

Original Article

Recombinant methioninase inhibits self-renewal and proliferation of gastric cancer stem cells

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Abstract: Objective: This study aimed to investigate the role of recombinant methioninase (rMETase) in the maintenance of gastric cancer stem cells (GCSCs) features and its molecular mechanism. Methods: GCSCs were isolated and enriched. GCSCs marker CD44 was detected by flow cytometry and immunofluorescence assay. The effect of rMETase on the GCSCs self-renewal was detected by the formation of the suspension cell spheres and the soft agar colony formation assay respectively. Cell survival rate was calculated with MMT assay. Cell apoptosis was analyzed by TUNEL method. The effect of silencing rMETase on the cancerigenicity of GCSCs in vivo was observed by subcutaneous transplantation cancer model in nude mice. The expression levels of stem cell associated genes Oct4, Nanog, bmi-1 and ttf-1 and PI3/Akt/mTOR signal pathway were detected by Real-time PCR method. Results: The number of suspended gastric cancer cells was significantly decreased in rMETase treatment group ($P < 0.01$). The results of soft agar colony formation test showed that the number of colons in rMETase treatment group was lower than that of control group (36.8 ± 9.0 vs 48.8 ± 10.5 , $P < 0.05$). The proportion of S phase GCSCs was significantly increased while G2 phase GCSCs was significantly decreased after rMETase treatment ($P < 0.05$). TUNEL results showed that rMETase treatment increased cell apoptosis. The expression levels of Oct4, Nanog, bmi-1 and ttf-1 decreased after rMETase treatment ($P < 0.05$). rMETase treatment also inhibited the expression of Akt, m-TOR and PI3K ($P < 0.05$). Conclusions: rMETase can inhibit the self renewal and proliferation of GCSCs, which maybe through PI3/Akt/mTOR signal pathway.

Keywords: Recombinant methioninase (rMETase), gastric cancer, stem cell, stem cell associated genes

Introduction

Cancer stem cells, or cancer initiating cells, are a group of cancer cells with self-renewal capacity and multiple differentiation potential [1]. Cancer stem cells play a great role in the occurrence, development, metastasis, resistance to chemotherapy and radiotherapy, they are also considered to be the source of cancer recurrence and high metastasis. The main reason for cancer stem cells to maintain their "stem" characters was their strong self renewal ability, which was different from common cancer cells. At present, the regulation mechanism of cancer stem cells self renewal is not clear [2, 3].

There was a significant difference between malignant cancers and normal cells in many metabolic processes, these differential metabolic phenotypes can provide new ideas for the

diagnosis and treatment of cancers. The most common difference of cancer cells on the metabolism of amino acid is methionine (methionine, Met), 80% of the malignant cancers were significantly inhibited in vitro or in vivo in the absence of Met [4]. Cancer had Met dependence and research on its mechanism has been deeply studied. It is generally accepted that cancer cells need much more Met than normal cells due to the increase of protein synthesis and methylation in cancer cells. At the same time, the methionine synthase activity in cancer cells was low, so most cancer cells showed an absolute dependence on exogenous Met [5].

Methioninase is a kind of homologous four polymer isolated from *Pseudomonas putida*. It can decompose methionine into methyl mercaptan, ammonia etc. Recombinant methioni-

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nase (rMETase) can be expressed in *E. coli*, it can split methionine inside or outside cells specifically. rMETase played the anti-cancer role by inhibiting the growth of cancer cells and inducing their apoptosis [6, 7]. Scholars have tried to use methioninase for gene therapy of cancer [8]. There was a synergistic effect between rMETase and chemotherapy drugs, rMETase can also reduce the toxicity of chemotherapy drugs [9].

rMETase induced apoptosis of cancer cells, but had no significant effect on normal tissue cells. Whether Met dependent played a role in the maintenance of the “stem” characters of cancer stem cells remains unclear. So this study aimed to investigate the role of rMETase in the maintenance of GCSCs stem features and its molecular mechanism.

Materials and methods

Isolation and culture of GCSCs

Gastric cancer cell line SGC7901 was purchased from ATCC. They were cultured with 5% CO₂ in RPMI-1640 medium containing 10% fetal bovine serum (FBS) at 37°C. The cells were digested with 0.25% trypsin and the supernatant was discarded after centrifugation. Then cells were cultured in RPMI-1640 medium containing 20 µg/l EGF and 10 µg/l bFGF without FBS to observe the formation of gastric cancer cell suspension ball. The cell spheres were collected when the number of suspended gastric cancer cell ball reached about 50 cells/ball. Single cell suspension of suspended gastric cancer cell was prepared after digestion and washing. Single cell suspension was inoculated into 96 well plate with 100/well. 5 mM rMETase and 0.2 ml RPMI-1640 medium without FBS were added into each well, PBS was added into control. The formation of two generation suspension cell ball was analyzed.

Soft agar colony formation test

The underlying agar (0.6%) was prepared with 1.2% low melting point agarose and cell culture medium with 1:1 volume ratio. They were added into 6 well plates for solidification at room temperature. The upper agar (0.3%) was prepared with 0.6% low melting point agarose and cell culture medium with 1:1 volume ratio. The upper agar (1 ml) and single cell suspension of

suspended gastric cancer cell (100 µl, 10⁴/ml) was added into each well. rMETase was added in experimental group and PBS was added in control group. Cell colony formation rate was calculated.

Detection of cell cycle distribution by flow cytometry

GCSCs were treated with 5 mM rMETase for 48 h and culture medium was removed, single cell suspension was prepared after washing and digestion. The cells was re-suspended and fixed with 70% ethanol. The cells (2×10⁶) was re-suspended in 300 µl 70% ethanol in each group and 150 µl PI was added and incubated avoidance of light at room temperature for 30 min. The cell cycle distribution was detected by flow cytometry.

Detection of cell apoptosis by TUNEL method

The cell apoptosis was detected by TUNEL method according to the kit manual. The data were analyzed with Image-Pro Plus 6.0 software.

Cancer formation in nude mice

A total of 12 Nu/Nu nude mice (4 weeks) were used in this study. Subcutaneous injection of SGC7901 (2×10⁵) in hind limb of nude mice was performed after routine disinfection of inoculated skin. The cancer tissues were separated when the maximum diameter of the subcutaneous cancer in nude mice was about 2 mm. The primary cells were isolated and cultured in 96-well plate. The formation of monoclonal cell ball was detected.

Effects of rMETase on the resistance of GCSCs

GCSCs were treated with oxaliplatin, 5-FU, oxaliplatin+ 5-FU, rMETase, rMETase+ oxaliplatin, rMETase+ 5-FU, rMETase+ oxaliplatin+ 5-FU respectively. The cell proliferation was detected with MTT method. Each group of cells was provided with five repeated holes, each of which was inoculated with 2000 cells. The plates were cultivated in the incubator after inoculation respectively take out a 96-well plate at the time of 0 h, 24 h, 48 h, 72 h. 50 ul 1×MTT (5 mg/ml) was added in each sample hole, and the cells were incubated for an additional 4 h at 37°C. 150 ul DMSO was added in

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Table 1. Real-time PCR primers

Gene	GenBank Accession NO.	Primer (5'-3')	Length	Tm
OCT4	NM_001285987.1	For: GCCCTGGAGTCCTACTTT	18	55.56
		Rev: TCCTTTCTGTCTTCGGTTA	19	42.11
PI3K	NM_006218.3	For: CAGTCAGGAAAGGTGGTG	18	54.84
		Rev: ATTCAAAAGGTTACCGGA	18	51.54
Akt	NM_001014432.1	For: ACCGTTTGTCTTCAGCG	18	52.13
		Rev: TGTTTGGATGGGTGGATT	18	51.04
ttf-1	U43203.1	For: ATCATCGAGTCGCCAAAC	18	55.17
		Rev: CCAAGGTAGTGCCTCTGC	18	58.12
bmi	NM_182916.2	For: CCACCTCTTCTGTTTGC	18	53.56
		Rev: CCAAGGTAGTGCCTCTGC	18	58.12
Nanog	NM_001297698.1	For: TTCTCCACAAGCCCTAG	18	53.37
		Rev: TTCTGCCTCTGAAATCTG	18	51.44
GAPDH	NM_014364	For: TGTGGGCATCAATGGATTGG	21	60.91
		Rev: ACACCATGTATTCCGGGTCAAT	21	61.40

each sample hole after the culture medium was discarded. The optical density (OD) value of each sample hole was measured by Microplate Reader at 490 nm after a 10 minutes shaking. The negative control group only containing medium.

RNA isolation and RT-qPCR

Total RNA was isolated using RNeasy Lipid Tissue mini kit (Takara, Japan) according to the manufacturer's protocol. Quality of RNA was assessed using Nanodrop 2000. Complementary DNA was synthesized using iScript cDNA Synthesis Kit according to the manufacturer's protocol. Quantitative reverse transcriptase polymerase chain reaction (PCR) was performed using SYBR Green. Quantifications of target genes mRNA was performed using the $2^{-\Delta\Delta Ct}$ method. RT-PCR reaction system: 2×SYBR Fast qPCR Mix: 10 µl; PCR Forward/Reverse Primer (10 Mm): 0.8-1.1 µl; 50×ROX Reference Dye II: 0.4 µl; cDNA template: 2 µl. Reaction parameter: Holding Stage 95°C, 30 s; Cycling Stage 95°C, 3 s; 60°C, 13 s. Cycles = 40. Primers were shown in **Table 1**.

Western-blotting detection

The cells were lysed in RIPA lysis buffer, the lysates were harvested by centrifugation and protein samples were isolated and quantified with BCA kit. Approximately 50 µg proteins were separated by electrophoresis in a 10% sodium dodecyl sulfate polyacrylamide gel and were transferred onto PVDF membrane. The mem-

branes were incubated with primary antibodies at 4°C overnight. Then they were washed three times with TBST buffer for 3 times and were probed with the second antibody at room temperature for 1 h. The membranes were developed using an enhanced chemiluminescence system. The levels of proteins were normalized to the level of GAPDH.

Statistical analysis

The data were shown in means \pm SEM. Statistical analysis of the results were performed using the SPSS 15.0 software (SPSS Inc, Chicago, IL, USA) and t test was conducted for comparison between groups. $P < 0.05$ was considered to be significant.

Results

GCSCs were successfully separated

The results of suspended ball cells formation were shown in **Figure 1A**, and the results of soft agar colony formation test were shown in **Figure 1B**. The results of CD44+ cells rate detected by flow cytometry were shown in **Figure 1C** (85.93%). All these results showed that GCSCs were successfully separated and enriched.

rMETase inhibited GCSCs self renewal ability

As shown in **Figure 2A**, the mean number of suspended ball cells of GCSCs in control group was significantly higher than that of rMETase treatment group (27.9 ± 8.4 vs 17.5 ± 7.5 , $P <$

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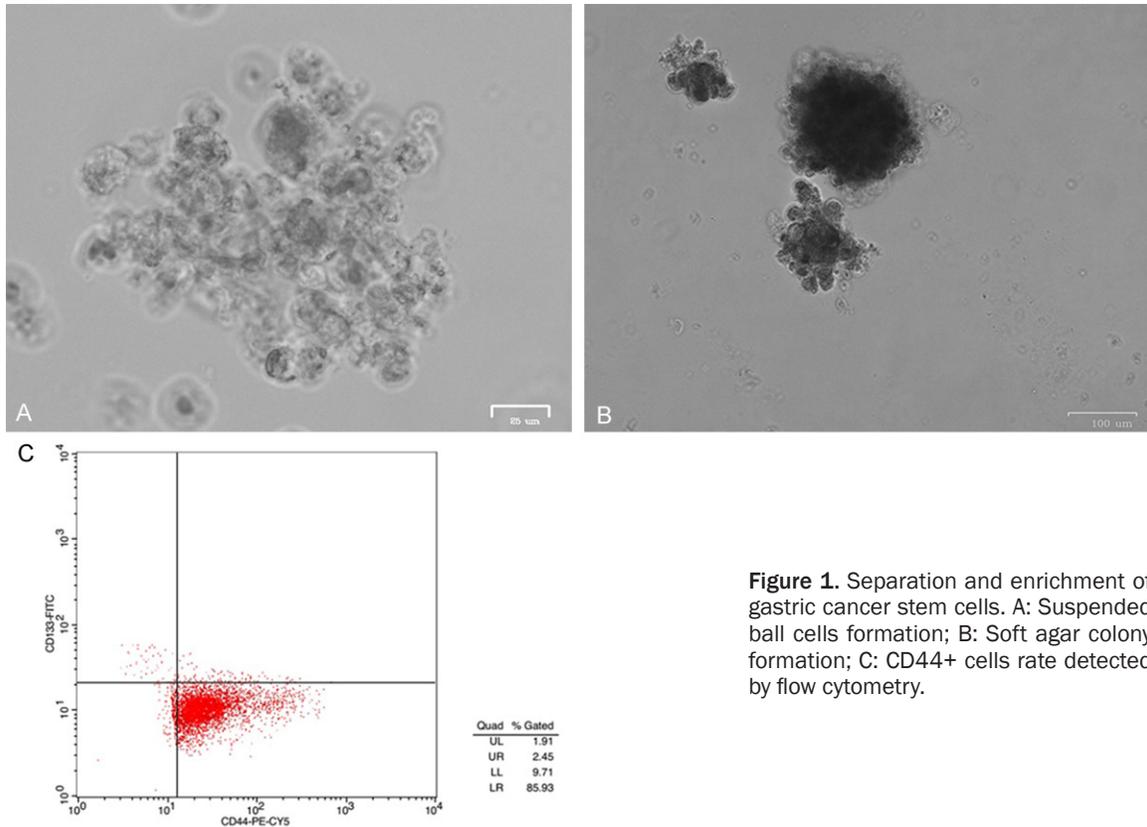


Figure 1. Separation and enrichment of gastric cancer stem cells. A: Suspended ball cells formation; B: Soft agar colony formation; C: CD44+ cells rate detected by flow cytometry.

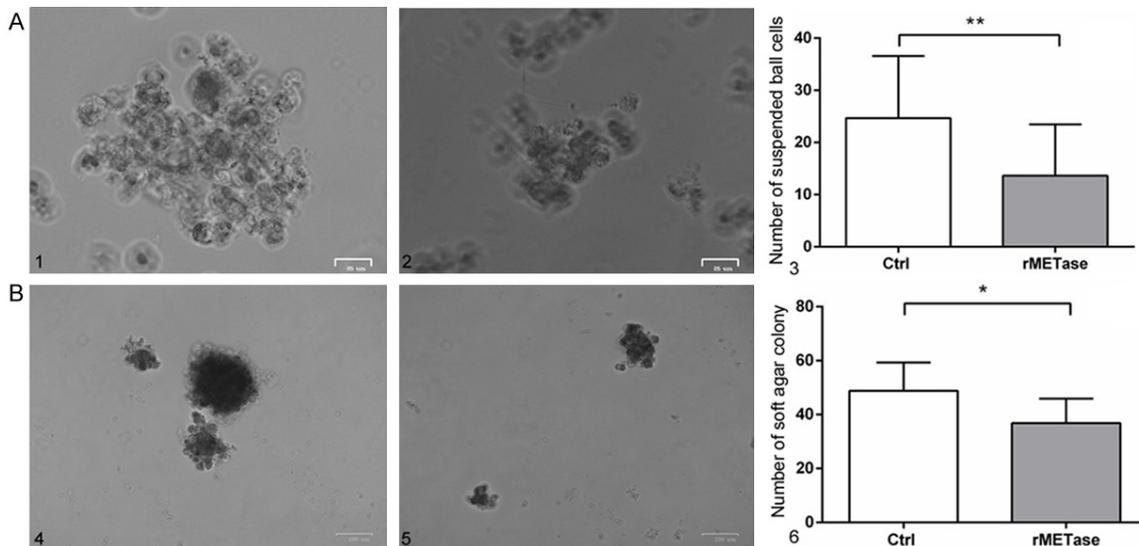


Figure 2. The effects of rMETase on GCSCs self renewal ability. A: Suspended ball cells formation; B: Soft agar colony formation; 1: Suspended ball cells formation in control group; 2: Suspended ball cells formation in rMETase treatment group; 3: Comparison of suspended ball cells formation number; 4: Soft agar colony formation in control group; 5: Soft agar colony formation in rMETase treatment group; 6: Comparison of soft agar colony formation number; vs Ctrl * $P < 0.05$, ** $P < 0.01$.

0.01). The result of soft agar colony formation test was shown in **Figure 2B**. It also showed that the mean number of colony formation of

GCSCs in control group was significantly higher than that of rMETase treatment group (48.8 ± 10.5 vs 36.8 ± 9.0 , $P < 0.05$).

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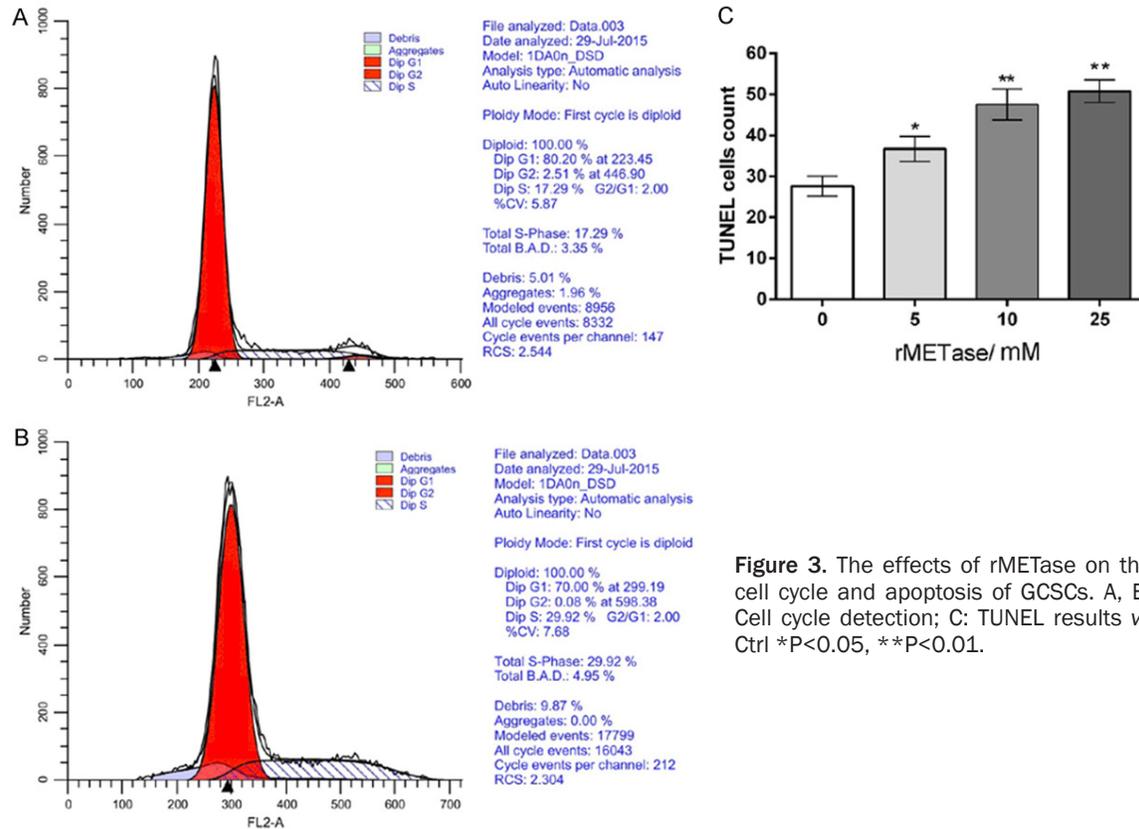


Figure 3. The effects of rMETase on the cell cycle and apoptosis of GCSCs. A, B: Cell cycle detection; C: TUNEL results vs Ctrl *P<0.05, **P<0.01.

rMETase promoted the apoptosis of GCSCs

The results of cell cycle distribution detected by Flow cytometry were shown in **Figure 3A, 3B**. They showed that the proportion of cells in S phase of GCSCs was significantly increased and the proportion of cells in G2 phase was significantly decreased in rMETase treatment group compared with control group (P<0.05). The apoptosis detection results were shown in **Figure 3C**. It showed that rMETase treatment obviously increased the number of apoptotic cells (P<0.05).

rMETase inhibited the proliferation of GCSCs

The MTT results were shown in **Figure 4**. It showed that rMETase treatment significantly inhibited cell proliferation (P<0.05).

rMETase inhibited the capacity of tumorigenicity of GCSCs

As shown in **Figure 5**, in primary cells isolated from tumor tissues, rMETase intervention could significantly inhibit the number of wells of GCSCs ball formation (P<0.05).

rMETase inhibited the expression of stem associated genes and PI3/Akt/mTOR signal pathway

RT-PCR results were shown in **Figure 6**. It showed that the expression levels of Oct4, Nanog, bmi-1 and ttf-1 in GCSCs significantly decreased after GCSCs were treated by rMETase (P<0.05). The rMETase treatment also inhibited the expression levels of Akt, m-TOR and PI3K in PI3/Akt/mTOR signal pathway. The western blotting results were shown in **Figure 7**. It showed the similar results with that of RT-PCR results.

Discussion

There was a significant difference between malignant cancers and normal cells in many metabolic processes, these different metabolic phenotypes can provide new ideas for the diagnosis and treatment of cancers. The most common differential metabolism of amino acids for cancer cells is Met, about 80% of the proliferation for malignant cancer is significantly inhibited in methionine deficiency environment in vitro or in vivo, so the cancer have methionine

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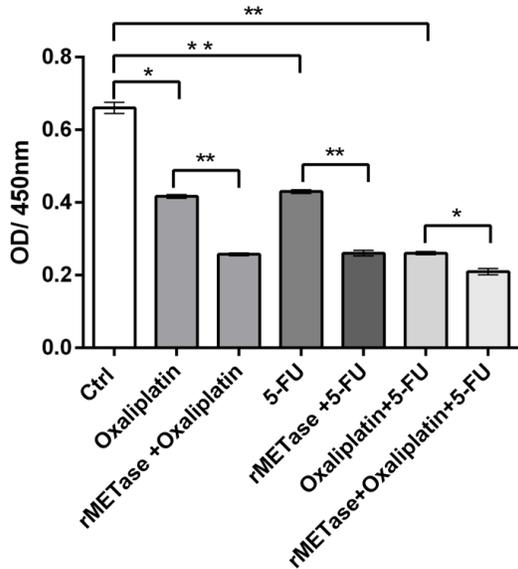


Figure 4. The effects of rMETase and antitumor drugs on the cell proliferation detected by MTT methods. * $P < 0.05$, ** $P < 0.01$.

dependence. Met plays a central role in the regulation of gene expression, the metabolism of all biological macromolecules and the maintenance of cell membrane integrity. The different metabolic phenotype of methionine was first found by Halpern 30 years ago, research on its mechanism has been carried out [10].

Methioninase is a kind of homologous four polymer isolated from *Pseudomonas putida*. It can decompose methionine into methyl mercaptan, ammonia etc and reduce the level of Met [11]. rMETase can split methionine inside or outside the cells specifically to inhibit the growth of cancer cells and induce their apoptosis. Therefore, rMETase is considered to be a promising drug for cancer therapy.

Gastric carcinoma is one of the multiple digestive tract tumors in China with high mortality rate. Surgical treatment is still the main way of treatment of gastric cancer currently. Peritoneal metastasis is the main metastasis and the main cause of death of gastric cancer. In recent years, the hypothesis of cancer stem cells (CSCs) provides a new idea for the prevention and treatment of malignant tumors [12, 13]. In this study, we explored whether it was possible to inhibit GCSCs by rMETase so as to achieve the purpose of inducing apoptosis of gastric cancer cells.

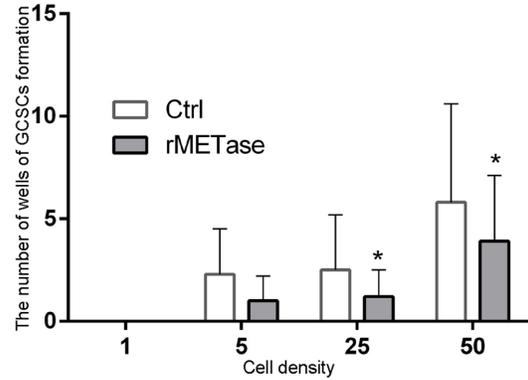


Figure 5. Effects of rMETase on capacity of tumorigenicity of GCSCs. * $P < 0.05$.

GCSCs still lacks the high specificity of stem cell markers and their surface markers are controversial [14, 15]. CD44 is considered to be the CSCs marker of prostate cancer, gastric cancer, colon cancer and other malignant tumors [16]. CD44+ cells obtained in this study in suspension cells ball reached 85.93%. The number of suspended cells was significantly decreased in rMETase treated group than that of control group ($P < 0.01$). Cell colony formation results also showed that the colony formation was significantly decreased in rMETase treated group than that of control group ($P < 0.05$). These results suggested that rMETase may inhibit the self-renewal capability of GCSCs. Flow cytometry results showed that the proportion of cells in S phase of GCSCs was significantly increased and in G2 phase was significantly decreased in rMETase treatment group compared with control group. Previous studies showed that Met metabolic disorders caused prostate cancer cells to block in the G2 phase [17, 18]. Apoptosis results showed that rMETase could significantly increase the number of apoptotic cells with a concentration gradient dependent. These results suggested that the occurrence of apoptosis was caused by abnormal metabolism of Met induced by rMETase, and then hindered the ability of GCSCs self-renewal. MTT results showed that GCSCs proliferation of rMETase treatment group was significantly inhibited and had an additive effect with anti tumor drugs. This could provide a new idea for the clinical application of rMETase.

Stem associated genes are essential for the homeostasis of stem cells, at the same time, they also maintain a strong self renewal ability

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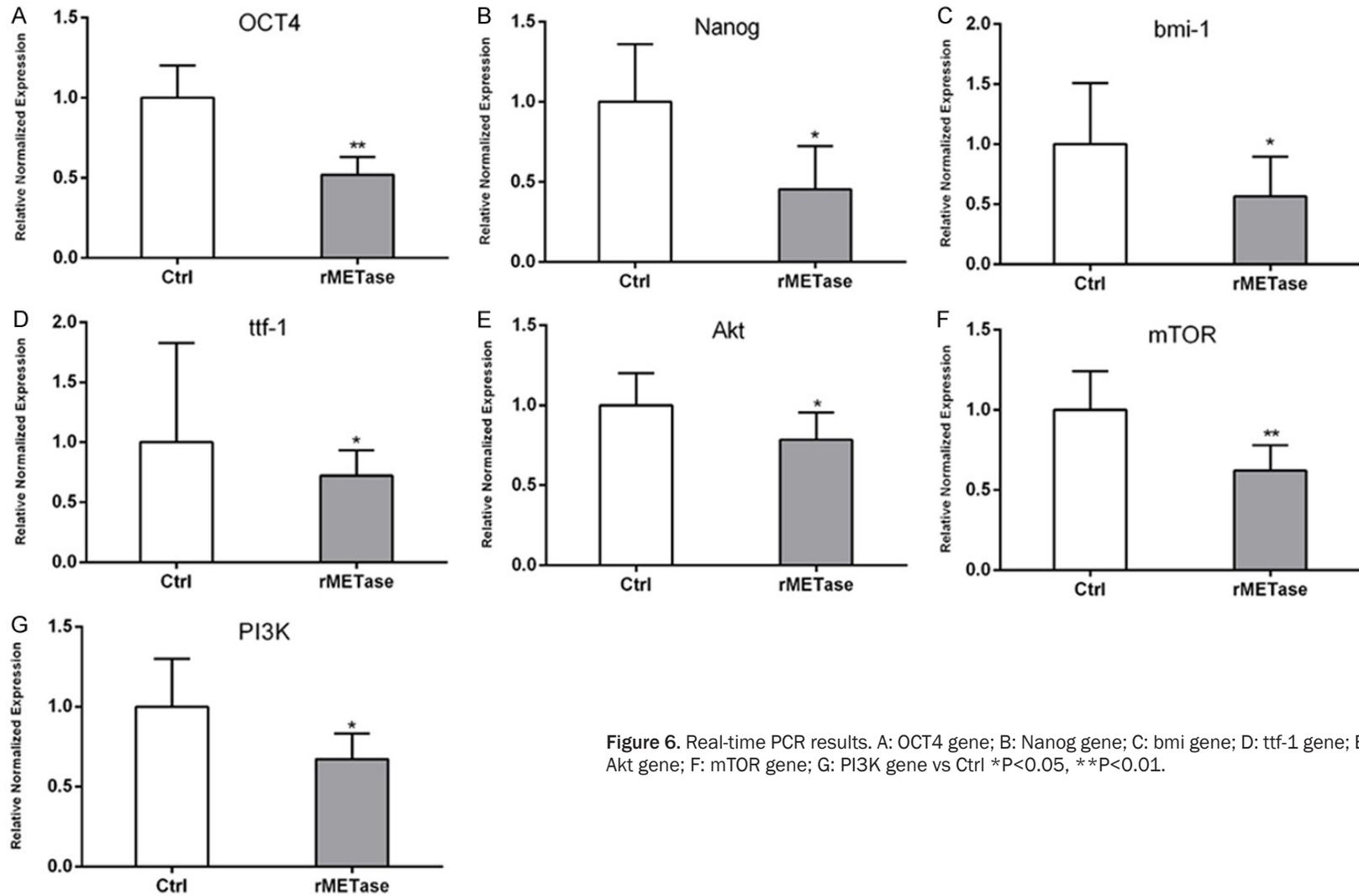


Figure 6. Real-time PCR results. A: OCT4 gene; B: Nanog gene; C: bmi gene; D: ttf-1 gene; E: Akt gene; F: mTOR gene; G: PI3K gene vs Ctrl *P<0.05, **P<0.01.

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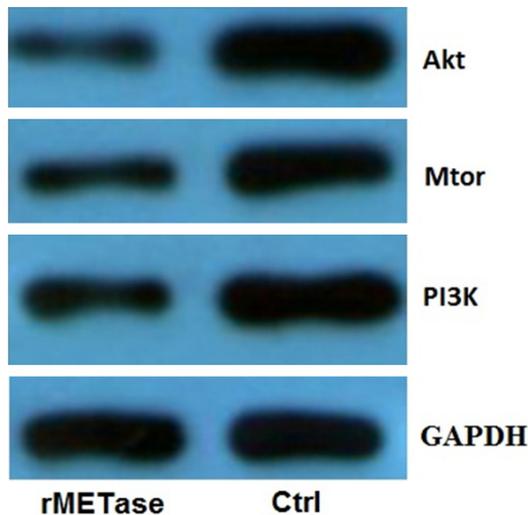


Figure 7. Western blotting results. rMETase treatment also inhibited the protein levels of Akt, M-tor and PI3K.

of stem cells. Transcription factors Oct4, Nanog and their network control system is an important regulation system for the maintenance of pluripotent stem cells, they maintain the stem characters of stem cells by regulating the expression and activity of a large number of differentiation related genes [19]. In this study we found that the expression levels of Oct4, Nanog, bmi-1 and ttf-1 in GCSCs significantly decreased after GCSCs were treated by rMETase (5 mM), which should be the main reason for rMETase to inhibit the self renewal ability of GCSCs.

The protein family of the phosphatidylcholine 3-kinase (PI3Ks) participated in cell proliferation, differentiation, apoptosis and glucose transport, the production of drug resistance and other cellular functions. Increased PI3K activity is associated with a variety of cancers including gastric cancer [19]. We found that the levels of m-TOR and Akt significantly decreased after GCSCs were treated by rMETase, so cell proliferation was inhibited and cell apoptosis was promoted by the negative regulation of PI3K/Akt signal pathway.

Conclusions

In a word, we believed that rMETase can inhibit the self-renewal ability of GCSCs, its molecular mechanism involved multiple stem associated genes and PI3/Akt/mTOR signal pathway.

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Disclosure of conflict of interest

None.

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