

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <https://orca.cardiff.ac.uk/id/eprint/173817/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Mao, Kemin, Wang, Xianghong, Hou, Yakun, He, Xiaowei, Geng, Shuo, Sadiq, Faizan Ahmed, Lian, Yunhe and Sang, Yaxin 2024. Integrated network pharmacology and transcriptomic approach reveal the role of equol in reducing colorectal cancer via regulating multiple cell cycle genes in HCT116 cells. International Journal of Biological Macromolecules 282 (Pt 2), 136832. 10.1016/j.ijbiomac.2024.136832

Publishers page: <https://doi.org/10.1016/j.ijbiomac.2024.136832>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See <http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



1 Integrated network pharmacology and transcriptomic approach reveal the role of
2 equol in reducing colorectal cancer via regulating multiple cell cycle genes in
3 HCT116 cells

4
5 Kemin Mao¹, Xianghong Wang¹, Yakun Hou¹, Xiaowei He¹, Shuo Geng¹, Faizan
6 Ahmed Sadiq², Yunhe Lian³, Yaxin Sang^{*1}

7
8 ¹ Department of Food Science and Technology, Hebei Agricultural University, Baoding,
9 Hebei, China

10 ² Advanced Therapies Group, School of Dentistry, College of Biomedical and Life
11 Sciences, Cardiff University, Cardiff, CF14 4XY, United Kingdom

12 ³ Chenguang Biotech Group Co., Ltd., Handan, Hebei, China

13 * Corresponding author, Yaxin Sang, yxsang1418@163.com, Tel, 86-13831222872,
14 permanent address, No.2596, Lekai South Street, Lianchi District, Baoding, Hebei,
15 China, CN 071000

Abstract

Equol is an isoflavone-derived metabolite known to exhibit strong estrogenic and antioxidant activities. The aim of this paper is twofold: first, to confirm the anticancer potential of equol against colorectal cancer, and second, to reveal the underlying mechanisms. After treatment with 40 µg/mL equol, cell proliferation, cell migration, and colony formation of HCT116 colon cancer cells were inhibited. Network pharmacology and transcriptomics analysis revealed the downregulation of genes related to DNA replication (CCND1, E2F1, CDC6, CDC45, MCM4), leading to G1/S cell cycle arrest and the induction of cell apoptosis, which was confirmed by flow cytometry. Genes associated with the G2-to-M transition (CDK1, CCNA2, CCNB1) were also downregulated. In addition, equol downregulated genes (FOXM1 and ASPM) that control cell migration and invasion. Our data indicate that equol can inhibit colorectal cancer by targeting multiple pathways, suggesting its potential as a key component in the adjuvant treatment of colorectal cancer.

Keywords: Equol; Network pharmacology; Anti-cancer

32



1. Introduction

Colorectal cancer is a type of cancer that often begins as a benign tumor in the colonic and rectal epithelial tissues. Colorectal cancer is the third most common malignancy and the second most common cause of cancer death in the world (Xi & Xu, 2021). If colorectal cancer has spread to the peritoneum, surgery in combination with chemotherapy is recommended as the treatment (Sánchez-Hidalgo, Rodríguez-Ortiz, Arjona-Sánchez, Rufián-Peña, Casado-Adam, Cosano-Álvarez, et al., 2019). Chemotherapy does not only cause unpleasant side effects, but it promotes cancer chemotherapy resistance which makes cancer cells resistant against the chemotherapy drugs, which means they can evade the effects of chemotherapeutics (Liotti, La Civita, Cennamo, Crocetto, Ferro, Guadagno, et al., 2021). The researchers have been trying to reduce the side effects of anti-cancer drugs by preparing precise systems for the targeted delivery of cancer therapeutics (e.g., smart drug delivery systems) that could target the tumor cells without affecting normal cells (Fan, Zhang, Wang, Lin, & Yang, 2020). Another approach is to identify alternative, potent anti-colorectal cancer agents with low toxicity, to help prevent the emergence of drug resistance.

Natural products are a major reservoir of potent therapeutic agents (Liu, Cui, Li, Xie, & Shi, 2023; Naseri, 2023). Flavonoids are important natural polyphenols, which are widely found in plants, vegetables, fruits, grains, tea, and wine. For instance, genistein is one of the most active natural flavonoids which exerts several biological effects in diseases including cancer. Recent structural and functional studies have

revealed that genistein acts as both an ion channel blocker and a gating modifier for TRPV6, a calcium-selective oncochannel implicated in cancer cell proliferation, providing new insights for structure-based drug design (Neuberger, Trofimov, Yelshanskaya, Nadezhdin, Krylov, Efremov, et al., 2023). Soy isoflavones and their metabolites have been extensively studied for their biological effects on different types of cancers (Sahin, Bilir, Ali, Sahin, & Kucuk, 2019). Equol is a metabolite that is produced from the soy isoflavone daidzein and it is known to exert various biological effects on human metabolism, especially because of its estrogen-like biological activity and anti-inflammatory potential (Mayo, Vázquez, & Flórez, 2019). Daidzein can be metabolized into equol in the human gut by special colonic bacterial species such as *Bifidobacterium animalis* (Kwon, Lim, Kim, Kim, & Kang, 2018; Soukup, Engelbert, Watzl, Bub, & Kulling, 2023). Functional studies have reported that equol can inhibit the proliferation of human cervical cancer cell Hela and mouse melanoma B16 cells in an estrogen receptor independent manner (Yamashita et al., 2022). Compared to its precursor daidzein, equol has shown higher anti-cancer activity because of its lipophilic character and higher bioavailability (Ariyani, Miyazaki, & Koibuchi, 2019; Ge, Wei, Wang, & Cao, 2020). In another trial, equol effectively suppressed choriocarcinoma cell proliferation, induced apoptosis, and reduced tumorigenesis through a mechanism involving TRIM21-mediated down-regulation of ANXA2 and inhibition of β -catenin activation (Liu, Wang, Wei, Song, & Ma, 2024). All the above-mentioned characteristics of equol implies its potential as an anticancer agent or a dietary

component of adjuvant therapy for the treatment of cancer. Studies have shown that equol can inhibit colon cancer cell proliferation in a dose-dependent manner (Cai, Zhang, Niu, Zou, & Ma, 2017), but the underlying mechanism is still not clear.

Previous studies have demonstrated molecular mechanisms underlying the anticancer activities of some flavonoids. For example, quercetin exerts anti-cancer effects by inhibiting heat shock factor 1 (HSF1), NF- κ B, and AP-1 (Danics et al., 2020). Apigenin exhibits anticarcinogenic activities through alternative splicing and by inhibiting Hsp70 that enhances TRAIL anticancer activity (Voss, Arango, Tossey, Villalona Calero, & Doseff, 2021). Network pharmacology is a new discipline that aims to understand drug actions and interactions with multi-pathways by taking into account metabolomics and systems biology and establishing “compound-protein/gene-disease” network (Chandran, Mehendale, Patil, Chaguturu, & Patwardhan, 2017). So, network pharmacology method may be appropriate for identifying the complex mechanisms of agents.

Our previous study showed that some specific dietary interventions, such as Tibit kefir milk, could increase the production of equol in the gut (Gao, Mao, Wang, Mi, Fu, Li, et al., 2021). So, the possibility of triggering equol production in the human body is not elusive. Despite a well-known anticancer potential of equol, the underlying mechanism remains unknown. The aim of this work is to elucidate the functions and related mechanism behind the anti-colorectal cancer activity. We have provided some unprecedented insights into the anti-colorectal-cancer mechanism of equol by

96 integrating network pharmacology and transcriptomics.

97 **2. Material and methods**

98 **2.1 Cell line culture**

99 The human colorectal cancer cell lines HCT116 (BNCC342188), HT29
100 (BNCC337732), SW480 (BNCC359300), and Caco2 (BNCC350769) were obtained
101 from BeNa Culture Collection (BNCC, Henan, China). All the cells were grown in
102 complete medium containing 90% Dulbecco's Modified Eagle Medium (DMEM,
103 Gibco, USA) and 10% fetal bovine serum (FBS, Wisent, Jiangsu, China), at 37°C in a
104 humidified atmosphere containing 5% CO₂.

105 **2.2 Cell proliferation assay**

106 The cell proliferation was measured using Cell Counting Kit-8 assay kit (CCK-8,
107 Sevenbio, Beijing, China). Briefly, 5×10^3 cells were seeded in 96-well culture plates
108 (Jet Biofil, Guangdong, China) with 100 μ L complete medium each well. After
109 overnight incubation, the cells were treated with equol (CAS: 94105-90-5, Aladdin,
110 Shanghai, China) at concentrations of 0, 20, 40, 60, 80, 100, 120 μ g/mL for 24 h and
111 48 h, respectively. Then, according to the manufacturer's instructions, the absorbance
112 of the solution in each well of plate was determined on a microplate reader (Multiskan
113 Spectrum, Thermo, USA) at 450 nm. All experiments were performed in sextuplicate.

114 **2.3 Cell scratch wound healing assay**

115 5×10^5 cells were seeded in 6-well culture plates with each well containing 2 mL
116 complete medium. Once an adherent confluent monolayer formed, a 10- μ L

micropipette tip was used to scratch the cell monolayer, and after washing with 0.01M phosphate-buffered saline (PBS, pH = 7.4, Sevenbio, Beijing, China), the medium was changed to 1% FBS medium containing equol with the following concentrations: 0, 20, 40, 80 $\mu\text{g/mL}$ for 24 h. The wound areas were micrographed before and after equol treatment. All experiments were performed in triplicate and software Image J software (Image J 1.53t, NIH, USA) were used to analyze the images.

2.4 Cell colony formation assay

A total of 10^3 cells were seeded in 6-well culture plates with 2 mL of complete medium each well. The cells were then cultured in complete medium containing equol at concentrations of 0, 20, 40, 80 $\mu\text{g/mL}$, with the medium refreshed every 2 days. After 14 days of cultivation, the cells were fixed with 4% paraformaldehyde (Solarbio, Beijing, China) and stained with 1% crystal violet ammonium oxalate solution (Solarbio, Beijing, China). The number of colonies was counted by software Image J (Image J 1.53t, NIH, USA). All experiments were performed in triplicate.

2.5 Cell cycle assay

HCT116 cells were seeded in 6-well plates at the density of 5×10^5 cells per well. After overnight incubation, the cells were exposed to equol for 24 h at the following concentration: 0, 20, 40, 80 $\mu\text{g/mL}$. Cells were then collected and fixed with 70% ethanol at 4°C overnight. After centrifugation, cells were stained with propidium (PI, Servicebio, Hubei, China) and RNase A solution (Servicebio, Hubei, China) for 30 min at 37°C in the dark. In the end, the cell cycle distributions were detected by flow

cytometry CytoFLEX (Beckman Coulter, USA).

2.6 Cell apoptosis assay

For cell apoptosis analysis, cells were cultured and treated exactly the same way as described above for cell cycle analysis. The apoptosis cells were determined by Annexin V-FITC/PI Apoptosis Kit (AP105, Lianke, Zhejiang, China) following the manufacturer's protocol. After 24 h of treatment, cells were collected and incubated with Annexin V-FITC and PI in the dark. Finally, cell apoptosis was analyzed by flow cytometry CytoFLEX (Beckman Coulter, USA).

2.7 Transcriptomics

HCT116 cells (5×10^5 cells/well) were seeded in 6-well plates and incubated overnight. Then, cells were exposed to 80 $\mu\text{g/mL}$ equol. After 24 h of treatment, total RNA was extracted using the TRIzol Reagent (Invitrogen, China). Bioanalyser (Agilent Technologies, Germany) and ND-2000 (NanoDrop Technologies, USA) were used to evaluate quality and quantities of RNA. RNA purification, reverse transcription, library construction and sequencing were performed at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China). Illumina NovaSeq 6000 sequencer (2 $\times$ 150 bp read length) was used to build the RNA-seq sequencing library.

To identify differentially expressed genes (DEGs) between two different groups, transcripts per million reads (TPM) method was used to calculate the expression level of each gene. RSEM was used to quantify gene abundances. The edgeR was used to calculate the gene expression values and $|\log_2(\text{Fold Change})| > 1$ and $p\text{-adjust} < 0.05$

were considered to be significantly different expressed genes. In addition, functional-enrichment analysis including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed to identify functional pathways using clusterProfiler (version 4.6.2).

2.8 Hub genes screening and transcription factors prediction

Genes related to cell proliferation, cell migration, colony formation, cell cycle, and cell apoptosis were collected from the GeneCards: The Human Gene Database (<http://www.genecards.org/>). The protein-protein interaction (PPI) was performed by the STRING database (<http://string-db.org/>) and drawn by Cytoscape (version 3.7.1, <https://cytoscape.org/>). Significant modules were identified from the PPI network using the molecular complex detection method with a score of ≥ 4 (Bader & Hogue, 2003). The hub genes were identified by Cytohubba plugin (<http://apps.cytoscape.org/apps/cytohubba>), and the transcription factors (TFs) were detected by iRegulon plugin (<http://apps.cytoscape.org/apps/iRegulon>).

2.9 Western blot analysis of key targets

The cells were incubated in 6-well plates (5×10^5 cells/well), when cellular density reached nearly 80%, the cells were exposed to different concentrations of equol (20, 40, 80 $\mu\text{g/mL}$) for 24 h. The cells were then washed with ice-cold PBS (Sevenbio, Beijing, China) and harvested by 0.25% trypsinization (Sevenbio, Beijing, China) followed by centrifugation. Cells were then lysed with lysis buffer (Bio-Rad, USA). Cells were recovered by spinning at 16000 rpm for 20 min and the cell pellet was used for protein

extraction. The bicinchoninic acid (BCA) assay kit (BCA Protein Assay Kit, Sevenbio, Beijing, China) was used to determine protein content.

Samples (10 µg/lane) were subject to 10-12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.45 µm polyvinylidene difluoride (PVDF, Millipore, USA) membranes. The PVDF membranes were blocked with 5% BSA (Sevenbio, Beijing, China) followed by overnight incubation at 4°C with primary antibodies: Anti-CDK1 (1: 10000, ab32094, Abcam, Shanghai, China), Anti-Cyclin A2 (1: 2000, ab181591, Abcam, Shanghai, China), Anti-Cyclin B1 (1: 2000, ab181593, Abcam, Shanghai, China), Anti-Cyclin D1 (1: 10000, ab134175, Abcam, Shanghai, China), Anti-E2F1 (1: 1000, Proteintech, Wuhan, China), and Anti-NFYA (1: 1000, ab139402, Abcam, Shanghai, China). The next day, the membranes incubated with the anti-rabbit secondary antibodies (1:5000, Proteintech, Wuhan, China) for 2 h at room temperature. Protein expression levels were determined using NcmECL Ultra reagents (New Cell & Molecular Biotech Co. LTD, Jiangsu China) and the grey value was evaluated by ImageJ (Image J 1.53t, NIH, USA).

2.10 Molecular docking of potential targets with equol

The target protein structure was obtained from Protein Data Bank (<https://www.rcsb.org/>) and UniProt Knowledge Database (<https://www.uniprot.org/>). Molecular docking study was performed to determine the molecular intervention using CDOCKER program in the BIOVIA Discovery Studio 2016 (Accelrys, Inc., San Diego, USA), and then the docking results were visualized by an open-source version PyMOL

2.4.0 (<https://github.com/schrodinger/pymol-open-source>).

2.11 Statistical analysis

All data were expressed as mean $\pm$ S.D. and analyzed using R programming language (version 4.0.0, R Core Team). A parametric test (one-way ANOVA followed by Tukey's post hoc test) was applied when normality assumptions and homogeneity of variance were satisfied. Nonparametric data were assessed using the Wilcoxon rank-sum test. $p < 0.05$ was considered significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Graphic presentations were generated using the R packages ggplot2 (<http://ggplot2.org>).

3. Results

3.1 Equol inhibited cell proliferation, migration and colony formation

CCK8 assay, colony formation assay, and wound healing test were performed to analyse the effect of equol on cell proliferation, migration and invasion of cancer cells. Equol inhibited proliferation in four human colorectal cancer cell lines (HCT116, HT29, SW480, and Caco2 cell) in a dose- and time-dependent manner (Figure 1a). Furthermore, the IC₅₀ values following 24 h treatment for HCT116, HT29, SW480, and Caco2 were 79.0 $\mu\text{g/mL}$, 57.6 $\mu\text{g/mL}$, 94.5 $\mu\text{g/mL}$, and 147.0 $\mu\text{g/mL}$, respectively. Additionally, HCT116 and HT29 colorectal cancer cell lines with lower IC₅₀ values were selected for wound healing assay and colony formation assay to test the effect of equol on tumor formation and cancer cell migration. Equol at the concentration of ≥ 40 $\mu\text{g/mL}$ and 80 $\mu\text{g/mL}$ inhibited migration of HCT116 and HT29 cells, respectively, after 24 h of incubation (Figure 1b). Colony formation assay showed a significant inhibition,

compared to the control, in the colony formation capacity of HCT116 and HT29 cells when subjected to 20 µg/mL of equol, whereas only negligible formation of colonies were observed when 40 µg/mL of equol was used (Figure 1c). Those results suggested that equol can inhibit colorectal cancer cell proliferation, migration and colony formation, and HCT116 cells were selected for further experiments due to its high sensitivity to equol.

3.2 Equol induced cell cycle arrest and promoted cell apoptosis

Flow cytometry was used to assess whether equol could induce cell cycle arrest and cell apoptosis. Figure 2a shows different phases of HCT116 cell population: G0/G1, S, and G2/M. Equol induced G0/G1 phase arrest, and different G0-G1 accumulation level can be seen induced by different doses of equol. In addition, the proportion of G2/M phase cells increased after equol intervention but not significantly. The flow cytometry results of apoptosis are represented in Figure 2b, there was a positive correlation between the apoptosis rate and the dose of equol. Those results demonstrated that equol could induce cell cycle arrest and promote cell apoptosis in a dose-dependent manner.

3.3 Equol induced a distinct gene expression pattern in HCT116 cells

To investigate the mechanism by which equol hinders cancer cell proliferation and induces cell cycle arrest, transcriptomic analysis was performed using RNA-sequencing. The RNA-seq data quality summary is provided in Supplementary Table S1. The genes from control group (CK) and equol treated group (EQ) are presented in the Venn

diagram (Figure 3a), a total of 14910 and 14812 genes were detected in the CK and EQ groups, respectively. There were 14070 genes that were expressed in both CK and EQ groups, while 840 and 742 genes were only associated with the CK and EQ groups, respectively. Based on the differential expression filter criteria ($|\log_2(\text{Fold Change})| > 1$ and $p\text{-adjust} < 0.05$), we identified 1685 DEGs (as shown in Supplementary Table S2) between EQ and CK groups, where 739 genes were upregulated and 946 genes were downregulated in EQ group compared with CK group (Figure 3b). PCA analysis was performed to discriminate samples with differential gene expression into different clusters. According to the PCA scores plot (Figure 3c), the samples were clearly separated into two clusters along the first principal component (PC1), which accounts for 84.87% of the variance. Thus, it became evident that equol-treated cancer cells (HCT116 cells) had altered gene expression. Hierarchical clustering and heat map analysis of differentially expressed genes is shown in Figure 3d. The distinct patterns visible in the heatmap demonstrate that equol treatment induces significant changes in the transcriptomics of HCT116 cells. This observation strongly supports our hypothesis that equol has a substantial impact on gene expression patterns in colorectal cancer cells.

3.4 GO and KEGG pathways enrichment analysis of difference expression genes

To understand the functions and pathways associated with DEGs, GO functional and KEGG enrichment analyses were performed. GO functional analysis covered three domains: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). GO term enrichment analysis of all DEGs showed significant enrichment of GO

terms associated with cell proliferation and cell cycle, such as organelle fission, nuclear division, DNA replication, chromosome segregation, regulation of mitotic cell cycle, and mitotic nuclear division (Figure S1a). GO enrichment analysis of downregulated genes specifically in EQ group showed that GO terms such as organelle fission, nuclear division, DNA replication, chromosome segregation, and mitotic nuclear division were enriched (Figure S1b). GO enrichment analysis of upregulated genes specifically in EQ group showed that GO terms related to regulation of endopeptidase activity, response to endoplasmic reticulum stress, response to unfolded protein, cellular response to unfolded protein, and intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress were enriched (Figure S1c).

KEGG pathway is a collection of manually drawn pathway maps that help to understand genetic pathways, signal transduction pathways and molecular interaction networks. For the cells treated with equol, KEGG pathways associated with all DEGs were related to glutamatergic synapse, HIF-1 signaling pathway, DNA replication, cushing syndrome, and cell cycle (Figure S1d). All downregulated genes could be assigned to progesterone-mediated oocyte maturation, Wnt signaling pathway, DNA replication, human T-cell leukemia virus 1 infection, and cell cycle (Figure S1e), whereas all upregulated genes could be assigned to epithelial cell signaling in *Helicobacter pylori* infection, rheumatoid arthritis, and NF- κ B signaling pathway (Figure S1f).

3.5 Screening of bioprocess targets in GeneCards database

To investigate the mechanism by which equol inhibits cancer cells proliferation, colony formation, and cell migration and induces cell cycle arrest and cell apoptosis, a bioprocess-target network was used to reveal the relationship between bioprocesses and targets. A total of 1569 genes related to cell proliferation, cell cycle, cell migration, colony formation, and cell apoptosis were obtained from the GeneCards database. A total of 168 shared genes were selected by taking the intersection of 1569 bioprocess targets and 1685 DEGs of transcriptomics (Figure 4a).

3.6 Biological-process-target network building and hub genes screening

To identify potential interactions of the 168 shared genes, a protein-protein interaction network (PPI) was constructed by STRING database. After removing free genes, a total of 112 genes from 168 candidate genes were contained into the network which included 112 nodes and 310 edges (Figure 4b). The higher the degree value of the node is, the larger the node is, and the darker the color of the node is. High degree of a node indicates importance of the node in the network because of its high number of interactions and possible role in the observed effect. The Cytoscape plugin MCODE was used to identify gene clusters with similar biological functions. As shown in Figure 4c, four significant modules were identified with MCODE score > 4 . Module 1 contained 13 nodes and 76 edges; Module 2, 3, and 4 contains 4 nodes and 6 edges each. CytoHubba plugin was used to explore the key nodes and sub-networks in the network. A total of 17 genes with maximal clique centrality (MCC) > 5000 were selected as

candidate genes, as shown in Figure 4d. The selected 17 genes belonged to Module 1 and Module 2. Results of MCODE and cytoHubba analysis indicated that 17 genes were key targets of equol in colorectal cancer treatment. Interactions between 17 hub genes are displayed in Figure 4e.

The results of the gene expression analysis of 17 hub genes are shown in Figure 4f. Among the 17 hub genes, the expression of genes related to cell apoptosis, such as CHEK1, CDK1, BIRC5, CCNA2, CCNB1, and MKI67 were decreased in EQ group. The expression of genes related to cell cycle, such as TOP2A, CDK1, CHEK1, CDCA8, AURKA, CCNB1, BIRC5, CDCA3, CDC6, CDC45, MKI67, BUB1B, MCM4, and CCNA2 reduced in EQ group. Colony formation related genes, such as CDK1, AURKA, and BIRC5, also showed reduced expression in EQ group. Genes related to cell migration (e.g., ASPM) also showed a decrease in expression in EQ group. Further, underexpression of genes promoting cell proliferation (e.g., CHEK1, CDK1, TPX2, TOP2A, AURKA, CCNB1, BIRC5, CCNA2, FOXM1, and MKI67) was observed in EQ group.

3.7 Predicted transcription factors of hub genes sets

The iRegulon plugin was used to identify the TFs of hub genes. The TF-gene target regulatory network is shown in Figure 4g, where 13 of 17 hub genes are shown to be regulated by TF nuclear transcription factor Y subunit alpha (NFYA). TF E2F1 was found to regulate 6 hub genes. The original gene expression values of TF NFYA and E2F1 are shown in Figure 4h. The two TFs were downregulated after the equol

treatment on cancer cells.

3.8 Western blot analysis of protein expression of hub genes

To further uncover the mechanism of equol on anti-colorectal-cancer, western-blot was used to analyze the expression of the initial factor of cell cycle protein CCND1, and several hub proteins CDK1, CCNA2, and CCNB1. Additionally, the expression of two TFs, NFYA and E2F1, were also detected by western blot. As shown in Figure 4i, the expression of CDK1, CCNA2, CCND1, E2F1, and NFYA decreased after subjecting the cancer cells to 80 µg/mL of equol, while CCNB1 showed a decrease in expression after being subjected to 40 µg/mL of equol.

3.9 Molecular docking analysis of potential targets and equol

Molecular docking was applied to predict the potential interactions between the 17 core proposed protein targets and equol. The information related to the binding of equol to multiple target proteins is shown in Table 1. Multiple H-bond interactions were found to be formed between equol and the surrounding amino acid residues from CDK1, CCNA2, BUB1B, ASPM, FOXM1, and CCND1. The numbers of H-bonds between target proteins and equol signifies a strong binding between the molecules. The docking results are shown in Figure S2.

4. Discussion

Currently, there is a growing interest in finding dietary components that can help to prevent and treat several cancers as functional food or drug elements (Zhang, Pan, Yang, Jiang, Zhang, Zeng, et al., 2020). Numerous studies have indicated that increased

soy food intake is linked to decreased risk of cancer (Fan, Wang, Li, Jiang, Shi, Shi, et al., 2022; Zhu, Yao, Shi, Everaert, & Ren, 2018). One of the reasons behind this is the presence of various type of isoflavones including genistein, daidzein, and glycitein in soy foods (Zhao, Zhao, Parris, Xing, & Yang, 2016). Daidzein can be metabolized into equol in the human gut by some special colonic bacterial species such as *B. animalis* (Kwon, Lim, Kim, Kim, & Kang, 2018). Equol has shown to inhibit proliferation of various types of tumor cell lines with a far greater antitumor potential compared to its precursor daidzein (Itsumi, Shiota, Takeuchi, Kashiwagi, Inokuchi, Tatsugami, et al., 2016). In this study, equol exhibited inhibitory effects on colorectal cancer cell proliferation, migration, and colony formation. Flow cytometry analysis found that equol arrested cell cycle and induced cell apoptosis, which are two mainly mechanisms in anticancer treatments (Orabi, Abaza, Luqmani, & Al-Attiah, 2021). This research investigated the underlying molecular mechanism behind the strong cytotoxic activity of equol against colorectal cancer HCT116 cells.

Limitless replicative potential of tumor cells, resistance to cell death, and evasion of growth suppressors and genomic instability are hallmarks of several cancers including colorectal cancer (Wang, Tang, Wu, Yang, Wang, Wang, et al., 2019). Therefore, hindering replication potential of cancer cells is an effective strategy for cancer therapy. As shown in Fig. 7, when the growth stimulation received at G1 stage, the D-type cyclins (CCND1, CCND2, CCND3) that promote cell cycle progression from G1 to S phase were upregulated and activated CDK4 and CDK6 by forming

368 cyclin-CDK protein complex. The cyclin-CDK complexes phosphorylate
369 retinoblastoma (Rb) protein which results in its inactivation that triggers uncontrolled
370 cell proliferation and this is one of the most fundamental events in cancer. This event
371 leads to the release of E2F proteins (E2F1–E2F3a, transcriptional activators; E2F3b–
372 E2F8, transcriptional repressors) to modulate cell cycle regulatory genes such as cyclin
373 E and cyclin A (Bury, Le Calvé, Ferbeyre, Blank, & Lessard, 2021; Suski, Braun,
374 Strmiska, & Sicinski, 2021). Cyclin E-CDK2 triggers DNA replication and promotes
375 the formation of pre-replication (preRC) complex, which consists of CDT1, CDC6, the
376 origin recognition complex (ORC) proteins and the DNA helicase MCMs (MCM2-7),
377 to license origins of replication (Lee, Fernandez, Alexandrou, Sergio, Deng, Rogers, et
378 al., 2020). ASK (activator of S phase kinase) is an activation subunit of Cdc7 kinase
379 activates MCM complex by phosphorylation and CDC45 recruits DNA polymerase α
380 to chromatin (Kurayoshi, Ozono, Iwanaga, Bradford, Komori, Araki, et al., 2018). In
381 this study, the CCND1 gene, as the initial factor of cell cycle, was downregulated after
382 equol intervention, which can reduce the synthesis of cyclin D-CDK4/6 complex,
383 causing a reduction in the release of E2F. Studies have shown that a decreased CCND1
384 expression is linked to the inhibition of cancer cell proliferation and decreased cisplatin
385 resistance, while some *in vivo* experiments have demonstrated that inhibiting
386 expression of CCND1 has little effects on the normal growth and development of mice
387 (Noel, Yeste-Velasco, Mao, Perry, Kudahetti, Li, et al., 2010; Zhu, Qin, Xu, Sun, Chen,
388 Zhao, et al., 2019). It means that CCND1 is a safe target for anticancer agents and equol

inhibits cell proliferation in a safe manner. A reduction in the expression of E2F1 would mean a reduction in the synthesis of proteins that are required by cells to entry into the S phase (Hwang & Clurman, 2005). The expression of CDC6, CDC45, and MCM4 was downregulated after equol intervention, illustrating the inhibition of preRC complex synthesis and recruitment of DNA Polymerase alpha primase, which compromise DNA replication. CDC6 is highly expressed in multiple types of cancer cells. A study reported a decrease in the cisplatin resistance after downregulation of the expression of CDC6 in bladder cancer cells (Song, Wang, & Liu, 2022). In addition, CDC6 and CDC45 are inextricably associated with colorectal cancer and thus they are perceived as potential targets for the diagnosis of colorectal cancer (Hu, Wang, Li, Wan, Shao, Wu, et al., 2019). NFYA is a transcription factor that regulates numerous genes involved in the cell cycle. One way by which NFYA regulates cell cycle is through the NFYA-CCND1/CDK4-E2F1 axis (Li, Xiao, Chen, Chen, Hu, & Yin, 2020). In this study, the expression of NFYA, CCND1, and E2F1 were downregulated after equol intervention, suggesting that the NFYA-CCND1/CDK4-E2F1 axis was blocked. All of those results suggest that equol inhibits the transition of cell cycle from G1 to S phase, as further confirmed by flow cytometry. Equol also has potential as an adjunct agent that may help improve cisplatin sensitivity.

Cyclin A is the activator of cyclin-dependent kinases: CDK1 and CDK2 during the S and G2 phases of the cell cycle. Cyclin A-CDK2 drives the transition from G1 to S phase, whereas cyclin A/B-CDK1 control the G2-to-M transition (Cheng, Peng, Zhu,

410 Zhou, Wang, Ju, et al., 2020; Markowitsch, Schupp, Lauckner, Vakhrusheva, Slade,
411 Mager, et al., 2020). Cyclin A/B-CDK1 protein complex phosphorylates several
412 proteins which are associated with mitotic progression and cell division. In this study,
413 downregulation of genes CDC25, CHEK1, and FOXM1 were observed after equol
414 intervention. Previous studies have shown that CDC25 can activate cyclin A/B-CDK1
415 (Liang, Cao, Zhang, Xia, Zheng, Li, et al., 2016), but the expression of CDC25 was
416 inhibited by CHEK1/2-mediated phosphorylation (Reinhardt & Yaffe, 2009).
417 Afterward, CHEK1/2 and FOXM1 accelerate the activation of cyclin B-CDK1 (Costa,
418 2005; Smits & Gillespie, 2015). Decreased expression of CDC25, CHEK1, and
419 FOXM1 indicated equol possibly inhibited the activation of cyclin A/B-CDK1. We
420 further observed significant down-regulation of genes CCNA2, CCNB1, and CDK1.
421 Inhibition of the synthesis of proteins associated with mitotic progression and cell
422 division caused cell cycle disruption at the G2/M phase. In summary, equol induces cell
423 cycle arrest, leading to an increase in apoptosis and a decrease in cell proliferation.

424 A previous study reported that BIRC5 knockout could induce cell apoptosis
425 (Narimani, Sharifi, & Jalili, 2019), and downregulation of BIRC5 leads to reduced
426 capillary formation (Tian, Fratz, Hou, Lu, Görlach, Hess, et al., 2011). Angiogenesis is
427 one of the therapeutic targets for cancer treatment (Farooq, Abutaha, Mahboob,
428 Baabbad, Almoutiri, & Wadaan, 2020). BIRC5 was downregulated after the
429 intervention with equol, indicating that equol intervention may have some potential
430 effects on controlling the oxygen and nutrients supply on tumor site. The tumor growth

also depends on cell migration and invasion. A growing body of evidence indicates that FOXM1 is highly expressed in solid tumors and that it regulates proliferation, migration, and invasion by activating the expression of ASPM (Zeng, Cheng, Wen, Wang, Chen, Zhao, et al., 2020). Equol also reduced the expression of FOXM1 and ASPM. In summary, equol has the potential to serve as an anticancer agent as it regulates cell proliferation, cell migration, cell cycle, and cell apoptosis.

Compounds that intervene and cause negative effect on cell cycle of cancer cells without causing any harm to normal cells are used for the development of new anticancer agents (Xi, Ma, Zhang, Yuan, & Wang, 2018). Multi-target kinase inhibitors, such as CDK, are being investigated as anti-cancer agents (Kwon, Lee, Kim, Jung, Kang, Hwang, et al., 2009; Law, Corsino, Narayan, & Law, 2015). In this study, we have predicted the interaction of equol and proteins related to cell cycle by molecular docking, the results have shown that equol could bind with multi-targets by forming hydrogen bonds which suggested the potential of equol as a multitarget anticancer compound.

5. Conclusion

In this study, we confirmed the anti-colorectal cancer activity of equol via various methods including a study on the effect of equol of cancer cells proliferation, colony formation, and cell migration. One of the underlying mechanisms by which equol exerts its anticancer effects is through regulation of the expression of cell cycle related genes. Additionally, we observed that equol also regulates genes related to cisplatin resistance

and capillary formation. In summary, equol has the potential to serve as an adjunct agent to improve cancer treatment.

Acknowledgments

This work was funded by the National Key Research and Development Program of China (2022YFD1600400).

Data Availability

The RNA-Seq data have been deposited in the NCBI Sequence Read Archive under accession number PRJNA1158419.

CRediT authorship contribution statement

Kemin Mao: Conceptualization, Methodology, Visualization, Writing - original draft. Xianghong Wang: Conceptualization, Methodology, Writing - review & editing. Yakun Hou: Methodology, Formal analysis. Xiaowei He: Software, Visualization. Shuo Geng: Visualization. Sadiq Faizan Ahmed: Writing - review & editing. Yunhe Lian: Methodology. Yaxin Sang: Funding acquisition, Project administration, Supervision, Writing - review & editing.

Conflict of Interest

The authors confirm that they have no conflict of interest to declare for this publication.

References

- Ariyani, W., Miyazaki, W., & Koibuchi, N. (2019). A Novel Mechanism of S-equol Action in Neurons and Astrocytes: The Possible Involvement of GPR30/GPER1. *Int. J. Mol. Sci.*, 20(20), 5178. <https://doi.org/10.3390/ijms20205178>
- Bader, G. D., & Hogue, C. W. (2003). An automated method for finding molecular complexes in large protein interaction networks. *BMC Bioinf.*, 4(1), 1-27. <https://doi.org/10.1186/1471-2105-4-2>

- 476 Bury, M., Le Calvé, B., Ferbeyre, G., Blank, V., & Lessard, F. (2021). New Insights
477 into CDK Regulators: Novel Opportunities for Cancer Therapy. *Trends Cell*
478 *Biol.*, 31(5), 331-344. <https://doi.org/10.1016/j.tcb.2021.01.010>
- 479 Cai, Y., Zhang, H., Niu, W., Zou, Y., & Ma, D. (2017). Effects of equol on colon cancer
480 cell proliferation. *Beijing da xue xue bao. Yi xue ban= Journal of Peking*
481 *University. Health Sciences*, 49(3), 383-387
- 482 Chandran, U., Mehendale, N., Patil, S., Chaguturu, R., & Patwardhan, B. (2017).
483 *Network Pharmacology: Innovative Approaches in Drug Discovery*, 127.
484 <https://doi.org/10.1016/B978-0-12-801814-9.00005-2>
- 485 Cheng, S., Peng, T., Zhu, X., Zhou, F., Wang, G., Ju, L., ... Wang, X. (2020). BORA
486 regulates cell proliferation and migration in bladder cancer. *Cancer Cell Int.*, 20,
487 1-10. <https://doi.org/10.1186/s12935-020-01392-8>
- 488 Costa, R. H. (2005). FoxM1 dances with mitosis. *Nat. Cell Biol.*, 7(2), 108-110.
489 <https://doi.org/10.1038/ncb0205-108>
- 490 Danics, L., Schvarcz, C. A., Viana, P., Vancsik, T., Krenács, T., Benyó, Z., ... Hamar, P.
491 (2020). Exhaustion of Protective Heat Shock Response Induces Significant
492 Tumor Damage by Apoptosis after Modulated Electro-Hyperthermia Treatment
493 of Triple Negative Breast Cancer Isografts in Mice. *Cancers*, 12(9), 2581.
494 <https://doi.org/10.3390/cancers12092581>
- 495 Fan, J., Zhang, Z., Wang, Y., Lin, S., & Yang, S. (2020). Photo-responsive degradable
496 hollow mesoporous organosilica nanoplatfoms for drug delivery. *J.*
497 *Nanobiotechnol.*, 18(1), 91. <https://doi.org/10.1186/s12951-020-00642-1>
- 498 Fan, Y., Wang, M., Li, Z., Jiang, H., Shi, J., Shi, X., ... Ma, L. (2022). Intake of Soy,
499 Soy Isoflavones and Soy Protein and Risk of Cancer Incidence and Mortality.
500 *Front. Nutr.*, 9, 847421. <https://doi.org/10.3389/fnut.2022.847421>
- 501 Farooq, M., Abutaha, N., Mahboob, S., Baabbad, A., Almoutiri, N. D., & Wadaan, M.
502 A. A. (2020). Investigating the antiangiogenic potential of Rumex vesicarius
503 (humeidh), anticancer activity in cancer cell lines and assessment of
504 developmental toxicity in zebrafish embryos. *Saudi J. Biol. Sci.*, 27(2), 611-622.
505 <https://doi.org/10.1016/j.sjbs.2019.11.042>
- 506 Gao, J., Mao, K., Wang, X., Mi, S., Fu, M., Li, X., ... Sang, Y. (2021). Tibet Kefir milk
507 regulated metabolic changes induced by high-fat diet via amino acids, bile acids,
508 and equol metabolism in human-microbiota-associated rats. *J. Agric. Food*
509 *Chem.*, 69(23), 6720-6732. <https://doi.org/10.1021/acs.jafc.1c02430>
- 510 Ge, Y. F., Wei, C. H., Wang, W. H., & Cao, L. K. (2020). The effect of sorghum
511 resistance resistant starch-mediated equol on the histological morphology of the
512 uterus and ovaries of postmenopausal rats. *Food Sci. Nutr.*, 8(8), 4055-4065.
513 <https://doi.org/10.1002/fsn3.1670>
- 514 Hu, Y., Wang, L., Li, Z., Wan, Z., Shao, M., Wu, S., & Wang, G. (2019). Potential
515 prognostic and diagnostic values of CDC6, CDC45, ORC6 and SNHG7 in
516 colorectal cancer. *OncoTargets Ther.*, 11609-11621.
517 <https://doi.org/10.2147/OTT.S231941>

518 Hwang, H. C., & Clurman, B. E. (2005). Cyclin E in normal and neoplastic cell cycles.
 519 *Oncogene*, 24(17), 2776-2786. <https://doi.org/10.1038/sj.onc.1208613>
 520 Itsumi, M., Shiota, M., Takeuchi, A., Kashiwagi, E., Inokuchi, J., Tatsugami, K., ...
 521 Yokomizo, A. (2016). Equol inhibits prostate cancer growth through
 522 degradation of androgen receptor by S-phase kinase-associated protein 2.
 523 *Cancer Sci.*, 107(7), 1022-1028. <https://doi.org/10.1111/cas.12948>
 524 Kurayoshi, K., Ozono, E., Iwanaga, R., Bradford, A. P., Komori, H., Araki, K., &
 525 Ohtani, K. (2018). The key role of E2F in tumor suppression through specific
 526 regulation of tumor suppressor genes in response to oncogenic changes. *Gene*
 527 *Expression and Regulation in Mammalian Cells-Transcription Toward the*
 528 *Establishment of Novel Therapeutics*, 143.
 529 <https://doi.org/10.5772/intechopen.74045>
 530 Kwon, J. Y., Lee, K. W., Kim, J.E., Jung, S. K., Kang, N. J., Hwang, M. K., ... Lee, H.
 531 J. (2009). Delphinidin suppresses ultraviolet B-induced cyclooxygenases-2
 532 expression through inhibition of MAPKK4 and PI-3 kinase. *Carcinogenesis*,
 533 30(11), 1932-1940. <https://doi.org/10.1093/carcin/bgp216>
 534 Kwon, J. E., Lim, J., Kim, I., Kim, D., & Kang, S. C. (2018). Isolation and identification
 535 of new bacterial stains producing equol from Pueraria lobata extract
 536 fermentation. *PloS One*, 13(2), e0192490.
 537 <https://doi.org/10.1371/journal.pone.0192490>
 538 Law, M. E., Corsino, P. E., Narayan, S., & Law, B. K. (2015). Cyclin-dependent kinase
 539 inhibitors as anticancer therapeutics. *Mol. Pharmacol.*, 88(5), 846-852.
 540 <https://doi.org/10.1124/mol.115.099325>
 541 Lee, C., Fernandez, K. J., Alexandrou, S., Sergio, C. M., Deng, N., Rogers, S., ...
 542 Caldon, C. E. (2020). Cyclin E2 Promotes Whole Genome Doubling in Breast
 543 Cancer. *Cancers*, 12(8), 2268. <https://doi.org/10.3390/cancers12082268>
 544 Li, Y., Xiao, X., Chen, H., Chen, Z., Hu, K., & Yin, D. (2020). Transcription factor
 545 NFYA promotes G1/S cell cycle transition and cell proliferation by
 546 transactivating cyclin D1 and CDK4 in clear cell renal cell carcinoma. *Am. J.*
 547 *Cancer Res.*, 10(8), 2446-2463.
 548 Liang, J., Cao, R., Zhang, Y., Xia, Y., Zheng, Y., Li, X., ... Lu, Z. (2016). PKM2
 549 dephosphorylation by Cdc25A promotes the Warburg effect and tumorigenesis.
 550 *Nat. Commun.*, 7(1), 12431. <https://doi.org/10.1038/ncomms12431>
 551 Liotti, A., La Civita, E., Cennamo, M., Crocetto, F., Ferro, M., Guadagno, E., ...
 552 Terracciano, D. (2021). Periprostatic adipose tissue promotes prostate cancer
 553 resistance to docetaxel by paracrine IGF-1 upregulation of TUBB2B beta-
 554 tubulin isoform. *Prostate*, 81(7), 407-417. <https://doi.org/10.1002/pros.24117>
 555 Liu, X., Cui, S., Li, W., Xie, H., & Shi, L. (2023). Elucidation of the anti-colon cancer
 556 mechanism of Phellinus baumii polyphenol by an integrative approach of
 557 network pharmacology and experimental verification. *Int. J. Biol. Macromol.*,
 558 253(Pt 6), 127429. <https://doi.org/10.1016/j.ijbiomac.2023.127429>
 559 Liu, X.-M., Wang, Z.-H., Wei, Q.-X., Song, Y., & Ma, X.-X. (2024). Equol exerts anti-

tumor effects on choriocarcinoma cells by promoting TRIM21-mediated ubiquitination of ANXA2. *Biology Direct*, 19(1), 78. <https://doi.org/10.1186/s13062-024-00519-5>

Markowitsch, S. D., Schupp, P., Lauckner, J., Vakhrusheva, O., Slade, K. S., Mager, R., ... Juengel, E. (2020). Artesunate inhibits growth of sunitinib-resistant renal cell carcinoma cells through cell cycle arrest and induction of ferroptosis. *Cancers*, 12(11), 3150. <https://doi.org/10.3390/cancers12113150>

Mayo, B., Vázquez, L., & Flórez, A. B. (2019). Equol: A Bacterial Metabolite from The Daidzein Isoflavone and Its Presumed Beneficial Health Effects. *Nutrients*, 11(9), 2231. <https://doi.org/10.3390/nu11092231>

Narimani, M., Sharifi, M., & Jalili, A. (2019). Knockout of BIRC5 gene by CRISPR/Cas9 induces apoptosis and inhibits cell proliferation in leukemic cell lines, HL60 and KG1. *Blood Lymphat. Cancer*, 9, 53-61. <https://doi.org/10.2147/BLCTT.S230383>

Naseri, G. (2023). A roadmap to establish a comprehensive platform for sustainable manufacturing of natural products in yeast. *Nat. Commun.*, 14(1), 1916. <https://doi.org/10.1038/s41467-023-37627-1>

Neuberger, A., Trofimov, Y. A., Yelshanskaya, M. V., Nadezhdin, K. D., Krylov, N. A., Efremov, R. G., & Sobolevsky, A. I. (2023). Structural mechanism of human oncochannel TRPV6 inhibition by the natural phytoestrogen genistein. *Nat Commun*, 14(1), 2659. <https://doi.org/10.1038/s41467-023-38352-5>

Noel, E. E., Yeste-Velasco, M., Mao, X., Perry, J., Kudahetti, S. C., Li, N. F., ... McIntyre, A. (2010). The association of CCND1 overexpression and cisplatin resistance in testicular germ cell tumors and other cancers. *Am. J. Pathol.*, 176(6), 2607-2615. <https://doi.org/10.2353/ajpath.2010.090780>

Ono, M., Ejima, K., Higuchi, T., Takeshima, M., Wakimoto, R., & Nakano, S. (2017). Equol Enhances Apoptosis-inducing Activity of Genistein by Increasing Bax/Bcl-xL Expression Ratio in MCF-7 Human Breast Cancer Cells. *Nutr. Cancer*, 69(8), 1300-1307. <https://doi.org/10.1080/01635581.2017.1367945>

Orabi, K. Y., Abaza, M. S., Luqmani, Y. A., & Al-Attiyah, R. (2021). Psiadin and plectranthone selectively inhibit colorectal carcinoma cells proliferation via modulating cyclins signaling and apoptotic pathways. *PloS One*, 16(6), e0252820. <https://doi.org/10.1371/journal.pone.0252820>

Reinhardt, H. C., & Yaffe, M. B. (2009). Kinases that control the cell cycle in response to DNA damage: Chk1, Chk2, and MK2. *Curr. Opin. Cell Biol.*, 21(2), 245-255. <https://doi.org/10.1016/j.ceb.2009.01.018>

Sahin, I., Bilir, B., Ali, S., Sahin, K., & Kucuk, O. (2019). Soy Isoflavones in Integrative Oncology: Increased Efficacy and Decreased Toxicity of Cancer Therapy. *Integr. Cancer Ther.*, 18. <https://doi.org/10.1177/1534735419835310>

Sánchez-Hidalgo, J. M., Rodríguez-Ortiz, L., Arjona-Sánchez, Á., Rufián-Peña, S., Casado-Adam, Á., Cosano-Álvarez, A., & Briceño-Delgado, J. (2019). Colorectal peritoneal metastases: Optimal management review. *World J.*

- Gastroenterol.*, 25(27), 3484-3502. <https://doi.org/10.3748/wjg.v25.i27.3484>
- Smits, V. A., & Gillespie, D. A. (2015). DNA damage control: regulation and functions of checkpoint kinase 1. *FEBS J.*, 282(19), 3681-3692. <https://doi.org/10.1111/febs.13387>
- Song, S., Wang, Y., & Liu, P. (2022). DNA replication licensing factors: Novel targets for cancer therapy via inhibiting the stemness of cancer cells. *Int. J. Biol. Sci.*, 18(3), 1211. <https://doi.org/10.7150/ijbs.67529>
- Suski, J. M., Braun, M., Strmiska, V., & Sicinski, P. (2021). Targeting cell-cycle machinery in cancer. *Cancer Cell*, 39(6), 759-778. <https://doi.org/10.1016/j.ccell.2021.03.010>
- Tian, J., Fratz, S., Hou, Y., Lu, Q., Görlach, A., Hess, J., ... Black, S. M. (2011). Delineating the angiogenic gene expression profile before pulmonary vascular remodeling in a lamb model of congenital heart disease. *Physiol. Genomics*, 43(2), 87-98. <https://doi.org/10.1152/physiolgenomics.00135.2010>
- Voss, O. H., Arango, D., Tossey, J. C., Villalona Calero, M. A., & Doseff, A. I. (2021). Splicing reprogramming of TRAIL/DISC-components sensitizes lung cancer cells to TRAIL-mediated apoptosis. *Cell Death Dis.*, 12(4), 287. <https://doi.org/10.1038/s41419-021-03567-1>
- Wang, Z., Tang, X., Wu, X., Yang, M., Wang, W., Wang, L., ... Wang, D. (2019). Cardamonin exerts anti-gastric cancer activity via inhibiting LncRNA-PVT1-STAT3 axis. *Biosci. Rep.*, 39(5). <https://doi.org/10.1042/bsr20190357>
- Xi, Y., Ma, Z., Zhang, H., Yuan, M., & Wang, L. (2018). Effects of *Clostridium difficile* toxin A on K562/A02 cell proliferation, apoptosis and multi-drug resistance. *Oncol. Lett.*, 15(4), 4215-4220. <https://doi.org/10.3892/ol.2018.7921>
- Xi, Y., & Xu, P. (2021). Global colorectal cancer burden in 2020 and projections to 2040. *Transl. Oncol.*, 14(10), 101174. <https://doi.org/10.1016/j.tranon.2021.101174>
- Yamashita, S., Lin, I., Oka, C., Kumazoe, M., Komatsu, S., Murata, M., ... Tachibana, H. (2022). Soy isoflavone metabolite equol inhibits cancer cell proliferation in a PAP associated domain containing 5-dependent and an estrogen receptor-independent manner. *J. Nutr. Biochem.*, 100, 108910. <https://doi.org/10.1016/j.jnutbio.2021.108910>
- Zeng, W. J., Cheng, Q., Wen, Z. P., Wang, J. Y., Chen, Y. H., Zhao, J., ... Chen, X. P. (2020). Aberrant ASPM expression mediated by transcriptional regulation of FoxM1 promotes the progression of gliomas. *J. Cell. Mol. Med.*, 24(17), 9613-9626. <https://doi.org/10.1111/jcmm.15435>
- Zhang, T., Pan, D., Yang, Y., Jiang, X., Zhang, J., Zeng, X., ... Guo, Y. (2020). Effect of *Lactobacillus acidophilus* CICC 6074 S-layer protein on colon cancer HT-29 cell proliferation and apoptosis. *J. Agric. Food Chem.*, 68(9), 2639-2647. <https://doi.org/10.1021/acs.jafc.9b06909>
- Zhao, Q., Zhao, M., Parris, A. B., Xing, Y., & Yang, X. (2016). Genistein targets the cancerous inhibitor of PP2A to induce growth inhibition and apoptosis in breast

644 cancer cells. *Int. J. Oncol.*, 49(3), 1203-1210.
 645 <https://doi.org/10.3892/ijo.2016.3588>
 646 Zhu, H., Qin, N., Xu, X., Sun, X., Chen, X., Zhao, J., ... Mishra, B. (2019). Synergistic
 647 inhibition of csal1 and csal3 in granulosa cell proliferation and steroidogenesis
 648 of hen ovarian prehierarchical development. *Biol. Reprod.*, 101(5), 986-1000.
 649 <https://doi.org/10.1093/biolre/ioz137>
 650 Zhu, Y., Yao, Y., Shi, Z., Everaert, N., & Ren, G. (2018). Synergistic effect of bioactive
 651 anticarcinogens from soybean on anti-proliferative activity in MDA-MB-231
 652 and MCF-7 human breast cancer cells in vitro. *Molecules*, 23(7), 1557.
 653 <https://doi.org/10.3390/molecules23071557>

Figure legends

Figure 1. The effects of equol on cell proliferation, cell migration, and colony formation.

(a) Cell viability of HCT116, HT29, SW480, and Caco2 after 24h and 48h of equol intervention; (b) Cell migration of HCT116 and HT29 after 24h of equol intervention (scale bar 200 μ m); (c) Colony formation of HCT116 and HT29 after 24h of equol intervention. Different letters mean significant difference ($p < 0.05$); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 2. Flow cytometric analysis of the changes on cell cycle and apoptosis of HCT116 cells.

(a) Cell cycle distribution of HCT116 under different equol concentrations for 24h; (b) Cell apoptosis of HCT116 under different equol concentrations for 24h, cells in the right lower quadrant (Annexin V+/PI-) represent early apoptotic cells, and cells in the right upper quadrant (Annexin V+/PI+) represent late apoptotic cells. Different letters mean significant difference ($p < 0.05$).

Figure 3. Analysis of the expression of genes.

(a) Venn diagram showing the overlap genes between control and equol-treated samples; (b) The volcano plot of DEGs; (c) Principal component analyses (PCA) of all expressed genes; (d) Heat map show DEGs in control and equol-treated samples. CK, control group; EQ, equol treated group.

Figure 4. Hub bioprocess targets screening and validation by western blot analysis.

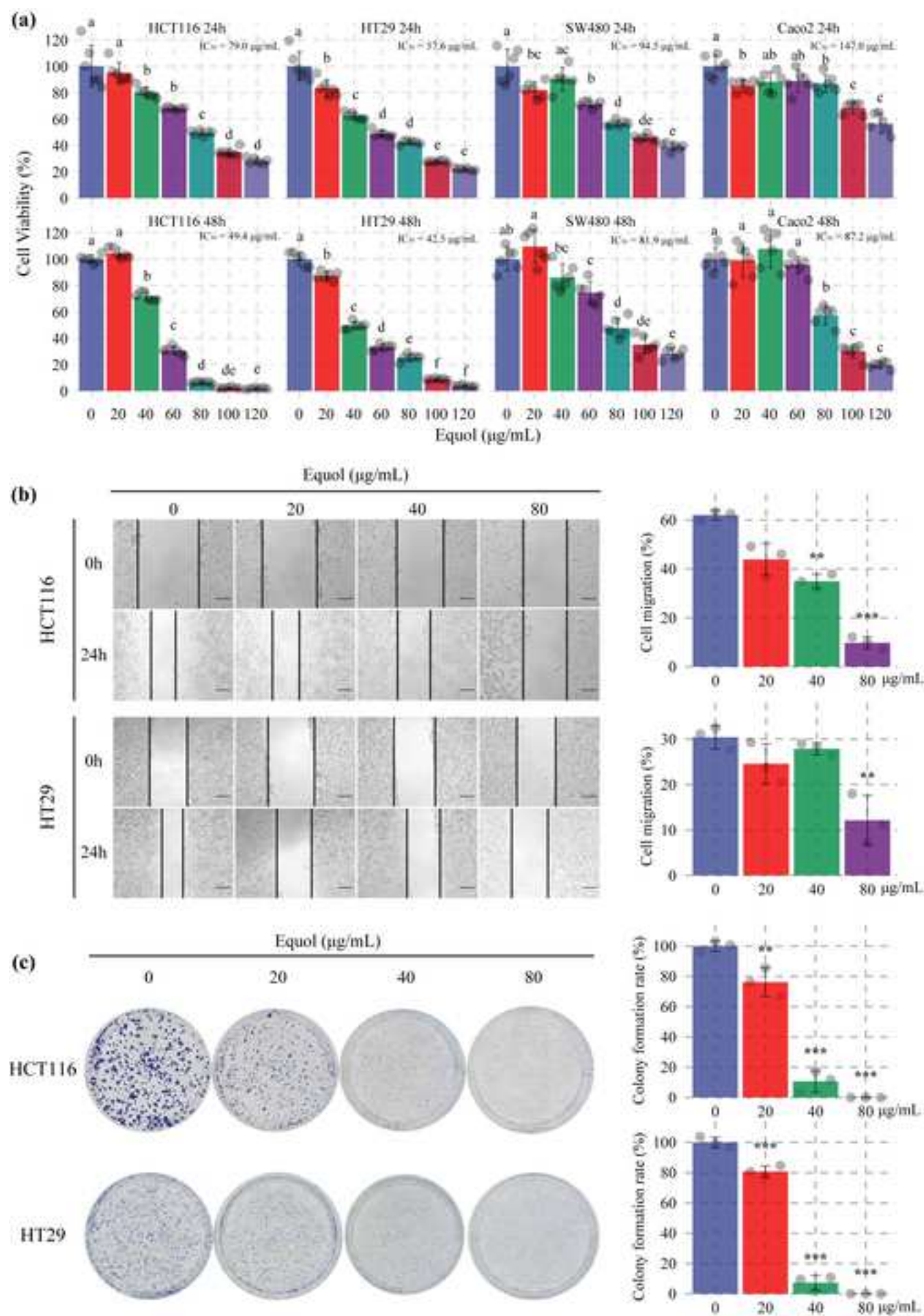
675 (a) Venn diagram showing the overlap genes between bioprocess targets (the left circle)
676 and DEGs from transcriptomics (the right circle); (b) PPI network diagram of 168
677 shared genes; (c) The four tightly connected network clusters obtained with MCODE
678 and rendered as separate modules; (d) The hub genes were obtained by MCC values of
679 cytoHubba; (e) the PPI network of hub genes which screened by MCODE and
680 cytoHubba; (f) Volcano plot displays the expression of 168 shared genes (EQ vs. CK)
681 and the hub genes are labeled with gene names; (g) iRegulon TF prediction of hub genes
682 correlating with NFYA and E2F1; (h) The gene expression of two TFs NFYA and E2F1;
683 (i) Western blot analysis of gene protein expression. *, $p < 0.05$; **, $p < 0.01$; ***, $p <$
684 0.001.

685 **Figure 5.** The overview of cell cycle and the effect of equol on regulating cell cycle.
686 Red color fonts represent the regulated genes after equol intervention.

Table 1 Molecular docking results of equol.

Gene Name	Protein Name	PDB ID	Total Interaction Energy (kcal/mol)	Amino acids forming H-bonds with equol
CDK1	Cyclin-dependent kinase 1	6GU7	-183.35	ASP146, LYS33, LYS89
CCNB1	G2/mitotic-specific cyclin-B1	4Y72	-128.22	LEU83, GLN132
CCNA2	Cyclin-A2	4BCM	-186.45	LYS56, HIS71, THR303
BUB1B	Mitotic checkpoint serine/threonine-protein kinase BUB1 beta	2WVI	-142.24	ARG169, THR204, GLN203
TOP2A	DNA topoisomerase 2-alpha	5NNE	-173.96	LYS141
AURKA	Aurora kinase A	1MQ4	-621.39	LYS162, ALA213
CDCA8	Cell division cycle-associated protein 8	6YIF	-217.30	GLU51, HIS80
TPX2	Targeting protein for Xklp2	6VPM	-159.87	ARG180
ASPM	Abnormal spindle-like microcephaly-associated protein	5OW5	-28.09	GLN579, GLY644, SER645, ALA646
BIRC5	Baculoviral IAP repeat-containing protein 5	2QFA	-595.34	LYS78
FOXM1	Forkhead box protein M1	3G73	-1179.74	ASP293, MET294, DT12
MKI67	Proliferation marker protein Ki-67	5J28	-681.66	TRY272, HIS66
CDCA3	Cell division cycle-associated protein 3	-	-95.30	-
CDC45	Cell division control protein 45 homolog	6CC2	-179.36	ASP210, ARG275
CHEK1	Checkpoint kinase-1	7SUG	-12.12	ASN135
MCM4	DNA replication licensing factor MCM4	4YWK	-206.44	LYS242
CDC6	Cell division control protein 6 homolog	4I5L	-161.73	ARG135, CYS266
CCND1	G1/S-specific cyclin-D1	2W96	-256.92	ARG61, GLU64, ARG87, LYS149
NFYA	Nuclear transcription factor Y subunit alpha	6QMP	-168.37	LYS49
E2F1	Transcription factor E2F1	6G0P	-25.95	ASP144

Note: PDB ID, Protein Data Bank identification; The 3D structure of CDCA3 was from UniProt database.



[Click here to access/download;Figure;Figure_2.tif](#)

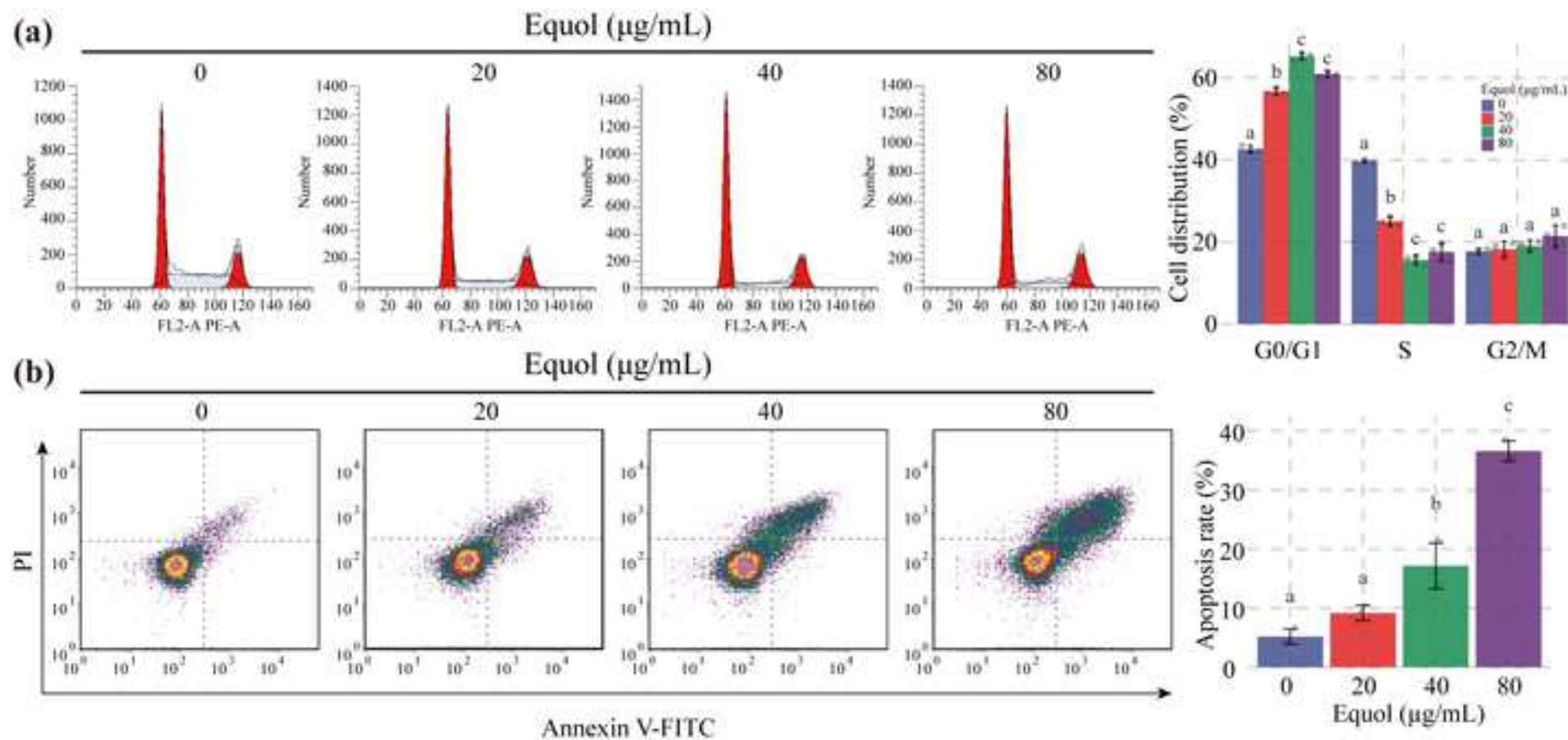
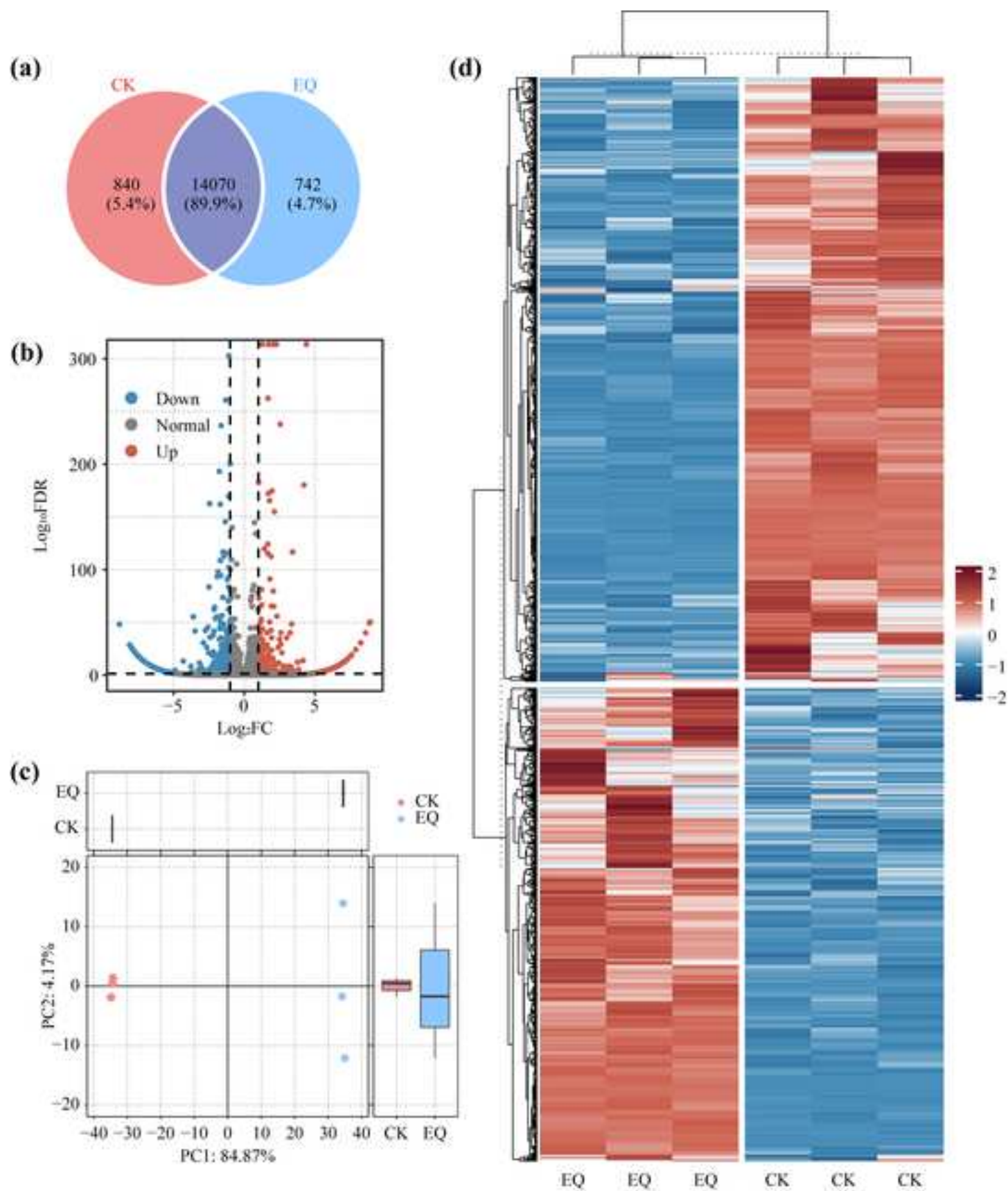
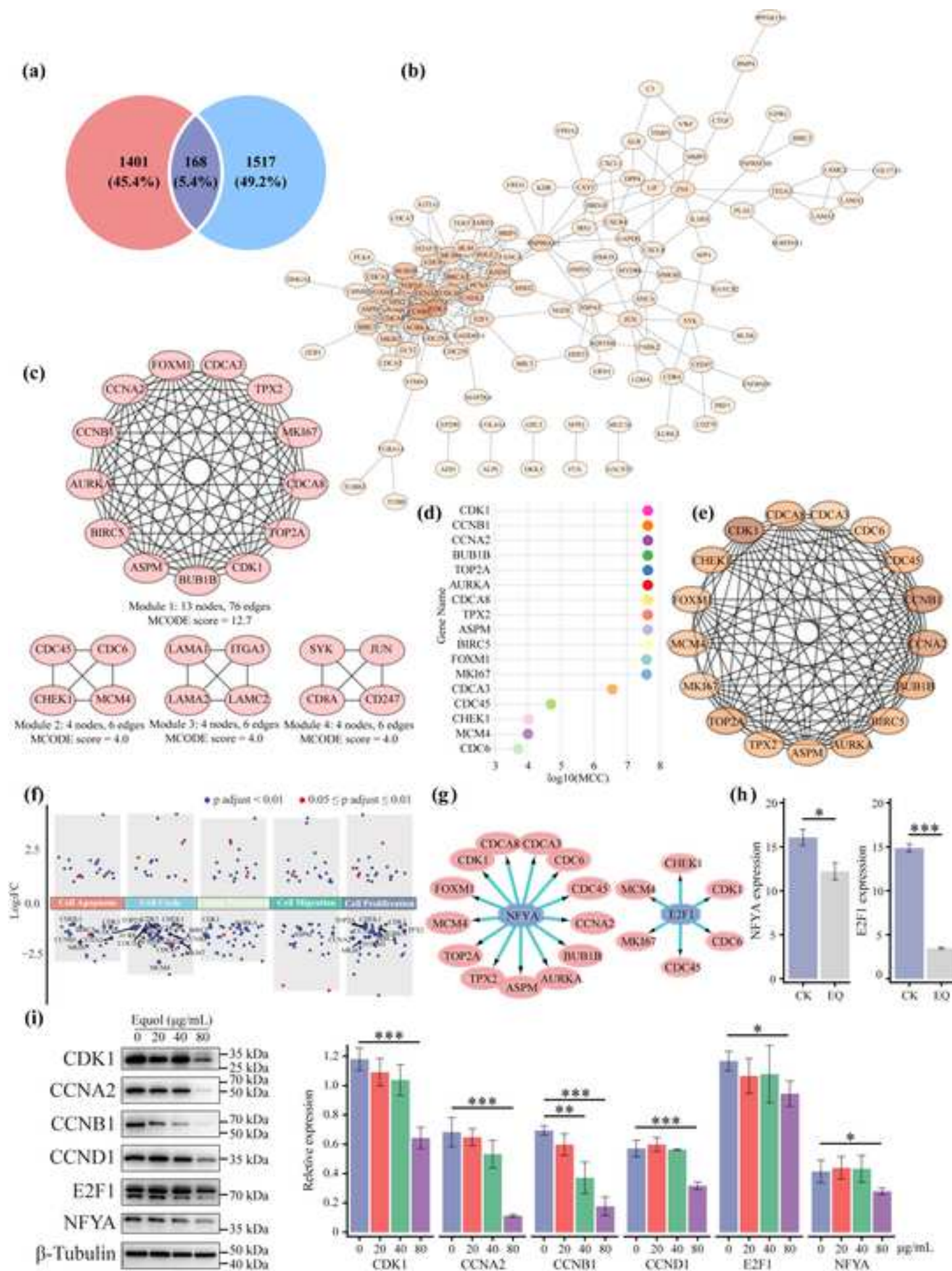
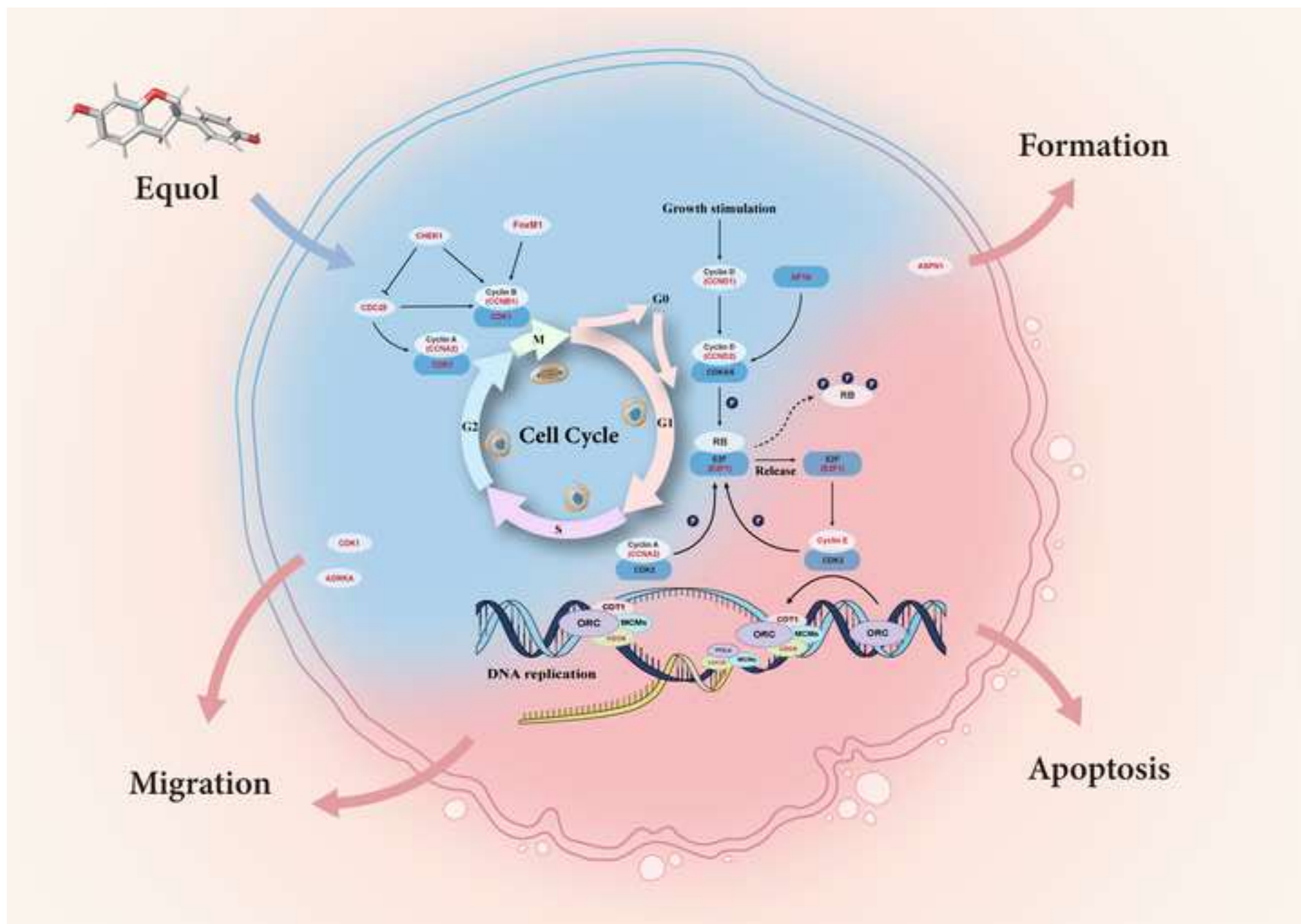


Figure 3

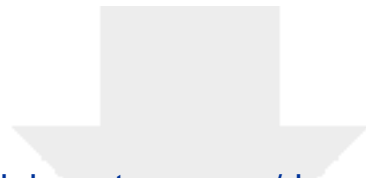
[Click here to access/download;Figure;Figure_3.tif](#)











[Click here to access/download](#)

Supplementary Material

Revised Supplementary material.docx

