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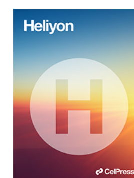
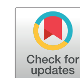


Research article

Long-term exposure to low levels of okadaic acid accelerates cell cycle progression in colonic epithelial cells via p53 and Jak/Stat3 signaling pathways



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HIGHLIGHTS

- Long-term exposure to low levels of OA accelerates cell cycles in *vitro* and in *vivo*
- OA induced changes in cell cycle by inhibiting the p53 signaling pathway
- OA induced changes in cell cycle by inducing the Jak/Stat3 signaling pathway

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ABSTRACT

As a major component of diarrheic shellfish poisoning (DSP) toxins, okadaic acid (OA) is widely distributed worldwide, and causes a series of serious public health problems. In colon tissue, previous studies have shown that high doses of OA can affect various intracellular processes, including destroy intercellular communication at gap junctions, induce cell apoptosis and trigger cell cycle arrest. However, there is a scarcity of studies on the effect and mechanism of action of low doses of OA in colonic tissues. In this study, we observed that exposure to low levels of OA altered cell cycle progression *in vitro* and *in vivo*. Investigation of the underlying mechanism revealed that OA induced alterations in the cell cycle by inhibiting the p53 signaling pathway or inducing the Jak/Stat3 signaling pathway. In conclusion, this study provides novel insights into the effect and mechanism underlying long-term exposure to low levels of OA.

1. Introduction

Okadaic acid (OA) is a typical acidic polyether toxin that is widely distributed worldwide [1, 2]. OA is one of the principal components of diarrhetic shellfish poisoning (DSP) toxins, and it has been identified that OA induces a series of gastrointestinal symptoms, including diarrhea, nausea, vomiting, and abdominal cramps in human subjects following the consumption of contaminated shellfish [3, 4]. For years, numerous studies have demonstrated that OA has variable effects at different concentrations. For instance, high doses of OA can induce the expression of c-fos mRNA, which is accompanied by the induction of apoptosis; however, low doses of OA induce DNA synthesis rather than apoptosis, and the expression of c-fos mRNA remains unaltered at low concentrations [5]. Serine/threonine protein phosphatases (PPs) play an important role in regulating intracellular cell events by dephosphorylating the serine and threonine residues of target proteins [6]. Type 1 (PP1) and type 2 (PP2) enzymes are the most representative PPs, and are inhibited by OA at high and low concentrations, respectively, in skeletal muscles [7]. Despite numerous reports on the effects of OA, there is a scarcity of studies on the varying toxic effects and mechanism of action of OA on the same organs at different concentrations.

A previous study by Matias *et al.* reported that OA is enriched in animal bodies, especially in the intestinal tract [8]. Studies by Fujiki *et al.* and Ito *et al.* have also demonstrated that the intestinal tract is the primary target organ of OA [9, 10]. Subsequent studies have been performed based on these findings for investigating the effects of OA on the

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intestinal tract, and have provided further insights into OA toxicity. For instance, Tripuraneni *et al.* reported that OA can increase the paracellular permeability of the colonic epithelium [11]. Liu *et al.* observed that long-term exposure to low doses of OA significantly alters the composition of gut microbes in rats [12]. The study by Traoré *et al.* demonstrated that intercellular communications at gap junctions are disrupted following treatment with OA [13]. It has been reported that OA has opposite effects on the proliferation of colonic epithelial cells at different concentrations [14, 15, 16]. In the study by Traoré *et al.*, the short-term exposure of a colonic epithelial cell line to high concentrations of OA resulted in DNA breakage and cell cycle arrest [14]. Conversely, exposure to sublethal doses of OA can facilitate cellular proliferation in colonic epithelial cell lines [15]. The variations in the effects of OA at different concentrations on cellular proliferation and the underlying molecular mechanisms are of immense interest.

OA is known to be a threat to human health at low concentrations, without causing acute toxicity [17, 18]; however, there is a scarcity of information regarding the sub-chronic toxicity of OA. Therefore, this study aimed to assess whether long-term exposure to low levels of OA can affect the proliferation of colonic epithelial cells, and subsequently elucidate the underlying mechanism. The results obtained in this study will aid in understanding the toxic effects of OA and elucidate the underlying mechanisms.

2. Materials and methods

2.1. Toxins

OA (purity grade: 98%) was purchased from LC laboratories (Woburn, USA) and dissolved in dimethylsulfoxide (DMSO). The toxins were properly diluted with a saline solution of 0.9% NaCl before use.

2.2. Experimental animal models

Female SD rats were purchased from Cyagen Biosciences Company (Suzhou, China) and kept in a controlled environment under a 12 h/12 h light/dark cycle at the experimental animal center of Xinqiao Hospital in Chongqing, China, and had *ad libitum* access to food and water. The animals in the treatment group (n = 8) and control group (n = 8) were matched for age (6 weeks) and weight (180–200 g). The rats were exposed to OA (100 μg/kg in 1 ml saline) by oral perfusion or by the administration of a saline solution of 0.9% NaCl once every three days for 90 consecutive days. The rats were sacrificed after 90 days and the colonic tissues were harvested. All the experiments on rats were approved by the Institutional Animal Care and Use Committee of Third Military Medical University (permit number: AMUWEC20211779).

2.3. Cell lines

The HCT116 (p53+/+) and HCT116 (p53−/−) colonic epithelial cell lines were obtained from Cellcook Bioscience Company (Guangzhou, China), and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, CA) at 37 °C in an atmosphere of 5% CO_2.

2.4. Transcriptome sequencing and bioinformatics analysis

Fresh colonic tissues were collected from the rats. The total RNA was extracted from the tissues using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and treated with RNase-free DNase I for removing genomic DNA contamination. The integrity, quality, and quantity of the extracted RNA were detected sequentially. The samples of RNA with high integrity, quality, and quantity were sent to Beijing Genomics Institute (BGI; Huada Genomics Institute Co. Ltd., Shenzhen, China) for further analysis by high-throughput sequencing as previously described [19].

Functional enrichment analyses based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were performed. The differentially expressed genes (DEGs) were mapped to the GO terms for functional categorization into the biological process (BP), cellular component (CC), and molecular function (MF) categories. KEGG pathway enrichment analysis identifies the significantly enriched metabolic pathways or signal transduction pathways related to DEGs. The GO terms and KEGG pathways with P value <0.05 were considered to be significantly enriched.

Gene Set Enrichment Analysis (GSEA) is a computational method that is used to determine whether a prior defined set of genes shows statistically significant concordant differences between two biological states. RNA-seq data can be ordered into a ranked list based on the differential gene expression between groups. Gene sets from C2. cp.KEGG v6.2. symbols.gmt (curated) and C2. cp.Reactome v6.2. symbols.gmt (curated) were used for enrichment analyses. The raw data is provided in Supplementary Tables 2 and S3.

2.5. Immunohistochemistry (IHC) analyses

The paraffin sections were incubated with an anti-Ki-67 antibody in an IHC autostainer (BenchMark GX, USA) for immunostaining. The experimental protocol used for IHC analyses has been previously described [20]. The sections were scanned using a ScanScope scanner (Leica, Wetzlar, Germany), and the samples were assessed in a blinded fashion by two analysts. The percentage of Ki-67-positive cells was quantified and the samples were classified into the 5 categories based on the score. Scores of 0, 1, 2, 3 and 4 were assigned when the percentage of Ki-67-positive cells was <10%, 10–25%, 26–50%, 51–75%, and ≥76%, respectively. The intensity of staining was quantified on a scale of 0 (no staining intensity) to 4 (highest possible staining intensity). The expression levels were calculated by determining the mean of the grades for the positive staining intensity.

2.6. Western blotting

Western blotting was performed as previously described [21]. The following primary antibodies were used for western blotting: p53 rabbit polyclonal antibody (1:500; Abcam), p21 mouse monoclonal antibody (1:1000; Santa Cruz Biotechnology), CCND2 (1:1000; Santa Cruz Biotechnology), CDK1 (1:1000; Santa Cruz Biotechnology), CDK2 (1:1000; Santa Cruz Biotechnology), CDK4 (1:1000; Santa Cruz Biotechnology), C-MYC (1:1000; Santa Cruz Biotechnology), Jak1 (1:1000; Santa Cruz Biotechnology), p-Jak1 (1:1000; Santa Cruz Biotechnology), Jak2 (1:1000; Santa Cruz Biotechnology), p-Jak2 (1:1000; Santa Cruz Biotechnology), stat3 (1:1000; Santa Cruz Biotechnology), p-stat3 (1:1000; Santa Cruz Biotechnology), and ACTIN monoclonal antibody (1:2000; Sigma). ACTIN was used as the loading control.

2.7. MTS assay

HCT116+/+ and HCT116−/− cells were plated on 96-well plates at a concentration of 5×10^3 cells/well, and treated with OA (0.50 nM) or DMSO. Cell vitality was detected using MTS Reagent (Promega, Madison, WI, USA) on days 1, 2, 3, 4 and 5 after transfection. The assay was performed in triplicate.

2.8. Cell cycle assay

The cells ($n = 5 \times 10^5$) were grown in 6-well plates, in triplicate for each condition, and treated with OA (0.50 nM) or DMSO. After 48 h of treatment, the cells were digested and collected for determining the alterations in cell cycle progression as previously described [20]. The experiments were performed in triplicate and repeated three times.

2.9. Plasmid construction, cell transfection, and luciferase reporter assay

For the overexpression studies, the expression vector encoding the full-length open reading frame of human p53 protein was synthesized and inserted into the pIRES2-EGFP expression vector. The pp53-TA-Luc and pSTAT3-TA-Luc reporter plasmids were purchased from Addgene.

The cells were transfected using Lipofectamine 2000 Reagent (Invitrogen Preservation, Carlsbad, CA, USA), according to the manufacturer's instructions. The cells ($n = 5 \times 10^4$) were inoculated in 24-well plates in triplicate for each condition and transfected with pp53-TA-luc or pSTAT3-TA-luc plasmids. The cells were collected and lysed after 24 h of transfection for monitoring the luciferase activity using a Dual Luciferase Reporter Gene Assay Kit (Beyotime, China). The experiments were performed in triplicate and repeated three times.

2.10. Statistical analyses

The data were analyzed using GraphPad Prism software, version 7.0 (GraphPad Prism, Inc, La Jolla, CA, USA). All the data were expressed as the mean ± standard deviation. Statistical comparisons between different groups were performed by Dunnett's test following One-Way ANOVA.

3. Results

3.1. Long-term treatment with low levels of OA induces proliferation of colonic epithelial cells in SD rat model

In order to determine whether the long-term exposure to low levels of OA can induce changes in cellular proliferation *in vivo*, we constructed a rat model of chronic OA toxicity via the oral administration of OA. The expression of the marker of proliferation, Ki-67, was detected in the colonic tissues of rats. Ki-67 is exclusively expressed in proliferating cells during the late G1, S, G2, and M phases of the cell cycle [22]. The results of IHC analyses revealed that the positivity and staining intensity of Ki-67 in the surface absorptive cells of the OA-treated group were higher than those of the control group (Figure 1A). The same results were observed in the crypt cells (Figure 1B). These results suggested that long-term treatment with low levels of OA was associated with increased cellular proliferation. As Ki-67 is a key regulator of the cell cycle, and cell cycle progression has a very significant influence on cellular proliferation, we speculated that low levels of OA might rapidly induce cell cycle progression.

3.2. Long-term exposure to low levels of OA is linked to p53 signaling in vivo

In order to verify the aforementioned speculation, we performed transcriptome sequencing in the collected colonic tissues, and the RNA sequencing data were subsequently analyzed by bioinformatics methods. The identified DEGs are depicted in Figure 2A and Supplementary Table S1. Based on the results of DEG identification, we generated a cluster heatmap of 1485 DEGs ($P < 0.05$). GO enrichment analysis of these 1485 DEGs was performed for determining whether cell cycle progression is associated with treatment with OA. The top 30 cell cycle progression terms comprised 5 cell cycle-related terms, including "cell cycle process", "cell cycle", "mitotic cell cycle", "mitotic cell cycle process", and "DNA replication" (Figure 2B). In order to determine the effect of OA on the cell cycle, cellular proliferation was investigated by treating the HCT116 human colon epithelial cell line with OA. As depicted in Figures 2C, 0.5 nM of OA markedly induced the proliferation of HCT116 cells. We further detected the alterations in cell cycle progression following treatment with OA (Figure 2D). These results suggested that treatment with OA was associated with the cell cycle; however, the mechanism underlying the effect of OA requires further exploration. Subsequent analyses with GSEA demonstrated that treatment with OA was associated with the "P53 SIGNALING PATHWAY" (Figure 2E and Table 1).

3.3. Low doses of OA expedited cell cycle progression in the colonic epithelial cell line by inhibiting p53 signaling

The p53 protein functions as a critical regulator by orchestrating a plethora of cellular responses, including the cell cycle [23]. In order to confirm whether the p53 signaling pathway functions downstream of OA

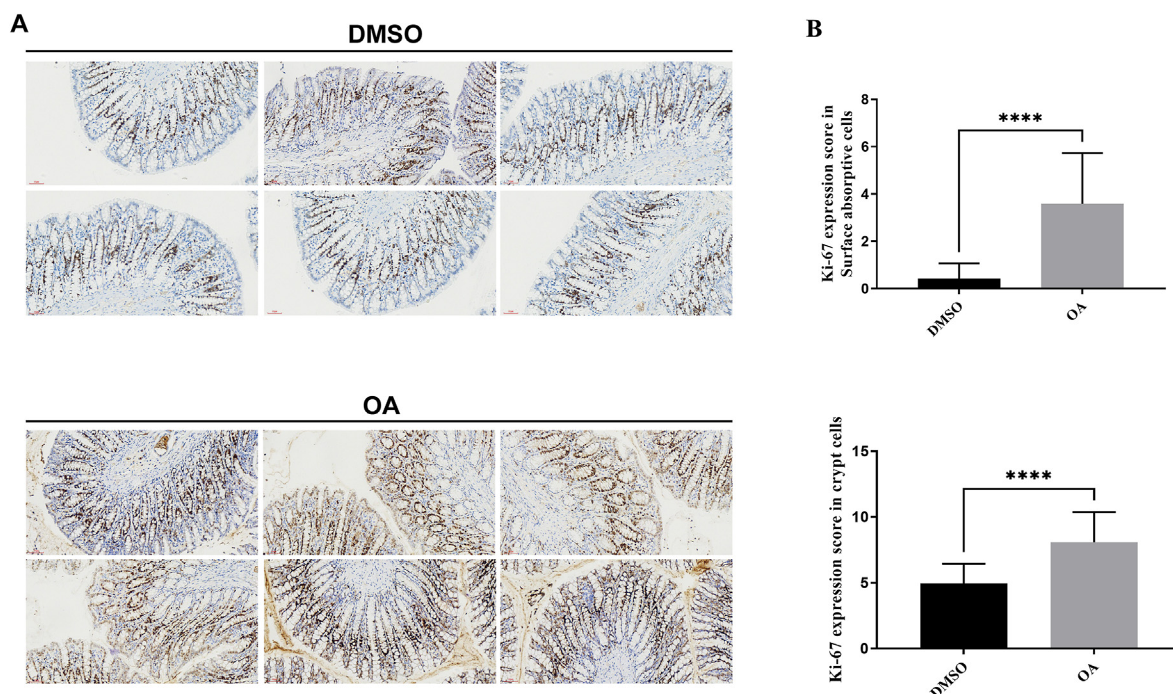

Figure 1. OA promoted the proliferation of the colonic epithelial cells of rats. A. Image obtained by IHC analysis of Ki-67 expression in the colonic epithelial cells of rats. B. IHC score of Ki-67 expression in the surface absorptive cells and crypt cells of rats treated with DMSO or OA. ****$P < 0.0001$.

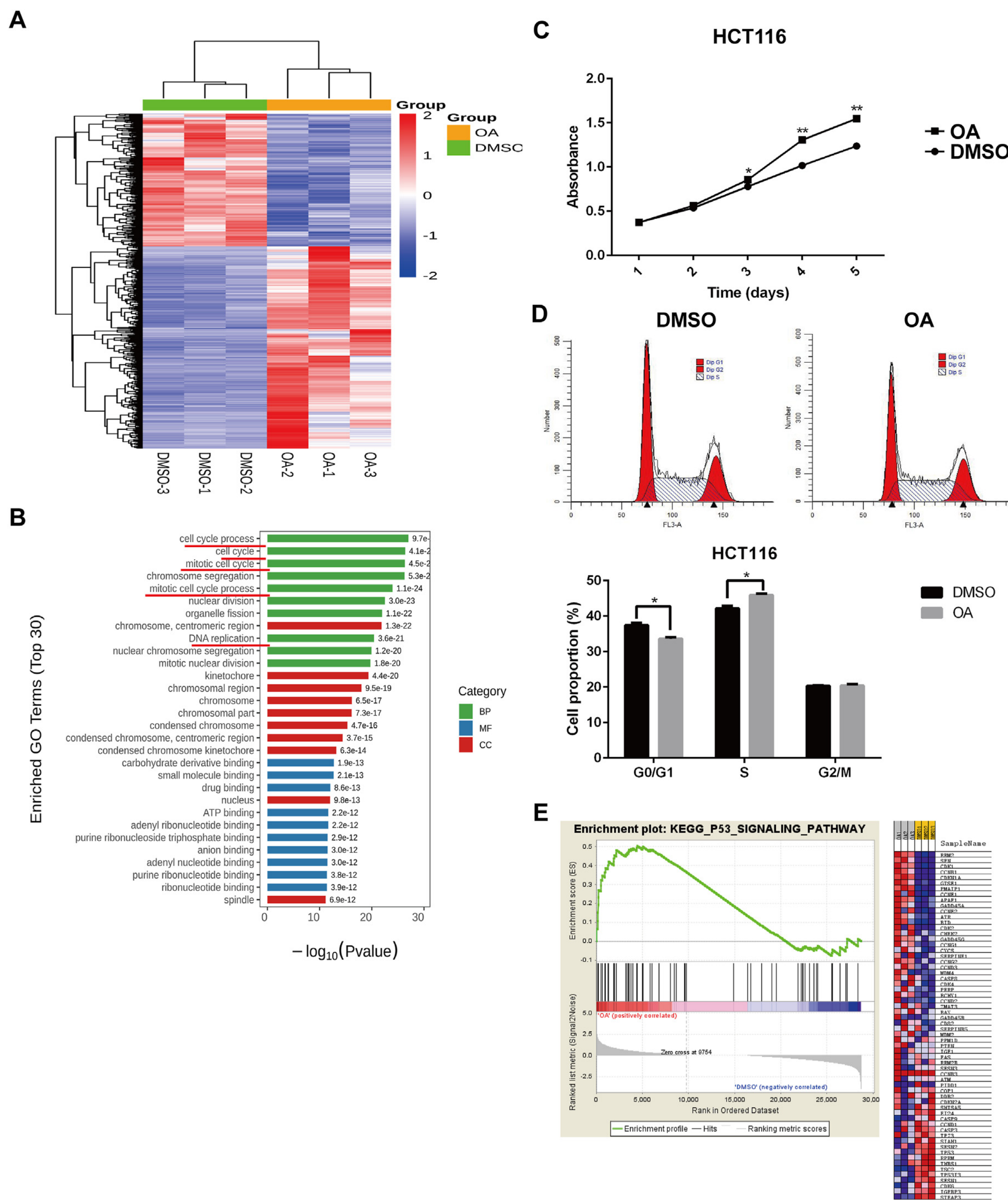

Figure 2. Treatment with OA accelerated cell cycle progression via the p53 signaling pathway in colonic epithelial cells. A. Heatmap of DEGs in the colonic tissues of rats treated with DMSO or OA. B. GO classification of the top 30 enriched terms of the altered mRNAs in the colonic tissues following treatment with OA. The results have been displayed as -$\log_{10}$ P values ($P < 0.05$). C. OA promoted the proliferation of HCT116 cells. **P < 0.01. D. OA promoted cell cycle transition in HCT116 cells. *P < 0.05. E. Association between OA exposure and the p53 signaling pathway.

Table 1. The parameter of GSEA for the correlation of OA treatment and p53 signaling pathway.

Pathway name	ES	NES	NOM P-value	FDR q-value	FWER P-value
p53 signaling pathway	0.50377	1.35373	<0.001	0.34110	0.909

Abbreviations: ES, enrichment score; NES, normalized enrichment score; FDR, false discovery rate; FWER, Family wise error rate.

during cell cycle alterations, we detected the changes in the protein levels of p53 and those of its target genes in the rat model and observed that OA markedly altered the expression of p53 and its target genes (Figure 3A). In order to confirm whether OA repressed the activity of p53, we used the p53 response reporter plasmid, pp53-TA-Luc, for determining the activation of the p53 pathway. The results demonstrated that p53 activity in HCT116 cells was attenuated following exposure to OA (Figure 3B). In order to investigate whether p53 functions downstream in this model, we overexpressed p53 in HCT116 cells during exposure to OA and examined the expression of p53 and its target genes. Analysis of the protein levels

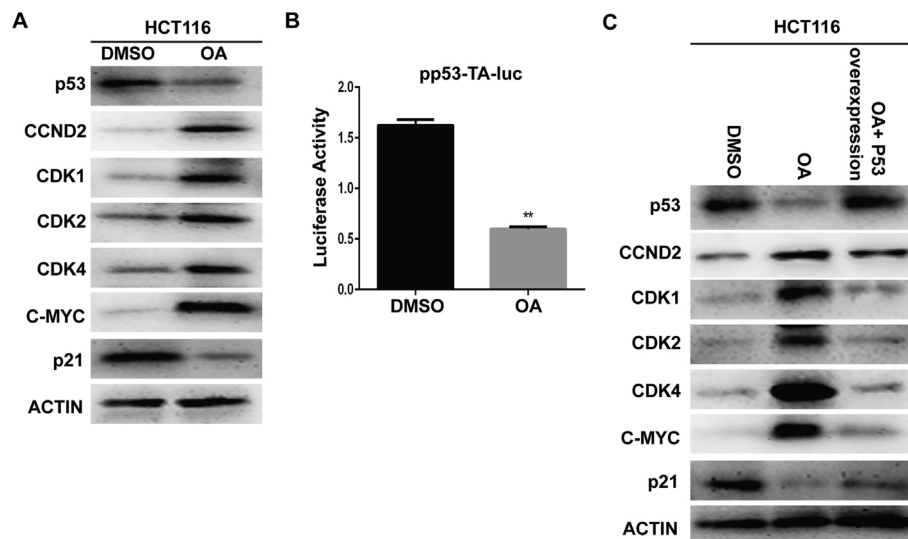

Figure 3. Treatment with OA inhibited the p53 signaling pathway in HCT116 cells. A. The target genes of p53 were detected by western blotting. B. Effects of treatment with OA on the p53 response reporter construct, pp53-TA-Luc, in HCT116 cells. **P < 0.01. C. The target genes of p53 were detected by western blotting following p53 overexpression.

revealed that the overexpression of p53 fully restored the expression of CDK1, CDK2, and CDK4; however, the expression of CCND2, C-MYC, and p21 was not completely restored (Figure 3C). These results suggested that another downstream signaling pathway was involved in OA-induced cell cycle acceleration.

3.4. The Jak/Stat signaling pathway serves as an alternative pathway in OA-induced cell cycle acceleration

In order to investigate whether OA regulates the cell cycle via a p53-independent pathway, we first detected the alterations in the cell cycle in another colonic epithelial cell line with *p53* knockout (HCT116 p53−/−) following treatment with OA (Figure 4A). The results demonstrated that exposure to OA induced the S/G2M transition in colonic epithelial cells (Figure 4B). GSEA was subsequently performed using the Reactome pathway database for identifying the alternate signaling pathway that is involved in OA-induced cell cycle progression. The results demonstrated that Jak/Stat signaling was enriched in the OA group (Supplementary Table S3). The Jak/Stat signaling pathway is a key regulator of the cell cycle and can promote cellular proliferation and expedite cell cycle progression by upregulating the expression of CCND2 and C-MYC and downregulating the expression of p21 [24, 25, 26]. We therefore speculated that OA might induce alterations in the cell cycle via the Jak/Stat signaling pathway. In order to verify this assumption, we monitored the changes in the expression of CCND2, C-MYC, and p21 in the HCT116 p53−/− cell line. The results demonstrated that OA treatment did not alter the protein levels, but increased the phosphorylation levels of Jak1, Jak2, and Stat3 (Figure 4C). Moreover, the expression of the target genes of Stat3, namely, *CCND2*, *C-MYC*, and *p21*, was significantly altered (Figure 4D). In order to verify whether OA activated Stat3 signaling, we determined Stat3 activation using a luciferase reporter vector. The results demonstrated that treatment with OA reinforced Stat3 signaling (Figure 4E). The expression of *CCND2, C-MYC*, and *p21* was restored following treatment with stattic (an inhibitor of Jak/Stat signaling) (Figure 4F). These results suggested that OA promoted cell cycle progression in the colonic epithelial cells via p53-dependent and p53-independent pathways through activation of Jak/Stat signaling (Figure 4G).

4. Discussion

Hormesis is defined as a beneficial dose-response effect, characterized by exposure to low doses of a toxin, which has an effect opposite to that observed at high doses. The concept of "hormesis" was first proposed by Southman and Erlich for describing the modest stimulatory functions of extracts of western red-cedar heartwood on cultured fungi that were strongly repressed by the same extract at high concentrations [27]. OA appears to follow this effect, as expected. Previous studies have demonstrated that high doses of OA induce apoptosis, cell cycle arrest, and have other genotoxic effects in colonic tissues [28, 29, 30]. Conversely, low doses of OA accelerated cell cycle progression in this study, and this opposite effect may have been caused by hormesis. Literature review revealed that the sub-G1 phase of colonic epithelial cells is decreased following treatment with high doses of OA [30]. The variations in the distribution of different phases of the cell cycle caused by high doses of OA are consistent with the variations observed following treatment with OA at low doses. However, OA has different effects on cellular proliferation at different doses. These results led us to explore the mechanisms underlying the changes in the cell cycle induced by low levels of OA by transcriptome sequencing.

The functional effects of OA on p53 expression are intricate. For instance, a study investigating the alterations in the mRNA levels of p53 revealed that treatment with OA reduces the transcription of p53 in A549 cells [31]. Paradoxically, studies on the protein levels of p53 have demonstrated that the stability of the p53 protein was increased in certain cell types but unaffected in other cell lines following treatment with OA [32, 33]. A study on the DNA binding activity revealed that the p53-mediated transactivation of MDM2 is enhanced in rat and mouse fibroblasts but reduced in the human Saos-2 cell line following treatment with OA [33]. Another study investigating cell growth revealed that OA causes hyperphosphorylation of p53 and growth arrest in rat embryonic fibroblasts with wild-type p53, but leads to aberrant mitoses in cells with mutated p53 [34]. Milczarek *et al.* demonstrated that low levels of OA induced p53-mediated cell cycle arrest but did not increase apoptosis [34]. In this study, we also observed that low levels of OA induced alterations in the cell cycle via the p53 signaling pathway. Similar to the results of Milczarek *et al.*, we observed that OA altered the expression of the target genes of p53 related to the cell cycle; however, the expression of the target genes of p53 related to apoptosis did not undergo significant alterations (Supplementary Table S1). These findings suggested that the functions of p53 in regulating cell cycle progression are critical, and are altered following treatment with OA. Ferron *et al.* confirmed that the differences in the status of p53 expression are responsible for the alterations in cell cycle progression in colonic epithelial cells following treatment with high doses of OA. The study further revealed that the

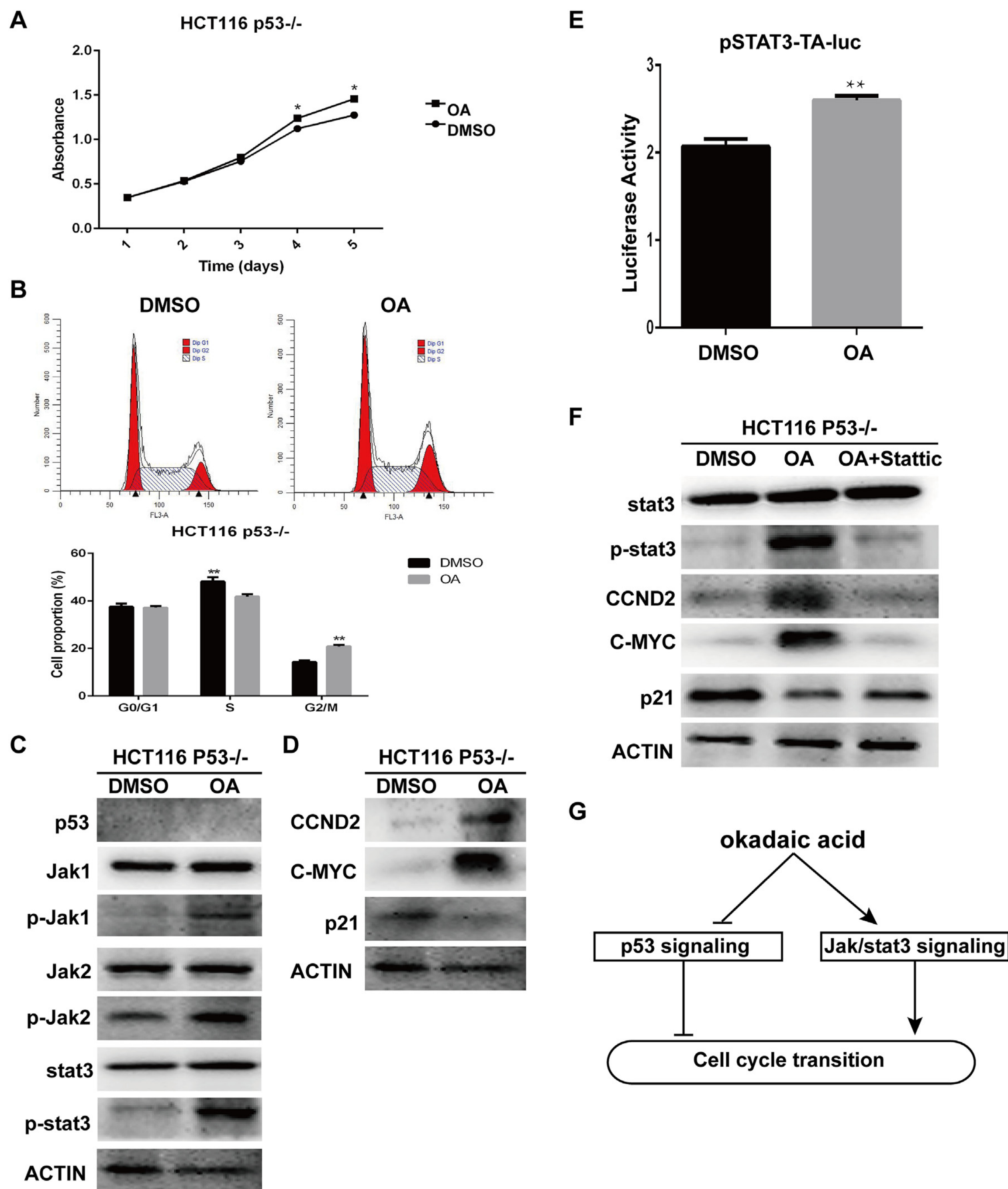

Figure 4. OA accelerated the cell cycle of HCT116 (p53−/−) cells by upregulating the Jak/Stat3 signaling pathway. A. OA promoted the proliferation of HCT116 (p53−/−) cells; *$P < 0.05$. B. OA promoted cell cycle transition in HCT116 (p53−/−) cells; **$P < 0.01$. C. The proteins related to the Jak/Stat3 signaling pathway were detected by western blotting. D. The proteins encoded by the target genes of Stat3 were detected by western blotting. E. Effects of treatment with OA on the Stat3 response reporter construct, pSTAT3-TA-Luc, in HCT116 (p53−/−) cells; **$P < 0.01$. F. The proteins encoded by the target genes of Stat3 were detected by western blotting following treatment with stattic. G. Schematic depicting the potential mechanisms underlying the OA-mediated acceleration of cell cycle transition in colonic tissues.

proportion of sub-G2 cells was higher in p53-deficient cells than in cells with aberrant p53, following treatment with high doses of OA [30]. In this study, we observed that the alterations in cell cycle progression varied in HCT116 p53+/+ and HCT116 p53−/− cells following treatment with low doses of OA. In the presence of p53, low doses of OA accelerated cell cycle progression by inducing G1/S transition in HCT116 cells. However, low doses of OA accelerated cell cycle progression by inducing S/G2/M transition in HCT116 cells following *p53* knockout. These results led us to investigate the relationship between the status of p53 expression and OA-induced alterations in the cell cycle. We aim to elucidate the molecular mechanisms underlying the OA-mediated alterations in cellular phenotype using cell lines with different *p53* expression patterns (deletion, mutation, and wild-type) in future studies.

Ever since its discovery, the Jak/Stat signaling pathway has been regarded as a key mediator of several human physiological processes, and diverse cellular functions of the Jak/Stat pathway, including the regulation of cellular differentiation, cellular transformation, cell cycle, and anti-apoptosis have been reported [35, 36, 37, 38]. Based on the

interactions of the Jak/Stat pathway with p53 [39,40], we determined that the Jak/Stat signaling pathway was altered following exposure to low doses of OA in a p53-deficient cell line, and observed that the Jak/Stat signaling pathway was upregulated. The findings indicated that low levels of OA activated Jak/Stat signaling via a p53-independent mechanism.

5. Conclusion

In summary, this study provides a novel insight into the cell cycle transition of colonic epithelial cells induced by the long-term exposure to low levels of OA. We observed that OA accelerated the cell cycle via the p53 signaling pathway, and also altered the progression of the cell cycle in a p53-independent manner via the Jak/Stat signaling pathway. To the best of our knowledge, this study is the first to report the association between the Jak/Stat signaling pathway and exposure to OA. Further studies are necessary for elucidating the potential molecular mechanism underlying the effect of OA in regulating cell cycle progression. These findings suggested the risk of ingestion of low levels of OA by human subjects, and provide valuable insights into the harmful effects of OA exposure on public health.

Declarations

Author contribution statement

Lu Huang: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ji Gong & Yan Hu; Qiu-Lin Tan: Performed the experiments.

Bo Liu & Xiao-Wen Yu: Analyzed and interpreted the data.

Xiang-Lin Hao: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Qiao-Nan Guo: Conceived and designed the experiments.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e10444.

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All the experiments on rats were approved by the Institutional Animal Care and Use Committee of Third Military Medical University (permit number: AMUWEC20211779).

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