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Transcriptional profiling analysis of Zearalenone-induced inhibition proliferation on mouse thymic epithelial cell line 1

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ABSTRACT

Zearalenone (ZEA) was a mycotoxin biosynthesized by a variety of *Fusarium fungi* via a polypeptide pathway. ZEA has significant toxic reaction on immune cells. Thymic epithelial cells (TECs) as a crucial constituent of thymic stroma can provide unique microenvironment for thymocyte maturation, but the mechanism of ZEA affecting the TECs is poorly understood. The basic data about gene expression differences for the ZEA on thymic epithelial cell line 1 (MTEC1) will help us to elucidate this mechanism. Here, cell viability and proliferation assay and transcriptome sequencing on MTEC1 treated with ZEA were performed. 4188 differentially expressed genes (DEGs) between ZEA treated and control groups were identified, confirmed and analyzed. Our results showed that 10–50 μg/ml ZEA significantly inhibited MTEC1 proliferation and arrested cell cycle at G2/M phase. Gene ontology and KEGG pathway analysis revealed that Chemokine, JAK-STAT and Toll-like receptor signaling pathway, were involved in the cell cycle pathway. 16 key genes involved in the cell cycle processes were validated and the results suggested that Mitotic catastrophe (MC) may take part in ZEA inhibition of METC1 cell proliferation. These data highlighted the importance of cell cycle pathway in MTEC1 treated with ZEA, and will contribute to get the molecular mechanisms of ZEA inhibition of MTEC1 cell proliferation.

1. Introduction

Zearalenone (ZEA), also named F-2 toxin, is a mycotoxin mainly produced by *Fusarium fungi*, which causes reproductive disorders of livestock or poultry and sometimes in hyperoestrogenic syndromes in humans. ZEA may produce responses in mammalian uterus or oviduct and also can cause alterations in both laboratory (guinea pigs, mice, hamsters, rats and rabbits) and domestic animals (Collins et al., 2006; Zinedine et al., 2007; Liu et al., 2017). ZEA can be retained in cereal products and tolerate standard decontamination procedures (Bullerman and Bianchini, 2007; Cano-Sancho et al., 2012). Mycotoxins produced by fungal affected approximately 25% food crops growth (Rotter et al., 1996). Humans are usually exposed to this F-2 toxin through intake of cereal food (Severino et al., 2006).

Thymus is the central lymphoid organ that generates T lymphocytes and plays a crucial role in both the development and maintenance of the immune system (Mor et al., 2001; Ma et al., 2013). Thymic epithelial cells (TECs) as a crucial constituent of thymic stroma can provide unique microenvironment for thymocyte proliferation and differentiation (Alves et al., 2009). Previous reports showed that changes of the TECs numeric can interpret the physiological process of age-related thymus involution (Gui et al., 2012; Odaka et al., 2013). In recent years, TECs had attracted more and more concern on cellular and molecular mechanisms controlling T-cell development, function, dysfunction, and regeneration. Therefore, we speculated that whether ZEA have effect on TECs and further impact on T cell development.

To explore the potential molecular mechanism of the ZEA inhibiting MTEC1 cell proliferation, cell viability and cell cycle analysis were detected. Transcriptome sequencing on MTEC1 treated with 25 μg/ml ZEA for 48 h were performed. Differentially expressed genes (DEGs) between ZEA treated and control groups were identified, confirmed and analyzed.

2. Materials and methods

2.1. Materials

Zearalenone (CAS no. 17924-92-4) was from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were from Gibco BRL (Grand Island, NY, USA). Cell counting kit-8 (CCK-8), cell cycle assay kit and DAPI dye were from Beyotime Biotechnology Co. Ltd. (Shanghai, China). EdU staining kit

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was from RiboBio Co. Ltd. (Guangzhou, China). TRIZOL reagent was from Takara Co. (Kusatsu, Japan).

2.2. Cell culture and ZEA treatment

MTEC1 cells were obtained from Peking University Health Science Center (Beijing, China) and cultivated in DMEM containing 10% FBS with a humidified atmosphere containing 5% CO_2 at 37 °C. ZEA was dissolved in DMSO as 5 mg/ml stock solution and then dissolved in DMEM medium and concentrations of the final compound were 1, 5, 10, 25 and 50 μg/ml. The control group added the same growth DMEM medium with a final DMSO concentration less than 0.1%.

2.3. Cell viability

MTEC1 cells were plated in 96-well at 4.5×10^3 cells/well and incubated for 15 h. Then, ZEA with different concentrations (0, 1, 10, 25, 50 μg/ml) was added to each well. After ZEA treatment for 6 h, 12 h, 24 h, or 48 h, cell viability was analyzed with CCK-8 reagents following the manufacturer's instructions.

2.4. Morphological observation

Cells were plated in 6-well at 7×10^4 cells/well and grown in growth DMEM medium with or without ZEA (1–50 μg/ml). After 24 h, cell morphology images were obtained with microscope equipment (Leica Microsystems, Germany).

2.5. Cell proliferation

Proliferation of METC1 was determined by EdU staining according to the manufacturer's instructions. Briefly, after 25 μg/ml ZEA treatment for 24 h, cells were incubated in phenol red-free DMEM plus 50 μM 5-Ethynyl -2′-deoxyuridine (EdU) for 2 h. Then, cells were washed twice with 0.02 M PBS (pH = 7.2) at 5 min intervals and immobilized with 4% paraformaldehyde for 30 min. Thereafter, cells were treated with glycine (2 mg/ml) for 5 min and washed with PBS for 5 min. Apollo staining was performed for 30 min using 1 × Apollo® staining solution, followed by 4′,6-diamidino 2-phenylindole (0.5 μg/ml) staining for 30 min and washed 3 times by PBS at 5 min intervals. Finally, fluorescence in cells was obtained with a Leica DM4000 B LED microscope equipment (Leica Microsystems, Germany).

2.6. Cell cycle analysis

MTEC1 cells were collected after incubating with different concentrations of ZEA (0, 1, 10, 25, 50 μg/ml) for 24 h, and then, cells were trypsinized for 1 min, centrifuged and washed once with PBS. Cells were fixed on ice for 15 min in 70% ethanol and followed by staining with propidium iodide (PI). The percentage of cells in different phases were analyzed by flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.7. DAPI staining

To reveal the nuclear division caused by ZEA in MTEC1 cells, DAPI assay was performed. Briefly, upon treatment with ZEA (25 μg/ml) as mentioned earlier, cells were incubated for 48 h and fixed at room temperature by 4% paraformaldehyde for 15 min, then washed thrice with PBS. 0.5% TritonX-100 closed at 37 °C for 15 min. Treated cells were stained with DAPI (Beyotime) at a concentration of 10 μg/ml in serum free media and incubated at 37 °C for 20 min, nuclear division was observed under fluorescence using florescent microscope (ZEES).

2.8. RNA extraction and Sequencing analysis

MTEC1 cells were plated into 55 cm^2 cell culture dish and grown in growth DMEM medium with or without ZEA (25 μg/ml ZEA). After 48 h, the total RNA extraction of both the treated and control group was performed as described in our previous reports (Ouyang et al., 2016; Guo et al., 2017). Then, the RNA was purified with the RNeasy Mini Kit and sent the processed samples to Majorbio (Shanghai). The total RNA concentration and purity were analyzed by Nanodrop2000. RNA integrity was detected by AGE and RIN number was measured by Agilent2100. Concentration ≥ 200 ng/μl, OD values between 1.8 and 2.2. And then Oligo dT enriched mRNA, fragmentation buffer fragmented mRNA, then the cleaved RNA fragments were reverse-transcribed to establish the final cDNA library, connected adaptor, and then we performed the paired-end sequencing on an Illumina Hiseq. 4000 (Illumina, San Diego, USA) according to the vendor's recommended protocol. There are three biological replicates and three technical replicates in each group.

2.9. Quantitative PCR

To validate gene expression changes measured by high-throughput sequencing analysis, quantitative real-time PCR was performed as our previous reported (Li et al., 2013; Ouyang et al., 2016; Guo et al., 2017). Briefly, Cells were plated in 6-well plate in triplicates and treated with ZEA (25 μg/ml) for 48 h. Total RNA was extracted and reverse-transcribed to cDNA in accordance with the manufacturer's instructions. Primers used for RT-PCR were shown in Table 1. The expression levels of the genes were normalized to the housekeeping gene β-actin by using $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). A Student *t*-test was performed for statistical analysis of the real-time PCR data.

Table 1
Primer pairs in RT-qPCR analysis of gene expression.

Gene	Primer (5′-3′)	GenBank accession no.
Gnai2	F: TTGCTTCTGTTAGGTGCTG R: ACTGGATGGTGTTGCTGT	NM_008138
Trpc1	F: ACACCTTCCACTCGTTCA R: TCGTCAGCACAATCACAA	NM_001311123
Shc2	F: GCATCTCAGTCAACATCTCC R: GACCTTCACAGCATTCCA	NM_001024539
Nras	F: GTAAGGGAGATACGCCAGT R: CAGGGATGTCAGAACCAG	NM_010937
Mapk3	F: GCTTTCTGACGGAGTATGTG R: GGATTTGGTGTAGCCCTT	NM_011952
Jak2	F: TACGAAGAATGAGAATGGAGA R: CTGAAGACGAGAAGGTTTGA	NM_001048177
Stat1	F: CTGCCTATGATGTCTCGTTT R: CTTGCTTTTCCGTATGTTGT	NM_001205313
Irf9	F: CAGCACACAGAGCAGTCA R: ATAAGAACCATCACAGAAGCA	NM_001159417
Bub1	F: AGGAGAGAAACCACAGGAA R: GCCACTTACAGGAACAGGA	NM_001113179.1
Bub1b	F: GCAAAGAAGAGGGAGGAG R: ACAGATGGAACAGGACAGAG	NM_009773.3
Ttk	F: ACAAGATGCCGAAGTGAG R: CTGGGCTGTTTAGAAGATTG	NM_001284272.1
Cenpe	F: GCTGTCTGTGTTCGTGTG R: CATTCTTCGTGGTTTCATTAC	NM_173762.4
Cdc20	F: AACATTCACTCAACATCAAGG R: ATCCACAGCACTCAGACAG	NM_023223.2
Cyclin B1	F: TGCTCTTGGAGACATTGG R: CAGGTTTTGGTAGGGCTT	NM_172301
Plk1	F: TTGAGGTGGATGTGTGGT R: ACTGGGTTGATGTGCTTG	NM_011121.4
Cdc25b	F: CGTTAGGATGGCACAGTAG R: CAGGAGAGGAGAGTAGACACA	NM_001111075.4
β-actin	F: CATCCGTAAAGACCTCTATGCCAAC R: ATGGAGCCACCGATCCACA	NM_007393.3