약학석사학위논문

A novel function of methionyl-tRNA synthetase in cyclin-dependent kinase 4 mediated cell cycle progression

Cyclin-dependent kinase 4(CDK4)를
매개로 하는 세포주기 진행에 있어서
methionyl-tRNA synthetase(MRS)의 새로운 기능 연구

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ABSTRACT

Methionyl-tRNA synthetase (MRS) is one of aminoacyl-tRNA synthetases (ARSs) which charge amino acids to cognate transfer RNAs (tRNAs) in translation. High expression of MRS in various cancers and its nuclear localization during cell proliferation has been reported. With this background information about MRS related to cancer, the present study was attempted to clarify the role of MRS on cell proliferation and cell cycle progression and further to reveal the signal pathway where MRS is involved.

A novel role of MRS for triggering cell cycle progression via the stabilization of cyclin-dependent kinase 4 (CDK4), one of cell cycle regulating factors, was shown. The protein level of CDK4 significantly decreased by MRS knock-down or activity inhibition. The protein levels of other cell cycle regulators such as CDK1, CDK2 and CDK6, however, were not affected. The deprivation of methionine or the treatment of methionine analogue reduced the binding of MRS to CDK4 and impeded the cell cycle progression without translation inhibition. It implied that the cell cycle regulating function of MRS was mediated by the interaction between MRS and CDK4 and the catalytic domain of MRS was in charge of the association. This study explains the mechanism of the increased

stability of CDK4 in cancer and suggests a novel function of MRS required for the cancer cell proliferation.

Key words: MRS (methionyl-tRNA synthetase), CDK4 (cyclin-dependent kinase 4), protein stabilization, enzyme activity, cell cycle progression

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CONTENTS

LIST OF FIGURES

INTRODUCTION

Aminoacyl-tRNA synthetases (ARSs) are key enzymes for charging transfer RNAs (tRNAs) with their cognate amino acids during the translation. Upon growth, stress and apoptotic stimuli, ARSs mediate various signaling pathways and perform critical roles in human diseases, especially in cancer (Lee et al., 2004).

It is notable that methionyl-tRNA synthetase (MRS) is an essential protein which is crucial for translation initiation. Beside of its well-known catalytic function, MRS is translocated into nucleus in response to growth signal like epithelial growth factor (EGF) or insulin and plays a role in the biogenesis of ribosomal RNA (Ko et al., 2000). It is known that ribosomal RNA synthesis is important for cell proliferation (Ruggero and Pandolfi, 2003), and MRS is highly expressed in a variety of cancers, therefore, the involvement of MRS in the cell proliferation in cancer was investigated .

Cell proliferation rate is regulated by proteins involved in the control of cell cycle, which is driven by cyclins and their associated kinases. The sequential activation of several cyclin-dependent kinase (CDK) / cyclin complexes establish the progression of the cell cycle through G1, S, G2 and M phases (Berthet and Kaldis, 2007). CDK4 is the essential kinase for cell cycle progression (Malumbres

and Barbacid, 2009). Once CDK4 is stabilized, it forms a complex with D type cyclins, especially cyclinD1, and phosphorylates Rb (Retinoblastoma) protein. Phosphorylation of Rb protein causes the bound E2F transcription factor to be released from Rb, promoting transcription of cell cycle transition and cell proliferation (Zhang et al., 2009). Therefore CDK4 is essential for the cell cycle transition and cell proliferation. It is also known that CDK4 is critical for the Ras-activated oncogenic transformation of mouse embryonic fibroblast (MEF), Erbb2 or HRas-driven mammary tumorigenesis and carcinogen- and Myc-induced skin cancers (Nevins, 2001). Also, homozygous *CDK4* null mutant mice are viable and are found to be very resistant to carcinogen-induced cancers (Reddy et al., 2005).

In this study, a novel function of MRS under growth condition was investigated and the relationship between MRS and CDK4 was found. MRS increased the stability of CDK4 in a protein level resulting in the activation of CDK4-cyclinD1-pRb axis with enhanced cell cycle progression.

MATERIALS AND METHODS

Materials

The antibodies against CDK1 (cell signaling, #9112P), CDK2 (cell signaling, 78B2), CDK4 (c-22: Santa Cruz biotechnology, sc-260, H-303: Santa Cruz biotechnology, sc-740, DCS-35: Santa Cruz biotechnology, sc-23896), CDK6 (B-10: Santa Cruz biotechnology, sc-7961), cyclinD1 (Millipore, 04-221), cyclinE (HE12: Millipore, 05-363), cyclinA (H-432: Santa Cruz Biotechnology, sc-751), cyclinB (H-433: Santa Cruz biotechnology, sc-752), Phospho-Rb (Ser780, C84F6, cell signaling, #3590), MRS (Abcam, ab50793), Myc (9E10: Santa Cruz biotechnology, sc-40), HA-probe (F-7: Santa Cruz biotechnology, sc-7392) and Flag (Sigma Aldrich, F3165) were used in this study. Secondary antibodies against mouse (Thermo Scientific, Cat. 31430) and rabbit (Thermo Scientific, Cat. 31460) IgG were used.

Cell culture

H460, A549, HCT116, MDA-MB-231 cells were cultured in RPMI medium

(with 25 mM HEPES and L-glutamine, Hyclone, USA) with 10% defined fetal bovine serum (defined FBS, Hyclone, USA) and 100 μg/mL of penicillin and streptomycin at 37°C in 5% CO_2 incubator.

293T cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (with 2.5 g Porcine trypsin, 4.0 mM L-glutamate, 400 mg/L glutamine and sodium pyruvate, Hyclone, USA) with 10% fetal bovine serum (FBS, Hyclone, USA) and 100 μg/mL of penicillin and streptomycin at 37°C in 5% CO_2 incubator.

MDA-MB-231 cells were also cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (with 2.5 g porcine trypsin, 4.0 mM L-glutamate, 400 mg/L glutamine and sodium pyruvate, Hyclone, USA) with 10% defined fetal bovine serum (defined FBS, Hyclone, USA) and 100 μg/mL of penicillin and streptomycin at 37°C in 5% CO_2 incubator.

RNA interference

SiRNA was transfected with Lipofectamine 2000 into the cells. All processes were performed according to the instruction of manufacturer. Each siRNA and Lipofectamine 2000 was diluted in serum free medium and incubated for 10 min at room temperature separately. Then, each solution was mixed by pipetting and incubated for 20 min. After 72 h of transfection, cells were harvested or assayed according to the experimental protocols. Stealth RNAi with medium GC (Invitrogen, 12935-300) was used as a negative control. SiRNA targeting MRS was purchased from Invitrogen and the sequences are 5'-CUACCGCUGGUUUAACAUUUCGUUU-3'.

Western blot

All cells were lysed by lysis buffer consisted of 50 mM Tris-HCl pH 7.5, 0.5% Triton X-100, 5 mM EDTA, 10% glycerol, 150 mM NaCl, and protease inhibitor for 30 min at 4°C. After then, lysates were collected in 1.5 mL EP tube and centrifuged at 13,200 rpm for 15 min at 4°C. The supernatant proteins were quantified by Bradford assay (BioRad, Cat.500-0006). Final sample was made by adding 5X laemmli sample buffer and lysis buffer. Samples were boiled for 7 min before gel running. After that, samples were loaded on SDS page gels and separated by electrophoresis. Proteins at the gel were transferred to poly vinyl dene fluoride (PVDF) membrane (Millipore, IPVH 00010) and run 13 mA per gel for 15 min. The membrane was incubated with 5% skim milk based 0.5% TBS-T for 1 h for blocking non-specific bindings. After removing skim milk solution, primary antibody was added to the membrane during overnight at 4°C. The membrane was washed with 0.5% TBS-T buffer for 5 min, 3 times at 4°C. Then, secondary antibodies were added and incubated for 1 h. The membrane was washed again with 0.5% TBS-T buffer for 5 min, 3 times at 4°C. ECL solution (EZ-Western Lumi Plus, DG-WPA200) was applied to detect target proteins. And for sensitive detection, ECL solution from Santa Cruz Biotechnology (ImmunoCruz, sc-2048) was applied.

Quantitative real time PCR (qRT-PCR)

Transcriptional levels of CDK4 and MRS (target molecules) and Papola

(internal control) were measured by qRT-PCR. RNA was extracted from A549 cells which were treated with si-control or si-MRS using miRNeasy mini kit (Qiagen, 217004) as the instruction of manufacturer. RNA (2 μg) was used for the synthesis of cDNA using M-MLV Reverse Transcriptase (Invitrogen), Random hexamer (Qiagen), dNTP (Takara), DTT (Invitrogen) and reaction buffer. The mixture was cycled (65°C for 5 min, 37°C for 2 min, 25°C for 10 min, 37°C for 50 min and 70°C for 15 min) and subjected to qRT-PCR with specific primers.

Sense primer: 5'-GAGGATGGGAAATTCGATAAGAGCCGCGGTGTG-3' and antisense primer: 5'-TTGGTTGCCATGTCGTCTTGGTGGGTACT-3' were used for the detection of MRS transcript, and sense primer: 5'-CCGGAATTCATGG CTACCTCTCGATATGAGCCA-3' and antisense primer: 5'-CCCCTCGAGTCAC TCCGGATTACCTTCATCCTT-3' were used for CDK4 mRNA detection. Then, qRT-PCR was performed with POWER SYBR GREEN master mix (Ab, 4367659) according to the protocol of manufacturer.

***In vitro* pull down assay**

GST-MRS fusion proteins were purified as followings. GST-MRS and GST-EV plasmids were transfected with BL21 competent cells and incubated in LB medium for around 6 h (until OD value became approximately 0.6). Isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was added to cells and incubated for 4 h at 18°C. Cells were harvested by centrifuging and then lysed with PBS based 0.5% triton-X 100 buffer by sonication of 50% Amp for 10 sec, 5 times. After centrifuging with 13000 rpm at 4°C, glutathione Sepharose 4B (GE healthcare Sepharose 4B) were added to supernatant and left for 4 h. To remove non-specific proteins, beads were washed for

about 5 min, 3 times. Then GST-pull down assay was performed in two ways. Buffer was changed for GST- pull down (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 0.05% NP-20). One of them which were incubated with GST-MRS or GST-EV proteins and 293T cell lysate (HA-CDK1, CDK2, CDK4 and CDK6 plasmids were transfected to 293T cell with Turbofect, then cells were lysed after 24 h for western blot assay) and the other was incubated with TNT lysate (TNT lysates were mixed with Flag-CDK4, Flag-MRS and [^{35}S]-methionine for around 90 min at 30°C) with same proteins. Then, the buffer was changed for GST- pull down (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 0.05% NP-20).

After incubated for 18 h, beads were washed for 5 min, 4 times. Then 1x sample buffer was added and boiled for 7 min to perform electrophoresis. After transfer to membrane, CDK4 was detected with HA antibody as western blot assay and quantified GST-EV or GST-MRS proteins by staining membrane with ponceaus. Gels were stained with Instant Blue to detect GST-EV and GST-MRS proteins and dried at 65°C for about 90 min. Then dried gel was exposed to be detected by autoradiography.

DNA transfection

Turbofect was used to transfect plasmids with 293T cell line as the instruction of manufacturer. DNA and reagent were diluted to 1:3 ratio with serum free DMEM medium and mixed to make a homogeneous mixture. After incubating for 15 min, the mixture was added to cells with fresh medium. And Lipofectamine 2000 was used to transfect plasmids with A549 cells as the manufacturer's instruction. 2 μg of DNA was diluted with serum free RPMI medium and mixed to make homogeneous mixture. Then 5 μl of Lipofectamine 2000 was diluted as the same way. Each mixture was incubated for 5

min. Then, each mixture was collected in one EP tube and incubated for 15 min. After incubating for 15 min, the last mixture was added to cells filled with fresh serum free RPMI medium and incubating for 4 h. After that, the serum free medium was exchanged with RPMI complete medium and cells were harvested after 24 h.

Immunoprecipitation

To observe the binding of MRS and CDK4 in FSMO treatment, immunoprecipitation (IP) assay was performed. IP buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 0.5% NP40, 50 mM NaF, 5 mM EDTA, 1 mM Na_3VO_4 and protease inhibitor) was added to plate to lyse cells and the cells were scrapped to collect lysate. Then the lysate was sonicated with a sonicator (1 sec pulse on, 3 sec pulse off, 25% Amp, 5 sec) for 3 times. Then cells were centrifuged for at 13200 rpm, for 15 min, and the protein extracts were incubated with beads and antibody for 4 h at 4°C (antibodies and beads were incubated for 4 h before cell lysis and types and amounts of antibodies were used as each pictures mentioned). After incubation, beads were washed for 5 min, 3 times with IP buffer, and 1x SDS sample buffer was added and boiled to elute proteins. Samples were separated by SDS-PAGE gel to detect each protein.

Stable cell line preparation

To prepare stably knocked-down MRS, MRS specific shRNA was synthesized and plasmids were transfected using Fugene reagent. After one day incubation of cells, cells were passed to 6 well plate to dilute confluency. From the next day, cells were

treated with G418 at the concentration of 800 μg/mL to select cells since plasmids had resistance marker to G418. Around one month of selection, some colonies could be picked up. Each colony was confirmed by western blot using MRS antibody.

BrdU incorporation

A BrdU cell proliferation assay kit (Cell Signaling) was used. MDA-MB-231 and H460 cells were seeded in 96 well plate at a density of 4000 cells per well. After adhesion, we cultured cells in methionine free DMEM media (10% dialyzed FBS added) for 9 h. After adhesion, cells were treated with Fmoc-Sec(Mob)-OH, methionine analogue #6 at 50 μM for 8 h in 2% serum media. Another set of 96 well plate was prepared and we cultured cells in methionine free DMEM media (10% dialyzed FBS was added) for 9 h. And 100 μl of 2% serum medium of BrdU solution was added to each well and incubated for 2 h. The medium was removed and 100 μl/well of the fixing/denaturing solution was added and incubated at room temperature for 30 min. Then, the solution was removed and 100 μl/well prepared detection antibody solution was added and incubated for 1 h at room temperature. After that, the plates were washed 3 times with wash buffer followed by the addition of 100 μl/well of prepared Horse Reddish Peroxidase (HRP)-conjugated secondary antibody solution and incubated for 30 min at room temperature. Then, the plates were washed 3 times with wash buffer and 100 μl of tetramethylbenzidine (TMB) substrate was added and incubated for 30 sec at room temperature. The amount of BrdU incorporated into the cells was determined at 450 nm by ELISA reader.

Methionine incorporation

MDA-MB-231 and H460 cells were seeded in 12 well plates and incubated for 1 day. Then, Fmoc-Sec(Mob)-OH, methionine analogue at 25, 50, 100 μM were treated for 8 h. Then, cells were incubated in Met-free media containing [^{35}S]Met for 2 h and harvested. The cells were lysed with lysis buffer and the amount of radioactive protein was measured by liquid scintillation.

RESULTS

MRS Knock-down inhibits cell proliferation, but not global translation

MRS could have a role of cell proliferation and association with some kinds of cancer was demonstrated (Ko et al., 2000). On the basis of the demonstration, the elucidation of the relationship between MRS and cell proliferation phenotypes was attempted, since up-regulation of proliferation is the one of the hallmarks of cancer (Hanahan and Weinberg, 2011). To suppress MRS function in cancer cells, MRS knock-down stable MDA-MB-231 cell line originated from breast cancer was adopted and BrdU cell proliferation assay which represents the amount of DNA synthesis was performed. The result showed that DNA synthesis was reduced in MRS-suppressed stable MDA-MB-231 cells compared to the control (Figure 1A). In addition to DNA synthesis, changes in cell cycle phase transition (G0, G1, S, G2, M phase) were also investigated by using FACS (Fluorescence-activated cell sorting). It was observed that G1 was arrested in the MDA-MB-231 cells whose MRS level was stably reduced (Figure 1A). It suggests that MRS is critically involved in the regulation of cell cycle control and cell proliferation

MRS is an enzyme which is important for the global translation (Green et al., 2009). Thus, the reduced cell proliferation caused by MRS-knockdown might be the consequence of the decrease in global protein synthesis. To clarify the question raised on this matter, MDA-MB-231 cell line from breast cancer and H460 cell line from lung cancer were adopted. The global translation in MDA-MB-231 and H460 cells treated with siRNA (targeting MRS) was monitored using methionine incorporation assay. The

amount of total translation seemed little affected (Figure 1D). Thus, it was concluded that the inhibition of MRS function might be caused by the suppression on cell cycle transition (G1-S) and not because of the diminishment of global protein synthesis. As the next step, it was investigated what happened if the cells were treated with siRNA (targeting MRS), and which molecules are related to cell cycle transition. Fortunately, it was found that among various molecules associated with cell- cycle (CDK1, CDK2, CDK4, CDK6, cyclin D, cyclin E, cyclin A, cyclin B), CDK4 was specifically down-regulated (Figure 1B) and CDK4 related regulators (cyclin D, phosphor-Rb) were reduced (Figure 1C).

MRS increases CDK4 stability at protein level

To confirm that MRS knock-down down-regulated CDK4 in MDA-MB-231cells from breast cancer, H460 and A549 cells from lung cancer, HCT116 cells from colorectal cancer and BT20 cells of another breast cancer cell line were also used. It was shown that MRS knock-down diminished CDK4 level in all above cell lines used (Figure 2A). In order to rule out the possibility of transcriptional control by MRS knock-down, quantitative-RT PCR was conducted to check whether the CDK4 mRNA level was also reduced or not (Figure 2B). Therefore, the possibility of transcriptional control by MRS was ruled out. As the next step, to get a hint whether CDK4 protein stability is affected or not by the MRS knock-down, cycloheximide (CHX) was adopted for the study. CHX is the chemical that blocks *de novo* protein synthesis. And two similar sets of experiment were designed. One was MRS knock-down condition and the other was MRS overexpressed condition. In MRS knock-down condition, CDK4 was specifically degraded in a time dependent manner during the CHX treatment (Figure 2C). On the other hand, in MRS overexpressed condition, CDK4 was stabler than the control (Figure

2D). Although, it was reported two major kinases, CDK2 and CDK4 were activated during G1 phase (Wang et al., 2002), it was found that MRS specifically regulated CDK4, but other cell cycle related kinases such as CDK1, CDK2 and CDK6 were little affected. From all the results obtained, it was concluded that MRS controlled the CDK4 level by the stabilization.

MRS inhibition reduces CDK4 mediated cell cycle progression

Since it was found that MRS knock-down or overexpression had effect on CDK4 level by regulating its stability, relationship between MRS and CDK4 was further investigated. It is broadly known that MRS is enzyme which has a role in protein synthesis (van Meel et al., 2013). Enzyme needs its substrate, and if the amount of substrate is insufficient, enzyme activity is reduced (Lazic et al., 2007). Thus, methionine was restricted in order to suppress MRS enzyme activity and BrdU cell proliferation assay and FACS analysis were followed. The methionine deprivation affected DNA synthesis and cell cycle transition from G1 to S (Figure 3A). When methionine was shortened, CDK4 protein level decreased as expected (Figure 3C). Among our methionine analogue library, Fmoc-Sec(Mob)-OH (FSMO), which effectively reduced translation, was chosen to reduce the MRS enzymatic activity. It was reconfirmed that the inhibition of MRS enzymatic activity by treating FSMO reduced DNA synthesis and induced G1 arrest in a same manner (Figure 3B). In the same context, if FSMO was treated in cell, CDK4 protein level also decreased (Figure 3D). To solve the problem with MRS activity and CDK4 stabilization effect, two types of experiments were performed (Figure 3E, 3F). First, the level of CDK4 was monitored from H460 cells treated with several doses of FSMO. Second, the protein synthesis was monitored via in vitro translation with treatment of FSMO. The results showed that FSMO decreased the level

of CDK4 in a dose dependent manner staring from 25 μM, while global translation was little affected at this concentration. That means that catalytic activity related domain (s) of MRS may be involved in the regulation of CDK4 stability and this function can be separated from the catalytic enzyme activity. In conclusion, in addition to the control of MRS level by using siRNA, inhibition of MRS enzymatic activity reduced the cell cycle proliferation via affecting CDK4 stability.

MRS directly interacts with CDK4

Since MRS-mediated CDK4 regulation was confirmed by two types of diminishment (MRS knock-down and MRS activity reduction), it was thought that the MRS-CDK4 interaction could be important part for this regulation. First of all, endogenous interaction between MRS and CDK4 was investigated (Figure 4A). Human MRS domains were already elucidated and it was reported that these domains were divided into 3 parts (DM1: GST-like domain, DM2: catalytic domain, DM3: transfer RNA binding domain) (Kwon et al., 2011). On the basis of this information, it is important to know which part (s) of domains is (are) necessary for binding to CDK4. The result of immunoprecipitation showed that domain 2 and 3 is important for binding to CDK4 (Figure 4B). Therefore, it is deduced that MRS domain 1 is obstacle for interaction with CDK4. Then, GST-pull down assay with FSMO was conducted *in vitro*. GST-MRS and the lysate from H460 cells transfected with flag-CDK4 plasmid were used in the assay. It was found that CDK4 bound to GST-MRS was reduced in a FSMO dose dependent manner. At the concentration of 50 μM of FSMO, the inhibitory effect was enormous (Figure 4C). Furthermore, the binding pattern in cell was investigated by performing immunoprecipitation assay. Surely, MRS-CDK4 interaction was blocked by the treatment of FSMO at the concentration of 50 μM as expected (Figure 4D).

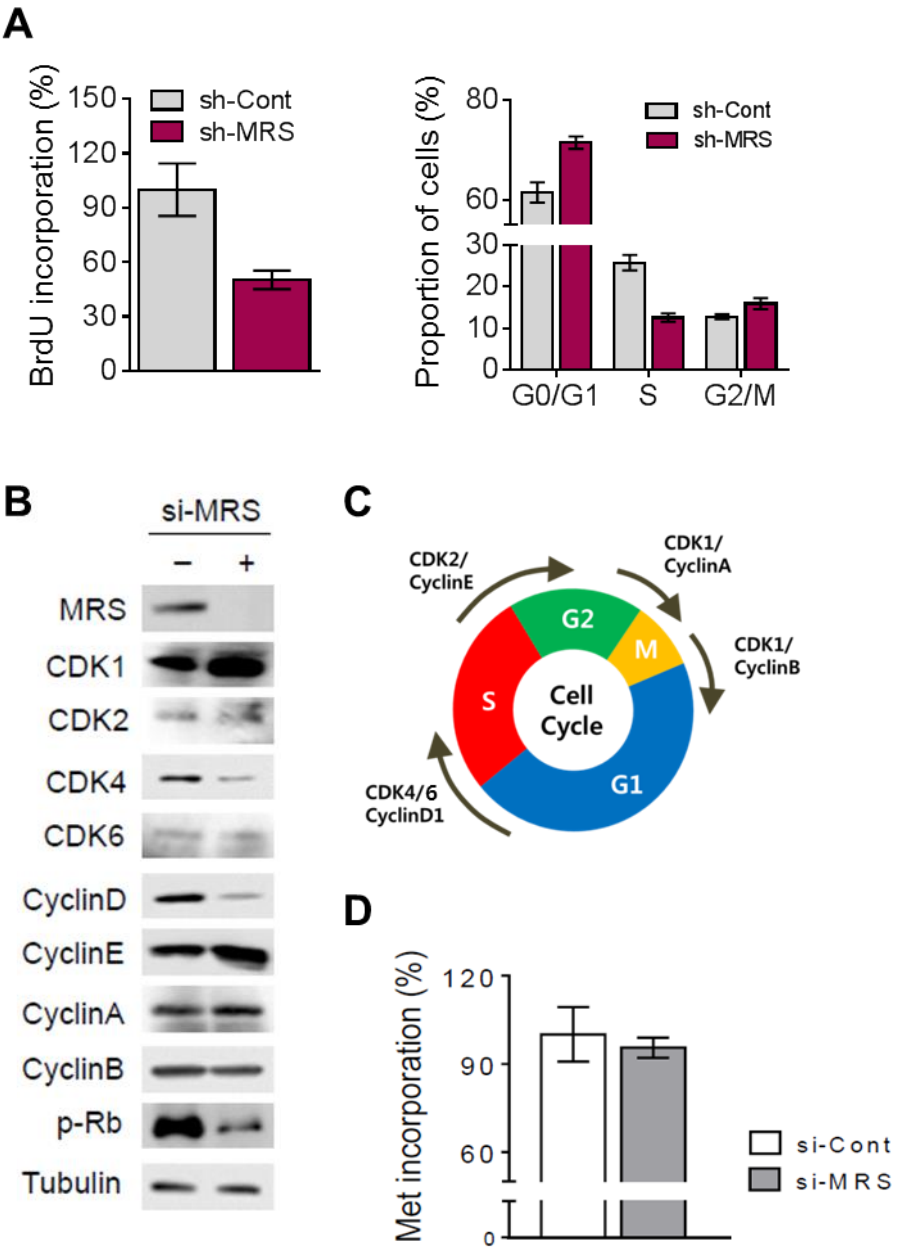
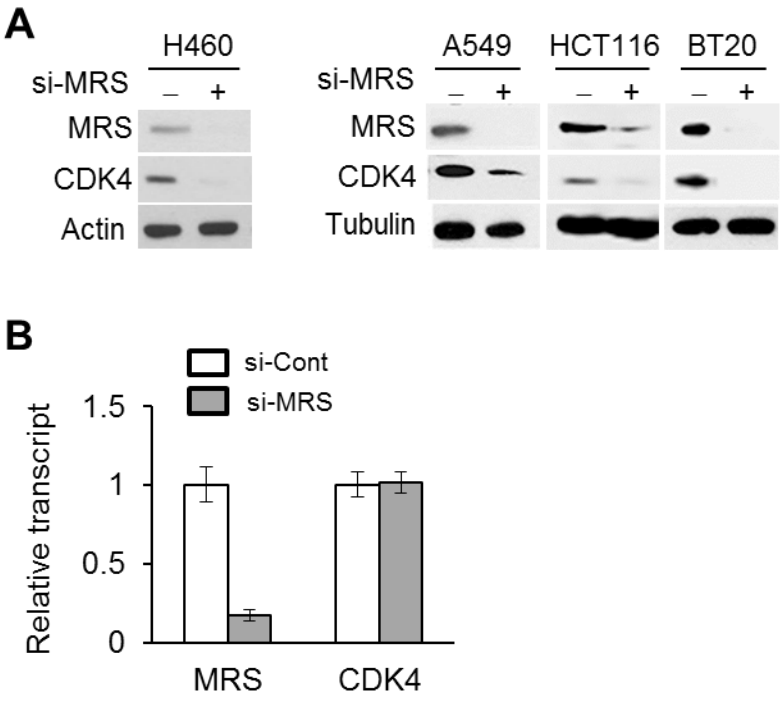

A
BrdU incorporation (%)
150
120
90
60
30
0
sh-Cont
sh-MRS
Proportion of cells (%)
80
60
30
20
10
0
sh-Cont
sh-MRS
G0/G1
S
G2/M
B
si-MRS
–
+
MRS
CDK1
CDK2
CDK4
CDK6
CyclinD
CyclinE
CyclinA
CyclinB
p-Rb
Tubulin
C
CDK2/
CyclinE
CDK1/
CyclinA
CDK1/
CyclinB
CDK4/6
CyclinD1
G2
M
S
G1
Cell
Cycle
D
Met incorporation (%)
120
90
60
0
si-Cont
si-MRS

Figure 1. MRS Knock-down modulates cell proliferation.

(A) For monitoring the significance of MRS on cell proliferation and DNA synthesis, BrdU cell proliferation assay and cell cycle analysis by propidium iodide (PI) staining followed by flow cytometry analysis were performed using sh-cont (control) and sh-MRS stable MDA-MB-231 cells in triplicates. (B) MDA-MB-231 cells were transfected with siRNA (targeting MRS) and harvested after 72 h. Each of proteins on the blot was detected by specific antibody. (C) Simple diagram which presents cell cycle relating molecules (G1, S, G2, and M) including CDK4. (D) Global *de novo* translation was checked by using [^{35}S]Met incorporation assay in the stable MDA-MB-231 cells expressing sh-cont (control) and sh-MRS.



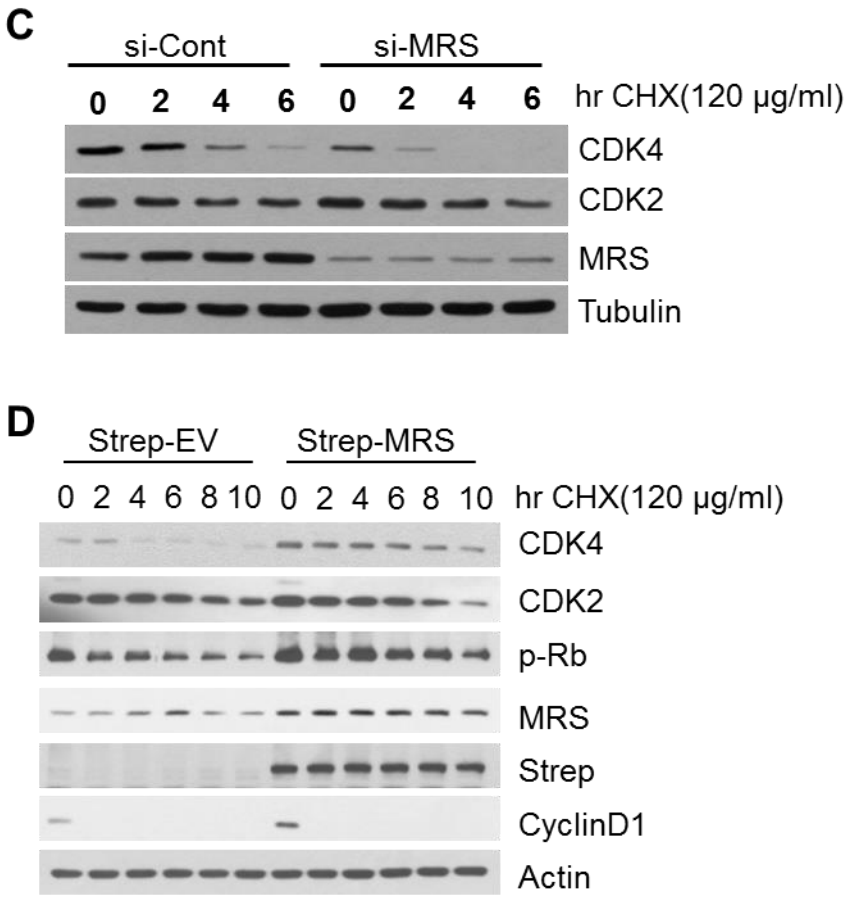
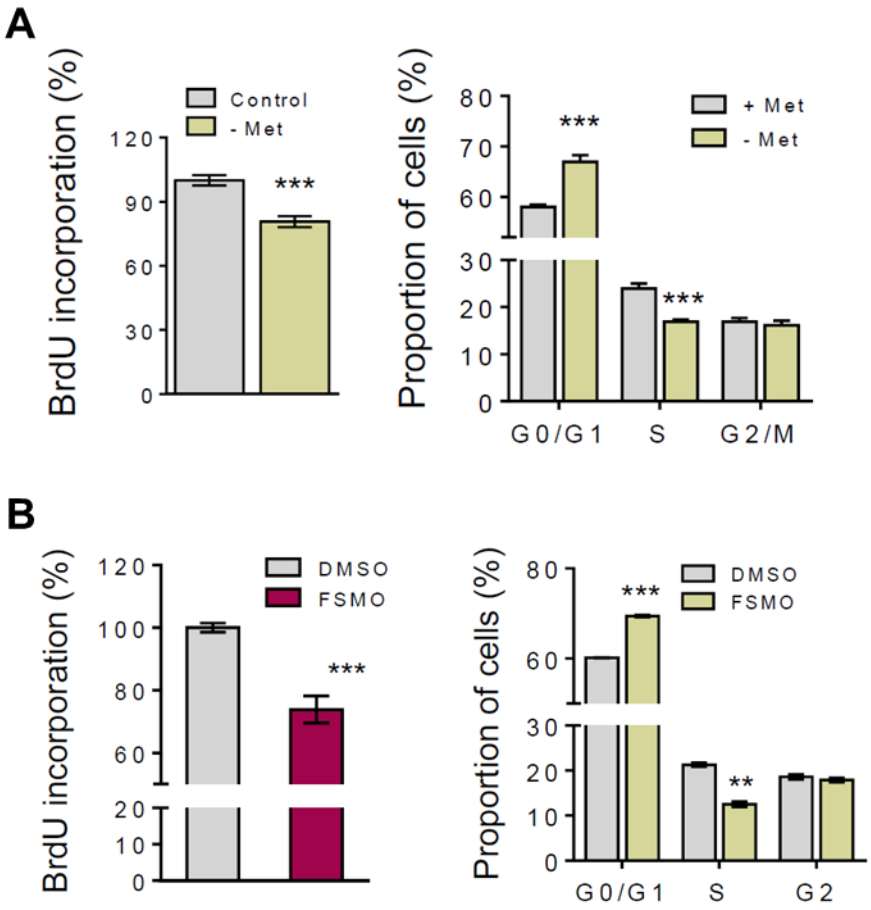


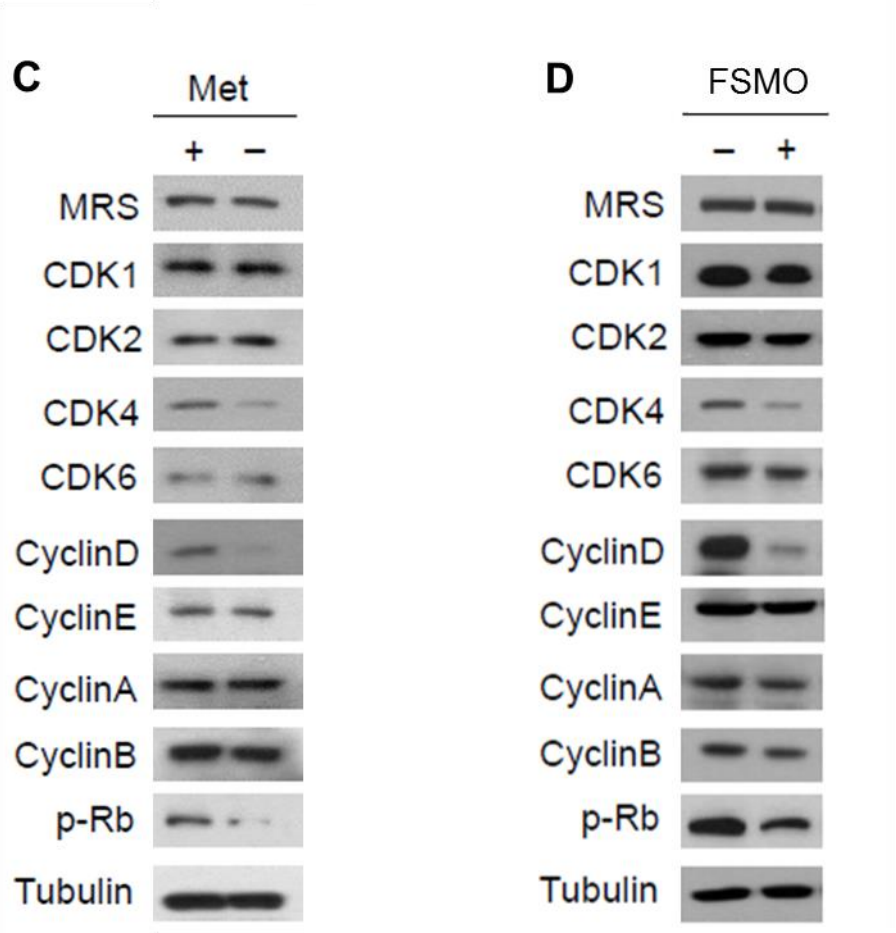
Figure 2. MRS specifically regulates CDK4 at protein level.

(A) To confirm the si-MRS effect on CDK4 in Figure 1C, A549 and H460 lung cancer cell lines and HCT116 colorectal cancer cells and BT20 breast cancer cells were transfected with siRNA (targeting MRS) and harvested after 72 h. (B) Total RNA was extracted from MDA-MB-231 cells transfected with si-MRS and qRT-PCR was performed to analyze the transcripts of MRS and CDK4.

(C) A549 cells were transfected with si-MRS for 72 h and cycloheximide (CHX) was treated to suppress *de novo* protein synthesis during indicated time and before harvest. The stability of CDK4 and CDK2 was monitored.

(D) MDA-MB-231 cells were transfected with onestrep-tagged MRS (strep-MRS) or empty vector (strep-EV) and treated with cycloheximide (CHX) to block *de novo* protein synthesis. The effect of MRS overexpression on CDK4 downstream molecules and CDK2 were investigated.




C
Met
+
−
MRS
CDK1
CDK2
CDK4
CDK6
CyclinD
CyclinE
CyclinA
CyclinB
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Tubulin
D
FSMO
−
+
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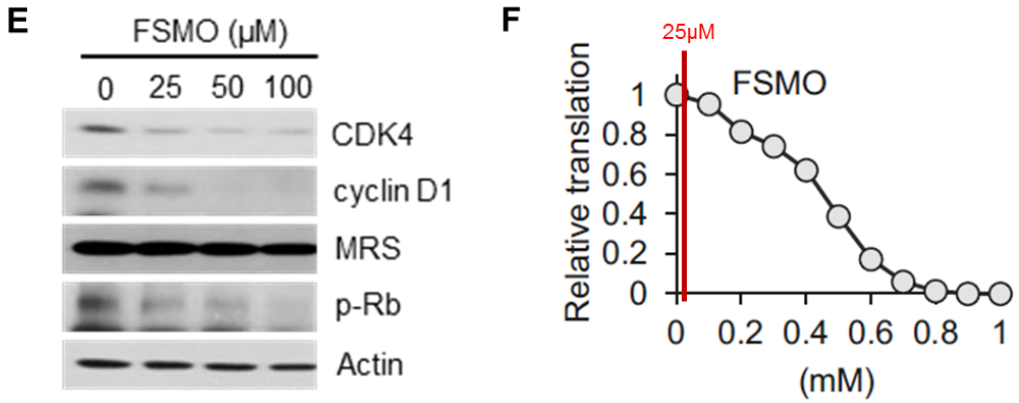
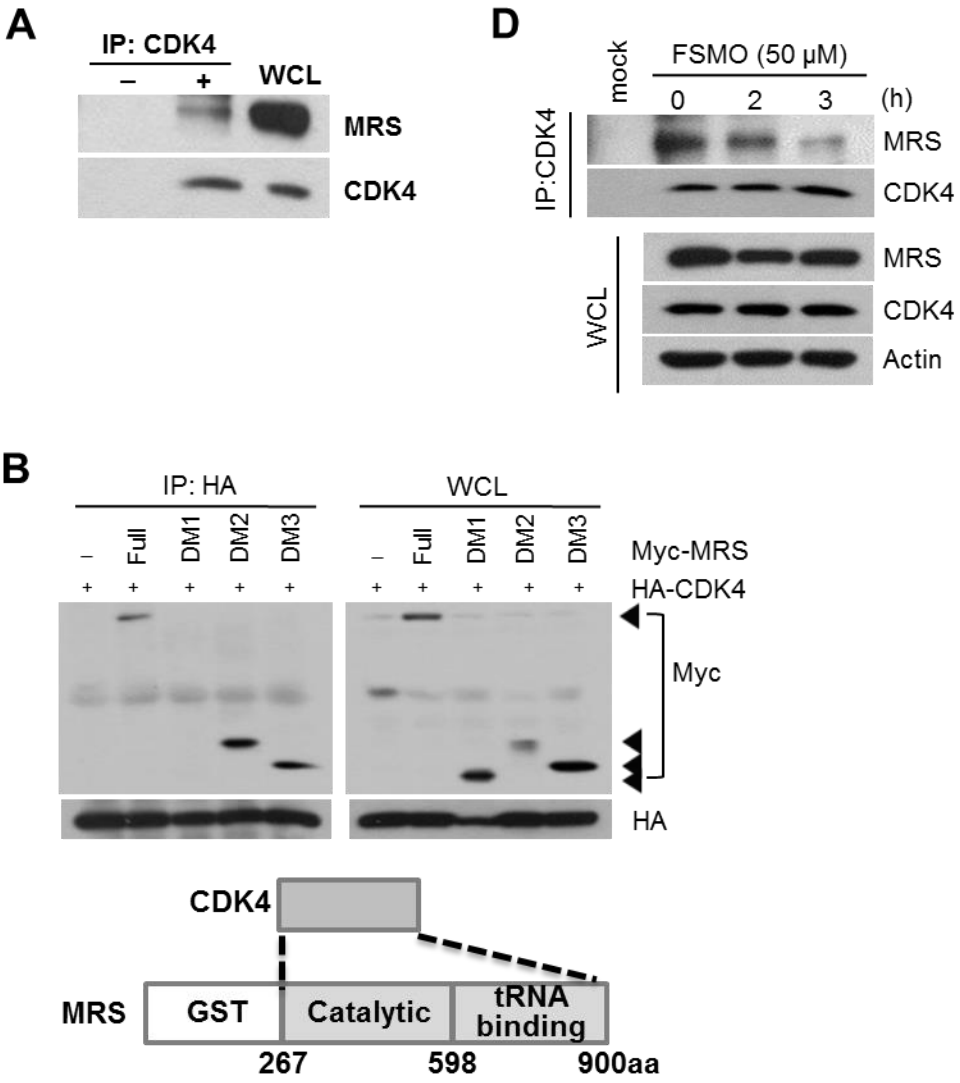

E
FSMO (μM)
0
25
50
100
CDK4
cyclin D1
MRS
p-Rb
Actin
F
25μM
FSMO
Relative translation
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
(mM)

Figure 3. MRS inhibition reduces CDK4 mediated cell cycle progression.

(A) To see the effect of methionine on the cell proliferation, BrdU cell proliferation assay (left) and cell cycle analysis (right) were performed using H460 cells incubated in Methionine-free DMEM media containing 10% dialyzed FBS for 9 h. (B) DNA synthesis (left) and Cell cycle progression (right) were investigated by BrdU assay and FACS analysis, respectively, using H460 cells treated 50 μM FSMO for 8 h. (C) Protein levels of cell cycle regulators involved in cell cycle progression were monitored by immunoblotting with lysates from MDA-MB-231 cells incubated in Met-free medium containing 10% defined FBS for 9 h. (D) Protein levels of cell cycle regulators involved in cell cycle progression were monitored by immunoblotting with lysates from H460 cells treated with 50 μM FSMO (Met analog) for 8 h. (E) CDK4 level was monitored in the H460 cell lines treated with FSMO. CDK4 level was decreased by FSMO in a dose dependent manner. (F) Global translation rate was investigated via in vitro translation reaction with FSMO treatment. The concentration of 25 μM which affected the level of CDK4 (E) was indicated (red line).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IP: CDK4
–
+
WCL
MRS
CDK4
D
mock
FSMO (50 μM)
0
2
3
(h)
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MRS
CDK4
WCL
MRS
CDK4
Actin
B
IP: HA
WCL
–
Full
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900aa

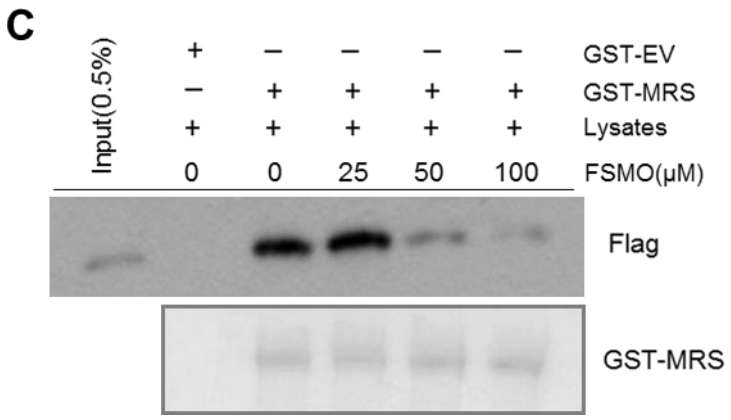

Figure 4. MRS directly interacts with CDK4.

(A) Endogenous interaction between MRS and CDK4 was monitored via immunoprecipitation.

(B) Myc-MRS fragments (F1, F2, and F3. See also Figure 2D) and HA-CDK4 were co-expressed in 293T and their interaction was detected by immunoprecipitation. (C) To monitor FSMO inhibitory effect on MRS-CDK4 binding pattern *in vitro*, GST-MRS and lysate from H460 transfected with flag-CDK4 plasmid were incubated with FSMO in dose dependent manner (0, 25, 50, 100 μM) and CDK4 level bound to GST-MRS was detected by immunoblotting. (D) To identify the effect of FSMO on MRS and CDK4 binding, endogenous CDK4 was immuneprecipitated with CDK4 specific antibody from H460 cell lysates treated with FSMO for 0, 2 and 3 h. Levels of MRS bound to CDK4 were determined by immunoblotting.

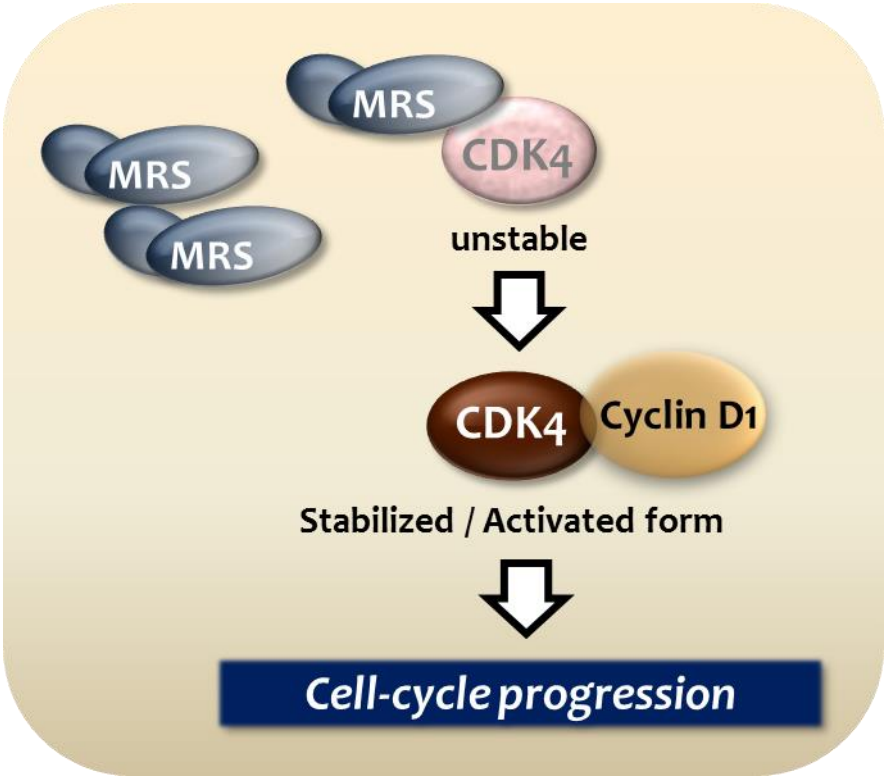

Figure 5. Schematic model

In cancer cells, MRS binds with unstable CDK4, and then CDK4 is stabilized and activated by forming a complex with cyclinD1. This activated complex promotes cell cycle progression.

DISCUSSION

MRS is a key regulatory enzyme of translation charging methionine to cognate Met-tRNA and initiating protein synthesis (Ghosh and Vishveshwara, 2008; Kwon et al., 2011). Beside the canonical function of MRS, there are several background information suggesting MRS has close relationship with regard to cell cycle, ribosomal RNA synthesis and cell proliferation (Ko et al., 2000). It was also reported that MRS might be associated with various diseases including cancer (Bharathkumar et al., 2015). In detail, MRS might have connection with CAGs (cancer associated genes) and MRS could mediate ribosomal RNA synthesis in nucleus in response to the stimulating signals for growth. On the basis of the information on MRS so far reported, the assumption that MRS might function as a cell cycle regulator as well as a translational enzyme has been raised by our group.

Thus, the present study was designed on the hypothesis that MRS might have an additional function for regulating the cell cycle progression. To reveal the hypothesis, it was attempted to clarify the role of MRS on cell proliferation and cell cycle progression. If the MRS was knocked-down by the treatment of si-MRS in MDA-MB-231 breast cancer cells, DNA synthesis and cell cycle transition from G1 to S were down-regulated. However, global protein synthesis was little affected by the treatment of si-MRS. The protein levels of the cell cycle related molecules were screened after the siRNA treatment to knock-down MRS. Interestingly, among various molecules associated with the cell cycle, only the proteins of CDK4 and cyclinD1 were reduced. Phosphor Rb protein which has been well-established with regard to Rb (retinoblastoma) protein (Shintani et al., 2000)

and E2F, transcriptional factor, was also reduced. Once CDK4 is stabilized and activated, it phosphorylates Rb protein bound to E2F (Scott et al., 2015). The inactivated and phosphorylated Rb protein, therefore, could activate E2F and promote the transcription of cell cycle relating proteins.

The MRS knock-down effect on CDK4 level was also confirmed using other cell lines such as lung cancer and colorectal cancer cell lines. The reduced protein level seemed not be caused by the diminishment of transcription. When the *de novo* protein synthesis was restricted by the treatment of cycloheximide, a blocker of the protein synthesis, in two-directional (knock-down and overexpression) condition, CDK4 was specifically degraded in MRS knock-down condition but was stabler in MRS overexpression condition. It could be deduced that MRS might control CDK4 stability since newly synthesized protein was restricted, so the protein left might undergo degradation. Therefore, it could be concluded that MRS has the function which stabilizes CDK4 at protein level. If MRS was insufficient, CDK4 might be attacked by a certain protease and the CDK4 level must be reduced.

The results obtained from this study also showed that the suppression of MRS activity down-regulated cell cycle progression and DNA synthesis (Qiao et al., 2015). It implied that CDK4 stability is affected by MRS level as well as MRS enzymatic function. It has been well known that some enzymes change their conformational structure and the change in molecular structure transits their original function (Crawford et al., 2015; Sousa et al., 2015). Thus, it could be deduced that once MRS catalytic activity was inhibited by the substrate shortage, MRS changed its conformational structure and then the binding affinity with CDK4 might be altered. Thus, MRS-CDK4 binding must be important to facilitate CDK4 stabilization. The starvation of methionine (Hoshiya et al., 1996) or treatment of methionine analogue, FSMO inhibited enzymatic activity of MRS and consequently MRS-CDK4 binding *in vitro* and in cell were inhibited.

Putting all the information together, diverse alterations of MRS function (knock-down or activity inhibition) regulated CDK4 level and MRS-CDK4 binding. It implied that MRS-CDK4 binding might be crucial for the stabilization of CDK4.

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요약 (국문초록)

Cyclin dependent kinase 4 (CDK4)를 매개로 하는 세포주기 진행에 있어서 methionyl-tRNA synthetase(MRS)의 새로운 기능 연구

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MRS (methionyl-tRNA synthetase) 는 ARS (aminoacyl tRNA synthetase) 효소 중의 하나인 단백질로 단백질 합성과정에서 아미노산을 해당 transfer RNA (tRNA)에 붙여주는 역할을 한다. MRS 는 잘 알려진 효소적인 기능 외에도, 암을 포함하여 다양한 질환의 발병에 관여한다는 보고가 있다. 암의 경우, 세포의 성장과 세포주기의 진행이 제대로 조절되지 않는다고 알려져 있다. MRS 에

대한 이와 같은 단서를 가지고, MRS 가 세포의 성장과 세포주기의 진행에 어떻게 영향을 미치는지 알아보았다. 또한, 구체적으로 어떤 물질이 이 현상을 매개하는지도 밝히고자 하였다.

본 연구에서 MRS 가 CDK4 (cyclin 의존성 인산화 효소 4)를 안정화시킴으로써 세포주기의 진행을 촉진시킨다는 MRS 의 새로운 기능을 규명할 수 있었다.

유방암 세포주 (breast cancer cell line)인 MDA-MB-231 세포와 폐암 세포주 (lung cancer cell line)들인 A549 와 H460 세포들에 siRNA 를 처리하여 세포내의 MRS 의 발현을 낮추면 세포주기 조절인자중의 하나인 CDK4 는 현저히 감소되었으나, 또 다른 세포주기 조절인자들인 CDK1, CDK2, CDK6 는 거의 영향을 받지 않는다는 것을 알 수 있었다. 이들 암 세포주에 메티오닌 (methionine)을 제한시키거나 메티오닌 유사체 (analogue)를 처리하면 MRS 와 CDK4 의 결합이 감소되고 세포주기의 진행이 지연되는 것을 알 수 있었다. 이러한 결과로부터 MRS 는 세포주기를 조절하는 기능이 있으며, MRS 의 세포주기 조절기능은 MRS 와 CDK4 의 결합에 의해 이루어진다는 것을 밝힐 수 있었다.

결론적으로 MRS 는 CDK4 와 결합하여 CDK4 를 안정화시킴으로써 세포주기의 진행을 촉진하며, 이 과정은 MRS 의 발현이나 MRS 의 효소적인 활성에 의해 영향을 받는다는 것을 본 연구에서 밝힐 수 있었다.

따라서, 본 연구는 CDK4 의 안정화를 통해서 세포성장을 촉진하는 MRS 의 새로운 기능을 밝힌 데 의의가 있다.

주요어: MRS(methionyl-tRNA synthetase), CDK4(cyclin-dependent kinase 4), 단백질 안정화, 효소활성, 세포주기 진행

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