

Formononetin Inhibits Metastatic Potential of Human Prostate Cancer Cells via Upregulating EGR1

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ABSTRACT

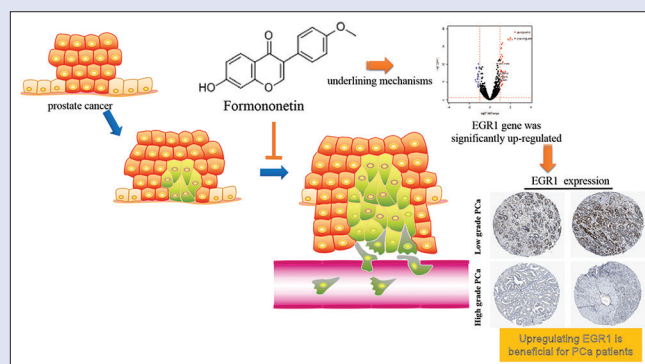
Objectives: Formononetin is a natural isoflavone compound and has been reported to have anti-cancer properties. However, the mechanism by which formononetin inhibits hormone-resistant prostate cancer (PCa) has not been reported. The present study investigates whether and how formononetin inhibits the epithelial-mesenchymal transition (EMT) of hormone-resistant PCa cell lines. **Materials and Methods:** The proliferation, migration and invasion of PCa cells were analyzed using RTCA system and CCK8 assays. Expression of EMT-related markers and MAPK pathway were analyzed using quantitative reverse transcription PCR (RT-qPCR), immunofluorescence staining and immunoblotting. Gene expression profile was observed by RNA-sequence analysis, and the EGR1 expression analysis in human PCa tissue was referred to the Cancer Genome Atlas database and The Human Atlas Database. **Results:** Formononetin inhibited the rate of proliferation, migration, and invasion of PCa cells, increased protein levels of E-cadherin, reduced protein levels of fibronectin and the phosphorylated ERK1/2 and JNK, as well as the mRNA levels of the fibronectin, slug and snail. Formononetin remarkably upregulated EGR1, as confirmed by bioinformatics analysis, RT-qPCR and western blotting. Furthermore, the EGR1 expression was lower in human PCa tissue versus its control, and in higher Gleason grade versus the lower Gleason grade PCa. **Conclusion:** Formononetin could ameliorate tumor aggressiveness via reversing the EMT of hormone-resistant PCa cells. The potential mechanisms involved are inactivation of MAPK signalling and subsequent upregulation of EGR1 expression.

Key words: EGR1, EMT, formononetin, MAPK, prostate cancer

SUMMARY

- The current study revealed that formononetin inhibits the epithelial-mesenchymal transformation on DU145 cell lines. Moreover, bioinformatics analysis, RT-qPCR and western blotting confirmed that EGR1 is remarkably upregulated by formononetin treatment. These results suggest that formononetin may exert anti-cancer activity by upregulating EGR1. Further, the western blot assay confirmed the inhibition effects of

formononetin on the phosphorylation of ERK1/2 and JNK, which explains the reason why EGR1 is upregulated. Together with the EGR1 expression in human prostate cancer tissue in The Human Protein Atlas database, we could easily know upregulating EGR1 is beneficial for prostate cancer patients.



Abbreviations used: PCa = Prostate cancer, EMT = Epithelial-mesenchymal transition, ADT = Androgen deprivation therapy, FDR = False discovery rate, AR = Androgen receptor; RNA-seq = RNA sequencing, EGR1 = Early growth response 1, ERK = Extracellular signal-regulated kinase; JNK = c-Jun N-terminal kinase

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INTRODUCTION

Prostate cancer (PCa) is one of the most aggressive human cancers, and androgen deprivation therapy (ADT) is a common therapy for PCa. However, cancer often transits from androgen sensitive to androgen resistant, and the molecular mechanisms are not clear. What's more, androgen deprivation can induce epithelial-mesenchymal transition (EMT).^[1] Recently, more and more evidence has indicated that EMT promotes the metastasis of hormone-resistant PCa, as well as contributes to the development of chemotherapy resistance.^[2,3]

Formononetin is an isoflavone that exists in plants such as *Astragalus membranaceus*, *Trifolium pratense* and *Glycyrrhiza*. Formononetin has been reported to have anti-cancer activity against many types of cancers including PCa, and the underlying mechanisms include inducing apoptosis and arresting cell cycle.^[4,5] Formononetin has also

been reported to increase the sensitivity of glioma cells to chemotherapy through preventing EMT.^[6] However, whether formononetin could affect the metastasis of hormone-resistant PCa cells via disturbing EMT has not been reported. In the present study, we investigated the effects of formononetin on hormone-resistant PCa cell line DU145, and results

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revealed that it could inhibit the metastatic potential of PCa by reversing EMT process.

MATERIALS AND METHODS

Reagents and antibodies

Formononetin was purchased from Chengdu Herbpurify Co. Ltd (Chengdu, China). The antibodies were as follows: anti-E-cadherin (#ab1416, dilution 1:1000), anti-fibronectin (#ab2413, dilution 1:1000) and anti-vimentin (#ab92547, dilution 1:2000) were purchased from Abcam (Cambridge, MA, USA); anti- α -tubulin (#5335, dilution 1:2000), MAPK family antibodies (#9926T, dilution 1:1000) and phospho-MAPK family antibodies (#9910T, dilution 1:1000) were purchased from Cell Signaling Technology (Beverly, MA, USA). The other reagents were purchased from Beyotime Institute of Biotechnology unless otherwise indicated.

Cell preparation and culture

The hormone-resistant PCa DU145 cell line used for *in vitro* experiment was obtained from American Type Culture Collection (ATCC, USA), and cultured by RPMI 1640 Medium (Gibco, catalog. 11875119), 10% fetal bovine serum (Catalog Number: 10270-106, Gibco, USA), and antibiotics (100 mg/L streptomycin, 100 KU/L penicillin) in an incubator (37°C, 5% CO₂ atmosphere).

Cell proliferation analysis

DU145 cells were seeded in E-Plate 16 (2×10^3 cells/well) and treated with 0, 25, 50, 100 μ mol/L of formononetin. The rates of cell proliferation were then detected by RTCA iCELLigence Analyzer in real time (ACEA Biosciences, USA). Cells were seeded in a 96-well plate (2×10^3 cells/well) and treated with 0, 25, 50, 100 μ mol/L of formononetin for 24 hr, then 10 μ M CCK-8 solution were added to the treated cells. The absorbance at 450 nm was recorded using a 96-well plate reader.

Migration and invasion detection

DU145 cells were seeded (2×10^3 cells/well) on CIM-Plate 16 (ACEA Biosciences, USA) for migration assay and on CIM-Plate 16 pre-coated with Matrigel (corning 356234, USA) for invasion assay. The upper chamber was filled with serum-free DMEM medium, while the lower chamber was filled with medium containing 10% fetal bovine serum. The cell movement was then continuously monitored in real time every 15 min for 24 hr by RTCA iCELLigence Analyzer (ACEA Biosciences, USA).

RNA extraction and RT-qPCR

DU145 cells were incubated with 100 μ M of formononetin or vehicle (DMSO) for 24 hr. Total RNA was extracted using the UNIQ-10 Column Trizol Total RNA Isolation kit (Sangon Biotech, China), and then was reverse-transcribed to cDNA using the PrimeScript RT reagent kit (Takara, Japan) according to the manufacturer's protocols. The gene-specific primers were as follows: Fibronectin (F) 5'-TCGCCATCAGTAGAAGGTAGCA-3', (R) 5'-TGTTATACTGAACACCAGGTTGCAA-3'; Snail (F) 5'-GCCTAGCGAGTGGTTCTTCT-3', (R) 5'-TAGGGCTGCTGGAAGG TAAA-3'; Slug (F) 5'-GAGCATTTGCAGACAGGTC A-3', (R) 5'-ACAGCAGCCAGATTCCCTCAT-3'; EGR-1 (F) 5'-GGTCAGTGGCCTAGTGAGC-3', (R) 5'-GTGCCGCTGAGTAAATGGGA-3'; GAPDH (F) 5'-TGGCAAAGTGGAGATTGTTGCC-3', (R) 5'-AAGATGGTGATGGGCTTCCCG-3'. Differential expression was calculated using the $2^{-\Delta\Delta C_q}$ method. Data are presented as the mean $\pm$ standard deviation (SD) of three independent experiments.

Immunoblotting

DU145 cells were seeded in a 6-well plate (2×10^5 cells/well) and treated with different doses of formononetin for 24 hr, following which whole-cell protein extracts were prepared with RIPA buffer supplemented with protease inhibitor cocktail (EASYPacks, Roche). The concentration of the total protein was determined by Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, catalog# 23225). 25 μ g of protein was separated using SDS-PAGE and was then transferred to polyvinylidene difluoride (PVDF) membrane. Next, the membranes were incubated with primary antibodies and respective secondary antibodies. Finally, protein bands were visualized using ECL reagent (Thermo Fisher Scientific, Inc.).

Immunofluorescence staining

DU145 cells were seeded on coverslips in 12-well plates and treated with different doses of formononetin for 24 hr. They were then washed with phosphate-buffered saline (PBS) for three times, fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.3% Triton X-100. Next, the cells were incubated with 5% bovine serum albumin (BSA) for 1 hr, Room Temperature (RT). Subsequently, the treated cells were first blotted with E-cadherin antibodies (1:400) and vimentin antibodies (1:400), then the secondary antibodies (1:200) for 1 hr, RT. The nuclei were stained by DAPI. Finally, samples were washed with PBS three times, mounted in Fluoromount-GTM (Invitrogen; Thermo Fisher Scientific, Inc.) and visualized using a confocal laser scanning microscope (LSM800, Zeiss).

Data from public database

The EGR1 expression comparison between normal prostate tissue and PCa tissue are cited from the following public website: <http://ualcan.path.uab.edu/cgi-bin/TCGAExResultNew2.pl?genenam=EGR1&ctype=PRAD>.^[7] The EGR1 expression of human PCa was cited from The Human Protein Atlas database: https://www.proteinatlas.org/ENSG00000120738-EGR1/pathology/prostate+cancer#imid_4891970, and the EGR1 expression of normal prostate glandular cells was also cited from The Human Protein Atlas database: <https://www.proteinatlas.org/ENSG00000120738-EGR1/tissue/prostate>.

Ethical statement

Not applicable.

Statistical analysis

All data were presented as mean $\pm$ standard deviation of three or more independent experiments. Differences were analyzed via a two-tailed unpaired student's *t* test using the GraphPad Prism (version 7). **P* < 0.05, ***P* < 0.01 were considered to be statistically different.

RESULTS

Formononetin inhibited the proliferation of hormone-resistant PCa cells

To evaluated the anti-cancer potential of formononetin in hormone-resistant PCa, DU145 cells were chosen and treated with 0, 25, 50, 100 μ mol/L of formononetin for 24 hr. Cell proliferation was inhibited by formononetin treatment as indicated by less amount of cells under a bright light microscope [Figure 1a], the lower growth rate in real-time monitoring [Figure 1b] and lower cell activity [Figure 1c]; moreover, the inhibition effects showed in a dose-dependent manner.

Formononetin inhibited the metastatic potential of hormone-resistant PCa cells

Migration and invasion occur at the initial stage of cancer metastasis and contribute to the spread of invasive malignant tumor cells to distant locations. To investigate the effects of formononetin on the migration and invasion of PCa cells, DU145 cell lines were selected and cultured with 0, 25, 50, 100 μM formononetin for 24 hr. The migration rate and invasion rate were significantly reduced by formononetin in a dose- and time-dependent manner [Figure 2a, c]. Moreover, the cell index significantly decreased by 100 μM formononetin treatment at the end time point in migration and invasion assay [Figure 2b, d]. These results suggested that a high concentration formononetin had an inhibitory effect on migration and invasion of hormone-resistant PCa cells.

Formononetin inhibited EMT of hormone-resistant PCa cells

EMT, which is a result in the development of therapy resistance, is the basic pathogenesis of metastasis. Thus, we further investigated whether the inhibition effects of formononetin on DU145 were

mediated by inhibiting EMT. EMT is associated with decreased expression of E-cadherin, and increased expression of vimentin and fibronectin. Interestingly, formononetin intervention enhanced the expression of E-cadherin and reduced the expression of fibronectin [Figure 3a–c]. Slug and Snail both are the transcriptional repressors of E-cadherin, so we studied their expression and found that formononetin decreased mRNA levels of snail, slug and fibronectin [Figure 3d–f]. Likewise, our immunofluorescence staining analysis demonstrated that formononetin intervention upregulated E-cadherin expression and downregulated vimentin expression significantly [Figure 3g, h].

Formononetin upregulated EGR1 and inhibited MAPK signaling in DU145

RNA sequencing was performed to explore the genes which had been affected by formononetin. Sequencing reads were aligned to the human genome. Difference expression analysis was performed using DESeq. Fold change ≥ 2 and false discovery rate (FDR) < 0.01 were considered to be differential expression. Results showed different gene expression profiles between 100 μM formononetin-treated DU145 and its control [Figure 4a]. Among the significantly

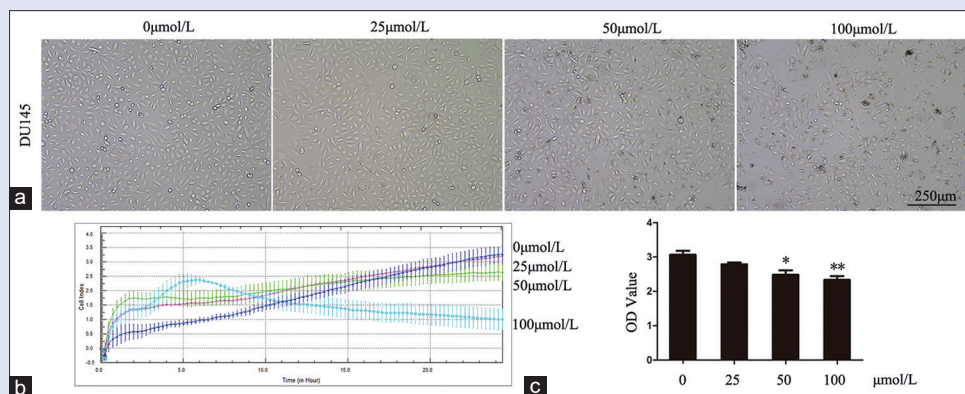


Figure 1: Formononetin inhibits the proliferation of DU145. DU145 was treated with 0, 25, 50, 100 μM /L formononetin for 24 hr. Cell morphological observation by microscope, original magnification, $\times 200$ (a); and RTCA iCELLigence Analyzer in real time (b); (c) Cell activities determined by CCK8. Data were expressed as mean $\pm$ standard deviation, $n = 3$, * $P < 0.05$; ** $P < 0.01$

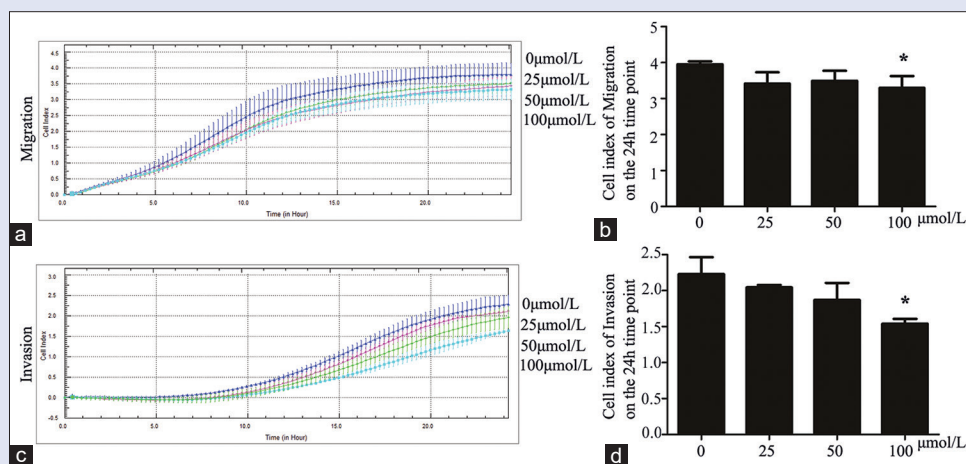


Figure 2: Formononetin inhibits the migration and invasion of DU145. DU145 treated with 0, 25, 50, 100 μM /L formononetin for 24 hr. Cell migration (a) and invasion (c) were determined using RTCA iCELLigence Analyzer in real time. Cell index at the end time point of migration examination (b) and invasion examination (d). Data are expressed as mean $\pm$ standard error, $n = 3$, * $P < 0.05$

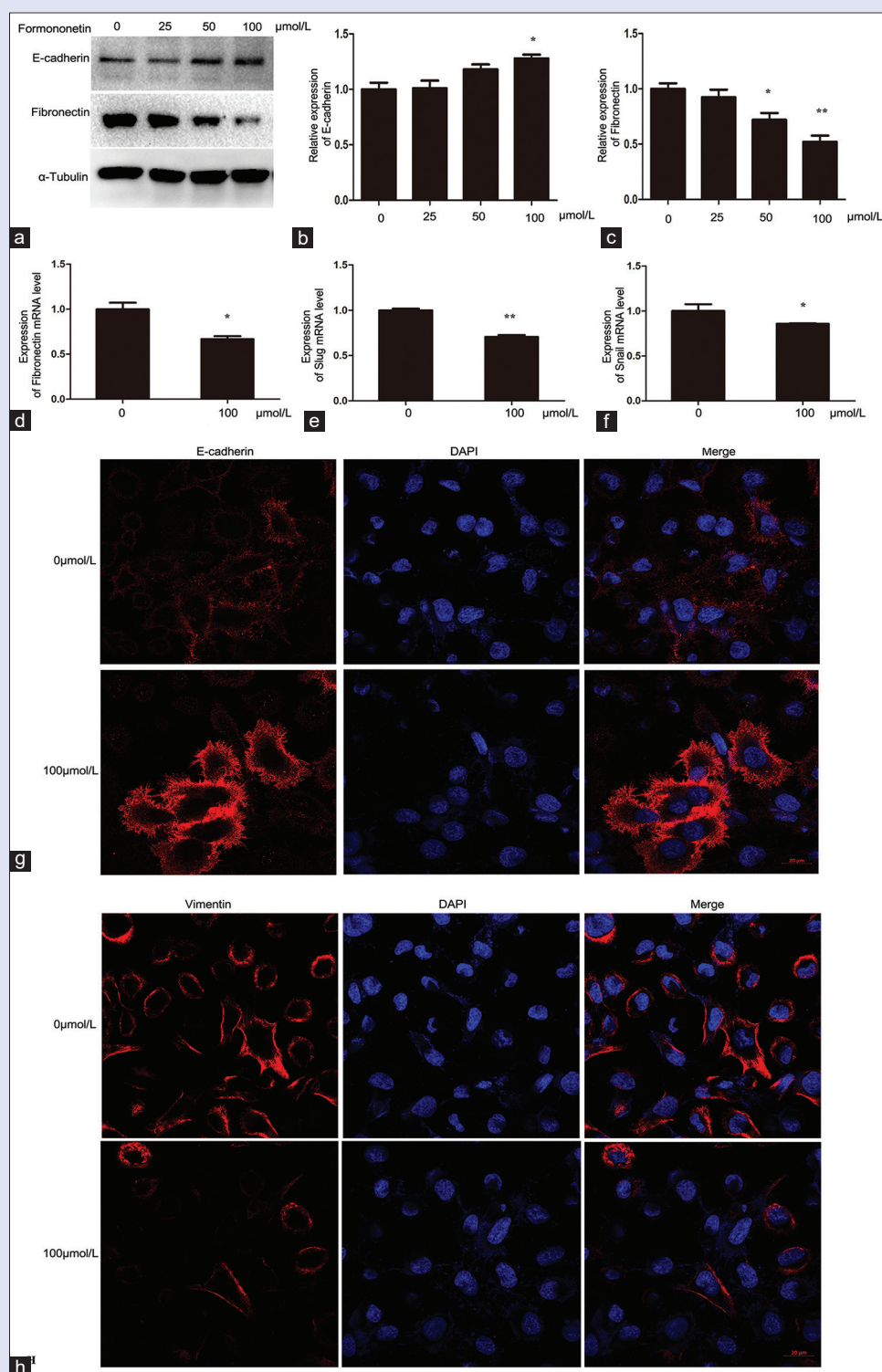


Figure 3: Formononetin represses EMT of DU145. The protein expression of fibronectin, E-cadherin, α -tubulin analyzed using western blot assay (a). Immunoblotting assay using α -tubulin as the internal reference (b–c), RT-qPCR assay (d–f) and immunofluorescence (g–h). Data are expressed as mean $\pm$ standard deviation, $n = 3$, * $P < 0.05$; ** $P < 0.01$

different expression genes, there was an EMT-related gene, EGR1, which was significantly upregulated [Figure 4b]. We then evaluated specific biological processes in which the differentially expressed genes participate. The gene ontology analysis, including molecular function, biological pathway, and cellular component,

were conducted. Results showed that the most enriched molecular function was mainly about MAP kinase activity and transcriptional activator activity [Figure 4c]. Moreover, the enrichment analysis of KEGG pathway suggested that the most remarkable changed pathway was MAPK signaling pathway [Figure 4d]. Thus, we checked

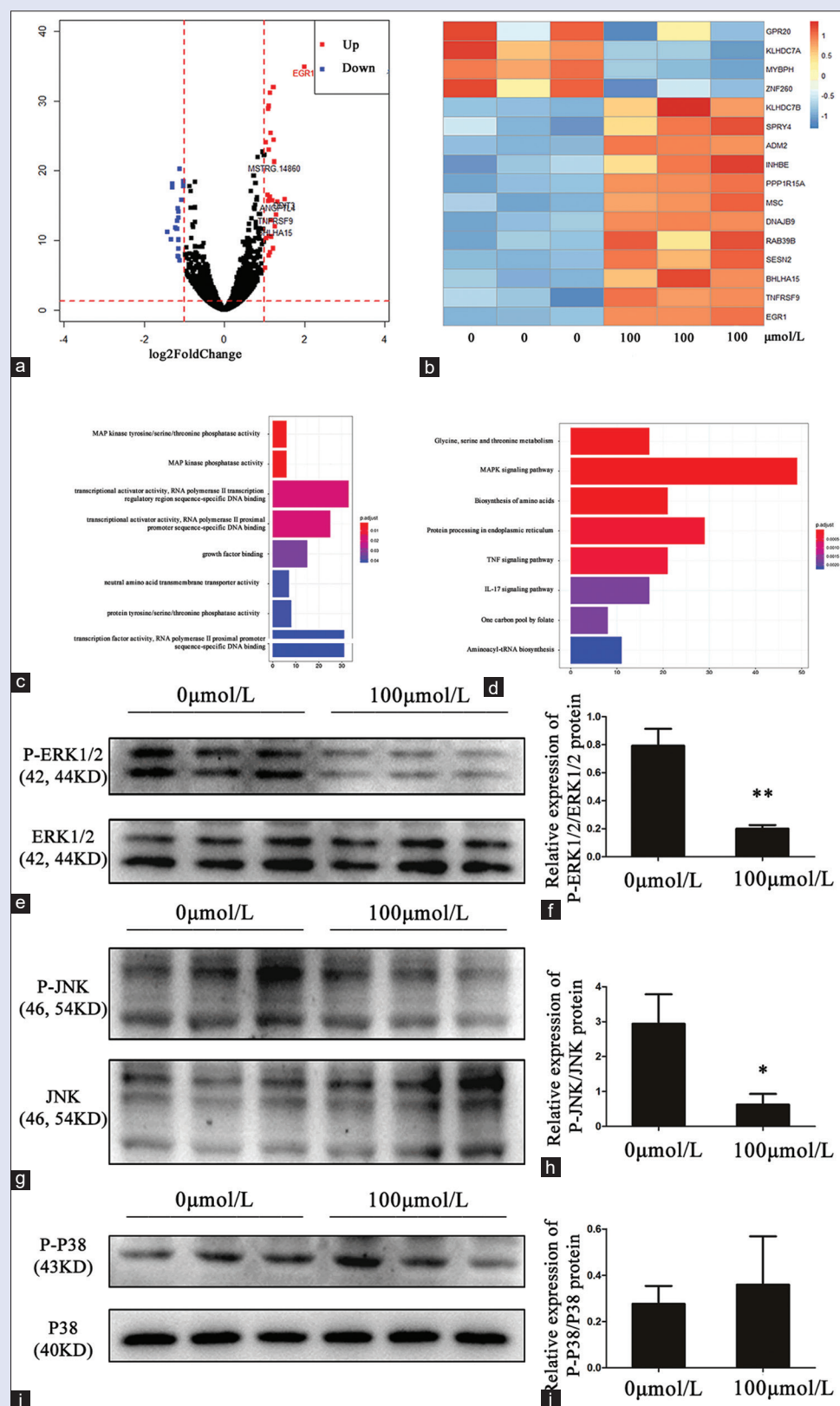


Figure 4: Formononetin upregulates EGR1 gene expression and inhibits MAPK pathway signaling of DU145. DU145 was treated with 100 μm formononetin or DMSO as control for 24 hr, and RNA-sequencing analysis and western blot were performed. Differentially expression genes shown by volcano map (a) and heat map (b). Remarkably changed biological process including molecular function (c), and KEGG pathway by gene ontology analysis (d). Phosphorylation level of ERK1/2 (e, f), JNK (g, h) and P38 (i, j) were analyzed by immunoblot and quantification. ***P* < 0.05, ****P* < 0.01

the expression of phosphorylated MAPK family protein ERK1/2, JNK and P38 in formononetin-treated DU145. The results showed that formononetin significantly decreased the phosphorylation of ERK1/2 [Figure 4e, f] and JNK [Figure 4g, h], while slightly increased phosphorylation of p38 [Figure 4i, j].

Formononetin increased the EGR1 expression in hormone-resistant PCa cells and EGR1 decreased during the human PCa progression

EGR1 is a zinc-finger transcription factor which modulates the androgen receptor (AR) signaling pathway. We found that EGR1 was significantly upregulated by formononetin using RNA sequencing (RNA-seq). The expression level of EGR1 gene was further confirmed via RT-qPCR and western blotting. Results showed that mRNA levels of EGR1 were significantly upregulated by formononetin [Figure 5a]. Meanwhile, the protein levels of EGR1 were also remarkably decreased in formononetin-treated DU145 [Figure 5b, c]. Inconsistent with the cell results, the data from TCGA showed that the expression of EGR1 was lower in PCa than normal prostate tissue [Figure 5d]. Furthermore, the data from The Human Protein Atlas database showed that the EGR1 expression decreased as PCa progressed on from low Gleason grade to high Gleason grade [Figure 5e].

DISCUSSION

EMT is a physiological and pathological process that is characterized by the loss of cell-cell adhesion signature in epithelial cells and meanwhile, acquiring mesenchymal attributes. Recent research has

shown that EMT involves many aspects of cancer progression, including apoptotic resistance,^[8] radiotherapy resistance,^[9,10] chemotherapy resistance,^[11] and stem cell-like self-renewing capacity.^[12] When EMT was stimulated, the expression of epithelial cell markers, including cell adhesion protein E-cadherin decreased; cytokeratin transformed into vimentin, while the expression of mesenchymal cell marker fibronectin increased. These changes enabled the cancer cells to be more mobile, resulting in distant metastasis and failure of treatment.^[13] Therefore, we first examined the effects of formononetin on cellular functions in a hormone-resistant PCa cell line DU145. Then we found that formononetin could inhibit the proliferation, migration and invasion of DU145.

The previous study showed that P62-promoted EMT has a close association with the metastasis of PCa cell line DU145, so we examined the classical EMT markers.^[14] Multiple proteins participate in the process of EMT, such as E-cadherin, fibronectin and vimentin. E-cadherin mediates cell-to-cell adhesion in normal epithelial cells. It decreases along with the progress of EMT, and its loss is considered as the most important marker of EMT during cancer progression. Besides, its expression was regulated by several transcription factors, such as snail and twist. Snail protein binds to the promoter of E-cadherin, then recruits several factors to coordinate histone hypermethylation and deacetylation, thus repressing the transcription and expression of E-cadherin, and triggering EMT in PCa.^[15-17] Fibronectin and vimentin are mesenchymal markers, which promote PCa-aggressive phenotype including tumor migratory capacity and invasiveness.^[18,19] Here, we found that formononetin significantly increased E-cadherin levels, and notably reduced fibronectin, vimentin, slug and snail levels.

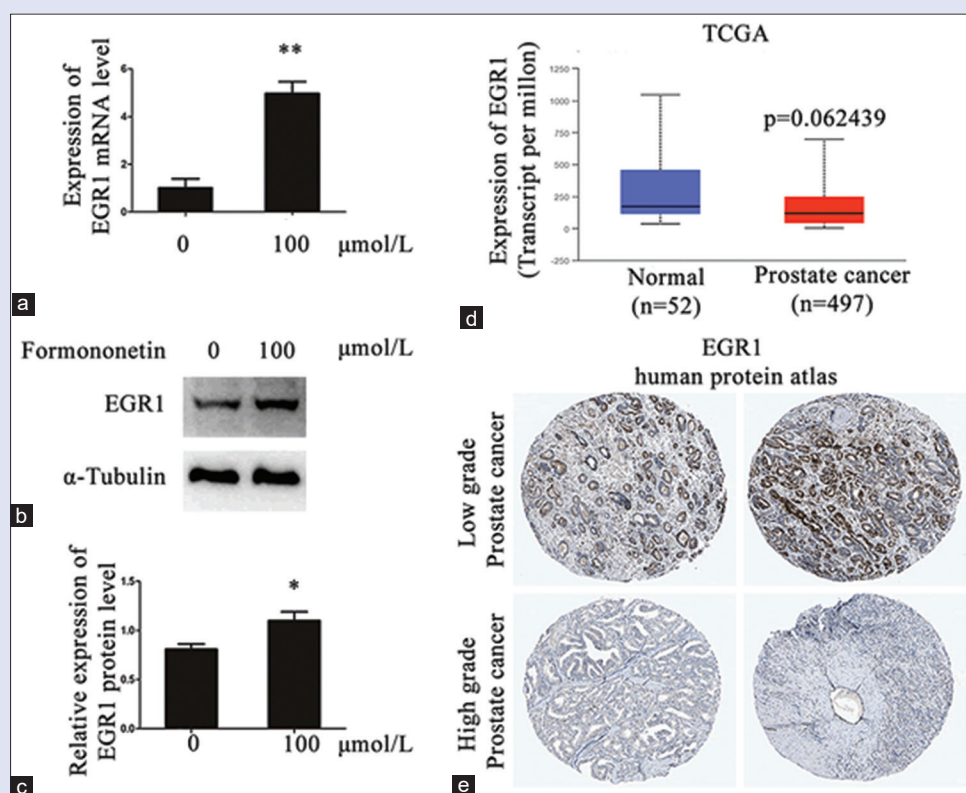


Figure 5: Formononetin increases EGR1 expression in DU145 and EGR1 decreases in the progression of human PCa. DU145 treated with 100 μm formononetin or DMSO as control for 24 hr, mRNA levels of EGR1 were measured by RT-qPCR (a) and immunoblot (b) and quantification (c). EGR1 expression in normal human prostate and PCa tissue from the TCGA database (d). EGR1 expression in low-grade and high-grade PCa tissue from The Human Protein Atlas (e). Data are expressed as mean ± standard error, $n = 3$. ** $P < 0.05$, *** $P < 0.01$

To explore its mechanism behind reversing EMT in DU145, RNA-sequencing was performed. We found the most differentially expressed gene EGR1, and the differential gene enrichment pathway, MAPK signaling pathway.

Early growth response 1 (EGR1), a transcription factor and an EMT-associated protein, is downregulated in PCa and considered to be a tumor suppressor gene.^[20] EGR1 was reported to inhibit the development of PCa by modulating the AR signaling pathway.^[21,22] In some types of cancer, EGR1 has a prominent suppressive activity that transactivates tumor suppressor factors such as fibronectin.^[23] Besides, formononetin has been reported to increase the EGR1 expression in hormone-dependent PCa cell line LNCaP.^[24] Inconsistent with the previous studies, we found that formononetin decreased fibronectin expression and increased EGR1 expression in DU145. Additionally, EGR1 levels had a negative correlation to the PCa progression based on data from the TCGA and The Human Protein Atlas. In addition to tumor suppression, EGR1 was also reported to have protectable effects on chemotherapy resistance.^[23] From all of these results, we speculated that EGR1 was an important target for preventing PCa progress from hormone-dependent to hormone-independent.

Many studies had proved that the tumor suppression role of EGR1 was dependent on MAPK signalling pathway.^[25] MAPK pathway was also one of the differential gene enrichment pathways in the current study. The typical MAPK pathway consists of the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase. It had been reported previously that formononetin attenuated the tumorigenesis of PCa via inhibiting the phosphorylation of ERK1/2 and activating p38.^[26,27] We confirmed these changes here again. Besides, we found that formononetin could also inhibit the phosphorylation of JNK, which hadn't been reported before. There is a clear causal relationship between EMT process and MAPK signalling pathway, as reported by many studies.^[28,29]

CONCLUSION

In summary, we found that formononetin could reverse EMT process, thus inhibiting the proliferation, invasion, and migration of the hormone-resistant PCa cells. Specifically, formononetin upregulated the expression of EGR1 via inhibiting the phosphorylation of ERK1/2 and JNK.

Author contributions

Xue Liang and Ting Liu conceived and designed the experiments. Xue Liang, Ziquan Lan, Yiqiao Huang, Ganggang Jiang performed the experiments. Xue Liang and Ziquan Lan analyzed and interpreted the data. Xue Liang and Ting Liu drafted the paper. Ganggang Jiang, Yiqiao Huang revised the paper. All authors read and approved the final manuscript.

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Conflict of interest

There are no conflicts of interest.

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