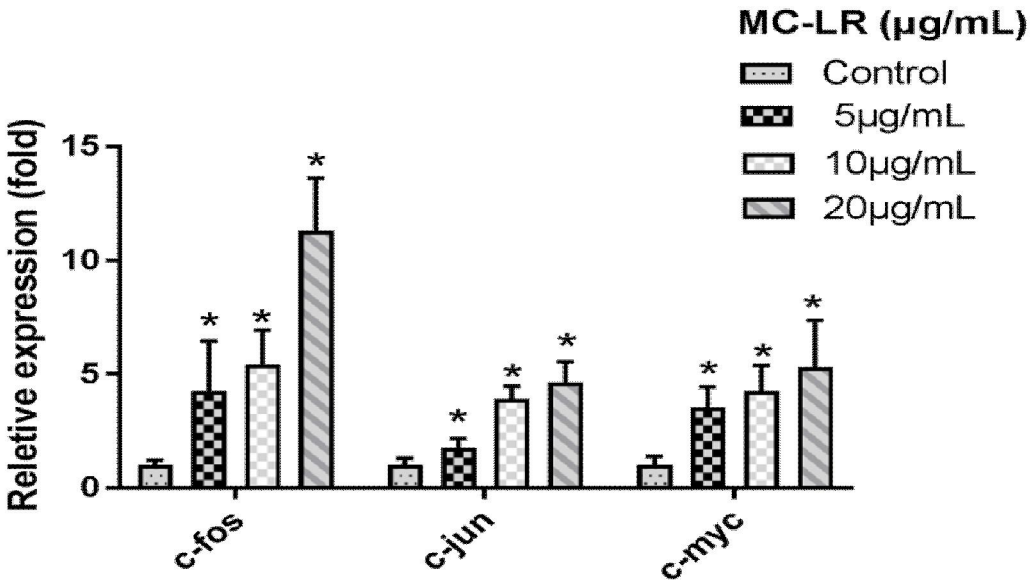

Figure 2. Effects of different concentrations of MC-LR on the expression of proto-oncogenes
Note: *P<0.05 vs. the control group.

3.3 MC-LR increases the expression of *Lnc-RNAs* in TM4 cells.

Cancer-associated *LncRNAs* (*LncRNA-THOR, LncRNA-GAS5, LncRNA-TUG1, LncRNA-Samd7*) were detected in TM4 cells after exposure to different concentrations of MC-LR. The results showed that the expression levels of the four *Lnc-RNAs* gradually increased with the increase of MC-LR exposure concentration. Among them, the elevation of *LncRNA-THOR* was the most obvious, which showed the same trend as the change of the proto-oncogenes. This suggested that the elevated expression of proto-oncogenes induced by MC-LR might be associated with the increased expression of *LncRNA-THOR* in TM4 cells (Figure 3).

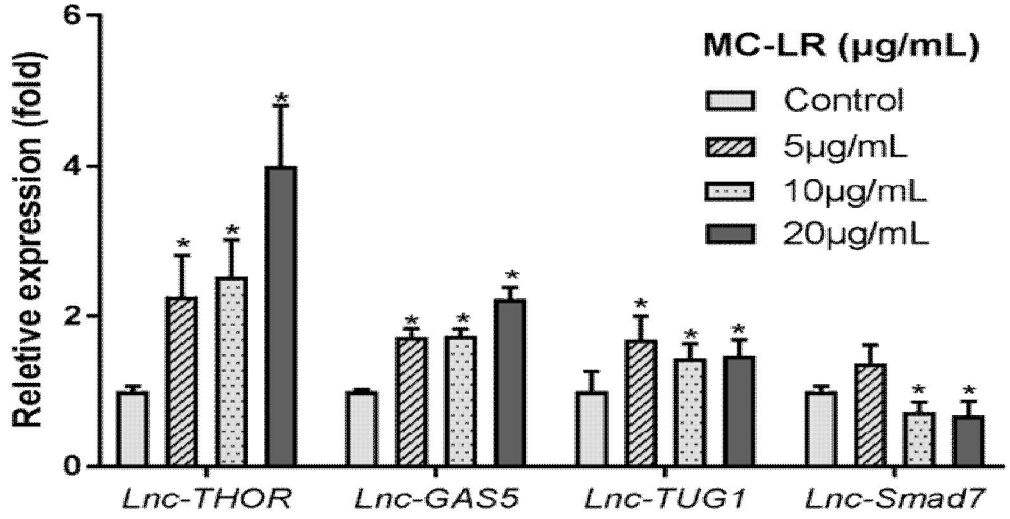

Figure 3. Effects of different doses of MC-LR exposure on *LncRNAs* in TM4 cells
Note: *P<0.05 vs. the control group.

3.4 MC-LR increases the expression of genes in the LncRNA-THOR/RAS pathway

To explore the mechanism of MC-LR regulating the abnormal expression of proto-oncogenes in TM4 cells through *LncRNA-THOR*, genes in the downstream pathway of *LncRNA-THOR* were detected. The results showed that *IGF2BP1*, a target gene that binding to *LncRNA-THOR*, was highly expressed (P<0.05). Moreover, the expression of *MAPK* and *ERK* genes in the downstream RAS signaling pathway were increased (P<0.05), suggesting that the RAS pathway was activated which might lead to the high expression of proto-oncogenes. (Figure 4).

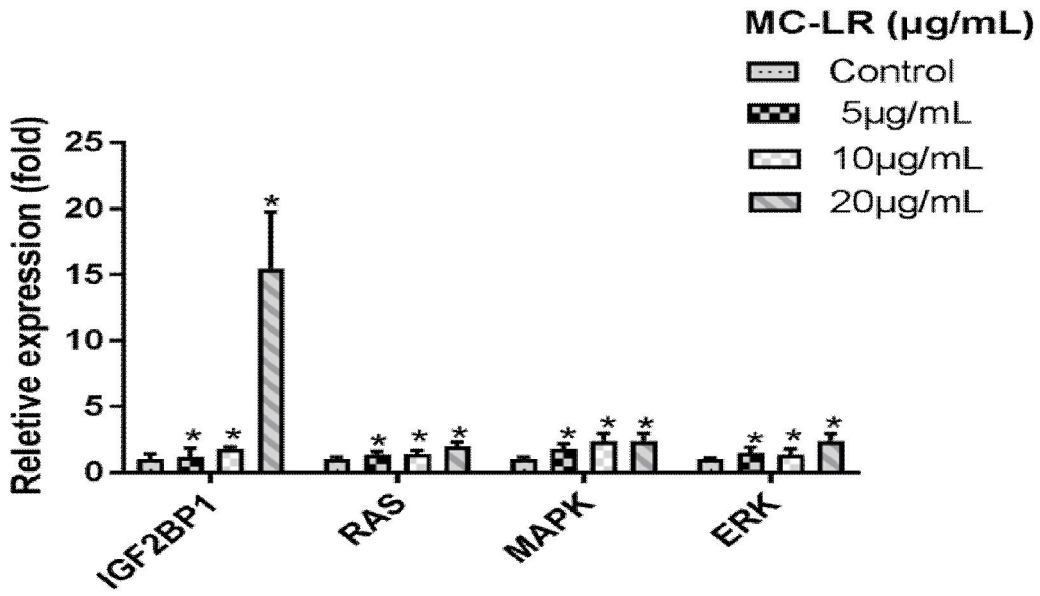

Figure 4. Effects of different doses of MC-LR exposure on gene expression in the LncRNA-THOR/RAS pathway in TM4 cells
Note: *P<0.05 vs. the control group.

4. Discussion

Cancer is a type of disease caused by the interaction of environmental and genetic factors, and the occurrence of cancer is associated with genetic alterations. Both proto-oncogenes and cancer suppressor genes play an important role in the regulation of cell growth and proliferation. In normal conditions, proto-oncogenes existing in the genome are at low or no expression and perform important physiological functions. However, under certain conditions, such as viral infection, chemical carcinogens or radiation, proto-oncogenes can be abnormally activated and induce cellular carcinogenesis. The accumulation of genetic damage in the form of activated proto-oncogenes and inactivated tumor suppressor genes is the driving force for the evolution of normal cells to malignant cells [11].

Epidemiological studies have found that the increase of testicular cancer incidence rate may be related to the content of MC-LR in the water [12]. Animal experiments have confirmed that MC-LR activates proto-oncogenes (*c-myc*, *c-jun*) in testis. Therefore, MC-LR may have carcinogenic effect on male reproductive organs. Our results showed that MC-LR could activate the proto-oncogene in TM4 cells, which suggested that TM4 cells might be the target cells for MC-LR to exert testicular tumor toxicity. Emerging evidence suggests that LncRNAs play an important part in tumor biology. The dysregulation of LncRNAs expression in cancer indicates the disease progression, and can be used as an independent predictor of prognosis. In terms of mechanism, most of the LncRNAs with well-defined characteristics have a functional transcriptional regulatory role in gene expression [13]. *LncRNA-THOR* is a conserved LncRNAs specifically expressed in testis. Studies have shown that overexpression of *LncRNA-THOR* can promote the gene expression of *IGF2BP1*, which indicates that *LncRNA-THOR* is involved in regulating the expression of *IGF2BP1* [14].

IGF2BP family proteins, which are composed of IGF2BP1/2/3, mediate RNA stability and translation by binding to many well-defined mRNA targets, and play an important role in regulating the translation and conversion of target transcripts [15, 16]. Our results confirm this conclusion that *IGF2BP1* is activated by high expression of *LncRNA-THOR*, demonstrating the regulatory role of *LncRNA-THOR* on *IGF2BP1*.

IGF2BP1 has high affinity for *RAS* mRNA and can regulate the expression of *RAS* in cells. It can bind to the coding region of *k-Ras* mRNA and 3'UTR. Overexpression of *IGF2BP1* increases the expression of *c-myc* and *RAS* and the proliferation of LIM2405 cells [17, 18]. Oncogene *Ras* has been identified in many human tumors and can activate proto-oncogenes at various stages of the oncogenic process [10]. The Ras promotes mitosis and carcinogenesis through activation of downstream protein kinase 1 (AP-1). AP-1 is a heterodimeric complex of Jun and Fos proteins, and it activates mitosis-inducible genes and is the main nuclear target of Ras. Ras can stimulate AP-1 activity by inducing the transcription of *c-fos* and *c-jun*. This process may be mediated by extracellular regulated protein kinases (ERK) 1 and -2 mitogen-activated protein (MAP) kinases [19]. In addition to inducing the transcription genes of *c-fos* and *c-jun*, the activation of the Ras signaling pathway can also promote the high expression of *c-myc*. *C-myc* is an oncogene, which participates in the carcinogenic transformation in normal cells and reprograms somatic cells into pluripotent stem cells [20]. In the present study, we found that MC-LR increased the expression of *RAS* and its downstream genes, which in turn caused the high expression of proto-oncogenes in TM4 cells. It suggested that MC-LR could cause the activation of the RAS signaling pathway in response to the high expression of *LncRNA-THOR* in TM4 cells.

In conclusion, our study found that MC-LR could induce high expression of *LncRNA-THOR* which in turn activated the RAS signaling pathway, resulting in increasing expression of proto-oncogenes in TM4 cells. Abnormal elevation of proto-oncogenes would potentially allow MC-LR to have an oncogenic effect on the reproductive system. This study elucidated the molecular mechanism of MC-LR activated proto-oncogenes in the reproductive system, which provided a new insight into the potential oncogenicity of MC-LR on the reproductive system.

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