Inhibition of fatty acid synthase by luteolin post-transcriptionally down-regulates c-Met expression independent of proteosomal/lysosomal degradation

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Abstract

The hepatocyte growth factor (HGF)/c-Met signaling pathway is involved in the progression of several cancers and associated with increased tumor invasion and metastatic potential. We determined previously that the polyphenol epigallocatechin-3-gallate inhibited HGF-induced c-Met phosphorylation in a variety of tumor cell lines in part by disrupting lipid rafts. Fatty acid synthase (FASN) is implicated in cancer progression and may regulate lipid raft function. We therefore examined the effects of luteolin, a potent FASN inhibitor, on c-Met signaling. Luteolin blocked HGF-induced c-Met phosphorylation and scattering of DU145 prostate cancer cells, but inhibition required at least a 4 h preincubation time. Western blot analysis indicated that inhibition of HGF-induced scattering by luteolin occurred coincident with reduction of total c-Met protein in DU145 cells. In addition, luteolin-induced c-Met down-regulation was mimicked by a pharmacologic inhibitor of FASN, C75, or short hairpin RNA knockdown of FASN. Consistent with a role for FASN, loss of c-Met in cells treated with C75 or luteolin was prevented by exogenous addition of palmitate. Luteolin-induced loss of c-Met primarily occurred at a post-transcriptional level and involved cell surface internalization but did not involve translation inhibition, nor was it dependent on the activity of the 26S proteosome or acidic lysosomes. Taken together, our study shows a novel connection between FASN activity and c-Met protein expression and suggests that luteolin could act as a novel HGF/c-Met inhibitor by reducing expression of this receptor. [Mol Cancer Ther 2009;8(1):214–24]

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Introduction

Metastatic cancer is the primary cause of patient mortality, and therapeutic approaches to block this process are urgently needed. A major step of the malignant process is the transition of the stationary cancer cells to a motile mesenchymal-like phenotype (1). This epithelial-to-mesenchymal transition is thought to be important in the loss of cell-cell adhesions and eventual invasion of the basement membrane, a prerequisite for metastasis (2). A major contributor to the promotion of a mesenchymal phenotype is the hepatocyte growth factor (HGF)/c-Met signaling axis. HGF is the only known ligand for the receptor tyrosine kinase c-Met. Binding of HGF to c-Met leads to autophosphorylation of tyrosine residues within the cytoplasmic domain, which function as docking sites for downstream effectors that mediate several cellular responses including proliferation, cell survival, actin remodeling, and motility (3, 4). Prolonged induction of these pathways accounts for the cancer-promoting properties of the HGF/c-Met signaling axis. Overexpression of c-Met and activating mutations are seen in several cancers, including prostate and breast, and are strongly associated with aggressive disease (5–8).

Recently, the interplay between distinct receptor tyrosine kinases has become better understood. For instance, c-Met overexpression leads to tumor cell resistance to epidermal growth factor receptor-targeted therapy through heterodimerization that reestablishes downstream signaling (9). Because of these unique receptor tyrosine kinase partnerships, it has become clear that single target therapies will not be sufficient to inhibit tumor progression and that multimodality therapy, targeting several stimulatory pathways simultaneously, will prove more efficacious.

Phytochemicals, such as flavonoids, represent a source of relatively nontoxic, orally available, and affordable compounds that are known to affect several different cancer-related pathways. Epidemiologic studies have shown a correlation between increased dietary intake of flavonoids with reduced risk of heart disease and cancer (10, 11). Several anticancer properties attributed to these compounds include acting as antioxidants, the ability to interfere with various cancer-promoting signaling pathways, and inhibition of growth factor receptors (11). We and others have also found that epigallocatechin-3-gallate (EGCG) appears to inhibit c-Met activation and epidermal growth factor receptor activation by disrupting lipid rafts (12).

Lipid rafts are important plasma membrane regions that regulate cellular signaling in part through compartmentalization of growth factor receptors. We have shown

that the active form of c-Met resides in lipid rafts,[1] suggesting that disruption of lipid rafts may lead to inhibition of c-Met signaling and its downstream effects. Fatty acid synthase (FASN) is the sole enzyme responsible for synthesis of long-chain saturated fatty acids and may play a role in regulating the activity of lipid rafts. In addition, many human cancers exhibit increased FASN expression correlating with advanced disease (13). FASN activity has been proposed to maintain membrane microdomain integrity and may promote cell survival involving a regulatory loop with the phosphatidylinositol 3-kinase (PI3K) pathway (14, 15). Brusselmans et al. ranked the inhibitory effects of a series of flavonoids on LNCaP prostate cancer cell lipogenesis. These effects correlated with growth arrest, induction of apoptosis, reduced synthesis of phospholipids and cholesterol, and selective cytotoxicity of malignant cells. Of the compounds investigated by the Swinnen laboratory, the flavonoid luteolin was determined to have the greatest inhibitory effect on lipogenesis (16). In addition, luteolin shares structural homology with known PI3K inhibitors, has been shown to inhibit FASN activity directly, and has strong antioxidant activity (16, 17).

Given the possible role of FASN activity in regulating lipid raft function and the localization of active c-Met in lipid rafts, we investigated the effects of luteolin on the HGF/c-Met signaling axis. In this report, we show that luteolin blocks HGF-induced scattering and motility of DU145 prostate cancer cells and is a very potent inhibitor of the PI3K pathway. Moreover, we show that luteolin can down-regulate c-Met expression through FASN inhibition, showing a novel link between FASN activity and c-Met protein expression.

Materials and Methods

Cell Culture

DU145, PC-3, H460, and MDA-MB-231 cell lines were obtained from the American Type Culture Collection and maintained in RPMI 1640 or DMEM/F-12 for 231s (Cellgro) containing 10% fetal bovine serum (Gemini) and penicillin/streptomycin (Cellgro). Cells were maintained in a 37°C incubator with 5.0% CO_2.

Western Blot Analysis

Western blot analysis was done as described previously (18). Primary antibodies used were phospho-Met (Y1234/Y1235), phospho-Met (Y1349), phospho-Met (Y1003), phospho-Akt (S473), phospho-Akt (S308), Akt, phospho-extracellular signal-regulated kinase, 4EBP1, phospho-c-Jun NH_2-terminal kinase, FASN (Cell Signaling Technology), phospho-S6 kinase, total extracellular signal-regulated kinase, total c-Met (C-28; Santa Cruz Biotechnology), phospho-focal adhesion kinase (BD Transduction Laboratories), actin (Sigma-Aldrich), and tubulin (Neomarkers) were used as load controls. Blots were probed with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), and enhanced chemiluminescence (Amersham Biosciences) was used for protein detection. Recombinant HGF and LY294002 were obtained from EMB Biosciences. Luteolin, C75, apigenin, EGCG, quercetin, taxifolin, lactocystin, and concanamycin A were obtained from Sigma-Aldrich. MG132 was obtained from Axxora. Palmitate (Sigma) was complexed to bovine serum albumin (Fischer) as described (16, 18, 19). In short, palmitate was dissolved in ethanol to 150 mmol/L and 1 volume was added to 4 volumes of a 4% bovine serum albumin solution in 0.9% NaCl and incubated for 1 h at 37% for a 30 mmol/L stock of bovine serum albumin-complexed palmitate.

Reverse Transcription-PCR

DU145 cells were plated and allowed to grow to confluency on a 10 cm dish in serum-containing medium. Cells were incubated with or without 25 μmol/L luteolin for 8 h. Cells were homogenized in Trizol (Invitrogen), and total RNA was isolated according to the manufacturer's protocol. RNA was subjected to reverse transcription-PCR for a range of cycles. The primer set for c-Met were purchased from Integrated DNA Technologies: forward 5′-AGGCACTAGCAAAGTCCGAGATGA-3′ and reverse 5′-GGAAACAATCTGGGTGTTCCAGCA-3′. PCR products were run on a 1% agarose gel. Glyceraldehyde 3-phosphate dehydrogenase PCR products were used as a loading control.

Immunofluorescence Microscopy

DU145 were grown on 4-well plastic slides (Nalge Nunc) in serum-containing medium. Following treatment, cells were fixed in 4% paraformaldehyde for 20 min and incubated for 1 h at room temperature with primary tubulin, phosphotyrosine (Cell Signaling), or c-Met (R&D Systems) antibody suspended in bovine serum albumin, saponin, and PBS. Wells were washed with PBS and Texas red-conjugated secondary antibody was applied suspended in bovine serum albumin, saponin, and PBS along with phalloidin. Wells were washed again and Antifade/4′,6-diamidino-2-phenylindole (Invitrogen) was added before setting coverslips. Fluorescent images were taken using an Olympus BX-50 epifluorescence microscope using MetaMorph software.

RNA Interference

DU145 cells stably expressing short hairpin RNA (shRNA) targeting FASN or nontarget shRNA were generated using Mission Lentiviral Transduction Particles from Sigma-Aldrich. Five separate cell lines were established using five lentivirus clones according to the manufacturer's protocol.

Scattering and Motility Assays

For the scattering assay, 4×10^4 DU145 cells, plated on a 24-well plate and grown in serum-containing medium, were pretreated with the indicated concentrations of luteolin for the appropriate time. HGF was then spiked into wells to give a final concentration of 33 ng/mL and incubated overnight. For the motility assay, 4.5×10^5 DU145 cells were plated on a 6-well plate to form a confluent monolayer in serum-containing medium. Cells

[1] Duhon et al., 2008, submitted for publication.

were pretreated for 1 h ± 25 μmol/L luteolin. Intersecting scratches were made across the plate with a pipette tip and washed with PBS to remove cell debris. Serum-free medium ± 15 μmol/L luteolin ± 33 ng/mL HGF was then added to the cells and incubated overnight. The scratch for a representative T_0 was made immediately before fixing. For each experiment, cells were fixed with paraformaldehyde and stained with phalloidin, and representative images were taken using an Eclipse TE300 inverted microscope (Nikon).

Results

Pretreatment with Luteolin Prevents HGF-Induced Scattering and Motility of Prostate Cancer Cells

We have shown that the addition of the flavonoid EGCG blocks the HGF-induced signaling and scattering of DU145 tumor cells via disruption of lipid rafts.[1] To determine if the flavonoid luteolin, a known FASN inhibitor, also affected the HGF-induced phenotypic change, DU145 cells were pretreated overnight with a range of luteolin concentrations before stimulation with HGF (33 ng/mL) for 18 h. Luteolin blocked HGF-induced scattering in a dose-dependent fashion, and this inhibition of scattering was more pronounced with overnight pretreatment compared with 1 h pretreatment (Fig. 1A). To address this pretreatment time-dependent effect, we pretreated cells with 25 μmol/L luteolin for varying periods from 1 to 12 h before HGF addition. An increase in inhibition of HGF-induced scattering was observed beginning at 4 h luteolin pretreatment, with a greater percentage of cells remaining adherent in colonies with ≥8 h pretreatment time (Fig. 1B).

In addition to blocking HGF-induced scattering, luteolin caused a change in cell morphology (compare Fig. 1A, *i*, with Fig. 1A, *v*). Cells appeared flatter and larger in diameter (Fig. 2A). To more closely examine the effects of luteolin on cell morphology, we performed immunofluorescence microscopy on DU145 cells treated with 25 μmol/L luteolin for 9 h. Cells were fixed and stained to visualize actin and tubulin. Figure 2A shows that luteolin caused a reduction in the number of F-actin stress fibers but had no apparent effect on microtubule distribution.

Actin stress fiber formation is an important contributor to cell motility; accordingly, we performed a wound-healing motility assay to determine the effects of luteolin on HGF-induced motility and wound closure. Confluent monolayers of DU145 cells were incubated with or without 25 μmol/L luteolin for 1 h, after which a scratch was made with a pipette tip to wound the monolayer. The medium was aspirated and fresh serum-free medium was added with or without 15 μmol/L luteolin and HGF at 33 ng/mL. Serum-free medium was used so that wound closure due to motility would not be obscured by closure due to normal cell growth and proliferation. A T_0 scratch was also made immediately before fixing the cells in paraformaldehyde (Fig. 2B, *i*). The control cells, not treated with HGF, failed to migrate into the cleared area (Fig. 2B, *ii*), whereas the HGF-stimulated cells almost entirely closed the wound area (Fig. 2B, *iii*). Luteolin, however, blocked the HGF-stimulated wound-closure (Fig. 2B, *iv*), suggesting that luteolin blocks HGF-mediated cell motility (Fig. 2B). Trypan blue staining revealed that luteolin had no affect on cell viability (results not shown).

Cell attachment to the substratum is mediated by focal adhesions, and dynamic focal adhesion turnover is essential for cell motility (20). To determine if luteolin inhibits cell migration by lowering the number of focal adhesion sites, DU145 cells were treated with 25 μmol/L luteolin for 9 h, and actin and phosphotyrosine residues were visualized by immunofluorescence microscopy. Focal adhesions were identified as areas densely populated with phosphorylated proteins localized at the tips of actin fibers. As shown in Fig. 2C, there was no difference in the size or quantity of focal adhesions between control and luteolin treated cells, suggesting that luteolin does not affect focal adhesion formation or stability.

Luteolin Pretreatment Is Required to Attenuate c-Met Activation by HGF, but Pretreatment Is Not Required for Inhibition of Akt Phosphorylation

To determine if luteolin affected HGF/c-Met signaling pathways, thus blocking cell scattering and motility, we treated DU145 cells with 25 μmol/L luteolin for 1, 4, 8, or 12 h before HGF treatment for 20 min. As shown in Fig. 3A, Western blot analysis of whole-cell lysates using antibodies specific for c-Met phosphorylation showed that HGF-induced c-Met phosphorylation at tyrosine residues 1234/1235 (the kinase domain) and 1349 (an adaptor docking site) was significantly attenuated but only after pretreatment with luteolin for >1 h (Fig. 3A); moreover, attenuation increased with pretreatment time. However, luteolin blocked phosphorylation of Akt at two separate residues within 1 h pretreatment. Additional experiments showed luteolin blocked phosphorylation of Akt when pretreated for only 10 min before the addition of HGF (results not shown). Conversely, HGF-mediated activation of two mitogen-activated protein kinases, c-Jun NH_2-terminal kinase and extracellular signal-regulated kinase, along with focal adhesion kinase was not affected by luteolin pretreatment (Fig. 3A).

Because Akt phosphorylation, indicative of PI3K activity, was sensitive to short preincubation times with luteolin, we further investigated the role of luteolin in HGF-dependent PI3K activity. To determine if luteolin affected the activity of PTEN, a phosphatase that negatively regulates PI3K signaling, a dose-response experiment was done using DU145 cells and the $PTEN^{-/-}$ prostate cancer cell line PC-3 (21, 22). Western blot analysis indicated that luteolin inhibited phosphorylation of Akt similarly in both cell lines with an IC_{50} of ~5 μmol/L, suggesting that luteolin acts directly as a PI3K inhibitor rather than by activating PTEN (Fig. 3B and C). Because luteolin pretreatment caused an apparent reduction of PI3K activity within 1 h followed by inhibition of c-Met phosphorylation beginning at 1 h, we tested if the

loss of PI3K activity negatively regulated c-Met phosphorylation. To test this hypothesis, we used the PI3K inhibitor, LY294002, to determine if prolonged inhibition of PI3K resulted in the reduction of HGF-induced c-Met phosphorylation. As shown in Fig. 3D, LY294002 pretreatment over a time course similar to luteolin pretreatment did not affect c-Met phosphorylation despite inhibiting HGF-induced Akt phosphorylation. Therefore, the ability of luteolin to attenuate c-Met phosphorylation is independent of its ability to inhibit PI-3K.

Luteolin Pretreatment Induces a Post-transcriptional Loss of c-Met Most Likely through Inhibition of FASN

To further investigate the mechanism of luteolin-mediated decrease in HGF-mediated c-Met activity, we performed Western blot analysis using whole-cell lysates from DU145 cells treated with luteolin for various times. Figure 4A shows that luteolin caused a reduction in the level of total c-Met protein beginning as early as 1 h, leading to a >90% loss by 12 h. The loss of c-Met expression over time mirrors the loss of HGF-induced c-Met phosphorylation seen in Fig. 3A, suggesting that the mechanism of luteolin-mediated down-regulation of c-Met activity may be primarily through decreased receptor expression. To confirm the generality of these findings, we treated several additional cancer cell lines, including the prostate tumor cell line PC-3, the breast cancer cell line MDA-MB-231, and the lung cancer cell line H460, with 25 μmol/L luteolin for 9 h. Luteolin treatment reduced c-Met expression in all cell lines (Fig. 4A, *right*), suggesting that the observed effects of luteolin on c-Met receptor levels are not cell context dependent.

Because luteolin is a known FASN inhibitor, we predicted that a specific FASN inhibitor, C75, might also induce a loss of c-Met expression (23). As seen in Fig. 4B, the addition of C75 resulted in the loss of c-Met with kinetics similar to that observed for luteolin treatment. To more directly determine the role of FASN in controlling c-Met levels, we expressed FASN-specific shRNA via lentivirus to generate stable cell lines. As shown in Fig. 4C, the FASN shRNA cell lines contained greatly reduced FASN levels, and this was paralleled by comparable decreases in c-Met levels. Finally, the addition of palmitate,

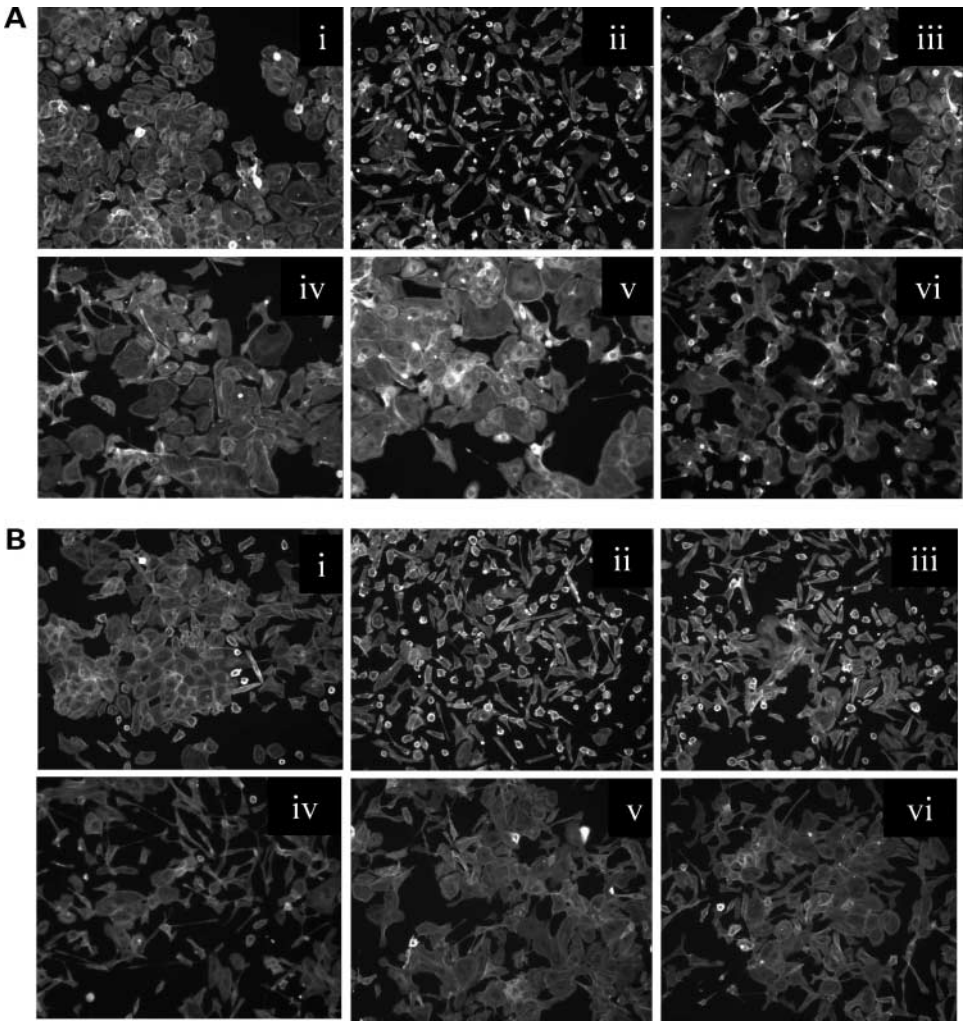

Figure 1. Luteolin pretreatment blocks HGF-induced cell scattering. **A,** DU145 prostate cancer cells were pretreated with DMSO (*i* and *ii*) or increasing luteolin concentrations (*iii,* 10 μmol/L; *iv,* 15 μmol/L; *v* and *vi,* 30 μmol/L) for 1 h (*vi*) or overnight (*iii-v*) before HGF stimulation (*ii-vi,* 33 ng/mL) for 18 h. **B,** DU145 cells were incubated with DMSO (*i* and *ii*) or 25 μmol/L luteolin for 1 h (*iii*), 4 h (*iv*), 8 h (*v*), or 12 h (*vi*) before HGF stimulation (*ii-vi*). Cells were fixed and stained for actin, and representative images were captured.

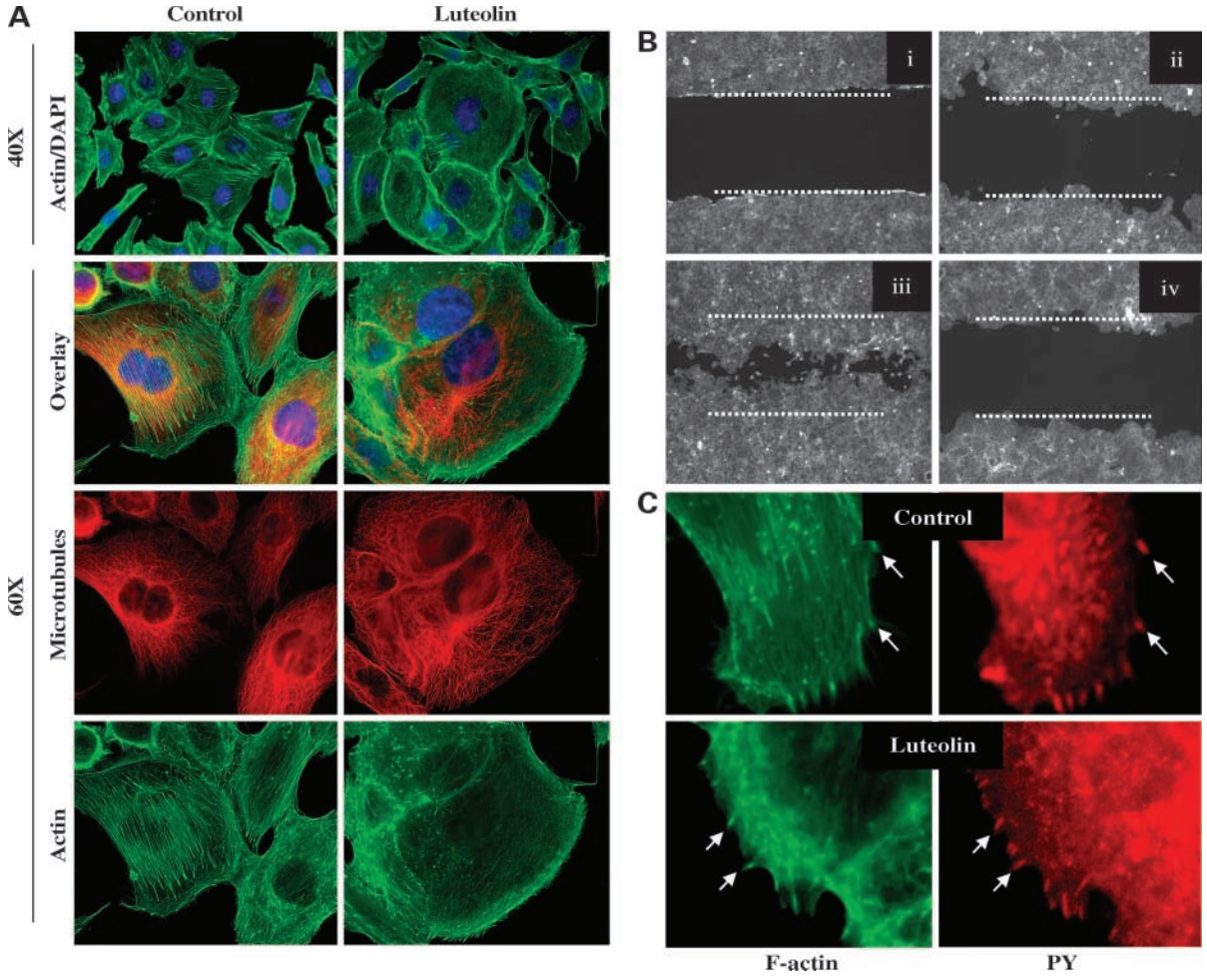

Figure 2. Luteolin disrupts actin stress fibers and blocks HGF-induced cell motility but does not affect focal adhesions. **A,** DU145 cells were treated with DMSO or 25 μmol/L luteolin for 12 h. Cells were fixed and stained for actin, 4′,6-diamidino-2-phenylindole, and microtubules, and representative ×40 and ×60 images were captured. **B,** confluent DU145 monolayers were pretreated with DMSO (*ii* and *iii*) or 25 μmol/L luteolin (*iv*) for 1 h. The monolayer was wounded and washed, and fresh serum-free medium containing 15 μmol/L luteolin (*iv*) or DMSO (*ii* and *iii*) was added. Cells were then incubated alone (*ii*) or with HGF (*iii* and *iv*, 33 ng/mL) overnight. The following day, one monolayer was wounded as an untreated control (*i*), after which all cells were fixed and stained for actin. **C,** DU145 cells were incubated with DMSO or 25 μmol/L luteolin for 12 h. Cells were fixed and stained for actin or phosphotyrosine, and representative images were captured. *Arrows,* focal adhesions represented by areas of actin and phosphotyrosine colocalization.

the end product of FASN catalytic activity, prevented luteolin-induced (Fig. 4D, *left*) and C75-induced (Fig. 4D, *right*) c-Met loss in a dose-dependent manner. These experiments suggest a potential novel link between FASN activity and c-Met expression levels.

To determine if luteolin affected c-Met transcription, thus accounting for loss of c-Met protein, we performed reverse transcription-PCR on RNA prepared from DU145 cells cultured alone or in the presence of luteolin for 8 h. Gel electrophoresis of PCR products at different cycle times indicated that luteolin treatment only reduced c-Met mRNA by ~20% in DU145 cells relative to glyceraldehyde 3-phosphate dehydrogenase RNA controls (Fig. 5A); real-time PCR confirmed this 20% reduction (data not shown). The modest loss of c-Met mRNA compared with the >90% loss of c-Met suggests a post-transcriptional level of control is involved. The PI3K/Akt pathway regulates cap-dependent translation in part by affecting 4EBP1 through mammalian target of rapamycin activation, leading to eIF4E availability to initiate translation of mRNAs with complex 5′-untranslated regions (24). The c-Met mRNA is predicted to have a 5′-untranslated region that might be eIF4E responsive.[2] Accordingly, we used rapamycin, a specific mammalian target of rapamycin inhibitor, and LY294002 to inhibit the PI3K/Akt/mammalian target of rapamycin pathway. Western blot analysis revealed that inhibition of this pathway, as indicated by the blocked phosphorylation of downstream S6 kinase and the shift of 4EBP1 to a hypophosphorylated form in cells exposed to HGF, did not result in a decrease in c-Met levels (Fig. 5B).

[2] Unpublished results.

This suggests that, in DU145 cells, c-Met mRNA translation is most likely not dependent on the mammalian target of rapamycin pathway and that luteolin is not lowering c-Met protein levels through inhibition of cap-dependent translation.

To further address the possible mechanism of action accounting for luteolin-induced loss of c-Met, we compared the rate of c-Met loss on treatment with the eukaryotic protein synthesis inhibitor, cycloheximide, alone or in combination with luteolin. Western blot analysis indicated an increased rate of c-Met loss in the presence of cycloheximide and luteolin compared with treatment with either agent alone (data not shown). If luteolin was inhibiting c-Met protein synthesis like cycloheximide, we would not observe this increased rate of c-Met loss when combined with cycloheximide. Increased doses of cycloheximide did not increase the rate of c-Met loss, suggesting that the dose used was optimal (data not shown). Together, these data suggest that luteolin promotes c-Met degradation of already synthesized c-Met protein.

Luteolin-Induced Loss of c-Met Is Independent of the Proteosomal and Lysosomal Degradation Pathways

When the c-Met receptor is activated by HGF, the juxtamembrane tyrosine residue 1003 is phosphorylated, which recruits the ubiquitin ligase Cbl. Ubiquitination prompts internalization of the receptor and trafficking for degradation via either the 26S proteosome or by trafficking to acidic lysosomes (25–29). Western blot analysis indicated that HGF treatment of DU145 cells resulted in loss of c-Met protein within 2 h, preceded by phosphorylation at Y1003 (Fig. 6A and B). In contrast, luteolin treatment did not result in phosphorylation at Y1003 (Fig. 6B). We used specific pharmacologic inhibitors to determine if luteolin-induced c-Met loss was dependent on the proteosomal or the lysosomal degradation pathway. MG132 and lactacystin are inhibitors of the 26S proteosome, and concanamycin A inhibits acidification of lysosomes, reducing proteolysis. DU145 cells were pretreated with the indicated inhibitors before HGF or luteolin treatment and probed for c-Met loss by Western blot analysis. HGF treatment resulted in a rapid loss of c-Met, which was partially prevented by inhibition of the 26S proteosome or by blocking lysosomal acidification (Fig. 6C). In contrast, luteolin-induced c-Met loss was not prevented in the presence of these inhibitors (Fig. 6C). In addition, inhibiting both the 26S proteosome and lysosomal acidification in combination prevented HGF-induced degradation (Fig. 6C, *bottom*), but again luteolin-induced c-Met down-regulation was not blocked by the combination of inhibitors (Fig. 6C, *bottom*). These experiments suggest the proteosomal and lysosomal pathways do not play a major role in luteolin-induced c-Met loss and suggest a potentially novel mechanism of action to account for the effects of this flavonoid on c-Met levels.

To confirm that a reduction in c-Met levels is the result of loss of c-Met from the cell surface, cells were treated with HGF or luteolin for the times indicated in Fig. 6D. Immunofluorescence microscopy using a c-Met antibody indicated that HGF treatment rapidly resulted in redistribution of c-Met from the cell surface to vesicles residing near the nucleus. Cell surface c-Met was also reduced by luteolin treatment, but the intracellular distribution was less vesicular, although c-Met did accumulate near the nucleus during the period levels were decreasing.

2,3 Double Bond in C-Ring of Flavonoids Is Important for c-Met Down-regulation

To determine if structurally related flavonoids have similar effects on c-Met levels when compared with luteolin, we treated DU145 cells for 9 h with the flavones luteolin and apigenin, the flavonol quercetin, the flavanone taxifolin, and the flavanol EGCG. Western blot analysis

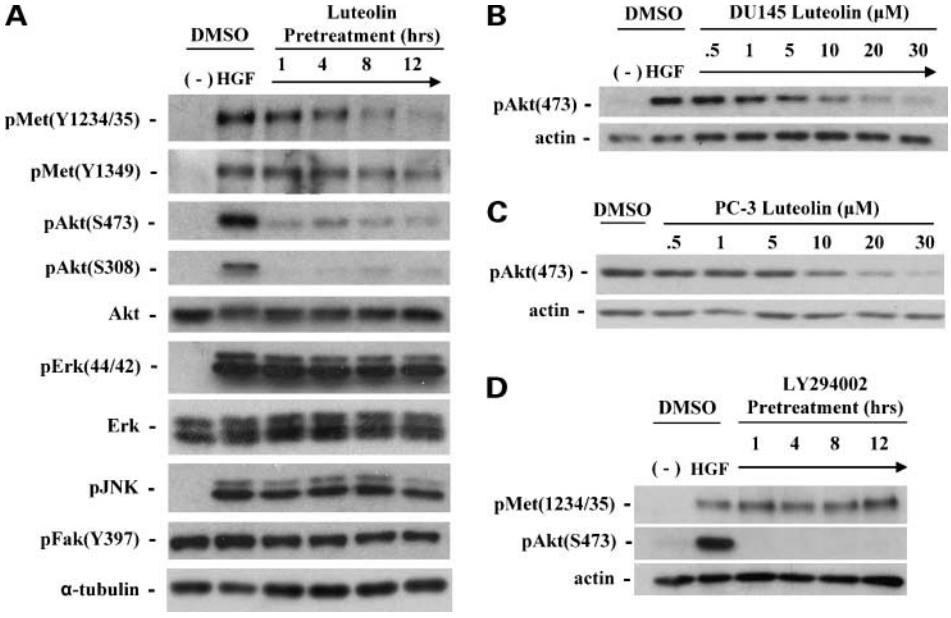

Figure 3. Luteolin inhibits PI3K and blocks HGF-induced c-Met phosphorylation with prolonged pretreatment. **A,** DU145 cells were pretreated with DMSO or 25 μmol/L luteolin for 1, 4, 8, or 12 h before HGF (33 ng/mL) stimulation for 20 min. **B,** DU145 cells were pretreated with DMSO or indicated luteolin concentrations for 1 h before HGF treatment for 20 min. **C,** PC-3 prostate cancer cells were treated with DMSO or indicated concentrations of luteolin for 1 h. **D,** DU145 cells were pretreated with DMSO or 20 μmol/L LY294002 for 1, 4, 8, or 12 h before HGF stimulation. For each experiment, whole-cell lysates were collected and probed by Western blot analysis using indicated antibodies. Actin or tubulin was also probed as a load control.

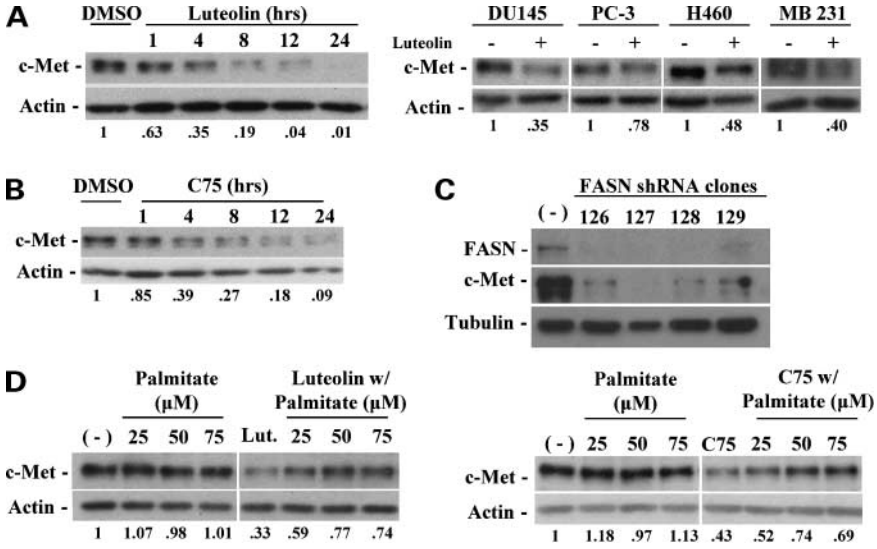

Figure 4. Luteolin reduces c-Met levels through inhibition of FASN. **A**, *left*, DU145 cells were treated with DMSO or 25 μmol/L luteolin for 1, 4, 8, 12, or 24 h; *right,* DU145, PC-3, the lung cancer cell line H460, and breast cancer cell line MDA-MB-231 were treated for 9 h with DMSO (−) or 25 μmol/L luteolin (+). **B,** DU145 cells were treated with DMSO or 25 μmol/L C75 for 1, 4, 8, 12, or 24 h. **C,** DU145 cells were stably transduced with lentivirus expressing nontarget shRNA (−) or one of four FASN-targeted shRNA clones (126, 127, 128, or 129). **D,** DU145 cells were pretreated with DMSO or 25 μmol/L luteolin (*left*) or 25 μmol/L C75 (*right*) before incubation with palmitate-bovine serum albumin for 9 h. For each experiment, whole-cell lysates were collected and probed by Western blot analysis using c-Met or FASN-specific antibodies. Actin was also probed as a load control. Densitometry was done on appropriate blots and shown as fold change of control.

indicated that luteolin, apigenin, and quercetin all reduced c-Met expression to similar levels but that taxifolin and EGCG had no significant effect on c-Met expression at these concentrations (Supplementary Fig. S1A).[3] Interestingly, each compound effective at lowering c-Met levels (luteolin, apigenin, and quercetin) contained a double bond between carbons 2 and 3 of the center six-member oxygen-containing C-ring. Comparatively, taxifolin, ineffective at lowering c-Met expression, lacks this double bond, which is the only difference from the molecular structure of quercetin (Supplementary Fig. S1B).[3] These data reveal that structurally similar flavonoids have similar effects on c-Met expression and that the 2,3 double bond of the C-ring is important for this effect.

Discussion

Some phytochemicals, such as EGCG from green tea extract, have potent inhibitory activity against growth factor signaling pathways, including HGF/c-Met (18, 30). In this report, we investigated the effects of the flavonoid luteolin on the HGF/c-Met signaling axis and found that it blocks c-Met signaling through a mechanism unique from EGCG. Our data suggest that one key mechanism of action is through inhibition of FASN, leading to a post-transcriptional reduction in c-Met protein levels.

In the presence of HGF, DU145 prostate cancer cells lose cell-cell adhesions and acquire a motile phenotype. EGCG blocks HGF-induced scattering with no preincubation time required (18); however, we report here that luteolin had a minimal effect added with HGF and that a preincubation time was required to block c-Met phosphorylation. This result suggests that the two compounds are working by different mechanisms. Also, luteolin caused changes in cell morphology (reduction in stress fibers and cell flattening) not observed with EGCG treatment. Similar effects by luteolin on the actin cytoskeleton were shown by Lee et al. in the context of HepG2 hepatoma cells (31). How luteolin affects these changes remains to be determined, but we speculate that luteolin disrupts actin-remodeling proteins, such as the Rho family of small GTPases and downstream effectors like Rho kinase (ROCK). This hypothesis is supported by Hendriks et al. who have shown previously that luteolin can inhibit RhoA activity in a monocyte system (32). Actin stress fibers are important for cell motility, and disruption of actin stress fibers caused by luteolin treatment may account for the ability of the compound to block HGF-induced scattering and motility, although this remains to be tested (33).

Luteolin also rapidly blocked HGF-induced Akt phosphorylation, whereas 1 h pretreatment was required to inhibit c-Met phosphorylation. We conclude that luteolin is working directly as a PI3K inhibitor rather than blocking ligand-induced receptor activation. Consistent with this possibility, others have found that luteolin can inhibit PI3K in an *in vitro* assay (34). Furthermore, luteolin was equally effective at blocking Akt phosphorylation in a $PTEN^{-/-}$ prostate cancer cell line, thereby ruling out the possibility that luteolin activated this phosphatase to negatively regulate PI3K activity. Loss or inactivation of PTEN is common in several cancers including prostate cancer, and aberrant signaling through the PI3K pathway has been implicated in tumor cells becoming resistant to receptor tyrosine kinase-targeted therapies (21, 22). Therefore, the ability of luteolin to target PI3K signaling independent of PTEN status emphasizes the potential therapeutic activity of this phytochemical.

Furthermore, the time-dependent attenuation of HGF-mediated c-Met phosphorylation by luteolin was independent of the effects on Akt phosphorylation, because

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

prolonged treatment of cells with LY294002 did not block the ability of HGF to induce c-Met phosphorylation. Activation of the extracellular signal-regulated kinase and c-Jun NH_2-terminal kinase signaling pathways were not affected by luteolin even after long preincubation when c-Met was no longer maximally activated by HGF. This suggests that sufficient active c-Met remained to induce phosphorylation of extracellular signal-regulated kinase and c-Jun NH_2-terminal kinase. Focal adhesion kinase was constitutively phosphorylated at high levels in DU145 cells, and phosphorylation was only minimally attenuated with prolonged luteolin preincubation.

Our results are consistent with Lee et al. who have shown that luteolin blocked HGF-induced Akt phosphorylation but only partially affected mitogen-activated protein kinases in hepatoma cells (31). In addition, however, our results show a requirement for pretreatment >1 h to affect c-Met phosphorylation, whereas only a short pretreatment was required to block phosphorylation of Akt. We speculate that the requirement for long pretreatment could reflect the length of time required to alter the concentration of a factor(s) regulating c-Met. One of these factors could be the levels of palmitate, which regulates lipid raft activity or integrity. One can rule out a delay in the compound crossing the cell membrane because of the rapid effects on PI3K activity.

Interestingly, the reduction of HGF-induced phosphorylated of c-Met was not the result of luteolin blocking ligand-induced activation of the receptor but instead appeared to be due to luteolin-mediated down-regulation of total c-Met protein. Reverse transcription-PCR analysis showed no dramatic change in total c-Met mRNA levels, suggesting that luteolin was acting primarily at a post-transcriptional level. This result was observed in multiple tumor cell lines. Similar to our results, it has been reported that apigenin and luteolin down-regulate HER-2 expression in HER-2/*neu*-overexpressing breast cancer cell lines, although the mechanism regulating this was not defined for luteolin (35). A subsequent report suggested that luteolin induced loss of HER-2 at a post-transcriptional level (36). We have observed similar effects of luteolin and apigenin on HER-2 expression in DU145 prostate cancer cells.[2]

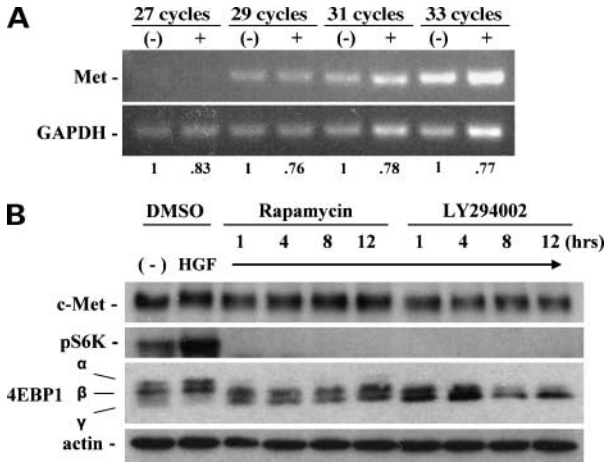

Figure 5. Luteolin post-translationally regulates c-Met levels. **A,** DU145 cells were treated with DMSO (−) or 25 μmol/L luteolin (+) for 8 h. RNA was then isolated and reverse transcription-PCR was done using primers for c-Met and for glyceraldehyde 3-phosphate dehydrogenase as control at different cycles. **B,** DU145 cells were pretreated with DMSO, rapamycin (100 ng/mL), or LY294002 (20 μmol/L) for indicated periods before HGF stimulation (33 ng/mL) for 20 min. Lysates were collected and probed by Western blot analysis using indicated antibodies. Actin was used as a load control.

C75, a specific pharmacologic inhibitor to FASN, as well as FASN-specific shRNA reduced the level of total c-Met protein in DU145 cells. FASN is the sole enzyme responsible for *de novo* synthesis of long-chain unsaturated fatty acids, primarily the 16-carbon fatty acid palmitate. The addition of exogenous palmitate to the system prevented the C75- and luteolin-induced loss of c-Met, further supporting a role or FASN in maintaining c-Met expression levels. Malignant cells have a much greater reliance on *de novo* synthesized fatty acids as opposed to exogenously derived fatty acids, suggesting that FASN could be a good therapeutic target (37). The need of malignant cells for high expression of FASN has been attributed to maintenance of the lipid supply required by highly proliferative cells, regulation of stimulatory signaling pathways through palmitoylation of proteins and stabilizing membrane domains, as well as restoration of oxidation potential through consumption of NADPH under hypoxic conditions (13). Several cellular receptors, including c-Met, require localization within ordered lipid microdomains (lipid rafts) for efficient signaling (38, 39).[1] Lipid rafts are rich in cholesterol and sphingolipids, products generated in tumors cells by FASN (15, 40). Our results suggest that higher FASN activity maintains lipid rafts, which may help stabilize levels of c-Met.

Our results suggest that, in DU145 cells, translation of c-Met mRNA was not regulated by the PI3K/Akt/mammalian target of rapamycin/eIF4E pathway; therefore, inhibition of PI3K is not the mechanism responsible for luteolin-induced c-Met loss. Our data also suggest that the loss of c-Met induced by luteolin occurred in part subsequent to c-Met synthesis but was independent of an active proteosome or acidic lysosomes.

The c-Met receptor, similar to other receptor tyrosine kinases, has been reported to undergo a mechanism of ligand-induced negative regulation through internalization and degradation (26, 28, 29). Using inhibitors to both proteosomal activity and acidification of lysosomes, we show that neither of these pathways was significantly involved in luteolin-induced c-Met loss. In contrast, Chiang et al. concluded that luteolin-induced loss of HER-2 in breast carcinoma cells was blocked by the addition of proteosomal inhibitors (36).

Another mechanism of c-Met down-regulation is receptor shedding, which is mediated by metalloproteinases that cleave the extracellular domain of the receptor promoting degradation of the cytoplasmic domain (41). Our results speak against this possibility for three reasons.

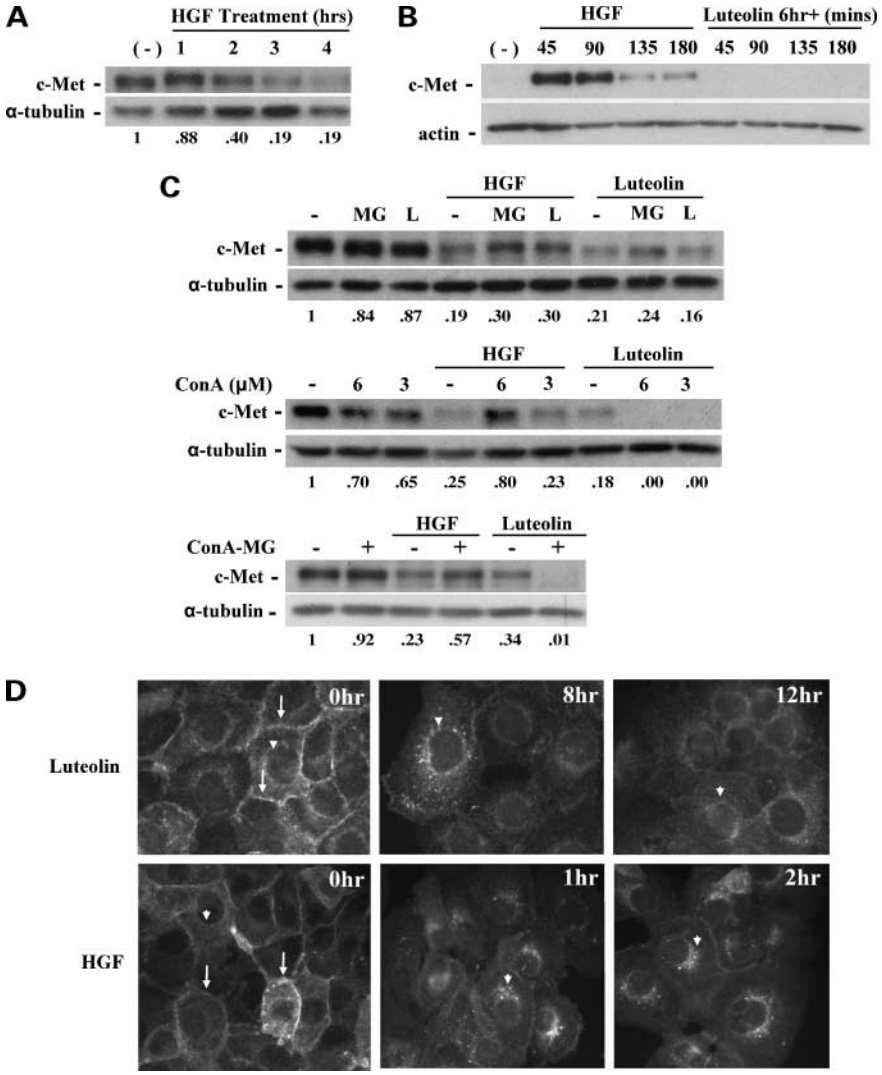

Figure 6. Luteolin down-regulates c-Met levels independent of the lysosomal or proteosomal pathways. **A,** DU145 cells were incubated alone (−) or treated with HGF (33 ng/mL) for 1, 2, 3, or 4 h. **B,** DU145 cells were incubated alone (−), treated with HGF for 45, 90, 135, or 180 min, or treated with 25 μmol/L luteolin for 6 h plus 45, 90, 135, or 180 min. **C**, *top,* cells were pretreated with DMSO or luteolin for 5 h before culture alone (−) or with 300 nmol/L MG132 (*MG*) or 20 μmol/L lactacystin (*L*) for 4 h with or without HGF stimulation. Cells were treated as described above but using 6 or 3 μmol/L concanamycin A (*ConA*; *middle*) or 300 nmol/L MG132 and 6 μmol/L concanamycin A in combination (*ConA-MG*; *bottom*). For each, whole-cell lysates were analyzed by Western blot analysis using the indicated antibodies. Actin or tubulin was used as a load control. Densitometry was done on appropriate blots and shown as fold change of control. **D,** DU145 cells were treated with 25 μmol/L luteolin for 8 and 12 h or with HGF for 1 or 2 h to stimulate loss of total c-Met. Cells were then fixed and stained with a c-Met-specific primary antibody and fluorescently labeled secondary antibody. Representative images are shown. *Arrowheads,* perinuclear distribution; *arrows,* cell membrane periphery.

Firstly, no c-Met protein was detected in the medium following luteolin treatment (data not shown). Secondly, similar trends of c-Met loss were detected with antibodies to both a cytoplasmic domain and an extracellular domain (data not shown). Finally, immunofluorescence microscopy indicated that reduction of c-Met levels was accompanied by internalization of the receptor into intracellular compartments.

An additional possibility for c-Met loss is that luteolin is stimulating an apoptotic response resulting in activation of caspases that subsequently degrade the receptor. However, no significant apoptosis was observed in treated cells during these time points by microscopy or protein analysis (data not shown). We conclude that luteolin is acting through a novel mechanism of receptor degradation unique from that induced by ligand or other known stimuli, although the mechanism remains to be defined.

We determined using a series of structurally similar compounds that the 2,3 double bond of the C-ring of luteolin conveys the activity required for down-regulation of c-Met. We show that the flavones luteolin and apigenin along with the flavonol quercetin, each containing the 2-3 double bond and having the most potent inhibitory activity toward FASN, as shown by Brusselmans et al., have the greatest effect on c-Met levels (16). Apigenin has been reported previously to down-regulate HER-2 by proteosomal degradation in breast cancer cells (42). We hypothesize that, by targeting FASN, these compounds can down-regulate the expression of several cancer-associated growth factor receptors that require membrane microdomains stabilized by FASN activity.

Our study provides evidence for a potential link between FASN activity controlling levels of c-Met perhaps by stabilization and that luteolin could be a potential therapeutic agent to down-regulate c-Met levels through inhibition of FASN. Overexpression of FASN has been reported to increase activity of the HER-1 and HER-2 receptors in breast cancer cells and may be important in

resistance to trastuzumab (43, 44). We hypothesize that increased expression of FASN could stabilize growth factor receptors localized in lipid rafts, such as c-Met, thereby promoting cancer progression. Our results, consistent with other published studies, show that luteolin inhibits PI3K activity in prostate cancer cells. FASN expression is, in part, controlled by a regulation loop with PI3K and is inversely correlated with PTEN expression (14, 45). Consequently, luteolin has the potential to target both PI3K and FASN activities to disrupt growth factor receptor stability and cancer progression, suggesting that it might be more effective than either PI3K inhibitors or FASN inhibitors given alone.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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