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14. ABSTRACT
The beneficial effects of soy on established breast cancer is controversial. The goal of this research is to investigate the potential molecular mechanisms by which soy isoflavones affect established breast cancers. A recent study by our laboratory reported that dietary genistein reduced tumor growth and metastasis and down regulated cancer promoting genes in a nude mouse model with tumors established from MDA-MB-435 metastatic cancer cells. In contrast, daidzein increased primary mammary tumor growth and metastasis, and significantly upregulated genes that regulate proliferation and protein synthesis including eukaryotic initiation factor eIF4F members eIF4G1 and eIF4E. The purpose of this study is to investigate the molecular mechanisms by which soy isoflavones genistein and daidzein disparately regulate protein synthesis initiation in established breast cancer. Our hypothesis is that soy isoflavones modulate breast cancer progression by specific regulation of the eIF4F eukaryotic initiation factor complex to affect the synthesis of cancer regulatory proteins. Using MDA-MB-435 metastatic cancer cell lines, we recently reported (de la Parra, et al, 2012, J. Biol. Chem.),that the metabolite of daidzein, equol, upregulated eIF4G and c-Myc, and specifically increased the protein expression of IRES containing cell survival and proliferation-promoting molecules. The elevated eIF4G was not associated with eIF4E or 4E-BP in cap affinity chromatography assays or co-immunoprecipitations. In dual luciferase assays, IRES-dependent protein synthesis was increased by equol. Polysomal associations demonstrated that equol specifically increased protein synthesis and the association of IRES-containing mRNAs of cancer promoting molecules. To validate a role for elevated eIF4G in the cellular responses to equol, eIF4G expression was knocked down using specific shRNA. Protein expression levels of IRES containing molecules Cyclin D, BCL-Xl and -p120, and IRES dependent translation in MDA-MB435 cells were reduced as a result of eIF4G knockdown. Therefore, the elevated protein synthesis of mRNAs with IRESs is due to the increased eIF4G in response to equol.

15. SUBJECT TERMS
breast cancer, metastasis, soy isoflavones, genistein, daidzein, glycitein, equol, protein synthesis initiation, eIF4G, eIF4E, 4E-BP, c-Myc, mTOR, cap-independent, polysomal profile, knock down gene expression

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INTRODUCTION

The **goal** of this research is to investigate the potential molecular mechanisms by which soy isoflavones affect established breast cancers. Although a large body of research has been generated on the role of soy compounds in cancer prevention, there are no definitive conclusions on the effect of soy foods and breast cancer progression. A previous study from our laboratory investigated the effect of dietary soy on mammary tumor progression in nude mice with mammary tumors established using an estrogen receptor (ER) (-) highly metastatic human cancer cell line MDA-MB-435. Dietary administration of genistein reduced tumor growth and metastasis and downregulated cancer promoting genes (1). On the contrary, individual daidzein and combined isoflavones (genistein:daidzein:glycitein, 5:4:1) increased metastasis and upregulated the expression of genes that promote cell proliferation and survival, including eukaryotic protein synthesis initiation factors *EIF4GI* and *EIF4E,* critical members of the eIF4F protein synthesis initiation factor complex. The eIF4F family initiation factors have been shown to be overexpressed in advanced cancer and to be essential for translation of a subset of proteins that regulate cellular bioenergetics, survival, and proliferation (2, 3). The **purpose** of this study is to investigate the molecular mechanisms by which soy isoflavones genistein and daidzein disparately regulate protein synthesis initiation in established breast cancer. Our **hypothesis** is that soy isoflavones modulate breast cancer progression by specific regulation of the eIF4F eukaryotic initiation factor complex to affect the synthesis of cancer regulatory proteins.

BODY

Specific Aim 1*: *Delineate the effects of genistein, daidzein, glycitein or combined soy isoflavones on mammalian target of rapamycin (mTOR) signaling. *The objective of this aim was to determine whether soy isoflavones affect the activity and expression of eIF4F initiation factors by determining changes to total and phospho (active) forms of the mTOR signaling pathway.* ***Completed*.**

In the previously submitted and accepted annual report, we demonstrated that soy isoflavones genistein, daidzein and glycitin did not significantly change the expression of the eIF4F family members or phosphorylation status of the mTOR target, the inhibitor of eIF-4E (4E-BP1). We analyzed the effect of the major isoflavones genistein,daidzein and glycitin in the ratio of 5:4:1 to mimic the composition of soy isoflavones consumed by humans, and at physiological concentrations that accumulate in the circulation following consumption of soy foods (4, 5). We used three metastatic human breast cancer cells, the low metastatic MDA-MB-231 (ERα-, ERβ+, EGFR+), SKBR3 (ER-, EGFR+, HER2++) and the highly metastatic MDA-MB435 (ER(-), EGFR (+), Her2(+++)). As was described in the previous annual report, none of the individual isoflavone compounds, in the three cell lines tested, changed the phosphorylation status of 4E-BP, which is a direct target of mTOR kinase (Fig.1), consequently we concluded that soy isoflavones do not regulate mTOR signaling. Treatment of MDA-MB-231 and SKBR3 cells with individual isoflavones genistein, daidzein, or glycitein, or in combination (gen:daid:gly) at physiological concentrations did not change eIF4E, eIF4G, or their phosphorylated form. However, we identified that similar to the *in vivo* result from MDA-MB-435 mammary fat pad tumors in nude mice (1) , where eIF4E was the only gene in

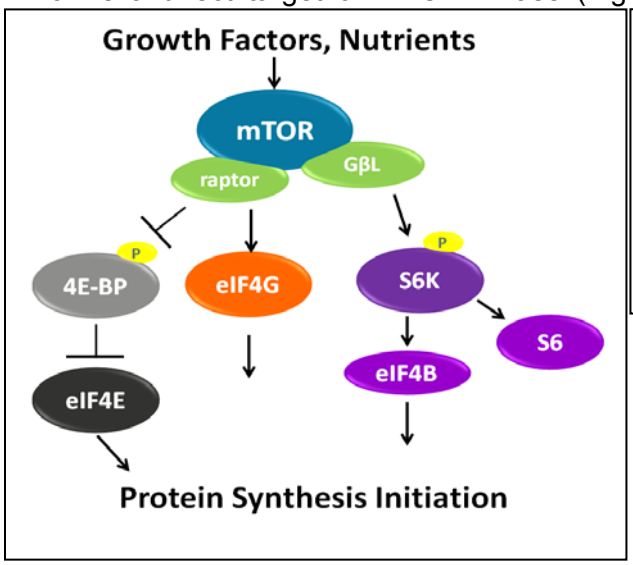

Figure 1. mTOR activity. Activation of mTOR by growth factors and nutrients results in mTOR-mediated phosphorylation of two targets: 4E binding protein (4E-BP) and p70S6 kinase (S6K). Phosphorylation of 4E-BP results in release of eIF4E to initiate cap-dependent protein synthesis with eIF4G. Phosphorylation and activation of S6K also results in ribosome biogenesis and protein synthesis initiation via phosphorylation of ribosomal S6 subunit (rpS6) and eIF4B.

the PI3-K pathway PCR array that was upregulated by combined soy isoflavones, the combined soy isoflavones increased eIF4F protein levels in MDA-MB-435 cells by ~1.7-fold.

An important finding from the first year of experiments of this award, described in the previous annual report, and complemented with data from the present report (recently published in the *Journal of Biological Chemistry*) (6), was that the described increase in MDA-MB-435 tumor growth and the up-regulation of the eIF4F family members eIF4G in response to dietary daidzein from the *in vivo* study (1), can be recapitulated *in vitro* by the daidzein metabolite equol. This metabolite has higher bioavailability and greater affinity for ER than its precursor daidzein, also rodents are efficient producers of equol, but, not all humans have the gut microflora necessary to convert daidzein to equol, and 30–50% of humans are equol producers (7, 8). The proportion of equol producers also vary with demographic, lifestyle factors, and ethnicity. The soy isoflavone equol significantly increased eIF4G expression in MDA-MB-231 and MDA-MB-435 cells. The protein levels of eIF4E and its inhibitory protein 4E-BP remained unchanged at all concentrations of equol tested, indicating a specific effect on eIF4G expression. Elevated eIF4F expression can impact cancer progression by increased protein synthesis even under the oxidative and nutrient stress conditions in solid tumors (7). Enhanced eIF4G levels can specifically initiate translation of mRNAs that contain an internal ribosome entry site (IRES). Interestingly, many mRNAs with IRESs code for several proteins related to cellular bioenergetics, survival, and proliferation (2, 9, 10). Even though the mTOR signaling is not regulated by soy isoflavones, the levels of eIF4E and eIF4G are increased by soy isoflavones at the transcription level. Therefore, we are continuing with testing the original hypothesis that soy isoflavones modulate breast cancer progression by specific regulation of the eIF4F eukaryotic initiation factor complex to affect the synthesis of cancer regulatory proteins (Fig. 2).

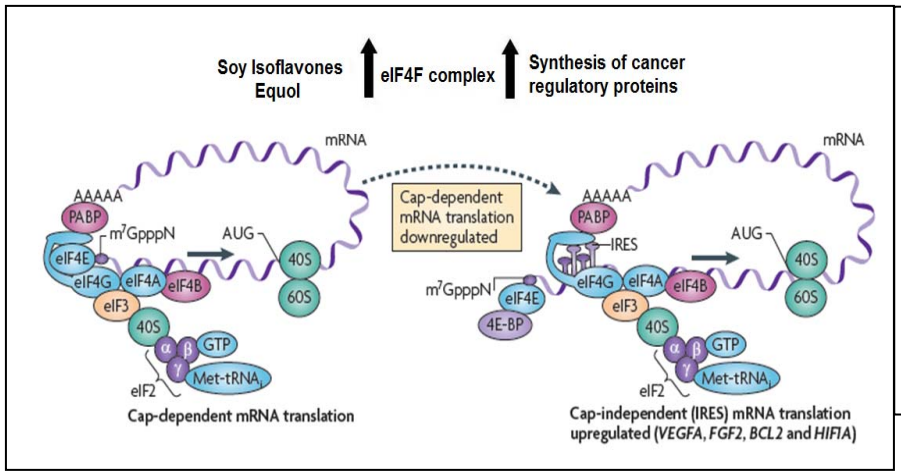

Figure 2. Working Hypothesis. Soy isoflavones increase members of the eIF4F eukaryotic initiation factor complex. Cap-dependent or cap-independent protein synthesis is predicted to be elevated; thus, synthesizing pro-cancer malignancy mRNAs with long structured 5' untranslated regions (UTR) or internal ribosome entry sites (IRES) that are limited in synthesis under normal conditions.

Specific Aim 2: ***Investigate the effects of genistein, daidzein, glycitein or combined soy isoflavones on expression of mRNAs that are specifically regulated by the eIF4F initiation complex.*** *The objective of this aim was* to investigate whether soy isoflavones direct preferential translation of cancer promoting genes by determination of the polysomal distribution and expression of specific mRNAs with structured 5'UTRs known to be sensitive to eIF4F levels.

As reported in the past annual report, we initiated this aim by investing the expression of pro-cancer molecules with internal ribosomal entry sites (IRES) containing mRNAs. High levels of eukaryotic initiation factors, specifically eIF4G1, have been correlated with increased cap independent translation of specific mRNAs that contain IRESs and long structured 5'UTRs (9, 10). Since equol treatment had a drastic and significant effect on eIF4G expression, which is related to enhanced cap-independent translation (Fig. 2), the effect of equol was analyzed on the expression of cancer promoting proteins in MDA-MB-435 cells. As we described in the past annual report, equol, upregulate protein expression of IRES containing pro-cancer molecules that regulate cancer cell proliferation and survival: c-myc, survivin, Bcl-2, Bcl-XL and Cyclin D, by ~1.3-1.7-fold compared to vehicle. During the second year of the award we further investigated the expression of cancer related proteins with IRES containing mRNAs, sensitive to eIF4G levels, such as p120, phospho-p120 and the angiogenesis promoter VEGF.

As controls, we compared the expression of proteins with mRNAs with short 5'UTR that do not contain IRESs JunB and GAPDH. Equol at 10, 25 and 50μM up-regulated protein expression of VEGF, and total and active p120 catenin in the MDA-MB-435 cells by ~1.3–2.0-fold compared with vehicle (Fig.3). The non IRES containing proteins JunB, GAPDH, and actin were not increased in response to all concentrations of equol tested (0–50μM).

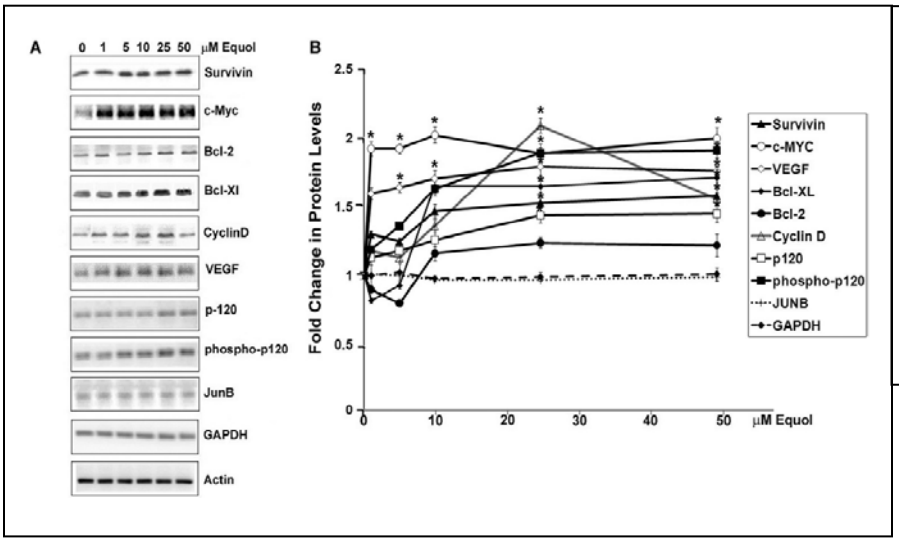

Figure 3. Expression of pro-cancer molecules following equol. Quiescent MDA-MB-435 cells were treated with vehicle or 1-50 μM equol for 24 h, lysed, and western blotted with antibodies to the indicated proteins. **A,** Representative western blots. **B**, Fold changes relative to actin as calculated by Image J analysis of positive bands from equol treatments (1-50 μM) compared to vehicle. N=3±SEM. Asterisk = $p \leq 0.05$.

We also investigated the effect of equol in protein expression of IRES-containing mRNAs and non IRES-containing mRNAs as a function of time (Fig.4). Levels of p120 catenin were increased at 6 h following equol treatment, while JunB levels remained unchanged. These findings, as well as other results to be described later in this second annual report, complemented by the data described in the first annual report, resulted in a publication in the high-impact peer-reviewed journal: *Journal of Biological Chemistry* (6).

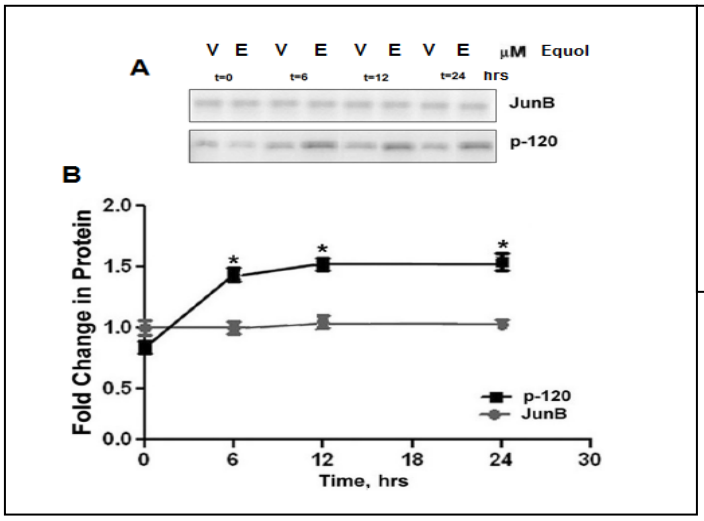

Figure 4. Expression of p120 and JunB as function of time following equol. Quiescent MDA-MB-435 cells were treated with vehicle or 25μM equol for various times as indicated and subjected to lysis and Western blotting for p120 catenin or JunB. Fold changes relative to actin were calculated by Image J analysis of positive bands from equol treatments compared with vehicle controls (n=3). An asterisk indicates statistical significance of $p<0.05$.

To investigate if the increased protein expression in response to equol was due to an increase in gene expression, we performed quantitative RT-PCR analysis following equol treatment. As previously shown in our first annual report, there was no significant change in mRNA levels of Bcl-2 or Cyclin D. This data was extended by RT-PCR analysis for the mRNA levels of VEGF, p120 (CTNND1) and EIF4G from cells treated with equol at 0,5,25 and 50 μM. The mRNA levels of VEGF and p120 did not change in response to equol treatment, the same tendency was reported for Bcl-2 and Cyclin D, however, gene expression of eIF4G was significantly increased following equol treatment (Fig 5).

Moreover, c-Myc mRNA levels, as was previously reported in the prior annual report, also increased significantly. The c-Myc transcription factor is one of the most important somatically mutated oncogenes in

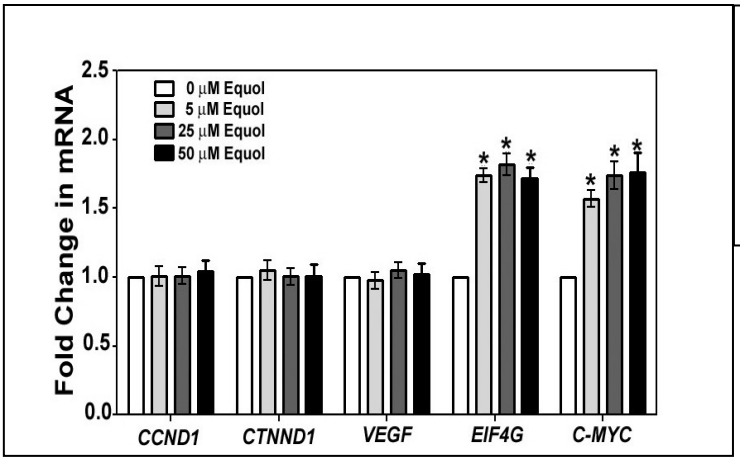

Figure 5. Effect of equol on gene expression of cancer promoting molecules. Quiescent MDA-MB-435 cells were treated for 24 h with 5-50 μM equol, and *CCND1*, *CTNND1*, *VEGF*, *EIF4G*, or *MYC* expression quantified by qRT-PCR. Fold changes in gene expression from cells treated with equol compared to vehicle (N=3). Asterisk= $p<0.05$.

human cancer and confers a selective advantage to cancer cells by promoting protein synthesis, proliferation, cell survival, differentiation, genetic instability, angiogenesis, hypoxia-mediated cancer progression, and metastasis (11-13). The transcription factor c-Myc upregulates eIF4E and eIF4G expression, and in turn becomes elevated by increased translation of c-Myc mRNA (14, 15). c-Myc also has an IRES site and is thus sensitive to elevated eIF4G and eIF4E levels (16-18). The initial equol-mediated elevation of MYC and EIF4G1 gene expression may result in further synthesis of eIF4G and c-Myc via IRES-driven mechanisms. Elevated eIF4G by equol increases protein expression of specific mRNAs with IRESs without affecting their gene expression. These data suggests that the changes in protein levels of molecules with mRNAs that have been shown to contain IRES sites (Fig. 3) are probably due to post-transcriptional regulation. The dual luciferase assays for cap-dependent and cap-independent IRES-mediated protein synthesis, performed and described in the first report, suggested that the excess eIF4G, that is not associated with eIF4E, specifically increased IRES-driven firefly luciferase activity by 1.6-fold compared with vehicle ($p <$ 0.01). Also, the results from synthetic *m7GTP* co-capture assays performed to isolate cap-bound eIF4E, eIF4G, and 4EBP demonstrated that equol treatment significantly decreased the amount of eIF4G co-captured with eIF4E in the m7GTP beads by ~75% compared to vehicle controls. However, there was a 3-fold increase in eIF4G levels recovered in the total cell lysate and the free pool of the supernatants, indicating that the elevated eIF4G in response to equol is recovered in the cytosol. This year we confirmed this experiment by performing eIF4G co-immuniprecipitations using an anti eIF4G to identify eiF4E recovered in the pulldowns. We found that the association of eIF4G and eIF4E is reduced by equol treatment by 32% in a statistically significant manner (Fig 6-B).

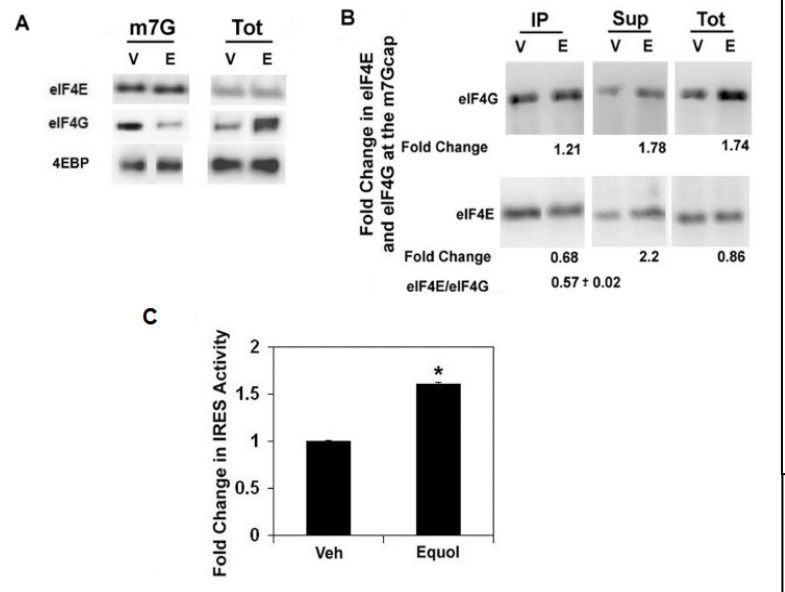

Figure 6. Potential regulation of IRES-dependent protein synthesis initiation by equol. Quiescent MDA-MB-435 cells were treated with vehicle (V) or 25 μM equol (E) for 24 h, lysed, and incubated with Sepharose 4B Conjugated 7-Methyl-GTP or immunoprecipitated using an anti-eIF4G. **A.** shown is a representative Western blot of m7G-bound proteins and the total protein (Tot) from cell lysates before pulldown assays.**B.** Representative Western blots stained with eIF4G or eIF4E are shown for immunoprecipitates (*IP*), supernatants (*Sup*), and total protein lysates (*Tot*). **C.** Relative IRES-dependent protein synthesis after equol treatment is shown.

Polysomal distribution and expression of mRNAs known to be sensitive to eIF4F levels: To determine whether the up-regulated eIF4G levels in response to equol resulted in increased protein synthesis initiation, we performed polysomal fractionations of cell lysates after vehicle or equol. Lysates from vehicle or the metabolite of daidzein equol were loaded onto 10-50% sucrose

gradients to isolate the ribosomal and polysomal fractions. Potential association of mRNAs was determined by A260 measurements. There was an increase of A260 measurements for the polysomal fractions from cells that were treated with equol compared to vehicle (Fig 7). This result indicates that the polysomes following equol treatment had a higher affinity for mRNA, thus suggesting enhanced protein synthesis.

The RNA isolated from polysomal fractions was subjected to real time RT-PCR assays to quantify the mRNA levels of p120 catenin (CTNND1), eIF4G and GAPDH using Beta-2 microglobulin (B2M), as an internal control. We found specific association of CTNND1 and EIF4G mRNA, but not GAPDH, with the heavier polysomal fractions by 1.7-fold from equol-treated cells compared with vehicle controls (Fig. 8). Similar to the

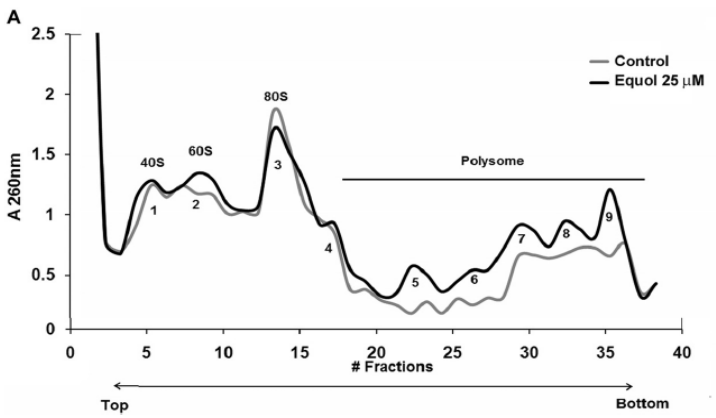

Figure 7. Analysis of polysome profiles. Equivalent amounts of total cell lysate from vehicle control or 25 μM equol-treated MDA-MB-435 cells were loaded onto10-50% sucrose gradients. The u.v. absorbance of pooled sequential gradient fractions was measured at 260 nm. The 40 S, 60 S, and 80 S fractions were classified as non-polysome fractions. All subsequent fractions were classified as polysome fractions. N=3.

results on protein expression (Fig. 3), association of the IRES-negative GAPDH mRNA was not changed by equol treatment. Therefore, the observed equol-mediated up-regulation of p120-catenin protein expression (Fig. 3) without changes in CTNND1 gene expression (Fig. 5) may indicate preferential synthesis of IRES containing mRNAs in equol-treated cells.

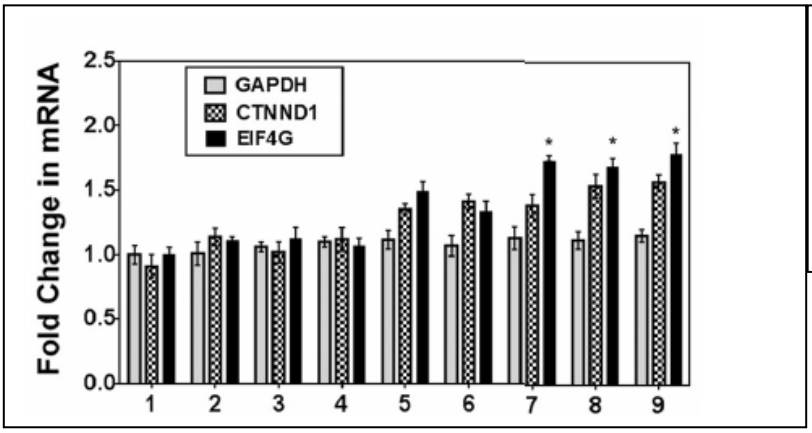

Figure 8. Analysis of polysome profiles. mRNA associated with polysome fractions was used to detect *GAPDH*, *CTNND1*, and *EIF4G*mRNA by qRT-PCR. B2M was used as an internal control. Results are shown as the -fold changes in equol-treated cells relative to vehicle controls (*n*=3). An *asterisk* indicates statistical significance of $p < 0.05$

Specific Aim 3: Demonstrate that differential regulation of eIF4E and eIF4G levels by soy isoflavones can directly contribute to breast cancer progression. *This Aim will determine whether soy isoflavones modulate breast cancer progression by direct modulation of eIF4E and (or) eIF4G by investigating the effects on cell cycle progression, cell survival/apoptosis, migration, and invasion in breast cancer cells expressing vector controls or siRNA targeted at eIF4E or eIF4G*.

In order to investigate if the regulation of eIF4G that is elevated in response to equol, contribute to breast cancer progression, we silenced eIF4G with short hairpin RNAs (shRNAs) that would yield small interfering RNA (siRNA) targeted at eIF4G. MDA-MB-435 cells were infected two times over 4 days with non-silencing (NS) or eIF4GI-silencing (eIF4GI) Adenovirus (Ad) shRNA vectors. This shRNA constructs were kindly provided by Drs. Deborah Silvera and Robert Schneider (New York University Langone Medical Center, NY). Using the eIF4G shRNA vectors, we were able to silence eIF4GI protein expression by ~60%, compared with control cells (Fig. 9). In order to investigate if the regulation of eIF4G that is elevated in response to equol, contributes to breast cancer progression, MDA-MB-435 cancer cells expressing control or eIF4G shRNA were treated with vehicle control or 25μM equol. The cells were analyzed for protein expression levels of members of eIF4F complex and pro-cancer molecules from IRES containing mRNA that were previously shown to be up-regulated by equol (Fig. 3). eIF4G silencing caused a marked reduction in protein expression levels of CyclinD, BCL-Xl, -p120 in both vehicle and equol treatmented cells. Lysates from vehicle presented a slight reduction,

and lysates from equol treatment presented a significant reduction by ~40%-50% compared to lysates from cells expressing control vector treated with equol. These results suggest that the overexpression of eIF4G is essential for the significant increase in protein expression of IRES-containing cancer promoting molecules by equol treatment. The elevated eIF4G levels in response to equol could be a major mechanism by which the daidzein metabolite regulates the expression of pro-cancer molecules with IRES containing mRNAs.

As expected, c-Myc protein levels were not affected by silencing eIF4G. Equol-mediated c-Myc protein levels increased by ~1.6-fold compared to vehicle, from MDA-MB-435 cells with eIF4G knockdown. Studies have shown that the key transcription factor c-Myc, upregulates eIF4E and eIF4G expression, and in turn becomes elevated by increased translation of c-Myc mRNA (14, 15). Fig.9 shows that equol or eIF4G silencing does not affect eIF4E expression. The protein expression of JunB and Actin from mRNA with short 5'UTR lacking an IRES were also not affected by eIF4G silencing.

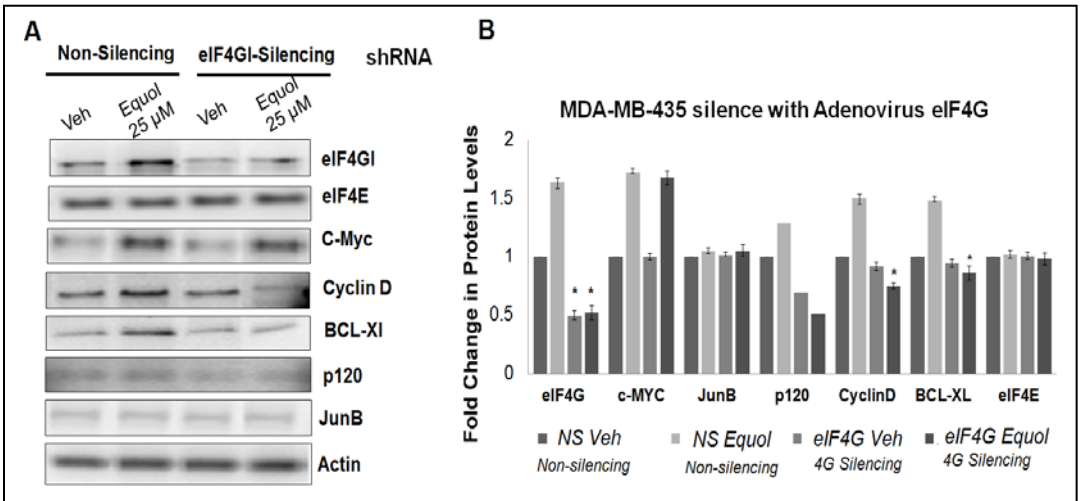

Figure 9. Expression of pro-cancer molecules following equol in MDA-MB-43 with eIF4G knockdown. MDA-MB-435 cells were infected two times over 4 days with non-silencing (NS) or eIF4GI-silencing (eIF4GI) Adenovirus (Ad) shRNA vectors, then treated with vehicle or 25μM equol for 24 h, lysed, and Western-blotted with specific antibodies to the indicated proteins.

Currently we are in the process of analyzing the mRNA levels of EIF4G ,C-MYC, CTNND1 (p120), CCND1 (CyclinD) using B2M as housekeeping gene control from MDA-MB-435 cancer cells expressing control or eIF4G shRNA, treated with vehicle control or 25μM equol. We expect to complete this analysis by the end of april 2013. We expect that if equol mediates up-regulation of eIF4G and regulates protein synthesis initiation of IRES-containing cell survival and pro-proliferation molecules, then the mRNA levels of CTNND1 (p120), CCND1 (CyclinD) should not be affected. However, mRNA levels of c-Myc will increase in response to equol, as we have shown that the increase in c-Myc protein levels is independent of eIF4G (Fig.9).

Effects on cell viability and cell cycle progression: Cell viability was determined using MTT assay in MDA-MB-435 cancer cells expressing control or eIF4G shRNA and treated with vehicle control or 25μM equol. The

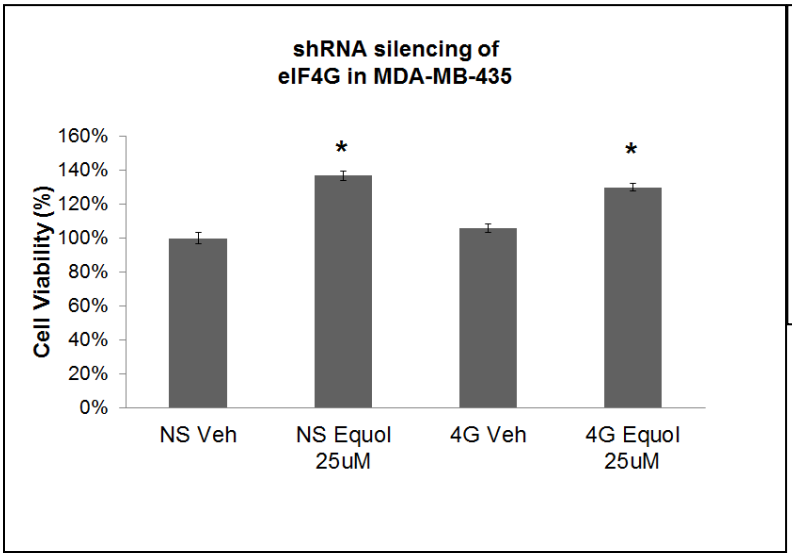

Fig 10. Effect of eIF4G silencing and equol on cell viability. MDA-MB-435 cells were infected two times over 4 days with non-silencing (NS) or eIF4GI-silencing (eIF4GI) Adenovirus (Ad) shRNA vectors , then treated with vehicle or 25μM equol for 24 h.Cells were lysed and subjected to a MTT assay. Results are shown relative to vehicle (100%). N=3 for all experiments. A single asterisk indicates statistical significance of $P \leq 0.05$.

eIF4G silencing had no effect on cell viability (Fig. 10). 25 μM equol treatments induced a ~1.3-fold increase in metastatic cancer cell growth. It has been shown in previously reports that eIF4G depletion can cause only a small reduction in protein synthesis, cell viability, and only slightly impaired cell

proliferation (19, 20). These results were not surprising, since the elevated levels of c-Myc transcription factor in response to equol can contribute to elevated cell growth in response to equol. Therefore, the elevated eIF4G in response to equol may specifically regulate the observed increases in metastasis in response to dietary daidzein, without affecting cell and thus, tumor growth.

IRES dependent translation after eIF4G silencing- Dual Luciferase Assays: In order to determine whether equol was regulated through the excess of eIF4G in the cytosol by cap-independent protein synthesis, we performed dual luciferase assays for cap-dependent and IRES-dependent protein synthesis on MDA-MB-435 cell expressing control or eIF4G shRNA, following equol treatment. The preliminary data shown in Fig.11 (n=1) suggests that the IRES dependent translation in MDA-MB435 cells were reduced as a result of eIF4G silencing. Vehicle treatment shows a slight decrease in IRES activity, but the equol treatment demonstrated a dramatic decrease of IRES-dependent translation. Therefore, the elevated protein syntehsis of mRNAs with IRESs is due to the increased eIF4G in response to equol.

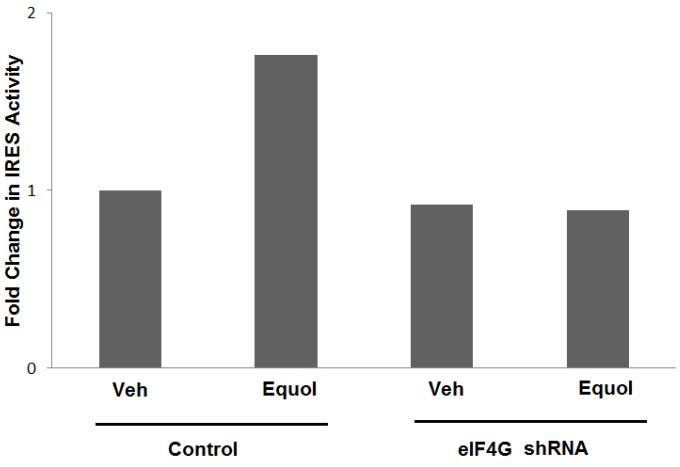

Fig 11. Effect of eIF4G silencing and equol on IRES-dependent protein synthesis. MDA-MB-435 cells with control or eIF4G knockdowns expressing a plasmid with a cap-dependent *Renilla* luciferase (RLuc) followed by a 5'UTR containing the p120 catenin IRES driving a firefly luciferase (FLuc) or control plasmid without an IRES, were treated with vehicle or equol for 24 h. Cells were lysed, and the relative IRES activity analyzed as 570 nm FLuc/480 nm RLuc. IRES activity was quantified relative to control activity for vehicle or equol treated cells.

KEY RESEARCH ACCOMPLISHMENTS:

1. The soy isoflavone equol, a metabolite of daidzein with higher bioavailability and greater affinity for ER, significantly increased in MDA-MB-435, the expression IRES containing molecules that are sensitive to eIF4G levels: survivin, Bcl-XL, c-myc, Cyclin D, VEGF, p120, p-p120. The non IRES containing proteins JunB, GAPDH, and actin were not increased.
2. mRNA levels of CCND1 (Cyclin D), CTNND1 (p120) and VEGF were not changed in response to equol treatments.
3. Gene expression of eIF4G and c-MYC was significantly increased following equol treatment.
4. eIF4G co-immuniprecipitation experiment confirmed the 5' methyl guanosine cap pulldowns and demonstrated that the association of eIF4G and eIF4E is reduced by equol treatment, suggesting that equol-mediated up-regulation of eIF4G directs cap-independent protein synthesis initiation of IRES-containing molecules, whereas eIF4E remains at the 5'cap, bound to 4E-BP.
5. Polysomal fractions of MDA-MB-435 cells following equol, increased the total mRNA associated with the polysomal fractions, indicating enhanced protein synthesis initiation.
6. Specific association of CTNND1 and EIF4G mRNA were found with the heavier polysomal fractions by 1.7-fold in from equol-treated cells compared with vehicle controls.
7. eIF4GI expression was successfully knocked down by 60% with an adenoviral vector containing short hairpin RNAs (shRNAs) for eIF4G in MDA-MB-435 cells.
8. eIF4G knockdown resulted in a significant reduction in protein expression levels of IRES containing molecules: CyclinD, BCL-Xl, -p120.
9. The c-Myc and eIF4E protein levels were not affected by silencing eIF4G.
10. Equol did not affect the viability of MDA-MB-435 cells with eIF4G knockdowns.
11. IRES dependent translation in MDA-MB435 cells decreased following eIF4G knockdown.

REPORTABLE OUTCOMES:

The PI has completed the following milestones in her training program and is on track with the timeline outlined in the original proposal.

Progress towards Ph.D. Degree

1. May 2012: Ph.D. Dissertation research proposal writing and presentation to thesis committee. PI, formally accepted as a Ph.D. Candidate.

Training

1. Dr. Robert Schneider, Ph.D. (Professor, New York University Langone Medical Center, NY), an expert in breast cancer and protein synthesis regulation, visited the PI's laboratory in Fall 2012. Dr. Schneider presented a seminar to the PI's Department and the PI presented her research to Dr. Schndeider and received valuable feedback. Supported partially by the DoD award, the PI will spend a month (May 2013) at the laboratory of Dr. Schneider performing experiments related to the present project, polysome profiling of on MDA-MB-435 cells expressing control or eIF4G shRNA following treatment with vehicle or equol.

Presentation of research at national and international conferences

1. The PI attended and presented a poster at the American Association of Cancer Research (AACR) 103rd Annual Meeting 2012, in Chicago, IL. April 2012, and will attend and present a poster at this year's Experimental Biology (EB) 2013 meeting in Boston, April 2013.

Abstracts

- American Association of Cancer Research *AACR* 103rd Annual Meeting *2012*. Chicago, IL.

  "Increased expression of eukaryotic protein synthesis initiation factor eIF4G by the daidzein metabolite equol may contribute to breast cancer malignancy".

  Authors: *Columba de la Parra*, Elisa Otero-Franqui , Surangani F. Dharmawardhane Flanagan . Univ. of Puerto Rico Med. Science Campus, San Juan, PR.

Manuscripts

- **de la Parra, C**., Otero-Franqui, E; Martinez-Montemayor, M; Dharmawardhane. 2012. The soy isoflavone equol may increase cancer malignancy via upregulation of eukaryotic protein synthesis initiation factor eIF4G. Journal of Biological Chemistry. Volume 50, Number, 10. pp.41640-50.

  See Appendix

CONCLUSION:

Results with highly metastatic cancer cell lines show that the effects of daidzein from mammary fat pad tumors in nude mice, can be recapitulated by its metabolite equol. The daidzein metabolite equol, significantly upregulates eIF4G and c-Myc, gene and protein expression. Equol increases protein expression of specific mRNAs with IRESs sensitive to eIF4G levels without affecting their gene expression, suggesting a post-transcriptional regulation mechanism. The preference for association with mRNAs that contain IRESs in the polysome fraction from equol-treated cells, indicates that equol increases protein synthesis of IRES containing mRNAs. eIF4G knockdown resulted in a significant reduction in protein expression levels of IRES containing molecules as well of the IRES dependent translation capacity without affecting cell viability or overall translation. Therefore, the elevated protein synthesis of mRNAs with IRESs by cap dependent protein synthesis is due to the increased eIF4G in response to equol. However, equol may exert additional pro-cancer effects on cell viability via increased c-Myc expression. In conclusion, we have shown that the daidzein metabolite equol may act as a potent regulator of the cancer-promoting effects of dietary daidzein. We have demonstrated that differential regulation of eIF4G levels by the isoflavone daidzein, equol, can directly contribute to breast cancer progression. Futures experiments during the third year of the award such as polysome profiling in response to equol in cells expressing control or eIF4G shRNA will corroborate this conclusion.

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APPENDICES

1- Manuscripts

- **de la Parra, C**., Otero-Franqui, E; Martinez-Montemayor, M; Dharmawardhane. 2012. The soy isoflavone equol may increase cancer malignancy via upregulation of eukaryotic protein synthesis initiation factor eIF4G. Journal of Biological Chemistry. Volume 50, Number, 10. pp.41640-50.

2- Poster Presentation Confirmations

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 50, pp. 41640–41650, December 7, 2012

The Soy Isoflavone Equol May Increase Cancer Malignancy via Up-regulation of Eukaryotic Protein Synthesis Initiation Factor eIF4G*

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Background: The molecular mechanisms of soy isoflavones in metastatic cancer remain to be elucidated.
Results: Equol, a daidzein metabolite, regulates eIF4G-mediated cap-independent protein synthesis initiation of proteins relevant for cancer malignancy.
Conclusion: Equol is a potent regulator of the cancer promoting effects of dietary daidzein.
Significance: Consumption of soy may not be advisable for patients with aggressive breast cancer.

Dietary soy is thought to be cancer-preventive; however, the beneficial effects of soy on established breast cancer is controversial. We recently demonstrated that dietary daidzein or combined soy isoflavones (genistein, daidzein, and glycitein) increased primary mammary tumor growth and metastasis. Cancer-promoting molecules, including eukaryotic protein synthesis initiation factors (eIF) eIF4G and eIF4E, were up-regulated in mammary tumors from mice that received dietary daidzein. Herein, we show that increased eIF expression in tumor extracts of mice after daidzein diets is associated with protein expression of mRNAs with internal ribosome entry sites (IRES) that are sensitive to eIF4E and eIF4G levels. Results with metastatic cancer cell lines show that some of the effects of daidzein *in vivo* can be recapitulated by the daidzein metabolite equol. *In vitro*, equol, but not daidzein, up-regulated eIF4G without affecting eIF4E or its regulator, 4E-binding protein (4E-BP), levels. Equol also increased metastatic cancer cell viability. Equol specifically increased the protein expression of IRES containing cell survival and proliferation-promoting molecules and up-regulated gene and protein expression of the transcription factor c-Myc. Moreover, equol increased the polysomal association of mRNAs for p 120 catenin and eIF4G. The elevated eIF4G in response to equol was not associated with eIF4E or 4E-binding protein in 5′ cap co-capture assays or co-immunoprecipitations. In dual luciferase assays, IRES-dependent protein synthesis was increased by equol. Therefore, up-regulation of eIF4G by equol may result in increased translation of pro-cancer mRNAs with IRESs and, thus, promote cancer malignancy.

Isoflavones found primarily in legumes and particularly in soy are a major class of phytoestrogens that are structurally and/or functionally similar to 17β-estradiol (1). These compounds have received increasing attention for their potential estrogenic or antiestrogenic effects, leading to concerns surrounding the use of phytoestrogen supplements in breast cancer patients who may overexpress estrogen receptors in the tumor tissue (2). Because soy foods have anticancer effects at early stages of carcinogenesis, most studies have focused on breast cancer prevention by soy isoflavones (3). However, the benefits of soy foods as chemopreventives for established breast cancer or as substitutes for hormone replacement therapies remain controversial (3–5).

The second most prominent isoflavone found in soybeans and soy products is the aglycone form daidzein. Intestinal bacteria are central to the absorption and metabolism of isoflavones. After oral ingestion, glucosidases metabolize the β-glycosidic isoflavone daidzin into the bioavailable aglycone daidzein (6). Daidzein can be further metabolized to equol; before final absorption, the intestinal microflora converts daidzein to equol or *O*-desmethylangolensin (Fig. 1). Rodents are efficient producers of equol. However, not all humans have the gut microflora necessary to convert daidzein to equol, and ~30–50% of humans are equol producers. The proportion of equol producers also vary with demographic, lifestyle factors, and ethnicity, and certain populations (*e.g.* Chinese) have been shown to be high equol producers (5, 7). This variation in equol production may explain the discrepancies found in epidemiological studies on the risks or benefits of dietary soy (5, 6, 8–10).

Unlike the metabolite *O*-desmethylangolensin, which has low biological activity (6, 11), equol is structurally similar to estrogen with 80 times more estrogen receptor-β (ERβ)[2] affinity than its precursor daidzein (11–13). Equol has been implicated with decreased prostate cancer cell proliferation and prostate cancer risk by acting as an antagonist for dihydrotest-

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[3] The abbreviations used are: ER, estrogen receptor; eIF, eukaryotic protein synthesis initiation factor; IRES, internal ribosome entry site; 4E-BP, 4E-binding protein; qRT-PCR, quantitative real-time reverse transcriptase-PCR; CCND1, Cyclin D1; m7GTP, 7-methyl-GTP; B2M, β2 microglobulin.

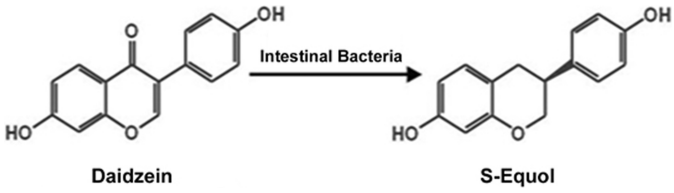

FIGURE 1. **Daidzein is metabolized to equol by intestinal bacteria.**

erone (14, 15). In ER (+) T47D and MCF-7 human breast cancer cells, equol increased estrogenic activity and cell proliferation, but dietary equol did not affect tumor growth in nude mice (16–19). Dietary daidzein also failed to reduce chemically induced mammary tumor growth in rats that demonstrated ~1 μM equol in the serum (20). Others have shown that equol inhibited growth and invasion of ERα (−) ERβ (+) human breast cancer cells and induced cell cycle arrest and apoptosis (14, 21–23). However, caution must be exercised when interpreting *in vitro* studies because the inhibitory effects of equol in breast cancer cells were observed at concentrations ranging from 50 to 100 μM (14, 22, 23), whereas low concentrations of equol ($\leq$1 μM) increased breast cancer cell proliferation (17, 24). Moreover, dietary soy, where genistein, daidzein, and equol were detected in serum samples, increased mammary epithelial cell proliferation of human subjects (25). Therefore, the association between equol production and cancer risk in humans remains to be adequately characterized (8, 26, 27). Overall, benefits from soy intake are associated with ER (+) breast cancer, and the effect of equol or soy isoflavones on ER (−) breast cancers or established aggressive breast cancers are not well understood (5, 28, 29).

Our recent data using ER (−) highly metastatic MDA-MB-435 human cancer cells reported that dietary daidzein and soy isoflavones (daidzein:genistein:glycitein, 5:4:1) increased mammary tumor growth and metastasis in nude mice (30). PCR analysis of mammary tumors demonstrated that dietary daidzein up-regulated the expression of a number of genes that regulate cell proliferation and survival including *CCND1*, *CTNNB1* (catenin (cadherin-associated protein) β1), *GRB2* (growth factor receptor-bound protein 2), *JUN* (Jun oncogene), *MAPK1* (mitogen-activated protein kinase 1), and *IRS1* (insulin receptor substrate 1). Of note was the significant up-regulation of eukaryotic initiation factor 4G (*EIF4G1*) and increased eIF4G and eIF4E protein levels in tumors after daidzein diets (30). Increased levels of eIF4F family members such as eIF4E, -G, and -B have been implicated with specific translation of tumor survival and malignancy-promoting proteins that have mRNAs with long structured 5′-untranslated regions (UTR) and/or internal ribosome entry sites (IRES) (31–33).

The present study was initiated to test the hypothesis that dietary daidzein promotes cancer progression via increased synthesis of cancer promoting molecules. We show that the isoflavone daidzein may promote cancer through the metabolite equol. Equol-mediated eIF4G up-regulation can contribute to non-canonical, eIF4E-independent and, thus, 5′-7-methylguanosine (M^7G) cap-independent protein synthesis via IRES sites (33, 34). Therefore, equol may specifically direct the synthesis of IRES-containing mRNAs that induce cell survival and cell proliferation and promote cancer malignancy.

EXPERIMENTAL PROCEDURES

Cell Culture—Metastatic variant of MDA-MB-435 (ER−) (gift of Dr. Danny Welch, The University of Kansas Cancer Center) and MDA-MB-231 (ERα-, ERβ+) metastatic human breast cancer cells (American Type Culture Collection, Manassas, VA) were maintained in complete culture medium: Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C in 5% CO_2.

Cell Treatment—Quiescent metastatic cancer cells were treated with 0 (vehicle, 0.1% DMSO), 1, 5, 10, 25, or 50 μM isoflavone daidzein (LC Laboratories, Woburn, MA) or metabolite (R,S) Equol (LC Laboratories, Woburn, MA) in DMEM and 5% FBS media for 24 or 48 h.

Tumor Model—The tumors were derived from our previous study (30). Briefly, female athymic nu/nu mice, 5 weeks old (Charles River Laboratories, Wilmington, MA), were inoculated at the mammary fat pad with green fluorescent protein (GFP)-tagged-MDA-MB-435 cells. After 1 week of tumor inoculation, vehicle (10% ethanol, 90% corn oil), 10 mg/kg body weight (BW) of daidzein or combined soy isoflavones 10 mg/kg BW genistein, 9 mg/kg BW daidzein, and 1 mg/kg BW glycitein were administered 3 times a week by oral gavage for 11 weeks. After necropsy, mammary tumors were excised and stored snap-frozen in liquid nitrogen.

Western Blotting—Cells and tumors were lysed and Western blotted as described in Ref. 30. Primary antibodies to eIF4E, phospho (P)-eIF4E$^{Ser\text{-}209}$, eIF4G, p-eIF4G$^{Ser\text{-}1108}$, 4E-BP1, p4E-BP1$^{Thr\text{-}37/46}$, c-Myc, p120 catenin, p-p120$^{Thr\text{-}916}$, β-catenin, survivin, Bcl-XL, Bcl2, vascular endothelial growth factor (VEGF), cyclin D, Jun B, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β actin (Epitomics, Burlingame, CA, Cell Signaling, Danvers, MA; Sigma) were used. Data from mouse mammary tumors were normalized to GFP expression to ensure quantification of proteins from GFP-MDA-MB-435 cells using anti-GFP antibody (Abcam, Cambridge, MA). The integrated density of positive bands was quantified using Image J software, as described in Ref. 30.

Cell Viability Assay—Cell viability was determined by the CellTiter 96 Non-Radioactive Cell Proliferation kit according to the manufacturer's instructions (Promega, Madison, WI). Briefly, quiescent 1×10^5 MDA-MB-435 cells were added to the wells of a 96-well plate and treated for 24 h with vehicle, 1, 5, 10, 25, or 50 μM equol. After equilibration, 15 μl/well MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reagent was added, and the plates were incubated at 37 °C for 4 h. Stop solution (100 μl) was added to each well, and the plates were incubated to facilitate solubilization of newly formed formazan salts. The absorbance at 570 nm was measured using an ELISA plate reader.

Quantitative Real-time Reverse Transcriptase Polymerase Reaction (qRT-PCR) Analysis—As described in Ref. 35, qRT-PCR analysis was performed from cells treated with vehicle or equol for 24 h. Total RNA was extracted using the Qiagen RNeasy Kit (Qiagen, Valencia, CA). RNA concentration was detected using a NanoDrop (Thermo Scientific, Wilmington, DE). RNA (0.5 μg) was used to synthesize cDNA using iScript cDNA synthesis kit (Bio-Rad). Real-time PCR primers were as

follows. *MYC*, forward (5′-TTCTCAGAGGCTTGGCGGGAAA-3′) and reverse (5′-TGCCTCTCGCTGGAATTACTACA-3′); β2 microglobulin (*B2M*) forward (5′-GGCTATCCAGCGTACTCCAAA-3′) and reverse (5′-CGGCAGGCATACTCATCTTTTT-3′); *GAPDH* (forward (5′-TTGCCATCAATGACCCCTTCA-3′) and reverse, (5′-CGCCCCACTTGATTTTGGA-3′); *CCND1* (forward (5′-TGGTGAACAAGCTCAAGTGGA-3′) and reverse (5′-TGATCTGTTTGTTCTCCTCCGCCT-3′); *VEGFA* forward, 5′-AGGCGAGGCAGCTTGAGTTAAA-3′ and reverse (5′-TTCTGTCGATGGTGATGGTGTGGT-3′); *EIF4G* forward (5′-TTGTGGATGATGGTGGCT-3′ and reverse (5′-TTATCTGTGCTTTCTGTGGGT-3′); CTNND1 forward (5′-TCCAGCAAACGATACAGTGG-3′) and reverse (5′-GAACCACCTCTGGCTGAAAT-3′). Real-time reactions were performed using iQ SYBR-Green PCR Master Mix (Bio-Rad). The amplification reaction was performed for 40 cycles (10 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C). B2M mRNA was used as an internal control. The -fold change was determined by the $2^{\Delta\Delta CT}$ method as described in Refs. 30 and 35.

Polysomal Fractionation—Sucrose density gradient centrifugation was used to separate the subpolysomal and polysomal fractions as in Refs. 32 and 36. MDA-MB-435 cells were treated with vehicle or equol (25 μM) for 24 h. Five minutes before harvest, 100 μg/ml cycloheximide was added to the culture medium. Cells were washed in ice-cold phosphate-buffered saline supplemented with 100 μg/ml cycloheximide and harvested in polysome lysis buffer (10 mM Tris-HCl at pH 7.4, 40 mM KCl, 3 mM $MgCl_2$, 5% glycerol, 0.2% Nonidet P-40, 150 μg/ml cycloheximide, 1 mM PMSF, 20 mM DTT, 200 μg/ml heparin). Samples were incubated on ice for 10 min and centrifuged at 12,000 × *g* for 10 min at 4 °C. The resulting supernatant was layered onto 10–50% sucrose density gradients and centrifuged in a Beckman SW41 rotor at 35,000 rpm for 3 h at 4 °C. The A_{260} of sucrose density gradient fractions (200 μl) was determined through the fractions collected from top to bottom. Consecutive fractions were pooled, generating a total of nine fractions. Sucrose density gradient fractions were resuspended in guanidine thiocyanate buffer containing 10% mercaptoethanol (RLT buffer, RNeasy Mini Kit, Qiagen). RNA was extracted using the RNeasy Mini Kit for isolation of total RNA (Qiagen) following the manufacturer's instructions. RNA preparations from each fraction were subjected to qRT-PCR for *CTNND1* (p120-catenin), *GAPDH*, and *EIF4G* as described above.

Cap Affinity Chromatography—Cell lysates, after vehicle or 25 μM equol treatment for 24 h, were incubated with 7-methyl-GTP (*m7GTP*) or control Sepharose 4B beads (Amersham Biosciences) for 1 h at 4 °C as described in Ref. 37–39). Total lysates, washed beads after m^7GTP co-capture, and the supernatants were Western-blotted for eIF4E, 4E-BP, or eIF4G.

eIF4G Immunoprecipitation—MDA-MB-435 cells were treated with vehicle or 25 μM equol for 24 h. Cells were lysed in radioimmune precipitation assay buffer (50 mM HEPES, pH 7.0, 2 mM EDTA, 250 mM NaCl, 50 mM NaF, 25 mM $Na_4O_7P_2$, 2 mM Na_3VO_4, 1 mM PMSF, 0.1 mM DTT, and 0.5% IGEPAL), and 500 μg each of total protein extracts were incubated with anti-eIF4G antibody (Cell Signaling) (1:50) or control antibody (1:50) for 2 h at 4 °C followed by incubation with protein A Sepharose (cell signaling) for an additional hour. Immunoprecipitates were washed and processed for SDS-PAGE and Western blotting as described in Ref. 40. Immunoprecipitates of eIF4G antibody or control monoclonal antibody, supernatants, and total lysates were immunoblotted with anti-eIF4G (top half of gel) or anti-eIF4E (bottom half of same gel) to visualize eIF4G as a 220-kDa band and eIF4E as a 25-kDa band.

Luciferase Reporter Assays—MDA-MB-435 cells were transfected with a bicistronic reporter system (a kind gift of Dr. Robert Schneider, New York University Langone Medical Center) or control plasmid containing the luciferase constructs without IRES using Lipofectamine 2000 (Invitrogen) as the per manufacturer's directions. As described in Ref. 32, this plasmid contains a cap-dependent *Renilla* luciferase followed by a 5′-UTR containing the p120 catenin IRES-mediated firefly luciferase. 24 h after transfection, cells were treated with equol for an additional 24 h. The relative IRES activity was analyzed as 570-nm firefly luciferase/480-nm *Renilla* luciferase in a luminometer using a dual luciferase assay kit (Promega) according to the manufacturer's instructions.

Statistical Analysis—Data were analyzed and reported as the mean ± S.E. in triplicate. Statistical analyses were done using Microsoft Excel and GraphPad Prism. Differences between means were determined using Student's *t* test, and $p \leq 0.05$ was considered significant.

RESULTS

Dietary Daidzein Up-regulates Expression of eIF4G and eIF4E and Increased Protein Levels of mRNAs with IRES Sites in Vivo but Not in Vitro—We recently reported that daidzein increased mammary tumor growth and metastasis in nude mice with mammary tumors established from the ER (−) highly metastatic human cancer cell line MDA-MB-435. Mammary tumors from mice treated with daidzein diets demonstrated a significant 2–3-fold up-regulation of *EIF4G1* gene and protein expression and a ~7.0-fold increase in eIF4E protein levels compared with vehicle controls. Combined soy treatment resulted in a 1.8-fold increase in *EIF4E* gene and a 2.5-fold increase in protein expression (30).

High levels of eukaryotic initiation factors, specifically eIF4G1, have been correlated with increased cap independent translation of specific mRNAs that contain IRESs and long-structured 5′-UTRs (32, 33, 41). To investigate the effect of the overexpressed eIF4F complex on translation of mRNAs sensitive to elevated eIF4F initiation factors, we analyzed protein expression levels of pro-survival, -angiogenesis, and -proliferation molecules known to have mRNAs with long UTRs and/or IRESs (32, 33, 42) from primary tumors of mice after daidzein or combined soy (genistein:daidzein:glycitein, 5:4:1) diets. As shown in Fig. 2, the pro-survival proteins survivin (2-fold), Bcl2 (2.2-fold), and Bcl-XL (2.3-fold) and total and active phospho-p120 catenin (8- and 4-fold, respectively) were significantly increased in tumors after daidzein diets. Combined soy also demonstrated significant increases in expression of Bcl-XL (4-fold) and VEGF (2.3-fold). However, the expression of β-actin, a constitutively expressed mRNA with a short 5′-UTR was not affected by dietary soy isoflavones. Both actin and GFP expression were used as standards for the analysis of -fold dif-

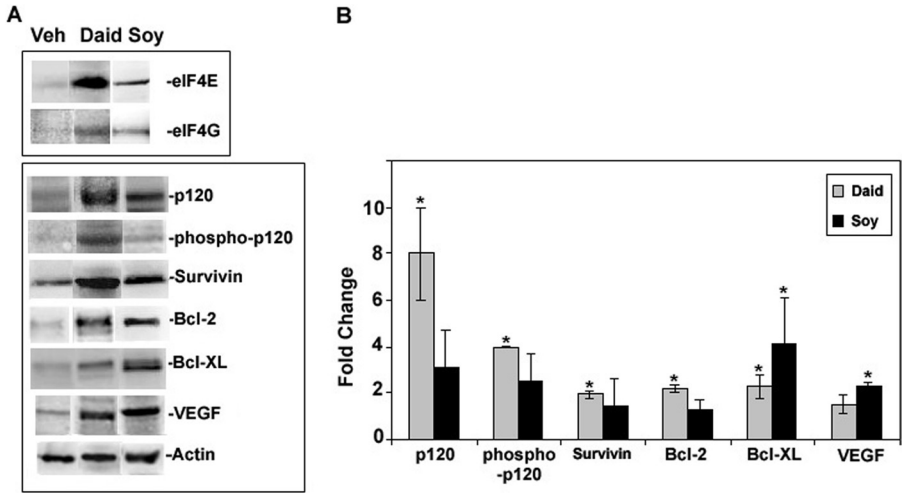

FIGURE 2. **Effect of soy isoflavones on protein expression in mammary fat pad tumors from mice treated with vehicle (*Veh*), genistein, daidzein (*daid*), or soy isoflavones (genitein:daidzein:glycitein (5:4:1).** Mammary fat pad tumors established from GFP-MDA-MB-435 cells from the study described in Ref. 30 were lysed, and the proteins were extracted. *A*, shown are representative Western blots from tumor extracts immunostained for cancer promoting molecules. These bands are representative of $n = 3–4$, derived from the same gel for all treatments. *B*, shown are -fold changes of protein expression compared with vehicle as calculated from the integrated density of positive bands from Western blots and normalized with actin and GFP expression. Values show the mean ± S.E. ($n = 3$). An *asterisk* indicates statistical significance of $p \leq 0.05$.

ferences of IRES containing molecules compared with vehicle controls to ensure analysis of GFP-MDA-MB-435 cells.

This data implicate dietary daidzein in eIF4F-controlled translation of proteins that regulate cancer progression. Therefore, the molecular mechanisms of daidzein action were further investigated *in vitro* using the same cell line from the *in vivo* study, ER (−) MDA-MB-435 cells as well as the ERβ (+) metastatic breast cancer cell line MDA-MB-231. Cells were treated with vehicle or daidzein at 0–50 μM. These concentrations fall within the range of 1–10 μM that has been shown to accumulate in the circulation after consumption of soy products (43). However, we did not detect any significant changes in eIF4E, p-eIF4E, eIF4G, or p-eIF4G in both cell lines after 24 or 48 h treatment of daidzein at all concentrations tested. There were slight increases in eIF4G and p-eIF4G in MDA-MB-231 cells treated with 25 and 50 μM daidzein; nevertheless, these increases were not statistically significant (Fig. 3).

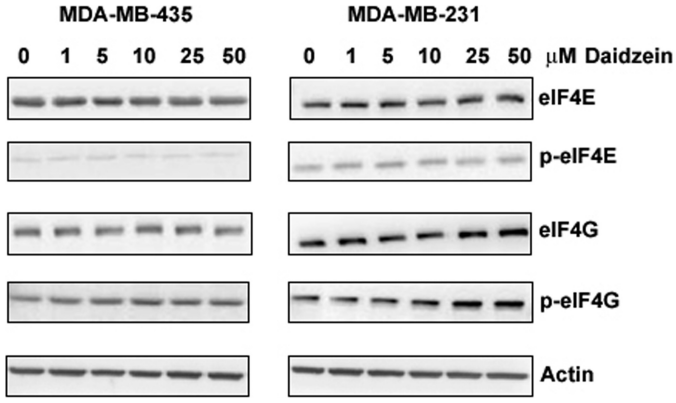

FIGURE 3. **Effect of daidzein on total and phospho (*p*) eIF4E and eIF4G expression in MDA-MB 435 and MDA-MB-231 cells.** Quiescent cells were treated with vehicle or daidzein (0–50 μM) in 5% serum for 24 h, lysed, and Western-blotted with mono-specific antibodies. *Left*, representative Western blots of MDA-MB 435 cell lysates ($n = 3$) are shown. *Right*, representative Western blots of MDA-MB-231 cell lysates ($n = 3$) are shown.

The Daidzein Metabolite Equol Up-regulates Gene and Protein Expression of eIF4G and c-Myc and Protein Expression of mRNAs with IRESs—Daidzein can be further metabolized to equol (70%) and *O*-desmethylangolensin (5–20%) (Fig. 1). In rodents, equol is the major circulating metabolite, and all rodents are equol producers (6, 11). Therefore, we reasoned that the daidzein effects on MDA-MB-435 metastatic cell lines *in vivo* may be due to the metabolite equol. MDA-MB-435 and MDA-MB-231 cells were treated with (*R*,*S*)-equol at different concentrations (0–50 μM) and tested for eIF4E and eIF4G expression by Western blotting.

Fig. 4 demonstrates that similar to dietary daidzein in mice, equol increased the expression of total and p-eIF4G in a concentration-dependent manner. Elevation of eIF4G preceded the p-eIF4G levels. Therefore, equol may not specifically affect phosphorylation of eIF4G, but only eIF4G expression. In MDA-MB-435 cells, equol at 25 and 50 μM significantly increased eIF4G protein levels by ~1.8-fold ($p \leq 0.05$) compared with vehicle controls. The increase in eIF4G in the MDA-MB-231 cell line was more modest (~1.3-fold) but consistent at similar concentrations (>10 μM). It is possible that the presence of ERβ in the MDA-MB-231 cell line may exert a differential effect on equol-mediated eIF4G expression. The protein levels of eIF4E and its inhibitory protein 4E-BP remained unchanged at all concentrations of equol tested in both cell lines, indicating a specific effect on eIF4G expression and not eIF4E expression or regulation.

Similar to the effect of dietary daidzein on mammary tumor growth in nude mice, we found that equol enhanced cancer cell viability. Treatment of equol (0–50 μM) to MDA-MB-435 cells increased cell viability starting at 1 μM in a concentration-dependent and statistically significant manner (Fig. 5). This increase in cell number reflects the effect of equol on gene and protein expression of eIF4G.

A recent study from ER (−) inflammatory breast cancer cells demonstrated that overexpression of eIF4G with no changes in the cap-binding protein eIF4E and its negative regulator

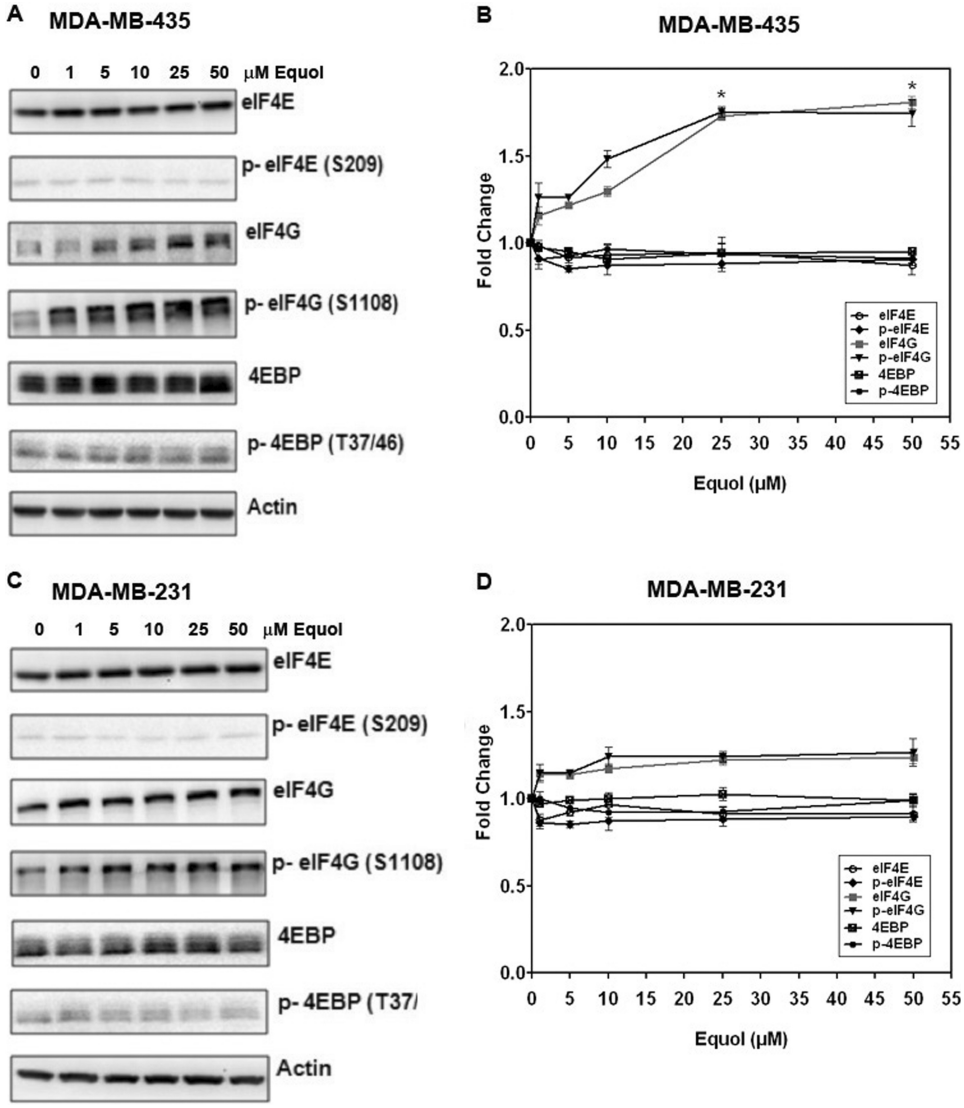

FIGURE 4. **Effect of equol on total and phospho (*p*) eIF4E, eIF4G, and 4E-BP expression in MDA-MB 435 and MDA-MB-231 cells.** Quiescent cells were treated with vehicle or equol (0–50 μM) for 24 h, lysed, and Western-blotted with mono-specific antibodies. *A* and *B*, shown are representative Western blots and -fold changes relative to vehicle, as quantified from Image J analysis of integrated density of positive bands of MDA-MB 435 cell extracts. *C* and *D*, shown are representative Western blots and -fold changes relative to vehicle, as quantified from Image J analysis of integrated density of positive bands of MDA-MB-231 cell extracts. Values show the mean ± S.E. ($n = 3$). An *asterisk* indicates statistical significance of $p < 0.05$.

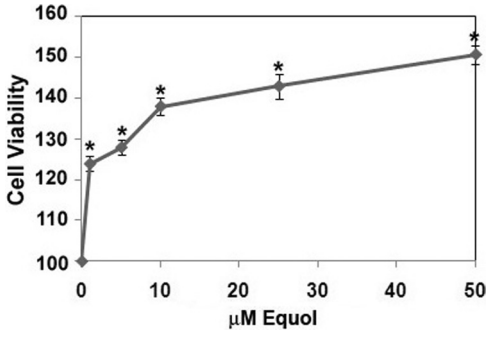

FIGURE 5. **Effect of equol on cell viability.** Quiescent MDA-MB-435 cells were treated with 1–50 μM equol or vehicle for 24 h. Cells were lysed and subjected to an MTT ((3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide)) assay. Results are shown relative to vehicle (100%). $n = 3$ for all experiments. An *asterisk* indicates statistical significance of $p \leq 0.05$.

4E-BP1 promotes cap-independent protein synthesis of IRES-containing mRNAs (32). The protein products of these mRNAs have been shown to regulate cancer cell survival and proliferation (44–48). To determine whether increased eIF4G in response to equol may drive IRES-dependent protein synthesis, we tested the protein and gene expression of mRNAs with or without IRESs.

Similar to the effect of dietary daidzein in MDA-MB-435 tumors, equol at 10, 25, and 50 μM up-regulated protein expression of IRES-containing mRNAs: survivin, c-Myc, Bcl-2, Bcl-XL, cyclin D, VEGF, and total and active p120 catenin in the MDA-MB-435 cells by ~1.3–2.0-fold compared with vehicle. Unlike the other proteins that demonstrated up-regulated expression after 5–50 μM equol, c-Myc protein expression was increased by ~1.8-fold at all concentrations of equol tested (1–50 μM). Equol treatment at 25 μM resulted in a 2.0-fold significant increase in protein expression of Cyclin D, but

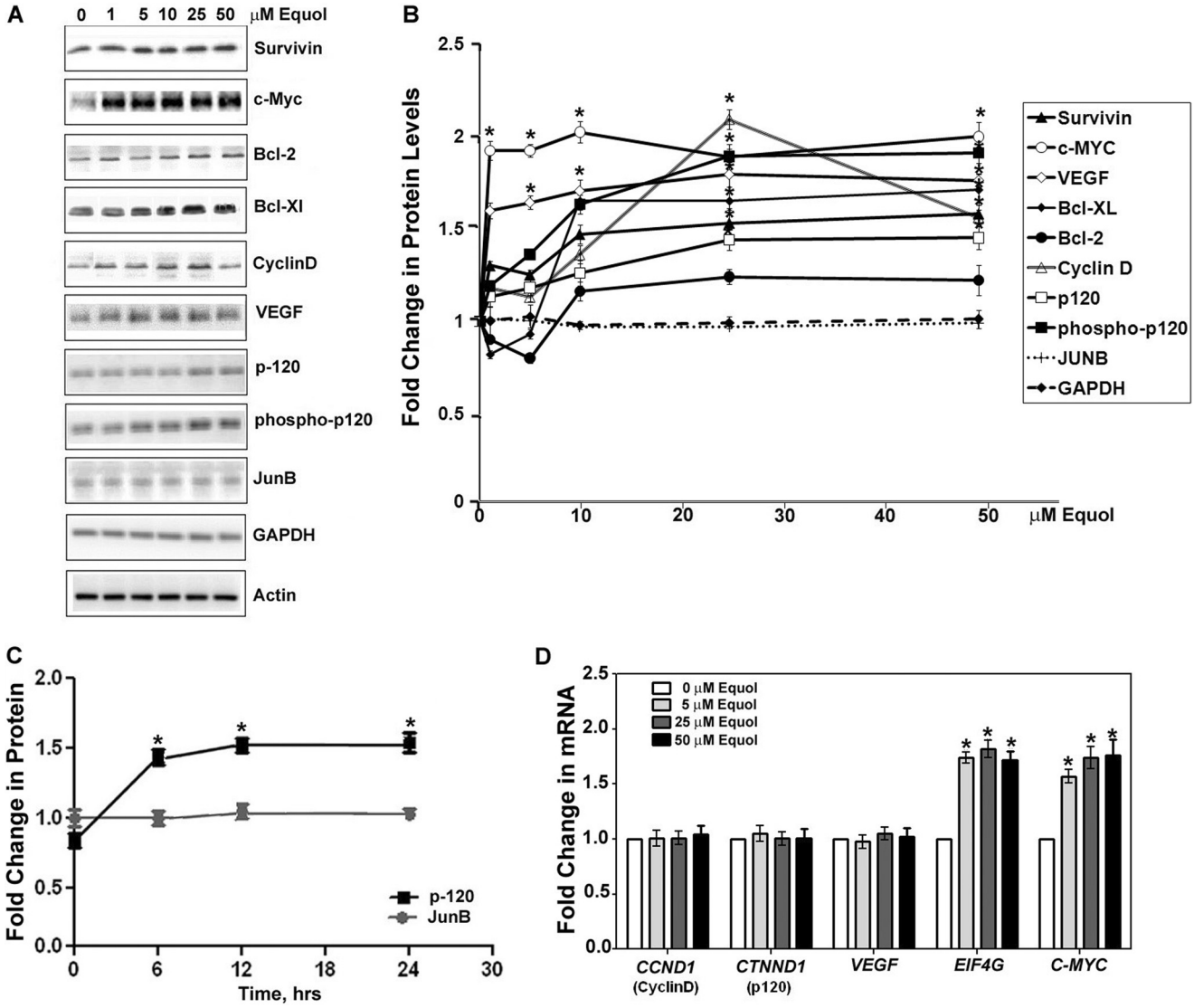

FIGURE 6. **Expression of cancer promoting molecules after equol treatment.** Quiescent MDA-MB-435 cells were treated with vehicle or 1–50 μM equol for 24 h, lysed, and Western-blotted with specific antibodies to the indicated proteins. *A*, representative Western blots are shown. *B*, shown are -fold changes relative to actin as calculated by Image J analysis of positive bands from equol treatments (1–50 μM) compared with vehicle controls. $n = 3$. An *asterisk* indicates statistical significance of $p < 0.05$. *C*, shown is -fold change in protein expression as a function of time in equol. Quiescent MDA-MB-435 cells were treated with vehicle or 25 μM equol for various times as indicated and subjected to lysis and Western blotting for p120 catenin or JunB. -Fold changes relative to actin were calculated by Image J analysis of positive bands from equol treatments compared with vehicle controls ($n = 3$). An *asterisk* indicates statistical significance of $p < 0.05$. *D*, shown is the effect of equol on gene expression of cancer-promoting molecules. Quiescent MDA-MB-435 cells were treated for 24 h with 5–50 μM equol, and *CCND1*, *CTNND1*, *VEGF*, *EIF4G*, or *MYC* expression was quantified by qRT-PCR. -Fold changes in gene expression from cells treated with equol are compared with vehicle ($n = 3$). An *asterisk* indicates statistical significance of $p < 0.05$.

this increase dropped off to ~1.4-fold at 50 μM equol (Fig. 6, *A* and *B*).

Proteins with mRNAs with short 5′-UTRs that do not contain IRESs (JunB, GAPDH, and actin (49, 50)) were not increased in response to all concentrations of equol tested (0–50 μM) (Fig. 6, *A* and *B*). In contrast to JunB, equol increased protein expression of p120-catenin, a molecule with an IRES containing mRNA, by 1.5-fold 6 h after treatment, which remained constant up to 24 h in equol (Fig. 6*C*).

To determine whether the increased protein expression in response to equol was due to an increase in gene expression, the expression of representative IRES-positive mRNAs was determined by qRT-PCR of cell lysates after vehicle or equol treatment. The IRES containing mRNAs CCND1 (cyclin D), *CTNND1* (p120 catenin), and *VEGF* did not change in response to equol. However, 5–50 μM equol up-regulated gene expression of eIF4G and c-Myc (Fig. 6*D*).

Because the effect of equol on increased protein levels of IRES-containing mRNAs saturated at 25 μM equol for 24 h, we selected these conditions for the subsequent *in vitro* assays. This concentration of equol has been found in the urine of humans after consumption of soy foods (51).

To determine whether the up-regulated eIF4G levels in response to equol resulted in increased protein synthesis initiation, we performed polysomal fractionations of cell lysates after vehicle or equol. The ribosomal and polysomal fractions were isolated from sucrose gradients, and potential association of mRNAs was determined by A_{260} measurements. Compared

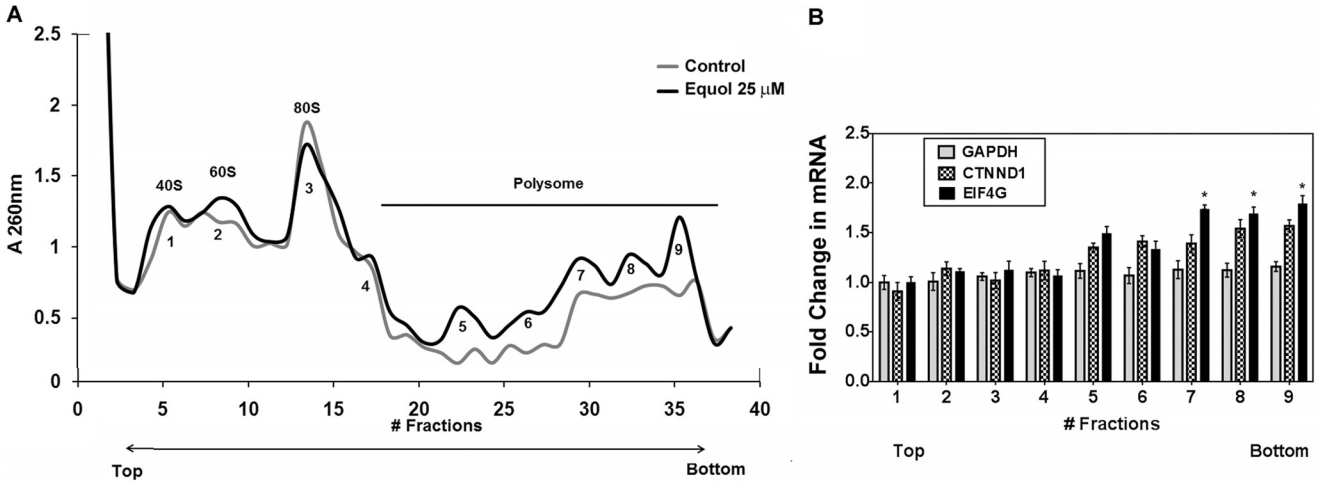

FIGURE 7. **Analysis of polysome profiles.** Equivalent amounts of total cell lysate from vehicle control or 25 μM equol-treated MDA-MB-435 cells were loaded onto10–50% sucrose gradients. The UV absorbance of pooled sequential gradient fractions (numbered consecutively) was measured at 260 nm. *A*, shown is the average A_{260} of fractions from the sucrose gradient for control or equol-treated cells. The 40 S, 60 S, and 80 S fractions were classified as non-polysome fractions. All subsequent fractions were classified as polysome fractions (4–9). $n = 3$. *B*, shown is mRNA associated with polysome fractions. These fractions were used to detect *GAPDH*, *CTNND1*, and *EIF4G* mRNA by qRT-PCR. B2M was used as an internal control. Results are shown as the -fold changes in equol-treated cells relative to vehicle controls ($n = 3$). An *asterisk* indicates statistical significance of $p < 0.05$.

with vehicle treatments, equol increased the total mRNA associated with the polysomal fractions (Fig. 7*A*). qRT-PCR analyses for *GAPDH*, *CTNND1*, and *EIF4G* (with *B2M* as the control) demonstrated that the IRES containing mRNAs *CTNND1* and *EIF4G*, but not *GAPDH*, were associated with the heavier polysomal fractions from equol-treated cells. *CTNND1* mRNA was increased by 1.5-fold, and *EIF4G* was significantly increased by 1.7-fold in heavier polysomal fractions from equol-treated cells compared with vehicle controls (Fig. 7*B*). Similar to the results on protein expression, association of the IRES-negative *GAPDH* mRNA was not changed by equol treatment. Therefore, the observed equol-mediated up-regulation of p120-catenin protein expression without changes in *CTNND1* gene expression (Fig. 6) may indicate preferential synthesis of IRES containing mRNAs in equol-treated cells. The enhanced affinity of *EIF4G* for the polysome fractions indicates that the *EIF4G* mRNA elevated in response to equol is translated into protein, thus accounting for the elevated eIF4G protein levels.

To determine if the increased eIF4G in response to equol was associated at the 5′ cap with eIF4E, synthetic m^7GTP co-capture assays were performed from MDA-MB-435 cell lysates treated with vehicle or equol. Fig. 8*A* demonstrates that total, m^7GTP-bound, or free eIF4E or 4E-BP levels remain unchanged after equol treatment. This is consistent with our results that showed no effect of equol on phospho or total eIF4E or 4E-BP protein expression (Fig. 4), indicating that equol does not affect eIF4E expression or activity. Intriguingly, equol treatment significantly decreased the amount of eIF4G co-captured with eIF4E in the m^7GTP beads by ~75% compared with vehicle controls. However, there was a 3-fold increase in eIF4G levels recovered in the total cell lysate and the free pool of eIF4G in the m^7GTP pulldown assays (supernatants). This result indicates that the equol-mediated elevated eIF4G is not associated with cap-dependent protein synthesis.

Fig. 8*C* shows the association of eIF4E and eIF4G from anti-eIF4G immunoprecipitates that pulled down equal amounts of eIF4G from vehicle or equol-treated cells. Equol treatment resulted in a statistically significant 32% less eIF4E co-immunoprecipitating with eIF4G. The excess eIF4E was recovered in the supernatants after equol, demonstrating reduced association of eIF4G and eIF4E after equol treatment. The increased eIF4G in response to equol was also recovered in the supernatant.

Dual luciferase assays for cap-dependent and cap-independent, IRES-mediated protein synthesis were performed to determine a function for the excess eIF4G that is not associated with eIF4E. As shown in Fig. 8*D*, equol treatment of MDA-MB-435 cells, specifically increased IRES-driven firefly luciferase activity by 1.6-fold compared with vehicle ($p \leq 0.01$).

DISCUSSION

eIF4F family initiation factors that include eIF4E and eIF4G are overexpressed in advanced cancers and have been shown to be essential for translation of a subset of proteins that regulate cellular bioenergetics, survival, and proliferation (32, 33, 41, 42, 44–48, 52, 53). These pro-cancer mRNAs often contain IRESs and are preferentially translated by elevated eIF4G in malignant cancer cells (32, 33, 54). Therefore, we hypothesized that our previously reported dietary daidzein and combined soy isoflavone-mediated increases in eIF4G and eIF4E expression in mammary tumors (30) may result in enhanced translation of IRES-containing pro-cancer mRNAs.

Results presented herein demonstrate that the effect of daidzein on eIF4G up-regulation could not be recapitulated *in vitro* with daidzein, but only with equol, the major daidzein metabolite in rodents. We also show that equol increased MDA-MB-435 cell viability similar to the previously reported increase in MDA-MB-435 tumor growth in response to dietary daidzein (30). Therefore, the observed effects of enhanced tumor growth and metastasis by dietary daidzein and soy isoflavones may, at least in part, be due to the metabolite equol.

Dietary daidzein significantly increased eIF4E protein levels and combined soy increased both gene and protein expression

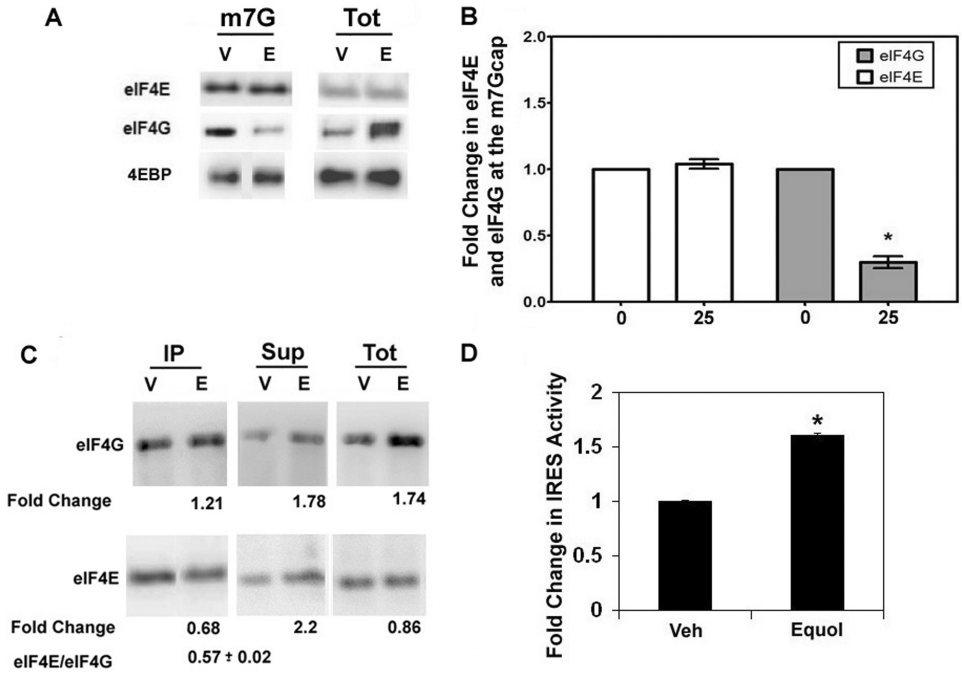

FIGURE 8. **Potential regulation of IRES-dependent protein synthesis initiation by equol.** *A* and *B*, *m7G* co-capture assays are shown. Quiescent MDA-MB-435 cells were treated with vehicle (*V*) or 25 μM equol (*E*) for 24 h, lysed, and incubated with Sepharose 4B-conjugated 7-methyl-GTP. The pulldown assays were washed and analyzed for eIF4G, eIF4E, and 4E-BP associated with the cap, supernatants, or total cell lysates. *A*, shown is a representative Western blot of m^7G-bound proteins and the total protein (Tot) from cell lysates before pulldown assays. *B*, shown are -fold changes in eIF4G and eIF4E after vehicle (0) or equol treatment at the m^7G cap relative to the total levels of eIF4G or eIF4E in cell lysate. $n = 3 \pm$ S.E. An *asterisk* indicates statistical significance of $p \leq 0.05$. *C*, association of eIF4G and eIF4E with anti-eIF4G immunoprecipitates in response to equol is shown. Quiescent MDA-MB-435 cells were treated with vehicle (*V*) or 25 μM equol (*E*) and lysed, and lysates with equal protein were immunoprecipitated using an anti-eIF4G. Representative Western blots stained with eIF4G or eIF4E are shown for immunoprecipitates (*IP*), supernatants (*Sup*), and total protein lysates (*Tot*). The average -fold changes ($n = 3$) from integrated densities of positive bands from equol-treated cells relative to vehicle are shown. *D*, relative IRES-dependent protein synthesis after equol treatment is shown. MDA-MB-435 cells expressing a plasmid with a cap-dependent *Renilla* luciferase followed by a 5′-UTR containing the p120 catenin IRES driving a firefly luciferase or control plasmid without an IRES were treated with vehicle or equol for 24 h. Cells were lysed, and the relative IRES activity analyzed as 570-nm firefly luciferase/480-nm *Renilla* luciferase. IRES activity was quantified relative to control activity for vehicle or equol-treated cells. Results show -fold change in IRES activity compared with vehicle for $n = 3 \pm$ S.E. An *asterisk* indicates statistical significance of $p \leq 0.05$.

of eIF4E (30). However, equol treatment *in vitro* did not affect eIF4E or its negative regulator 4E-BP expression or activity. Thus, soy consumption may have more profound effects on protein synthesis initiation in cancer cells than the effects of equol described in this report.

Equol has been implicated with daidzein activities *in vivo* after ingestion of soy foods and has been shown to be more potent than daidzein *in vitro* (8, 55, 56). Equol is a chiral molecule capable of existing in two enantiomeric forms: *R*-(+) equol and *S*-(−) equol; the latter is the natural diasteromer produced by intestinal bacteria (6, 11). Both equol enantiomers show better uptake and have higher bioavailability (65–83%) than the isoflavones daidzein (30–40%) or genistein (7–15%) (57). Both enantiomers bind ERs, with *R*-equol showing a preference for ERβ (13).

The racemic (*R*,*S*)-equol used in this study demonstrated a more pronounced response in up-regulation of eIF4G in ER (−) MDA-MB-435 cells compared with ERβ (+) MDA-MB-231 cells. This result suggests that the effect of equol on eIF4G expression is not ER-dependent or that the presence of ERβ has a protective effect on the cancer promoting action of equol. To our knowledge *EIF4G* does not have an estrogen response element and, therefore, cannot be directly under the regulation of ER.

A key finding is that Equol up-regulated gene and protein expression of c-Myc at all concentrations tested. The c-Myc transcription factor is one of the most important somatically mutated oncogenes in human cancer and confers a selective advantage to cancer cells by promoting protein synthesis, proliferation, cell survival, differentiation, genetic instability, angiogenesis, hypoxia-mediated cancer progression, and metastasis (33, 33, 47, 62–66). Studies have shown that c-Myc up-regulates both eIF4E and eIF4G gene expression (58). Myc also has an IRES site and thus, in turn, is sensitive to elevated eIF4G and eIF4E levels (48). In MDA-MB-435 cells, c-Myc and eIF4G levels were up-regulated by equol without a concomitant increase in eIF4E. Therefore, similar to a previous report where c-Myc did not affect the eIF4E mRNA or protein levels in a human B cell line (59), it is possible that c-Myc may not regulate eIF4E expression in our system.

MYC has an estrogen response element, and its expression is known to be regulated by estrogen and estrogen mimetics (60) as well as by a plethora of signaling pathways and mechanisms (61). Because the MDA-MB-435 cells are negative for ERα and ERβ but may still express estrogen-related receptors as well as other steroid receptors, equol may activate these receptors to up-regulate *MYC* expression.

The initial equol-mediated elevation of *MYC* and *EIF4G1* gene expression may result in further synthesis of eIF4G and c-Myc via IRES-driven mechanisms, where eIF4G itself has an IRES site (42, 67). We show that mRNAs for eIF4G are preferentially associated with the polysomal fractions from equol-treated cells, indicating enhanced protein synthesis of the elevated *EIF4G* mRNA by equol. Moreover, the majority of the equol-mediated up-regulated eIF4G is phosphorylated, thus suggesting it is functional and available for kinase activity in the cytosol. The functional consequence of phosphorylation of eIF4G in translation is not well established. Recent reports have implicated phosphorylation of eIF4G by PAK2 in inhibition of cap-dependent translation but not IRES-driven translation (77), thus implicating elevated p-eIF4G in response to equol in IRES-dependent protein synthesis initiation.

Our results from equol-treated breast cancer cells substantiate the hypothesis that elevated eIF4G by equol increases protein expression of specific mRNAs with IRESs without affecting their gene expression. Of the proteins that were elevated in response to equol, IRES-containing cyclin D1 and c-Myc up-regulation are hallmarks of cancer that have been directly associated with eIF4G up-regulation (32, 33, 42, 68–70). The cell survival genes survivin, Bcl-2, and Bcl-XL and the angiogenesis promoter VEGF are also sensitive to eIF4G levels, have IRES sites, and are elevated in aggressive cancers (71–74).

In MDA-MB-435 cells, p120 catenin protein expression, but not gene expression, was affected by equol. Moreover, p120-catenin mRNAs from equol-treated cells had a higher affinity for the heavy polysomal fractions from sucrose density gradients. p-120 catenin and phosphorylated $p^{Thr\text{-}916}$-p120 catenin have been shown to stabilize the E-cadherin axis at cell adhesions and are implicated in regulation of Rho GTPase function leading to increased cancer cell invasion (75). However, the E-cadherin axis is lost in the metastatic cancer cells, used in our study, that have undergone epithelial to mesenchymal transition. Therefore, the elevated p120 catenin in response to equol may contribute to cancer progression via enhanced nuclear transcription regulated by free p120 catenin (76).

We also show that more mRNAs were associated with the polysome fraction from equol-treated cells, indicating enhanced protein synthesis initiation. However, equol treatment disassociated eIF4G from eIF4E in 5′ caps, and the excess eIF4G synthesized in response to equol was not associated with eIF4E. Moreover, equol treatment preferentially increased the expression of IRES-driven luciferase relative to a cap-dependent luciferase. Therefore, taken together, our data suggest that equol-mediated up-regulation of eIF4G directs cap-independent protein synthesis initiation of IRES-containing cell survival and pro-proliferation molecules, whereas eIF4E remains at the 5′ cap, bound to 4E-BP (Fig. 9). Nevertheless, these data do not rule out additional effects of equol on cap-dependent protein synthesis initiation, protein stability, or gene expression.

In conclusion, we have shown that the daidzein metabolite equol may act as a potent regulator of the cancer-promoting effects of dietary daidzein. Therefore, consumption of soy foods may not be advisable for patients with ER (−) breast cancer; however, more research needs to be conducted before definitive dietary recommendations.

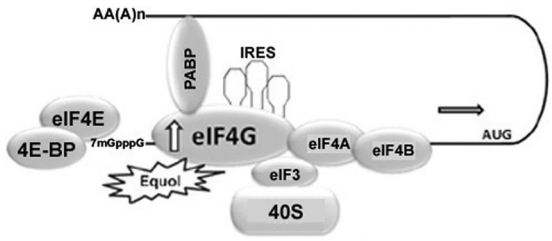

FIGURE 9. **Potential role of equol in protein synthesis regulation.** Increased eIF4G expression by equol is expected to result in enhanced IRES-dependent mRNA translation, whereas eIF4E and 4E-BP remains at the m^7G cap. Poly(A)-binding protein (*PABP*) interacts with the poly(A) tail of the mRNA and eIF4G. eIF4A, eIF4B, and eIF3 interact with eIF4G. eIF3 binds the scaffolding protein eIF4G and the 40 S ribosomal subunit at the IRES.

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Abstract Title: Increased protein synthesis of IRES containing mRNAs, that induce cell survival and proliferation, by the daidzein metabolite equol may contribute to breast cancer malignancy.
First Author: Columba Parra
Poster Session Title: RNA Function and Protein Synthesis
Day of Presentation: Sunday April 21, 2013
Program Number: 551.5
Poster Board Number: C83
Poster Manning Time: Authors must be present at their posters from 12:25 PM - 1:55 PM
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