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Polystyrene Microplastics Stimulate the Wnt Pathway to Cause Colorectal Cancer

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Abstract. Polystyrene is one of the most commonly used plastics in daily life. After being discarded, it can be degraded into polystyrene microplastics under the action of the environment and enter the human body with the food chain. Polystyrene microplastics as a growing plastic threat, we focused on its role in the occurrence of colorectal cancer. Mechanistically, we focused on the interaction between polystyrene and the Wnt pathway, and determined whether the Wnt pathway was activated by detecting the expression of APC gene, MYC protein and β-catenin. It is hoped that through our experiments, the public can realize the harm of polystyrene microplastics to public health.

Keywords: Polystyrene microplastics, colorectal cancer, Wnt / β-catenin signaling pathway

1. Introduction

Global plastic production is forecast to reach 34 billion tons by 2050, but less than 10 % is now recycled [1]. After a large amount of plastic is discarded, it is decomposed into microplastics under the action of environmental factors, and enters the food chain through various ways [2]. Polystyrene microplastics (PS-MPs) are one of the more typical and have become a global pollution problem. The accumulation of polystyrene microplastics within organisms can lead to a wide range of detrimental effects. These include weight loss, increased mortality, pulmonary disease, neurotoxicity, transgenerational issues, oxidative stress, altered metabolism, ecotoxicity, immunotoxicity, and numerous other physiological dysfunctions [3-7]. The poisonousness of polystyrene microplastics in humans has also been studied, such as the destruction of the blood-testis barrier by polystyrene microplastics [8]. Furthermore, bone cells can absorb polystyrene microplastics, leading to cell toxicity. This process also triggers oxidative stress from a buildup of reactive oxygen species (ROS), ultimately causing DNA damage and halting the cell cycle [9]. In addition to the above toxicity, it is worth noting that polystyrene microplastics have been shown to exhibit high persistence and accumulation in colorectal cancer cell lines, which may indicate that polystyrene microplastics are a potential colorectal cancer catalyst [10].

Further exploring the relationship between the toxicity of polystyrene microplastics and the development of CRC ,What we found is that nano-scale microplastics (NPs) are more toxic than MPs because NPs are more easily absorbed into cells [11]. And studies have found that in AOM / DSS mouse model and cell model, The exposure to PS-NPs was found to activate the PI3K/AKT/mTOR cascade, a key signaling pathway. (Phosphatidylinositol 3-kinase / AKT /

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mammalian target of rapamycin), thereby promoting the occurrence and deterioration of colorectal cancer [12]. This has further sparked our interest in the link between polystyrene microplastics and colorectal cancer. The Wnt pathway is an important pathway in the development of colorectal cancer and its interaction with polystyrene has not been explored, so this will be the focus of our research.

Colorectal cancer has become a major malignant tumor endangering human beings. In 2022, the global incidence rate ranks third (9.6 %) and the mortality rate ranks second (9.3 %) [13,14]. Usually, the occurrence of CRC follows the adenoma-carcinoma-metastasis [15]. For that above process, The inactivation of APC tumor suppressor gene on chromosome 5q21-q22 is very important [16]. APC mutations lead to abnormal Wnt / β-catenin signaling, therefore inducing the development of CRC [17,18].

The Wnt signaling pathway is a signaling pathway that plays an important role in the normal physiological function of the human body and the occurrence of cancer. The canonical Wnt signaling pathway (Wnt / β-catenin signaling pathway) is the most representative inducer of colon cancer. When the Wnt ligand not binding to the receptor, cytoplasmic β-catenin is phosphorylated by a complex consisting of glycogen synthase kinase 3β (GSK3β), casein kinase I (CKI), Axin, and adenomatous polyposis protein (APC), which is subsequently broken down in the cytoplasm through the ubiquitin-mediated protein degradation pathway [19]; When the Wnt ligand binding to the receptor , it blocks or destroys the formation of Axin / GSK3 / APC complex by recruiting cytoplasmic (Dvl) proteins, and activates Wnt signals, therefore hindering the breakdown of β-catenin and triggering the accumulation of β-catenin in the cytoplasm. Subsequently, β-catenin can be translocated to the nucleus, interact with TCF / LEF, and activate Wnt target genes, such as c-MYC, CCND1, (cyclin D1-encoding gene), EGFR and leucine-rich repeat receptor 5 (LGR5), thereby exerting carcinogenic effects [19,20] Among them, the expression of c-MYC, in particular, as a positive prognostic marker for CRC [21], is an important test object in our experiment.

In summary, we know that polystyrene microplastics can promote and stimulate colorectal cancer. Through the understanding of the mechanism of colorectal cancer, we know that the Wnt / β-catenin pathway is the most important signaling pathway in colorectal carcinogenesis. The mutation of APC gene can lead to the failure of the formation of β-catenin phosphorylation complex, which leads to the accumulation of β-catenin in cells, and finally leads to the abnormal activation of Wnt / β-catenin pathway. MYC protein can promote the occurrence of colorectal cancer by acting as an important carcinogenic factor downstream of the Wnt / β-catenin pathway.

2. Hypothesis

We predict that increasing concentrations and treatment durations with Polystyrene microplastics inhibits the expression of the APC gene, increases phospho-beta catenin and increases expression of MYC protein, increasing colorectal cancer in mice.

3. Methods and materials

3.1. Chemicals

The diameter of polystyrene microspheres (PS-MPs) was 4μm and the concentration was 5 % (w / v) [22].Studies had shown that humans intake about 0.1-0.5g of MPs per week, or even more than 5g [23]. Assuming that the weight ratio of human to mouse was 50 kg : 25 g, and each mouse drank 4 ml of water daily, the weekly intake of PS-MPs in the 10 μg / ml and 100 μg / ml experimental groups was 0.28 mg and 2.8 mg, respectively, and the corresponding human intake was 0.56 g and

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5.6 g. Subsequently, they were prepared into 10 μg/ml ,40μg/ml,70μg/ml and 100μg/ml PS-MPs. Store in a refrigerator at 4 degrees; AOM was prepared into 10 mg / kg solution and DSS was prepared into 1%

3.2. Animal and experimental design

A group of male BALB/c mice weighed between 14 and 18 grams The mice were housed in individually ventilated cages under specific-pathogen-free (SPF) conditions. They were kept in a controlled environment with a 12-hour light/dark cycle, a temperature of 23 ± 1 °C, and a humidity level of 50% ± 5% [22].. These mice were given a common diet and free access to water for 3 weeks as the adaptation period. After that, the mice were randomly and equally divided into experimental group and control group, as followed: Negative control (free access to water for 6 weeks), Positive control (AOM was intraperitoneally injected and 1 % DSS solution was freely drunk for 4 days, continuing for 4 cycles. Then free access to water), A group (free access to water with 10 μg / ml PS-MPs for 6 weeks), B group (free access to water with 40 μg / ml PS-MPs for 6 weeks), C group (free access to water with 70 μg / ml PS-MPs for 6 weeks) and D group (free access to water with 100 μg / ml PS-MPs for 6 weeks). At the end of the experiment after 6 weeks, the mice were euthanized and their colorectal tissues were collected. According to the above process and grouping, the culture time was divided into 6 weeks, 8 weeks, 10 weeks and 12 weeks with the feeding time of mice as the variable.

3.3. qrtPCR

The colorectal tissue was quickly frozen in liquid nitrogen preventing RNA degradation. The frozen tissue was placed in a mortar and quickly ground to fine powder, then transferred to a 1.5 ml centrifuge tube. Added 1 mL TRIzol reagent (50-100 mg tissue), immediately vortexed and mixed, stood at room temperature for 5 minutes. Centrifuged at 4°C (12,000 × g, 15 min), divided into three layers (upper water phase containing RNA, middle protein, lower organic phase). Carefully absorbed the upper water phase to the new tube, added an equal volume of isopropanol, mixed upside down, and stood at room temperature for 10 minutes. Centrifuged at 4°C (12,000 × g, 10 min), discard supernatant, white RNA precipitate visible. The precipitate was washed with 75 % ethanol, centrifuged at 4°C (7,500 × g, 5 min), discarded ethanol, and dried in air for 5 min. Reverse transcription with 1 μg of total RNA used a reverse transcription kit (e.g. PrimeScript RT Master Mix). Subsequently, the cDNA was amplified by qrtPCR. In this process, the experimental group and the control group were labeled separately to facilitate the distinction, and the expression of APC gene was the object of comparison between the two.

3.4. Western bot

Took 50-100 mg powder, added 1 mL RIPA lysis buffer (containing protease inhibitor), and lysed on ice for 30 min. Subsequently, ultrasonic crushing (10 sec × 3 times, interval 30 sec, ice operation) was performed. Centrifuged at 4°C, 12,000 × g for 15 min, and took the supernatant. Then, determined protein concentration using a BCA protein assay kit. After protein extraction, load equal amounts of protein (30 μg) onto SDS-PAGE gels and perform electrophoresis and transfer the proteins to a PVDF membrane. Block the membrane with 5% non-fat milk in TBST for 1 hour at room temperature. Incubated the membrane overnight at 4°C with primary antibodies against MYC protein (1:1000), β-catenin (1:000) and GAPDH (1:5000). Washed the membrane with TBST and

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incubated with HRP-conjugated secondary antibodies (1:2000) for 1 hour at room temperature. Washed the membrane again and detected protein bands using ECL detection reagent. Used a chemiluminescence imager (e.g. Bio-Rad ChemiDoc) for Signal acquisition. In this process, the experimental group and the control group were labeled separately to facilitate the distinction, and the content of MYC protein and β-catenin was the object of comparison between the two.

3.5. Immunofluorescence microscopy

The collected colorectal tissues were paraffin sectioned, fixed in 4 % paraformald -ehyde (PFA) at room temperature for 15 min and permeabilized in 0.3 % Triton X-100 / PBS for 10 min. The sections were blocked with 5 % BSA at room temperature for 1 h. Incubated the section at room temperature for 2h with primary antibodies against cell signaling #8480(1:200) for β-catenin, Abcam ab32072(1:500)for MYC and Thermo Fisher MA5-14520(1:200) for ki-67. Incubated the section at room temperature for 1h with secondary antibodies with Alexa flour 488(1:500), Cy3(1:500) and Alexa flour 647(1:500). Subsequently, the nuclei were stained with DAPI (1 μg / mL, 5 min). Confocal microscopy for imaging. In this process, the experimental group and the control group were labeled separately to facilitate the distinction, and the fluorescence intensity of the specific protein molecule and the location of the fluorescence distribution in the cell were the comparison objects between the two.

3.6. Statistical analysis

The experiment would be repeated for 5 times. Student T test was used to analyze the statistical significance of all numerical data obtained from qrtPCR, immunofluorescence microscopy and Western blot, and $p < 0.05$ was the significance criterion.

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4. Results

Table 1. The combination of possible results

Combination of possible results (CR)	PS-MPs decrease the expression of APC gene by qrtPCR?	PS-MPs increase the β-catenin by the western blot?	PS-MPs increase the MYC protein by western blot?	PS-MPs increases the incidence of colorectal cancer by immunofluorescence microscopy?	Support of hypothesis
CR 1	+	+	+	+	Yes
CR 2	+	+	+	-	Partial
CR 3	+	+	-	+	Partial
CR 4	+	-	+	+	Partial
CR 5	-	+	+	+	Partial
CR 6	+	+	-	-	Partial
CR 7	+	-	+	-	Partial
CR 8	-	+	+	-	Partial
CR 9	+	-	-	+	Partial
CR 10	-	+	-	+	Partial
CR 11	-	-	+	+	Partial
CR 12	+	-	-	-	Partial
CR 13	-	+	-	-	Partial
CR 14	-	-	+	-	Partial
CR 15	-	-	-	+	Partial
CR 16	-	-	-	-	No

Regarding Table 1, it presents the various possible outcomes of the experiments we set up, demonstrating the multiple potential pathogenic effects of PS-MPs on the development of colon cancer. The "+" sign means that the heading phenomenon is observed similar to the positive control and opposite to the negative control in the experiments and is statistically significant. The "-" sign means that the phenomenon observed is neither similar to the positive control group and nor opposite to the negative control or is not statistically significant. Negative control (free access to water for 6 weeks). Positive control (AOM (10 mg / kg) was intraperitoneally injected and 1 % DSS solution was freely drunk for 4 days, continuing for 4 cycles.

Combination of possible results 1 (CR1): In qrtPCR, the expression of APC gene was significantly decreased. Western blot showed that the content of MYC protein and β-catenin increased. Immunofluorescence showed colorectal cancer occurred.

Combination of possible results 2 (CR2): In qrtPCR, the expression of APC gene was significantly decreased. Western blot showed that the content of MYC protein and β-catenin increased. But the immunofluorescence didn't indicate colorectal cancer occurred.

Combination of possible results 3 (CR3): In qrtPCR, the expression of APC gene was significantly decreased. Western blot just showed that the content of β-catenin increased, while the MYC protein not. Immunofluorescence showed colorectal cancer occurred.

Combination of possible results 4 (CR4): In qrtPCR, the expression of APC gene was significantly decreased. Western blot just showed that the content of MYC protein increased, while the β-catenin not. Immunofluorescence showed colorectal cancer occurred.

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Combination of possible results 5 (CR5): In qrtPCR, the expression of APC gene was not decreased. Western blot showed that the content of MYC protein and β-catenin increased. Immunofluorescence showed colorectal cancer occurred.

Combination of possible results 6 (CR6): In qrtPCR, the expression of APC gene was significantly decreased. Western blot just showed that the content of β-catenin increased, while the MYC protein not. immunofluorescence didn't indicate colorectal cancer occurred.

Combination of possible results 7 (CR7): In qrtPCR, the expression of APC gene was significantly decreased. Western blot just showed that the content of MYC protein increased, while the β-catenin not. immunofluorescence didn't indicate colorectal cancer occurred.

Combination of possible results 8 (CR8): In qrtPCR, the expression of APC gene was not decreased. Western blot showed that the content of MYC protein and β-catenin increased. immunofluorescence didn't indicate colorectal cancer occurred.

Combination of possible results 9 (CR9): In qrtPCR, the expression of APC gene was significantly decreased. But Western blot didn't show that the content of MYC protein and β-catenin increased. Immunofluorescence showed colorectal cancer occurred.

Combination of possible results 10 (CR10): In qrtPCR, the expression of APC gene was not decreased. Western blot just showed that the content of β-catenin increased, while the MYC protein not. immunofluorescence indicated colorectal cancer occurred.

Combination of possible results 11 (CR11): In qrtPCR, the expression of APC gene was not decreased. Western blot just showed that the content of MYC protein increased, while the β-catenin not. immunofluorescence indicated colorectal cancer occurred.

Combination of possible results 12 (CR12): In qrtPCR, the expression of APC gene was significantly decreased. western blot didn't show that the content of MYC protein and β-catenin increased. immunofluorescence didn't indicate colorectal cancer occurred.

Combination of possible results 13 (CR13):In qrtPCR, the expression of APC gene was not decreased. Western blot just showed that the content of β-catenin increased, while the MYC protein not. immunofluorescence didn't indicate colorectal cancer occurred.

Combination of possible results 14 (CR14): In qrtPCR, the expression of APC gene was not decreased. Western blot just showed that the content of MYC protein increased, while the β-catenin not. immunofluorescence didn't indicate colorectal cancer occurred.

Combination of possible results 15 (CR15): In qrtPCR, the expression of APC gene was not decreased. western blot didn't show that the content of MYC protein and β-catenin increased. immunofluorescence indicated colorectal cancer occurred.

Combination of possible results 16 (CR16): In qrtPCR, the expression of APC gene was not decreased. western blot didn't show that the content of MYC protein and β-catenin increased. immunofluorescence didn't indicate colorectal cancer occurred.

5. Possible results for the variables of concentration and treatment duration

For qrtPCR experiments, too short treatment time or too high concentration of PS-MPs may lead to sufficient expression of APC gene. At the same time, with the increase of treatment time and concentration, the expression of APC gene will gradually decrease. For western blot experiments, the amount of β-catenin and MYC protein may increase with the increase of treatment time and concentration. For immunofluorescence experiments, the incidence of colorectal cancer may increase with the increase of treatment time and concentration, or may be flat in the high concentration and longtime experimental groups.

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6. Discussion

The purpose of this experiment is to study the role of PS-MPs in promoting the occurrence of colorectal cancer through the Wnt pathway, specifically by measuring the expression of APC gene, MYC protein and β-catenin content, and immunofluorescence techniques. The table lists 16 possible combinations of experimental results for discussion.

Combination of possible results 1 (CR1) strongly supported the hypothesis, suggesting that PS-MPs can indeed promote the occurrence of colorectal cancer by stimulating the abnormal activation of the Wnt pathway. This, together with previous studies using polystyrene nano plastics to stimulate the P13k / AKT / mTOR pathway leading to colorectal cancer [11], illustrated the important role of polystyrene microplastics in the environment for colorectal cancer. On the basis of the correct hypothesis, the future hypothesis should be to study how polystyrene microplastics inhibit APC gene expression (Such as genetic mutations, epigenetic silencing or abnormal transcriptional regulation.)

Combination of possible results 2 (CR2) partially supported the hypothesis. The occurrence of colorectal cancer was not observed by immunofluorescence technique. This may be because there are too few targets selected. In this experiment, β-catenin, MYC protein and ki-67 were selected. Increasing the relevant targets as detection criteria (such as E-cadherin or CD31) can enhance the sensitivity of colorectal tumorigenesis. On the basis of the above, the next experiment should study whether the Wnt pathway stimulated by polystyrene microplastics induces carcinogenic effects or other pathogenic effects (such as fibrotic diseases, metabolic diseases, etc.).

Combination of possible results 3 (CR3) partially supported the conclusion. Although APC gene expression decreased, β-catenin content increased and colon cancer occurred, the expression of MYC protein did not change. This indicates that the activation of Wnt pathway promoted by PS-MPs does not induce the expression of MYC protein, a downstream protein, but may lead to colorectal cancer through other downstream carcinogenic proteins (such as Cyclin D1, EGFR, etc.). On the basis of the above, the next experiment should study the expression of other downstream proteins in tissues, so as to determine the specific mechanism of PS-MPs activating Wnt pathway.

Combination of possible results 4 (CR 4) partially supported the hypothesis. The decrease of APC gene expression and the increase of MYC protein content indicated that Wnt pathway was indeed activated. This may be due to the increased expression of AXIN2 (negative feedback regulator) after Wnt activation, which may promote the degradation of β-catenin and keep its total level unchanged, but there is still enough to enter the nucleus to activate MYC. On this basis, the following experiments will study the reasons for the decrease of β-catenin. Specifically, by measuring negative feedback regulators such as AXIN2 and DKK1, we can determine whether β-catenin is inhibited by them.

Combination of possible results 5 (CR 5) partially supported the hypothesis that APC gene plays an important role by expressing APC protein and other proteins (such as GSK3β, CKI and Axin) to form a complex that degrades β-catenin. The increase in the content of known β-catenin indicates that the complex is not formed or lost. Therefore, PS-MPs may inhibit the expression of other proteins that make up the complex, thereby abnormally activating the Wnt pathway. On this basis, the following experiments will study the expression of other proteins that constitute the complex in tissues.

Combination of possible results 6 (CR 6) partially supported the hypothesis. The decrease of APC gene and the decrease of β-catenin indicated the activation of Wnt pathway. The absence of changes in MYC protein content and the absence of colorectal cancer may indicate that the main function of the Wnt pathway stimulated by polystyrene microplastics is not to induce cancer, but other

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pathogenic effects. Similar to CR2, the next experiment should study the expression of other disease-related proteins in tissues.

Combination of possible results 7 (CR 7) partially supported the hypothesis. The decrease of APC gene expression and the increase of MYC protein content indicate the activation of Wnt pathway. Therefore, the reason why β-catenin did not change and colorectal cancer was not detected may be the same as CR4 and CR2, respectively. The next experiment is to combine the two.

Combination of possible results 8 (CR 8) partially supported the hypothesis. The activation of Wnt pathway cannot be judged by the increase of MYC protein and β-catenin protein content. The activation of other pathways such as PI3K / AKT / mTOR pathway and NF-κB pathway can also directly or indirectly increase the content of the two proteins. On this basis, the following experiments will study the activation of other pathways such as PI3K / AKT / mTOR in tissues.

Combination of possible results 9 (CR 9) partially supported the hypothesis. In the case of decreased APC gene expression, the reason for the unchanged content of β-catenin may be the same as that of CR4, and the content of MYC did not change, which may be the same as that of CR3. The next experiment is to combine the two.

Combination of possible results 10 (CR 10) partially supported the hypothesis. Similar to CR8, the activation of Wnt pathway by PS-MPs cannot be judged by the change of β-catenin content and the occurrence of colorectal cancer. For example, PI3K / AKT / mTOR can also achieve the same effect.On this basis, the following experiments will study the activation of other pathways such as PI3K / AKT / mTOR in tissues.

Combination of possible results 11 (CR 11) partially supported the hypothesis. Similar to CR10, the increase of MYC protein content and the occurrence of colorectal cancer cannot be used to judge that PS-MPs activate the Wnt pathway. On this basis, the following experiments will study the activation of other pathways such as PI3K / AKT / mTOR in tissues.

Combination of possible results 12 (CR 12) partially supported the hypothesis. Only the expression of APC gene was inhibited, indicating that PS-MPs may only affect the expression of APC protein, but not affect the degradation of β-catenin by the complex. Therefore, the following experiments can test the expression of other proteins that make up the complex and the ability of the complex to degrade β-catenin.

Combination of possible results 13 (CR 13) partially supported the hypothesis. Only the increase of β-catenin did not indicate that PS-MPs activated the Wnt pathway and no colorectal cancer was detected. The future hypothesis is that polystyrene microplastics stimulate the PI3K / AKT / mTOR pathway to trigger an increase in β-catenin. The next experiments will study the activation of the PI3K / AKT / mTOR pathway in tissues and detect the occurrence of related diseases (such as metabolic diseases and neurodegenerative diseases).

Combination of possible results 14 (CR 14) partially supported the hypothesis. Similar to CR13, the increase of MYC protein content alone could not judge that PS-MPs had the effect of activating Wnt pathway.The following experiments will study other possible pathways that trigger MYC protein expression (such as Ras / MAPK pathway and PI3K / AKT / mTOR pathway).

Combination of possible results 15 (CR 15) partially supported the hypothesis. Only colorectal cancer was detected, indicating that PS-MPs may promote cancer through other pathways. The future hypothesis is that polystyrene microplastics stimulate the PI3K / AKT / mTOR pathway and cause colorectal cancer.

Combination of possible results 16 (CR 16) are completely contrary to the hypothesis. It is possible that the diameter of PS-MPs used in this experiment do not reach the level that can activate the Wnt pathway in mice, or that PS-MPs have other toxicological properties.

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7. Discussion of the possible results for the variables of concentration and treatment duration

In this experiment, the concentration and time of treatment with polystyrene microplastics meant the severity of mice exposed to environmental toxins. Too low concentration and treatment time may lead to the expression of some proteins difficult to be detected, thus affecting the hypothesis test, such as CR3.At the same time, too high concentration and treatment time may lead to the loss of specificity of the experimental results. If the low concentration and time treatment showed no effect, while the high concentration and time showed high effect, indicating that the toxicity of polystyrene microplastics has a threshold effect. If both low and high concentrations show no obvious effect, it is necessary to consider whether there are flaws in the experimental design or material selection in this experiment, such as the diameter of microplastics is too large to be absorbed by cells. In summary, the systematic treatment of pus and time is conducive to our comprehension of the toxicity of PS-MPs.

8. Conclusions

Our research focused on the effect of polystyrene microplastics on the Wnt signaling pathway and the development of CRC. The experiment focused on the expression of APC gene, the content of β-catenin and MYC protein, and the occurrence of colorectal cancer. A variety of results show the complex toxicity of polystyrene microplastics and make up for the knowledge gap between polystyrene and the Wnt pathway.

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