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Original Article

MAPK1 up-regulates the expression of MALAT1 to promote the proliferation of cardiomyocytes through PI3K/AKT signaling pathway

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Abstract: The previous studies identify mammalian heart is terminal differentiation organs without regenerative capacity. Recently, there is some evidence point that cardiomyocytes are not terminally differentiated cells and cell proliferation may be stimulated in the pathologic heart. The aim of this study is to discover the possible mechanism which involved in cardiomyocytes proliferation process. In this study, the proliferation assay and cell cycle assay showed the proliferation of cardiomyocytes was inhibited when the cells treated with MAPK1 inhibitor. Moreover, the bioinformatics analysis revealed MAPK1 was positively correlated with MALAT1. Meanwhile, the expression of MALAT1 in H9C2 cells with the treatment of MAPK1 siRNA was obvious lower than scramble siRNA treated group. Finally further study suggested H9C2 cells treated with Wortmannin in combination with LY294002 (PI3K/AKT signaling pathway inhibitor), the expression of MALAT1 was dramatically decreased. These results indicated that MAPK1 was able to increase the proliferation of cardiomyocytes via up-regulating the expression of MALAT1 through PI3K/AKT signaling pathway.

Keywords: Cardiomyocytes, proliferation, MAPK1, MALAT1

Introduction

Previously, the accepted common view in cardiac biology is considered the mammalian heart is terminal differentiation organs without regenerative capacity [1]. The loss of myocardial cells impairs the function of heart, in server case, resulting in the occurrence of heart failure [2]. However, the recent studies revealed that an increase in myocyte number occurs with severe myocardial hypertrophy [3, 4]. Moreover, severe myocardial scarring consists of multiple sites of replacement fibrosis and diffuse interstitial fibrosis in ischemic and idiopathic dilated cardiomyopathies patients [5-7]. Apoptosis does not lead to tissue fibrosis, dying myocytes are removed from neighboring cells in the absence of an inflammatory reaction [8]. These phenomena, indicating severe ongoing necrotic and apoptotic myocyte death, point to the possibility that myocytes are not terminally differentiated and cell proliferation may be stimulated in the pathologic heart.

The mitogen-activated protein kinases (MAPK) constitute an essential signal transduction cascade that plays crucial role in proliferation, differentiation, transcription regulation and development [9]. Mitogen-activated protein kinase 1, the downstream signal of MAPK, also known as MAPK1, p42MAPK, and ERK2, is an enzyme that in humans is encoded by the MAPK1 gene [10]. The previous studies have discovered that ERK activation can partially antagonize apoptosis [11]. On the other hand, the suppression of ERK signaling was demonstrated to increase daunomycin-induced cardiomyocytes apoptosis in vitro [12], while in a model of ischemia/reperfusion in the intact heart, the activation of ERK1/2 was shown to weaken the amount of apoptosis subsequent to reperfusion injury [13]. Although the ERK1/2 activation is able to protect cardiomyocytes against apoptosis, it remains uncertain whether ERK activation benefits the proliferation of cardiomyocytes.

Metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is a large, infrequently spliced non-coding RNA, which is highly conserved amongst mammals and highly expressed in the nucleus [14]. It regulates the expression of metastasis-associated genes [15]. A mass of

studies have demonstrated that MALAT1 is aberrantly highly expressed in multiple tumors, such as, gastric cancer [16], colorectal cancer [17], lung cancer [18], and these results suggest that MALAT1 function as a promoter of cancer cell proliferation. Therefore MALAT1 may regulate cells' proliferation.

The purpose of this manuscript is to reveal the possible mechanism of cardiomyocytes proliferation, we hypothesis that MAPK1 was able to accelerate the proliferation of myocardial cells through up-regulating the expression of MALAT1. In this study, we explored the proliferation behavior and cell cycle after cardiomyocytes treated with MAPK1 inhibitor. Then the positive correlation between MAPK1 and MALAT1 was analyzed by bioinformatics and in vitro test, and the possible mechanism which involved in the process of MAPK1 regulated the expression of MALAT1 was detected.

Materials and methods

Materials

Cell Counting Kit-8 was obtained from Dojindo (Japan). Cell culture plates were ordered from Corning (NY, USA). RNeasy mini kit was purchased from Qiagen (Valencia, CA). RIPA lysis buffer and PVDF membrane were obtained from Bio-Rad (Hercules, CA, USA). DMEM medium, fetal bovine serum, glutamine, and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). The primary antibodies pMAPK1, Total-MAPK1 and GAPDH was acquired Cell Signaling Technology (Beverly, MA). DMSO, LY2228820, LY294002 and Wortmannin were purchased from Aladdin (Shanghai, China). The scramble siRNA and MAPK1 siRNA were commercial synthesized from Funeng Company (Shanghai, China).

Cell lines

The rat myocardial cell line H9C2 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco Modified Eagle Medium (DMEM) media plus 10% fetal bovine serum, 1% glutamine and 1% penicillin-streptomycin at 37°C and 5% CO_2.

CCK-8 assay

H9C2 cells were seeded in a 96-well plate at a concentration of 5×10^3 cells/well for 24 hours. Then the cultured medium was replaced by conditional medium, which was added with DMSO, 10 nM LY2228820, 100 nM LY2228820, 1 uM LY2228820 respectively, and continually cultured for 2, 4, 6 days. At each time point, 10 ul CCK-8 was added and continually cultured for 4 h. Then the optical density (OD) was determined using an enzyme-linked immunosorbent assay plate reader (Bioreader,) with a reference wave length of 450 nm.

Cell cycle assay

Cell cycle analysis was carried out by flow cytometry according to a standard protocol. H9C2 cells with the treatment of DMSO, 10 nM LY2228820, 100 nM LY2228820, 1 uM LY-2228820 respectively, then washed with cold PBS, and fixed with cold 70% ethanol for 12 hours. After this the fixed cells were stained with PI solution consisting of 50 μg/mL PI, 20 μg/mL RNase A, and 0.1% Triton X-100. After 0.5 h incubation in the dark, the stained cells were detected in a FACScan flow cytometer. The distribution of cells in the different cell cycle phases was analyzed using Multicycle software (Phoenix Flow Systems, San Diego, CA).

Western blot

H9C2 cells were treated with DMSO, 10 nM LY2228820, 100 nM LY2228820, 1 μM LY2228820 for 24 hours, and then the cells were harvested, and lysed with ice-cold lysis buffer supplemented with protease inhibitors. Proteins were electrophoretically resolved on 10% Tris-glycine gels and transferred onto a nitrocellulose membrane. After blocking with skim milk, the membrane was incubated with the primary antibody at 4°C overnight. Membranes were washed 3 times and then incubated with the peroxidase-conjugated secondary antibody. After washed 3 times, the specific protein bands were detected using the enhanced chemiluminescence reagents.

Bioinformatics

We obtained the GSE4386 and GSE1145 microarray date from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). Then the correlation analysis was performed.

RT-PCR

H9C2 cells were used to isolate total RNA by using RNeasy kit according to the manufactur-

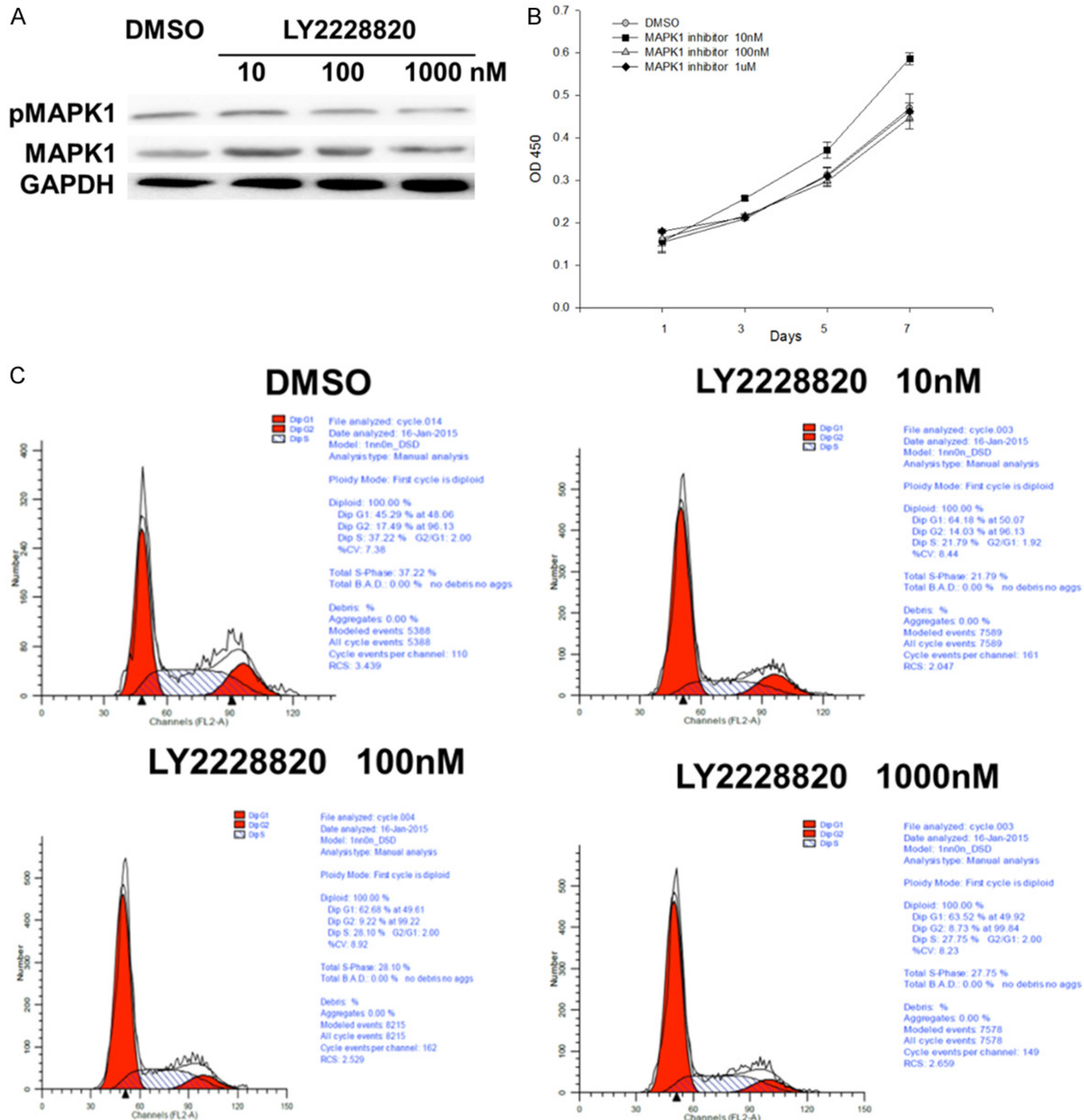
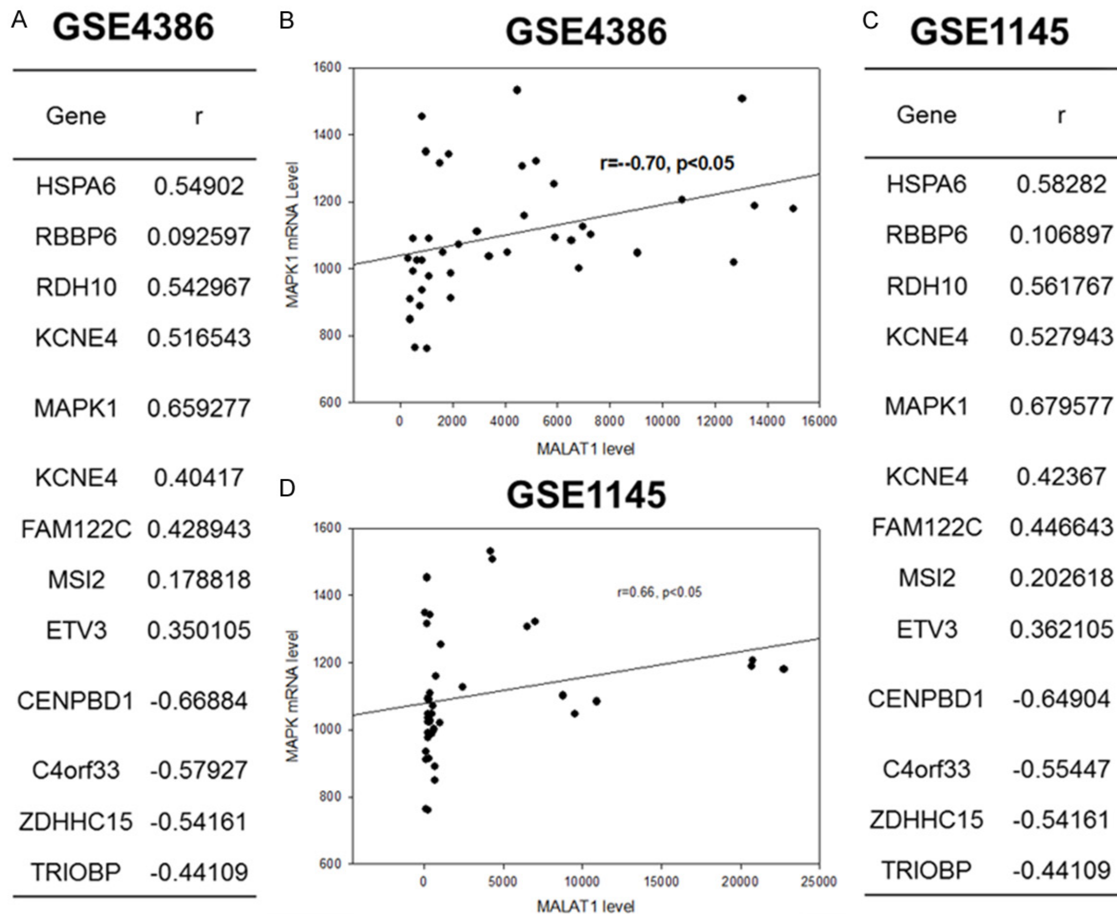


Figure 1. MAPK1 promotes the proliferation of myocardial cell. A. The phosphorylation of MAPK1 in myocardial cells with the treatment of DMSO, 10 nM, 100 nM, 1000 nM LY2228820 respectively. B. The proliferation of myocardial cells with the treatment of DMSO, 10 nM, 100 nM, 1000 nM LY2228820 respectively. C. The cell cycle analysis of myocardial cells with the treatment of DMSO, 10 nM, 100 nM, 1000 nM LY2228820 respectively.

er's protocol (Qiagen, Valencia, CA). Briefly, first-strand cDNA was reverse-transcribed from 1 μg total RNA using the Super-Script First-Strand cDNA System (Invitrogen), and was amplified by Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). A master mix was prepared for each PCR reaction, which included Platinum SYBR Green qPCR SuperMix-UDG, forward primer, reverse primer, and 10 ng of template cDNA. PCR conditions were 5 min at 95°C, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The forward and backward primer sequences for MALAT1, forward: CCGCTGCTATTAGAATGC reverse: CTTCAACAATCACTACTCCAA.

Statistical analysis

All data were expressed as mean ± S.D. Differences between the groups were analyzed using one-way analysis of variance (ANOVA) using GraphPad Prism version 4.00 for Windows



A GSE4386

Gene	r
HSPA6	0.54902
RBBP6	0.092597
RDH10	0.542967
KCNE4	0.516543
MAPK1	0.659277
KCNE4	0.40417
FAM122C	0.428943
MSI2	0.178818
ETV3	0.350105
CENPBD1	-0.66884
C4orf33	-0.57927
ZDHHC15	-0.54161
TRIOBP	-0.44109

C GSE1145

Gene	r
HSPA6	0.58282
RBBP6	0.106897
RDH10	0.561767
KCNE4	0.527943
MAPK1	0.679577
KCNE4	0.42367
FAM122C	0.446643
MSI2	0.202618
ETV3	0.362105
CENPBD1	-0.64904
C4orf33	-0.55447
ZDHHC15	-0.54161
TRIOBP	-0.44109

Figure 2. MAPK1 was positively correlated with the expression of LncRNA MALAT1. A, B. Linear correlation was applied to analyze the relationship between MAPK1 and MALAT1 by bioinformatics in GSE4386 gene chip. C, D. Linear correlation was applied to analyze the relationship between MAPK1 and MALAT1 by bioinformatics in GSE1145 gene chip.

software. *P-values* less than 0.05 were considered statistically significant.

Results

MAPK1 promotes the proliferation of myocardial cell

In order to explore whether MAPK1 was able to accelerate myocardial cells proliferation, we used different concentrations of MAPK1 inhibitor LY2228820 to treat myocardial cells and detected the effects of LY2228820 on the cells' proliferation and cells' cycle. As shown in **Figure 1A**, the phosphorylation of MAPK1 was gradually reduced with the increase of LY2228820 concentration, suggesting the phosphorylation of MAPK1 was successfully inhibited with the treatment of LY2228820. We also found that the proliferation of myocardial cells in 10 nM LY2228820 treated group was significant higher than 100 nM and 1 μM LY2228820 treated group (**Figure 1B**). The cell cycle analysis further showed that cell cycle was arrested in G1 phase and the cell population in S phase was reduced with the treatment of LY2228820 when compared to DMSO treated group (**Figure 1C**). These results revealed that the phosphorylation of MAPK1 can increase the proliferation of myocardial cells.

Positive correlation is in existence between MAPK1 and LncRNA MALAT1

In order to determine whether MAPK1 was able to regulate the expression of LncRNA MALAT1, we used bioinformatics to analysis the MALAT1 related genes in GSE4386 and GSE1145 gene chips. As shown in **Figure 2A** and **2B**, In GSE4386 gene chip, there was a strong positively correlation between the MAPK1 mRNA level and MALAT1 level (r=0.70, $P<0.05$).

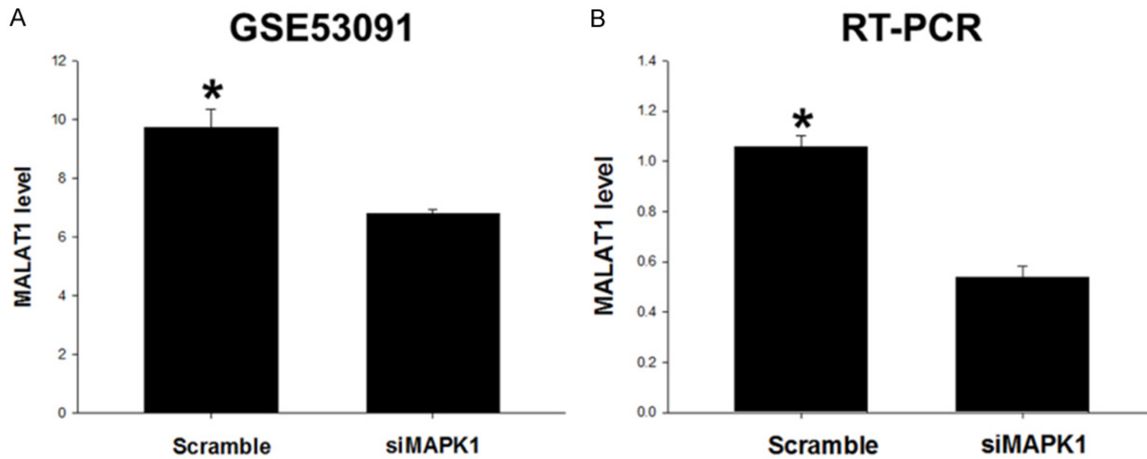


Figure 3. MAPK1 up-regulates the expression of MALAT1. A. The expression of MALAT1 in GSE53091 gene expression microarray data when the samples treated with scramble siRNA and MAPK1 siRNA respectively. B. The expression of MALAT1 in H9C2 cells treated with scramble siRNA and MAPK1 siRNA respectively. *P<0.05.

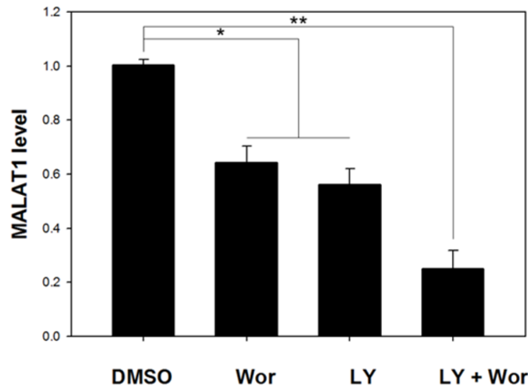


Figure 4. The expression of MALAT1 in H9C2 cells with the treatment of DMSO, Wortmannin, LY294002, Wortmannin in combination with LY294002 respectively. *P<0.05, **P<0.01.

Similarly, In GSE1145 gene chip, MAPK1 mRNA level was positively correlated with MALAT1 (r=0.66, P<0.05) (**Figure 2C** and **2D**). Then results revealed that MAPK1 was positively correlated with the expression of LncRNA MALAT1.

MAPK1 up-regulates the expression of MALAT1

To further ascertain whether MAPK1 was able to regulate the expression of MALAT1, the GSE53091 gene expression microarray data was analyzed. As shown in **Figure 3**, we found that there was a significant difference in MALAT1 expression between scramble siRNA treated group and MAPK1 siRNA treated group (P<0.05). Similarly, the expression of MALAT1 in H9C2 cells with the treatment of MAPK1 siRNA was obvious lower than scramble siRNA treated group. The result revealed that MAPK1 was able to increase the expression of MALAT1.

MAPK1 regulates the expression of MALAT1 via activating PI3K/AKT signaling

Finally we further explored the possible mechanism which involved in the process of MAPK1 regulated the expression of MALAT1. As **Figure 4** showed, the expression of MALAT1 was inhibited in Wortmannin and LY294002 (PI3K/AKT signaling pathway inhibitor) treated group when compared with DMSO treated group (P<0.05). Moreover, when H9C2 cells treated with Wortmannin in combination with LY294002, the expression of MALAT1 was dramatically decreased (P<0.01). Suggesting MAPK1 regulates the expression of MALAT1 via activating PI3K/AKT signaling pathway.

Discussion

The dogma, heart is incapable of self-renewal, has been undermined by lots of evidences that the heart of adult mammals contains its own resource of progenitor (or stem) cells, cardiomyocytes will proliferate in case of injury [1, 19, 20], and cardiomyocytes in the human heart are renewed throughout the life [21]. The previous study reported formation of new cardiomyocytes may come from the proliferation of dedifferentiated myocytes [22]. However, the detailed mechanism of cardiomyocytes proliferation was still unclear. In this study, we

revealed a possible mechanism that MAPK1 (ERK2) promotes the proliferation of cardiomyocytes through up-regulating the expression of MALAT1.

In this study, we showed that cell cycle was arrested in G1 phase and the cell population in S phase was reduced with the treatment of MAPK1 (ERK2) inhibitor, we speculated that this phenomenon own to the inactivation of ERK2.

ERK1/2 are members of the MAPK super family which can regulate cell proliferation [23]. The activation of ERK1/2 sustain until late G1 for successful S-phase entry [24] and ERK1/2 translocation to the nucleus is essential for G1 to S phase progression [25]. Additionally, upon translocation to the nucleus, activated ERK1/2 phosphorylates the ternary complex factors TIF-IA, Sap-1a, and Elk-1 [26-28]. Phosphorylation of Elk-1 on the C-terminus increases its affinity for the serum response factor and enhances transcription of growth related proteins, such as c-Fos [29, 30].

We also discovered that MAPK1 was positively correlated with the expression of LncRNA MALAT1; moreover, MAPK1 was able to up-regulate the expression of MALAT1 via activating PI3K/AKT signaling in H9C2 cells. Similarly, Wu et al. reported that MALAT1 might serve as an oncogenic LncRNA that promotes proliferation of gallbladder cancer cells and activates the ERK/MAPK pathway [31]. In addition, Dong et al. suggested that knockdown of MALAT1 inhibited the osteosarcoma cell proliferation both in vitro and in vivo, and knockdown of MALAT1 led to the inactivation of the PI3K/Akt pathway [31]. Thus these results indirectly demonstrate our conclusion.

In summary, in this study, we found that the proliferation of cardiomyocytes was inhibited when the H9C2 cells treated with MAPK1 inhibitor. And the bioinformatics analysis revealed MAPK1 was positively correlated with MALAT1. Moreover the further study indicated MAPK1 was able to increase the expression of MALAT1 via PI3K/Akt signaling pathway. This study provided a possible mechanism for cardiomyocytes proliferation research.

Disclosure of conflict of interest

None.

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