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The estrogen metabolite 2-methoxyestradiol regulates eukaryotic initiation factor 4E (eIF4E) and inhibits protein synthesis in MG63 osteosarcoma cells

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Abstract Osteosarcoma is a primary bone tumor that affects children and young adults. The estrogen metabolite 2-methoxyestradiol (2-ME) induces cell death in osteosarcoma cells. To determine whether 2-ME actions involve the control of protein synthesis, we studied the effect of 2-ME on eukaryotic initiation factor 4E (eIF4E) and eIF4E-binding protein 1 (4E-BP1) in MG63 osteosarcoma cells. Our results show that 2-ME treatment increases the association of eIF4E with 4E-BP1 in osteosarcoma cells. Also, 2-ME decreases the binding of eIF4E protein to 7-methyl-guanosine cap structure, indicating that 2-ME treatment results in the inhibition of translational initiation. These findings are further supported by the inhibition of protein synthesis in 2-ME-treated osteosarcoma cells. Taken together, our studies show that 2-ME-mediated antitumor effects in osteosarcoma cells involve the regulation of protein synthesis, and translational machinery could serve as a target in the treatment of osteosarcoma.

Introduction

Osteosarcoma is a pediatric malignancy. Even though a combination of surgery and chemotherapy has improved the survival rate, the mortality rate remains high for this disease. 2-Methoxyestradiol (2-ME) is a metabolite of 17β-estradiol that exerts antitumor effects in a number of tumor cells, including osteosarcoma.[1–7] We have previously

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shown that 2-ME induces apoptosis in osteosarcoma cells, but not in normal osteoblasts.[5,8]

Protein synthesis regulation has been implicated in the control of tumor cell proliferation and apoptosis. Protein synthesis comprises three steps: initiation, elongation, and termination. The majority of the regulation takes place at the level of translation initiation involving eukaryotic translation initiation factor (eIF) 4E. eIF4E is a part of a multiprotein eIF4F complex, which consists of three proteins: eIF4E, which binds the 5′ cap structure of the mRNA; eIF4A, an ATP-dependent RNA helicase that is expected to unwind the secondary structures in mRNA; and eIF4G, a protein that interacts with eIF4E, eIF4A, and other initiation factors to form a scaffold.[9–11] The eIF4E serves as a rate-limiting factor of the eIF4F complex and is required by almost all mRNAs to be translated into proteins. eIF4E binds to 7-methyl guanosine triphosphate cap, which is present in most mRNAs, and thereby brings them to ribosomes and regulates cap-dependent protein synthesis.[12] The eIF4E activity is regulated by eIF4E-binding proteins (4E-BPs) that function as translational inhibitors.[12–15] The 4E-BP binding to eIF4E is determined by its phosphorylation status. The dephosphorylated 4E-BP binds to eIF4E and thereby blocks its activity.[12,13] RNA dependent protein kinase (PKR) is another protein which has been implicated in the regulation of protein synthesis and induction of apoptosis in a variety of different tumor cells.[16–18] PKR has been shown to act through the phosphorylation of eIF-2α.[8,16–18] Previous studies demonstrate the involvement of translational control and the components of eIF4F complex in malignant transformation and progression.[12,19] The goal of this study was to investigate the role of initiation factors eIF4E and 4E-BP1 in 2-ME-mediated actions in osteosarcoma cells.

Materials and methods

Cell culture and metabolite treatment

MG63 osteosarcoma cells and normal human osteoblast (HOB) cells[5] were maintained in Dulbecco's modified eagles medium (DMEM)/F12 containing 10% charcoal-stripped fetal bovine serum and supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin and maintained at 37°.[4,20] Cells were treated with 10 μM 2-ME (Sigma Chemical Co., St. Louis, MO) or the vehicle (70% ethanol) for indicated periods of time.

Preparation and analysis of cytoplasmic extract

Cells were harvested after vehicle and 2-ME treatment and lysed in cell lysis buffer, as described.[21,22] Following centrifugation, the supernatant was collected, and protein concentration was determined by as described.[21] Cytoplasmic extracts (60 μg protein) were analyzed by Western blot using anti-eIF4E, anti-eIF4E-BP1, antiphospho-eIF4E-BP1 (Thr37/46), anti-nonphospho-eIF4E-BP1 (Thr46) (Cell Signaling, Danvers, MA), and anti-actin antibodies (Sigma) as described in our earlier reports.[21,22]

Immunoprecipitation analysis was carried out as described.[22] Briefly, cytoplasmic extracts containing 60 μg protein were used for immunoprecipitation with anti-4E-BP1 antibodies. Bound proteins were purified using protein A Sepharose and analyzed by western blot hybridization using anti-4E antibodies. The levels of proteins on the western blots were quantitated using densitometer and Quantity one 4.5.2 software (BioRad, Hercules, CA).

Cap-binding assay

Cap-binding assay by m^7GTP-Sepharose chromatography was performed as described.[23] About 250 μL cell lysate (100 μg protein) was added to 20 μL of packed m^7GTP-Sepharose beads to capture eIF4E and its binding partner proteins. Captured proteins were eluted with buffer containing 50 mM Tris (pH 8.0); 150 mM NaCl; 1mM EDTA; 0.1% IPEGAL and 0.2% gelatin and analyzed by western blot using anti-eIF4E directly or after immunoprecipitation with anti-eIF4E-BP1 antibodies.

Protein synthesis (^{3}H-leucine incorporation)

The rate of protein synthesis was measured as described.[15] Briefly, protein synthesis was studied following vehicle and 2-ME (10 μM) treatment and by pulse labeling of cells for 1 h with 10 μCi/mL of ^{3}H-labled leucine. The cells were harvested and washed in 5 mL of cold phosphate buffered saline, and then the cells were lysed and precipitated with 10% trichloroacetic acid (TCA). The precipitate was filtered and washed with 5% TCA, and the TCA-insoluble radioactivity was determined by scintillation counting.

Statistical analysis

All values are expressed as means ± standard error. The data are representative of four independent experiments. Significant differences between groups were determined by Fisher's protected least significant difference post hoc test for multiple-group comparisons following detection of significance by one-way analysis of variance.

Results and discussion

Effect of 2-ME on initiation factors eIF4E and 4E-BP1 in osteosarcoma cells

To determine the effect of 2-ME on protein synthesis, we have investigated whether 2-ME regulates the binding of protein synthesis initiation factor eIF4E to its inhibitor protein 4E-BP1. We have analyzed the cytoplasmic extracts from vehicle and 2-ME-treated MG63 cells by co-immunoprecipitation studies which show that 2-ME treatment increased the binding of 4E-BP1 to eIF4E at 16 and 24 h (Fig. 1A). Furthermore, the cap-binding assays carried out show that 2-ME treatment resulted in decreased binding of eIF4E to cap structure (Fig. 1B). In eukaryotic cells, eIF4E associates with ribosomes and facilitates cap-dependent mRNA translation.[12,14,24,25] Studies suggest that cancer cells depend more on cap-dependent translation than normal cells, and cap-dependent translation is essential for

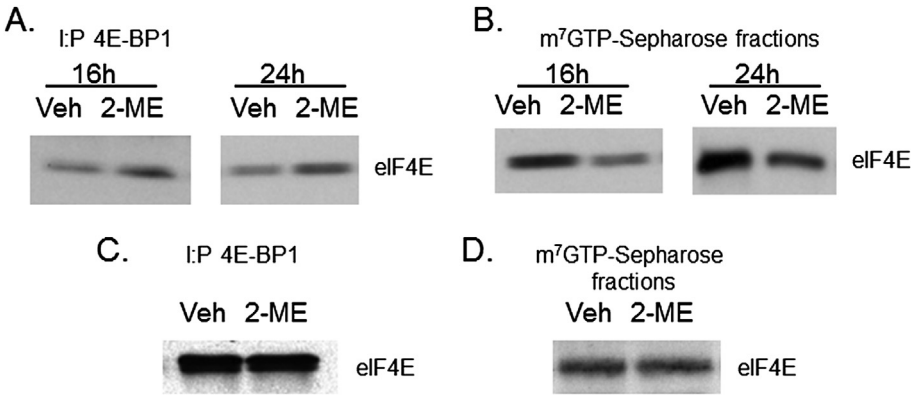

Figure. 1 2-ME regulates eIF4E and 4E-BP1 functions in osteosarcoma cells. Cytoplasmic extracts were prepared from MG63 osteosarcoma cells (A, B) at 16 and 24 h and normal human osteoblast (HOB) cells (C, D) at 24 h following vehicle (Veh) and 2-ME treatment. The extracts were subjected to immunoprecipitation (IP) with anti-4E-BP1 antibodies (A, C) or cap-binding (B, D) and analyzed by western blot analysis using anti-eIF4E antibodies.

the synthesis of tumorigenic proteins.[26] In addition, eIF4E is required for the transport of specific mRNAs. The eIF4E-mediated regulation of gene expression exhibits different gene specificities, as some genes are being regulated at the level of translation and others at the level of both transport and translation.[12,25,27] Deregulated transport of mRNAs of oncogenes and growth regulatory genes appears to be the cause for the oncogenic functions of eIF4E.[12,27] Our current results show that an increased association of eIF4E and 4E-BP1 is accompanied by decreased binding of eIF4E to 7-methyl guanosine cap structure in the presence of 2-ME treatment. Furthermore, our results demonstrate that these effects are specific to osteosarcoma cells, and 2-ME treatment does not affect the binding of eIF4E to 4E-BP1 (Fig. 1C) and cap structure (Fig. 1D) in normal HOB cells.

The western blot analysis shows that 2-ME treatment does not affect the levels of eIF4E and control actin, but modulates 4E-BP1 protein levels in MG63 cells (Fig. 2). The results show that overall band intensity ratio in vehicle and

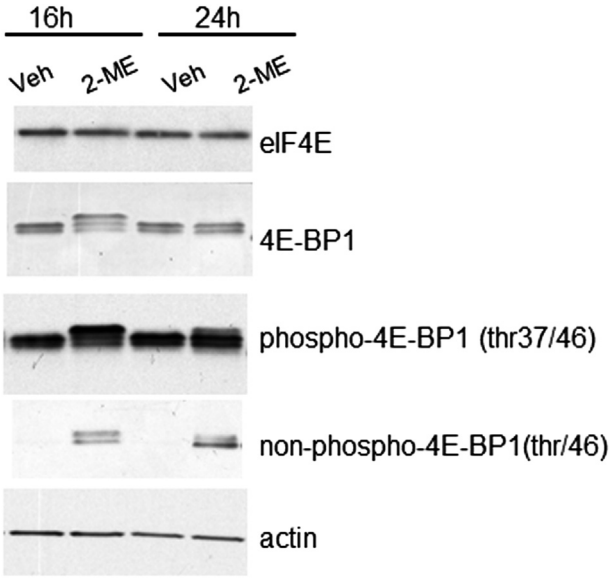

Figure. 2 Effect of 2-ME treatment on eIF4E and 4E-BP1 expression levels. Cytoplasmic extracts were prepared following 16 and 24 h of vehicle and 2-ME treatment from MG63 cells and analyzed by western blot using anti-eIF4E, anti-4E-BP1, antiphospho-4E-BP1 (Thr37/46), anti-nonphospho-4E-BP1 (Thr46), and anti-actin antibodies.

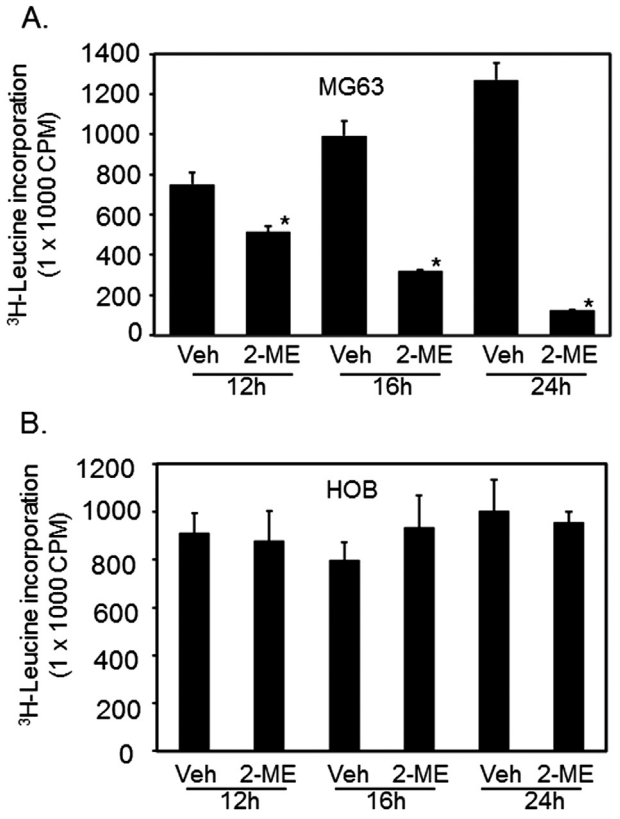

Figure. 3 2-ME treatment downregulates protein synthesis in osteosarcoma cells. Protein synthesis was measured following vehicle and 2-ME treatment in MG63 (A) and HOB (B) cells at 12, 16, and 24 h through pulse labeling with ^{3}H-leucine. *$P < 0.01$ *vs* vehicle.

2-ME-treated conditions at 16 h were 1.2:1.0; 1.0:1.5 and 1:20, respectively, when probed with the anti-4E-BP1, phospho-4E-BP1, and non-phospho-4E-BP1 antibodies. Similarly, the ratios at 24 h were 1.4:1.0; 1.0:1.6 and 1: 60 when probed with the anti-4E-BP1, phospho-4E-BP1, and non-phospho-4E-BP1 antibodies. Thus, these results point out that 2-ME treatment increases the non-phospho-4E-BP1 levels more compared to phospho-4E-BP1 protein levels. Published reports indicate that eIF4E and 4E-BP1 interaction is controlled by phosphorylation of the 4E-BPs.[14,15] There are 3 different types of 4E-BPs, and the hypophosphorylated forms of 4E-BPs interact with high affinity to competitively inhibit the binding of eIF4G to eIF4E.[28] Conversely, phosphorylation of 4E-BPs decreases the affinity of the protein for eIF4E and facilitates the binding of eIF4E to cap structure and continuous protein synthesis in cells.[14,24] Current studies show that higher levels of non-phosphorylated or hypophosphorylated forms of 4E-BP1 are present in the presence of 2-ME treatment compared to vehicle controls. 4E-BPs could be phosphorylated at multiple sites, and hyperphosphorylation has been shown to be required for the dissociation of eIF4E and activation of eIF4E functions.[13,23] Also, a large number of studies report that protein kinase mTOR (mammalian target of rapamycin) is the major protein responsible for the 4E-BP phosphorylation.[13,25–28] While our results clearly show an increase in non-phospho 4E-BP1 protein, additional investigations are required to establish the involvement of mTOR, and to delineate the various forms of the phosphorylated and non-phosphorylated forms of 4E-BP1 in 2-ME-treated osteosarcoma cells.

Changes in the state of phosphorylation of the 4E-BP and in the extent of its association with eIF4E could occur in response to stimulation of cell death in cells. Previous reports show that the growth-inhibiting apoptotic activities of cytokines and drugs have an impact on the eIF4E/4E-BP system. TNFα has been shown to inhibit overall translation in breast cancer cells by mechanisms that involve decreased phosphorylation of 4E-BP1 and increased association of eIF4E and 4E-BP1.[29] This observation is in agreement with the increased association of eIF4E with 4E-BP1 and decreased binding of eIF4E to cap structure observed in 2-ME-treated osteosarcoma cells. Hence, this indicates that the antitumor actions of 2-ME, in part, could be at the level of eIF4E-dependent translation initiation. Our result is corroborated by the absence of eIF4E regulation in HOB cells that are resistant to 2-ME-mediated antigrowth effects.

Effect of 2-ME on protein synthesis

To determine whether 2-ME-mediated inhibition of eIF4E has any effect on protein synthesis, we have followed the rate of protein synthesis. Our findings from ^{3}H-labeling studies show that the rate of protein synthesis is significantly ($P < 0.1$) reduced in MG63 cells to 68%, 32%, and 9.8%, respectively, at 12, 16, and 24 h in the presence of 2-ME compared to controls (Fig. 3A). However, 2-ME treatment does not affect protein synthesis in HOB cells (Fig. 3B) and it is in agreement with the absence of regulation of eIF4E and 4E-BP1 in HOB cells. Thus, the inhibition of eIF4E through sequestration by inhibitory protein 4E-BP1 leads to a block in protein synthesis in the presence of 2-ME. We have also observed that 2-ME inhibits protein synthesis in a number of osteosarcoma cell lines (A. Maran, K. Shogren,

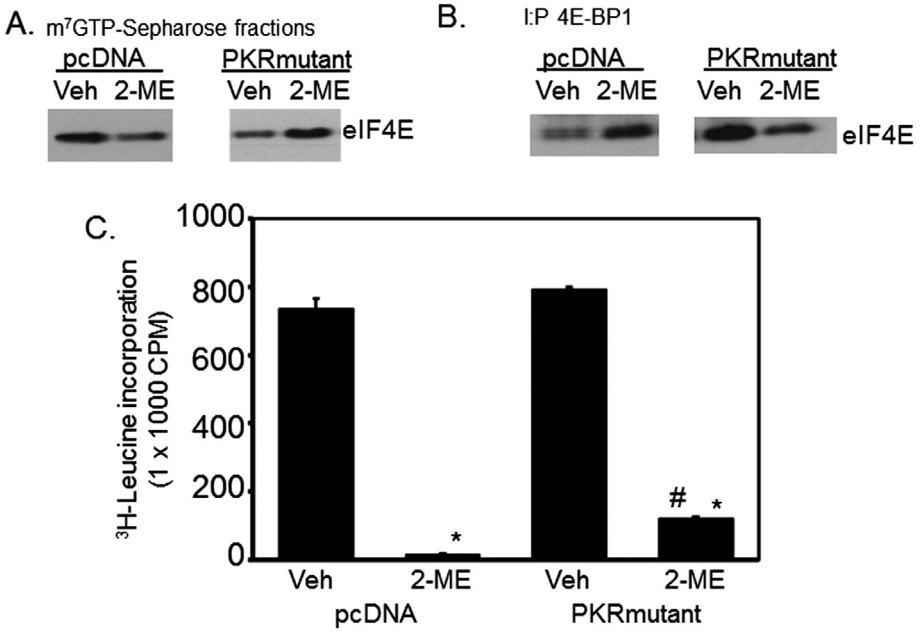

Figure. 4 2-ME effect on protein synthesis is decreased in cells expressing PKRmutant protein. MG63 osteosarcoma cells expressing vector (pcDNA) and PKRmutant protein were treated with vehicle and 2-ME for 24 h. The cytoplasmic extracts prepared were subjected to cap-binding (A) or IP with anti-4E-BP1 antibodies (B) and analyzed by western blot analysis using anti-eIF4E antibodies. Protein synthesis was measured through pulse labeling with ^{3}H-leucine following 24 h of vehicle and 2-ME treatment (C). *$P < 0.01$ *vs* Veh, $^{\#}P < 0.01$ *vs* pcDNA 2-ME.

M.J. Yaszemski, unpublished observations). Future investigations are necessary to identify the associated mechanism and the target mRNAs that undergo translational block in the presence of 2-ME treatment.

Effect of 2-ME on MG63 cells expressing negative dominant mutant PKR protein

MG63 osteosarcoma cells expressing PKRmutant protein are resistant to 2-ME-mediated antigrowth effects.[4,8,20] We have studied the effect of 2-ME on eIF4E binding to cap structure and to the inhibitory protein 4E-BP1 in cells expressing negative dominant mutant PKR (PKRmutant) and the control vector (pcDNA). Our results show that 2-ME treatment decreased the association of eIF4E with 4E-BP1 (Fig. 4A) and increased the binding of eIF4E to cap structure in control pcDNA cells but not in PKRmutant osteosarcoma cells (Fig. 4B). Furthermore, the studies reveal a partial reversal in 2-ME-mediated inhibition of protein synthesis in PKRmutant cells (Fig. 4C). Our results show that, compared to vehicle, 2-ME treatment decreases protein synthesis to 2% and 15% in MG63 cells expressing pcDNA and PKRmutant, respectively (Fig. 4). Our previous studies show that 2-ME treatment regulates the initiation factor, eIF2α, and induces phosphorylation of eIF2α through the activation of PKR.[8] Current studies indicate that PKR could be involved in additional translational regulations in 2-ME-treated osteosarcoma cells involving eIF4E in addition to already demonstrated initiation factor eIF-2α-dependent mechanisms.

Conclusion

In this report, we have shown a direct relationship between the assembly of the cap-dependent translation initiation apparatus and the antitumor effects of 2-ME. Our results show that 2-ME treatment in osteosarcoma cells resulted in a) increased association of eIF4E with eIF4E-BP1; b) decreased binding of eIF4E to 7-methyl guanosine cap structure; c) increased levels of hypophosphorylated 4E-BP1 protein; and d) decreased protein synthesis. Previous work demonstrates that 2-ME exerts antigrowth and antitumor effects in osteosarcoma cells but not in normal osteoblasts.[4,5,30] Current findings reveal that 2-ME inhibits cap-dependent protein synthesis in cancerous MG63 cells without affecting protein synthesis in normal HOB. Thus, our studies suggest that eIF4E/4E-BP system could play a major regulatory role in 2-ME-mediated cell death, and could be further explored as a potential target in the treatment of osteosarcoma.

Conflicts of interest

All authors have none to declare.

Acknowledgments

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References

1. Fotsis T, Zhang Y, Pepper MS, et al. The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumour growth. *Nature*. 1994;368:237—239.
2. Ganapathy M, Ghosh R, Jianping X, et al. Involvement of FLIP in 2-methoxyestradiol-induced tumor regression in transgenic adenocarcinoma of mouse prostate model. *Clin Cancer Res*. Mar 1 2009;15:1601—1611.
3. LaVallee TM, Zhan XH, Johnson MS, et al. 2-methoxyestradiol up-regulates death receptor 5 and induces apoptosis through activation of the extrinsic pathway. *Cancer Res*. 2003;63:468—475.
4. Maran A, Shogren KL, Benedikt M, Sarkar G, Turner RT, Yaszemski MJ. 2-methoxyestradiol-induced cell death in osteosarcoma cells is preceded by cell cycle arrest. *J Cell Biochem*. 2008;104:1937—1945.
5. Maran A, Zhang M, Kennedy AM, et al. 2-Methoxyestradiol induces interferon gene expression and apoptosis in osteosarcoma cells. *Bone*. 2002;30:393—398.
6. Mukhopadhyay T, Roth JA. Induction of apoptosis in human lung cancer cells after wild-type p53 activation by methoxyestradiol. *Oncogene*. 1997;14:379—384.
7. Pribluda VS, Gubish Jr ER, Lavallee TM, Treston A, Swartz GM, Green SJ. 2-Methoxyestradiol: an endogenous antiangiogenic and antiproliferative drug candidate. *Cancer Met Rev*. 2000;19:173—179.
8. Shogren KL, Turner RT, Yaszemski MJ, Maran A. Double-stranded RNA-dependent protein kinase is involved in 2-methoxyestradiol-mediated cell death of osteosarcoma cells. *J Bone Miner Res*. Jan 2007;22:29—36.
9. Gross JD, Moerke NJ, von der Haar T, et al. Ribosome loading onto the mRNA cap is driven by conformational coupling between eIF4G and eIF4E. *Cell*. Dec 12 2003;115:739—750.
10. Rogers Jr GW, Komar AA, Merrick WC. eIF4A: the godfather of the DEAD box helicases. *Prog Nucleic Acid Res Mol Biol*. 2002;72:307—331.
11. Sonenberg N. eIF4E, the mRNA cap-binding protein: from basic discovery to translational research. *Biochem Cell Biol*. Apr 2008;86:178—183.
12. Siddiqui N, Sonenberg N. Signalling to eIF4E in cancer. *Biochem Soc Trans*. Oct 1 2015;43:763—772.
13. Gingras AC, Kennedy SG, O'Leary MA, Sonenberg N, Hay N. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev*. Feb 15 1998;12:502—513.
14. Clemens MJ. Translational regulation in cell stress and apoptosis. Roles of the eIF4E binding proteins. *J Cell Mol Med*. Jul—Sep 2001;5:221—239.
15. Constantinou C, Clemens MJ. Regulation of translation factors eIF4GI and 4E-BP1 during recovery of protein synthesis from inhibition by p53. *Cell Death Differ*. Mar 2007;14:576—585.
16. Barber GN. The dsRNA-dependent protein kinase, PKR and cell death. *Cell Death Differ*. 2005;12:563—570.
17. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Ann Rev Biochem*. 1998;67:227—264.
18. Garcia MA, Meurs EF, Esteban M. The dsRNA protein kinase PKR: virus and cell control. *Biochimie*. 2007;89:799—811.
19. Mamane Y, Petroulakis E, Rong L, Yoshida K, Ler LW, Sonenberg N. eIF4E—from translation to transformation. *Oncogene*. Apr 19 2004;23:3172—3179.
20. Yang C, Shogren KL, Goyal R, Bravo D, Yaszemski MJ, Maran A. RNA-dependent protein kinase is essential for 2-methoxyestradiol-induced autophagy in osteosarcoma cells. *PLoS One*. 2013;8:e59406.

21. Benedikt MB, Mahlum EW, Shogren KL, et al. 2-methoxyestradiol-mediated anti-tumor effect increases osteoprotegerin expression in osteosarcoma cells. *J Cell Biochem*. Apr 1 2010;109:950—956.
22. Kennedy AM, Shogren KL, Zhang M, Turner RT, Spelsberg TC, Maran A. 17beta-estradiol-dependent activation of signal transducer and activator of transcription-1 in human fetal osteoblasts is dependent on Src kinase activity. *Endocrinology*. Jan 2005;146:201—207.
23. Constantinou C, Clemens MJ. Regulation of the phosphorylation and integrity of protein synthesis initiation factor eIF4GI and the translational repressor 4E-BP1 by p53. *Oncogene*. Jul 14 2005;24:4839—4850.
24. Gingras AC, Raught B, Sonenberg N. Regulation of translation initiation by FRAP/mTOR. *Genes Dev*. Apr 1 2001;15:807—826.
25. Karaki S, Andrieu C, Ziouziou H, Rocchi P. The eukaryotic translation initiation factor 4E (eIF4E) as a therapeutic target for cancer. *Adv Protein Chem Struct Biol*. 2015;101:1—26.
26. Jia Y, Polunovsky V, Bitterman PB, Wagner CR. Cap-dependent translation initiation factor eIF4E: an emerging anticancer drug target. *Med Res Rev*. Jul 2012;32:786—814.
27. Clemens MJ. Targets and mechanisms for the regulation of translation in malignant transformation. *Oncogene*. Apr 19 2004;23:3180—3188.
28. Gingras AC, Raught B, Sonenberg N. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem*. 1999;68:913—963.
29. Jeffrey IW, Bushell M, Tilleray VJ, Morley S, Clemens MJ. Inhibition of protein synthesis in apoptosis: differential requirements by the tumor necrosis factor alpha family and a DNA-damaging agent for caspases and the double-stranded RNA-dependent protein kinase. *Cancer Res*. Apr 15 2002;62:2272—2280.
30. Wimbauer F, Yang C, Shogren KL, et al. Regulation of interferon pathway in 2-methoxyestradiol-treated osteosarcoma cells. *BMC Cancer*. 2012;12:93.