Bioinformatics and In Vitro Studies Reveal the Importance of p53, PPARγ and Notch Signaling Pathway in Inhibition of Breast Cancer Stem Cells by Hesperetin

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Abstract

Purpose: The failure of chemotherapy in breast cancer is caused by breast cancer stem cells (BCSCs), a minor population of cells in bulk mammary tumors. Previously, hesperetin, a citrus flavonoid, showed cytotoxicity in several cancer cells and increased cytotoxicity of doxorubicin and cisplatin. Hesperetin also inhibited osteogenic and adipocyte differentiation, however, a study of the effect of hesperetin on BCSCs has not yet been performed.

Methods: In this study, we combined bioinformatics and in vitro works. A bioinformatic approach was performed to identify molecular targets, key proteins, and molecular mechanisms of hesperetin targeted at BCSCs, and genetic alterations among key genes. In addition, an in vitro study was carried out to measure the effects of hesperetin on BCSCs using the spheroids model of MCF-7 breast cancer cells (mammospheres).

Results: Using a bioinformatics approach, we identified P53, PPARγ, and Notch signaling as potential targets of hesperetin in inhibition of BCSCs. The in vitro study showed that hesperetin exhibits cytotoxicity on mammospheres, inhibits mammosphere and colony formation, and inhibits migration. Hesperetin modulates the cell cycle and induces apoptosis in mammospheres. Moreover, hesperetin treatment modulates the expression of p53, PPARγ, and NOTCH1.

Conclusion: Taken together, hesperetin has potential for the treatment of BCSC by targeting p53, PPARγ and Notch signaling. Further investigation of the molecular mechanisms involved is required for the development of hesperetin as a BCSC-targeted drug.

Introduction

Drug discovery in the era of information technology has become easier, faster and directed to molecularly targets with the aid of artificial intelligence, cheminformatics, and data mining, as well as high throughput screening.1 One application is the use of an integrated bioinformatics approach to obtain molecular targets, identification of key proteins, and molecular mechanisms of a drug candidate.2,3 Hence, drug development for certain diseases, such as cancer, can be performed in a faster and more strategic way using integrated bioinformatics analysis.

Breast cancer is the leading cause of death among women worldwide.4 The failure of chemotherapy in breast cancer is caused by breast cancer stem cells (BCSCs), a minor population of cells in bulk mammary tumors.5 BCSCs are considered to possess stem-cell characteristics, which are self-renewal and differentiation, and thus are responsible for tumor relapse and metastasis.6 Targeted BCSC therapy has proven to be effective as a companion to chemotherapy in breast cancer, namely combination therapy.7 Candidate compounds for combination therapy derive mostly from natural ingredients that exhibit potent cytotoxicity toward cancer cells but low toxicity to normal cells.8

One natural compound that can be developed for combination with chemotherapy is hesperetin (Figure 1A), a flavonoid that is found in many citrus species. Previous studies have shown that hesperetin exhibited cytotoxicity by inducing apoptosis and modulating the cell-cycle in various types of cancer cells, such as breast cancer cells,9 cervical, colon, prostate cancer cells,10 leukemia cells,11 gastric cancer cells,12 esophageal cancer cells,13 skin carcinoma cells,14 and hepatocellular carcinoma cells.15 Moreover, hesperetin exhibited anticancer activity in animal cancer models, such as rat colon cancer,16 DMBA-induced rat mammary tumor,17 DMBA-induced hamster buccal pouch carcinogenesis,18 and benzo(a)pyrene-induced lung carcinogenesis in Swiss albino mice.19

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In addition, hesperetin has also been shown to increase the effectiveness of chemotherapy such as that mediated by doxorubicin and cisplatin, on various cancer cells. Hesperetin increased cytotoxicity of doxorubicin on MCF-7 breast cancer cells. Furthermore, hesperetin showed synergism to platinum-based chemotherapy by inhibiting UGT1A3 and increasing levels of reactive oxygen species (ROS) in lung adenocarcinoma cells. Hesperetin also has an effect on stem cells, which inhibit osteogenic differentiation and adipocyte differentiation. However, a study on hesperetin-targeted BCSCs has not yet been conducted.

In this study, we combined bioinformatics and in vitro work. A bioinformatic approach was performed to identify molecular targets, key proteins, and molecular mechanisms of hesperetin targeted at BCSCs. In addition, an in vitro study was carried out to measure the effects of hesperetin on BCSCs using the spheroid model of MCF-7 breast cancer cells (mammospheres). This study is expected to be the basis for the development of hesperetin as a BCSC-targeted drug for overcoming chemotherapy resistance in breast cancer therapy. We identified possible specific molecular targets of hesperetin in BCSC inhibition. This analysis suggested that p53, PPARG, and Notch signaling could be developed as targets of hesperetin for targeting BCSCs.

Materials and Methods

Acquisition of direct target proteins, indirect protein targets, and BCSC regulatory genes

Direct target proteins (DTPs) of hesperetin were searched from STITCH (http://stitch.embl.de). The indirect protein targets (ITPs) of each DTP were obtained from the STRING database (https://string-db.org), with a minimum interaction score of 0.7 and the maximum number of interactors as 20. The ITPs of all DTPs were generated after removing repetitive proteins. BCSC regulatory genes were retrieved from PubMed with the keywords “breast cancer stem cells”. A Venn diagram between all ITPs, DTPs and BCSC regulatory genes was constructed using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/). The overlapping genes were considered as hesperetin targets (HTs) in BCSCs.

Protein-protein interaction (PPI) network, gene ontology and KEGG-pathway enrichment of the HT

PPI network analysis among HTs was conducted with STRING-DB v11.0 with confidence scores >0.7 and visualized by Cytoscape software. Genes with a degree score of more than 10, analyzed using CytoHubba plugin, were selected as hub proteins. Analysis of gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were conducted using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 with P < 0.05 selected as the cut-off value.

Analysis of genetic alterations among hub proteins

The genetic alterations of selected genes were analyzed using cBioPortal (http://www.cbioportal.org). Protein genes including TP53, PPARG, PCNA, HES1, and MYC were screened for genetic alterations in all breast cancer studies available in the cBioPortal database. The breast cancer study with the highest number of genetic alterations was chosen for Oncoprint and connectivity analysis.

Cell culture and mammosphere generation

MCF-7 cells were cultured using DMEM high glucose media containing 10% fetal bovine serum (FBS) and penicillin-streptomycin, incubated in CO2 incubators at 37°C. The formation of mammospheres was carried out with modifications from previous studies. Briefly, MCF-7 cells were seeded (40 000 cells/mL) on a 50 mg/mL poly-HEMA-coated plate. Mammospheres were grown in DMEM media containing 10 ng/mL epidermal growth factor (EGF), 10 ng/mL basic fibroblast growth factor (bFGF), 5 µg/mL insulin, and penicillin-streptomycin, and incubated in a CO2 incubator at 37°C. Mammospheres were allowed to grow for a maximum of 7 days before being tested with hesperetin (HST, Sigma-Aldrich) and other compounds (doxorubicin or DXR, Sigma-Aldrich; metformin or MET, Sigma-Aldrich).

Cytotoxicity test

Cytotoxicity tests were carried out using MTT methods. Briefly, cells were seeded in a 96-well plate to form monolayer cells and mammospheres. Cells were then treated with hesperetin and incubated for 72 hours. At the end of incubation, the MTT solution was added followed by incubation for 3 hours. After that, a 10% SDS in 0.01 M HCl solution was added until formazan crystal completely
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Sex hormone-binding globulin
Adiponectin
Sterol O-acyltransferase 2;
37
Neurogenic locus notch homolog protein 1
Microsomal triglyceride transfer protein large subunit
Diacylglycerol O-acyltransferase 1
Peroxisome proliferator-activated receptor gamma
Sterol O-acyltransferase 1
Cyclin-dependent kinase 2
Cyclin-dependent kinase 4

Gene name

Peroxisome proliferator-activated receptor alpha

with trypsin to obtain a single-cell suspension. Apoptotic
percent of cell population in G0/G1, S, and G2/M phase.
Cells were then analyzed using the BD FACSCalibur flow
cytometry system. Cell cycle profile was presented as
propidium iodide (PI), 50 mg/mL RNAse, and triton X.

conditions using a solution containing 100 mg/mL of
cell conditions were carried out at intervals of 0, 18, 24,

cells/well) into 24-well plates and incubated for 24 hours.

Mammosphere-derived MCF-7 cells were seeded (10000
cells/well) in poly-HEMA-coated 96-well plates and

Mammosphere-forming potential
The effect of hesperetin in mammosphere forming was
examined based on a previous study. Briefly, cells were
pre-treated with hesperetin for 72 hours. The medium
was changed with fresh medium and cells were then
incubated for the following 24 hours. Recovered cells
were then seeded (40 000 cells/well) in poly-HEMA-
coated 96-well plates and incubated for 96 hours. At the
end of the observation, the number of mammospheres
formed was calculated manually. Results are expressed as
mammosphere-forming potential (MFP).

Colony formation assay
Cells were seeded (1000 cells/well) in a 6-well plate and
incubated for 24 hours. Cells were then treated with drugs
for 72 hours. At the end of treatment, the medium was
changed with a fresh medium and followed by 14 days of
incubation. At the end of the incubation, the cells were
fixed with paraformaldehyde and stained with a Gentian
violet solution. The surviving colonies were counted and
analyzed with ColonyArea.36

Wound-healing assay
Mammosphere-derived MCF-7 cells were seeded (10 000
cells/well) into 24-well plates and incubated for 24 hours.
Starvation was carried out by replacing culture media
with starvation media containing only 0.5% of FBS and
incubated for the following 24 hours. Scratching was

Analysis of cell cycle
Cell cycle observations were carried out as in a previous
study with modification. Briefly, cells were seeded,
incubated, and treated with hesperetin for 72 hours.
At the end of treatment, cells were harvested with trypsin
to obtain a single-cell suspension. Cells were then fixed
with cold methanol, and stained for 20 minutes in dark
conditions using a solution containing 100 mg/mL of
propidium iodide (PI), 50 mg/mL RNAs, and triton X.
Cells were then analyzed using the BD FACSCalibur flow
cytometry system. Cell cycle profile was presented as
percent of cell population in G0/G1, S, and G2/M phase.

Apoptosis assay
Cells were seeded, incubated, and treated with hesperetin
for 96 hours. At the end of treatment, cells were harvested
with trypsin to obtain a single-cell suspension. Apoptotic
observations were carried out using the Annexin-V-
FLUOS staining kit according to the manufacturer’s
instructions. Briefly, cell suspensions were prepared by
tryptsinization, and Annexin-V-FLUOS staining kit
containing binding buffer, Annexin and PI was added and
the mixture incubated in the dark for 10 minutes. Cells
were then examined using the BD FACSCalibur flow
cytometry system to measure the percentage of cells
undergoing apoptosis.

q-RT PCR
Cells were seeded, incubated, and treated with hesperetin
for 96 hours. Total mRNA was isolated using GeneJet RNA
Purification Kit (Thermo Fisher Scientific), according
to the manufacturer’s instructions. Next, cDNA was
synthesized using RevertAid First Strand cDNA Synthesis
Kit (Thermo Fisher Scientific). SsoFast EvaGreen
Supermix (Bio-Rad) was used to quantify PCR. Expression
of regulatory genes was conducted using selected primers
(Supplementary file 1, Table S1). GAPDH was used as a
housekeeping gene. The results were analyzed using the
comparative threshold cycle (ΔΔCT method).

Statistical analysis
All statistical analyses were conducted with GraphPad
Prism 5.0 software.

Results and Discussion

Acquisition of DTPs, ITPs and BCSC regulatory genes
This study aimed to explore the molecular target of
hesperetin in the inhibition of BCSCs using integrated
bioinformatics and in vitro studies. We obtained 11 DTPs
of hesperetin, including PPARA, PPARG, ADIPOQ, DGAT1,
MMTP, SOAT2, SOAT1, CDK4, CDK2, NOTCH1, and
SHBG (Figure 1B, Table 1). We showed interactions
among the DTPs, including CDK4-NOTCH1-CDK2,
PPARG-ADIPOQ, DGAT1-ADIPOQ, MMTP-ADIPOQ,
and PPARA-DGAT1. These interactions indicated that
proteins played a critical role in the molecular function

| Gene symbol | Protein name                        |
|-------------|------------------------------------|
| SOAT1       | Sterol O-acyltransferase 1         |
| SOAT2       | Sterol O-acyltransferase 2         |
| MTTP        | Microsomal triglyceride transfer protein large subunit |
| SHBG        | Sex hormone-binding globulin      |
| CDK2        | Cyclin-dependent kinase 2         |
| NOTCH1      | Neurogenic locus notch homolog protein 1 |
| CDK4        | Cyclin-dependent kinase 4         |
| PPARG       | Peroxisome proliferator-activated receptor gamma |
| ADIPOQ      | Adiponectin                        |
| PPARA       | Peroxisome proliferator-activated receptor alpha |
| DGAT1       | Diacylglycerol O-acyltransferase 1 |
mediated by hesperetin. In total, we retrieved hesperetin mediated proteins consisting of 11 DTPs and 98 ITPs (Table S2), and 1041 BCSC regulatory genes from PubMed (Table S3). A Venn diagram generated 43 hesperetin targets in BCSCs or HT (Figure 1C, Table S4).

**Protein-protein interaction network analysis, gene ontology, and KEGG-pathway enrichment of the HT**

The PPI network of HT consists of 43 nodes, 234 edges, an average node degree of 17, and a high-confidence interaction (0.7; Figure 2A). Furthermore, hub proteins were selected from the PPI network based on their degree score (Table 2) including one DTP (PPARG). These results indicated that the biological effect of hesperetin is strongly correlated with PPARG. GO analysis of HT was classified into three groups, consisting of biological process, cellular component and molecular function (Table S5). HT was found to participate in the biological processes of cell cycle and cell proliferation. The HT is located in the nucleoplasm, cytosol extracellular matrix, and cytoplasm. Moreover, the HT has a molecular function in cyclin-dependent protein kinase regulator and inhibitor activity. KEGG-pathway enrichment analysis showed 21 pathways regulated by HT (Table S6), including the cell cycle, and TGF-beta, PPAR, Wnt, and Notch signaling pathways.

**Genetic alterations among the hub proteins**

A total of five HT were analyzed using cBioportal to explore their genomic alterations across breast cancer studies. HT consists of TP53, PPARG, MYC (based on highest score degree), TP53, PCNA (based on KEGG pathway enrichment results in the cell cycle), PPARG (based on KEGG pathway enrichment results in PPAR signaling pathway), and HES1 (based on KEGG pathway enrichment analysis in Notch signaling pathway). Among twelve breast cancer studies, a study namely METABRIC by Pereira et al was selected for further analysis. Oncoprint analysis showed that genetic alterations of HT occur in 1.2 to 34% of patients samples (Figure 3B), in which amplification is the most common gene alteration. Additional analysis of the interactive relationship between five selected genes and altered genes in the METABRIC study revealed a network contains five queries and neighbor genes (Figure 3C). In addition, TP53 and MYC were the genes with the highest number of neighbor genes. Moreover, TP53 and MYC were the main targets of most cancer drugs (Figure 3D), thus indicating the potential of p53 and Myc to be hesperetin targets in BCSCs.

**Mammosphere generation and characterization**

We successfully generated mammospheres to enrich BCSC properties in MCF-7 cells. Cells were cultured in poly-HEMA-coated plates using serum-free medium supplemented with insulin, EGF, and B27, as described in the methods section. BCSC characterization using q-RT PCR showed upregulation of BCSC markers, including CD133, OCT4, NANOG and ALDH1 (Figure 4A). Taken

| Gene symbol | Degree score |
|-------------|-------------|
| TP53        | 25          |
| CCND1       | 22          |
| MYC         | 22          |
| RB1         | 19          |
| INS         | 19          |
| IL6         | 17          |
| PPARG       | 17          |
| CDKN1A      | 16          |
| CDKN1B      | 16          |
| E2F4        | 15          |
| LEP         | 15          |
| ESR1        | 15          |
| CEBPB       | 14          |
| TNF         | 14          |
| IGF1        | 14          |
| SMAD3       | 14          |
| CCND2       | 13          |
| RBL1        | 12          |
| CCNE1       | 12          |
| CDKN2A      | 12          |

**Figure 2.** (A) PPI network of hesperetin protein targets. (B) Top 20 hub proteins based on degree score, analyzed by CytoScape.
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Together, the mammospheres were enriched with BCSC properties and could be used for further analysis.

**Hesperetin exhibits cytotoxicity, inhibits mammosphere and colony formation and hampers migration**

Cytotoxicity assay with MTT showed that hesperetin exhibited cytotoxicity in MCF-7 cell monolayers (M) and mammospheres (MS) at a concentration of 100 µM (Figure 4B). We performed cytotoxicity assay at a different time point, i.e., 24, 48 and 72 hours. However, we only showed the data of 72 hours experiment, since hesperetin did not exhibit cytotoxicity on MS in 24 and 48 hours, shown by cell viability value above 90%.

A compound for combination therapy (i.e., alongside chemotherapy) should be potent, but also less toxic toward normal cells. Previous acute and chronic toxicity studies showed that hesperetin, a glycoside form of hesperetin, is not toxic in animals. Thus, those findings support the use of hesperetin as a combination agent in BCSC therapy. Hesperetin also inhibited mammosphere forming based on MFP (Figure 4C). The clonogenic assay revealed that hesperetin inhibited colony formation from MCF-7 cells (Figure 4D). Mammosphere forming is a standard assay to measure the frequency of tumor-initiating cells in cancer cell lines, stem cell activity, and self-renewal. The colony formation assay is a standard assay of measuring the capability of single cells to grow into a colony and is a sensitive indicator of undifferentiated CSCs. One of the hallmarks of BCSCs is promoting migration and metastasis. Hesperetin hampers migration in mammosphere-derived MCF-7 cells (Figure 4E).

**Figure 3.** (A) Overview of changes in TP53, PPARC, PCNA, HES1, and MYC in genomics dataset from 12 studies of breast cancer. (B) Summary alterations of TP53, PPARC, PCNA, HES1 and MYC across breast cancer samples (based on a study by Pereira et al).

**Figure 4.** (A) Upregulation of BCSC markers in mammospheres from MCF-7 cells. Gene expression was measured by q-RT PCR. Results are shown as relative to monolayer cells and are expressed as mean±SD (n=3). (B) Hesperetin showed cytotoxicity on MCF-7 monolayers and mammospheres. Cytotoxicity was measured by MTT and shown as % cell viability compared to control (mean±SD; n=3). (C) Hesperetin inhibits mammosphere formation from MCF-7 cells. Results are expressed as mammospheres-forming potential (MFP) as mean±SD (n=3). (D) Hesperetin inhibits colony formation. The surviving colonies were counted and analyzed with ColonyArea, and the results are shown as mean±SD (n=3). (E) Hesperetin inhibits the migration of mammosphere-derived MCF-7 cells. Observation and documentation of cells were carried out from 0 to 48 hours. Results are expressed as mean±SD (n=6). Statistical analyses were conducted using Student’s t test. *** P<0.001, **** P<0.0001.
Collectively, these findings highlighted the potential of hesperetin as a BCSC-targeted drug.

**Hesperetin inhibits cell cycle and induces apoptosis in mammospheres**

The cytotoxicity of hesperetin was further examined by measuring cell cycle and apoptosis profiles. Cell-cycle analysis results showed an increase in G0/G1 arrest in untreated mammospheres compared to monolayer MCF-7 cells (Figure 5A). In addition, hesperetin treatment increased G0/G1 arrest in monolayer cells. Moreover, hesperetin treatment showed a similar cell-cycle profile as untreated mammospheres. Apoptosis assay results revealed that hesperetin induced apoptosis in both monolayers and mammospheres (Figure 5B). In addition, the apoptosis population in monolayer cells is higher than in mammospheres.

**Effect of hesperetin on stemness properties and BCSC regulatory genes**

We determined the expression of cell cycle, apoptosis, and stemness regulatory genes by q-RT PCR. To validate the results of bioinformatics analysis, we performed qRT-PCR on p53, NOTCH1, HES1, PPARG, and CMYC. Gene-expression analysis with q-RT PCR revealed the downregulation of p53, NOTCH1, HES1, PPARG, and CMYC upon hesperetin treatment in MCF-7 monolayer cells and MCF-7 mammospheres (Figure 5C). This result is supported by a previous study that demonstrated increased G1 arrest and p21 expression in mammospheres from MCF-7 cells.

Hesperetin treatment significantly reduced MMP9 mRNA levels in monolayer cells (Figure 5D). In mammospheres, hesperetin treatment reduced the mRNA level of vimentin, β-catenin, ALDH1, and MMP9, and increased the mRNA level of E-cadherin (Figure 5E). Hesperetin treatment increased mRNA expression of
apoptosis regulatory genes caspase 7 and BAX in monolayer cells and reduced the mRNA level of BCL2, survivin, and p21 (Figure 5F). In mammospheres, hesperetin increased the mRNA level of BAX and reduced the mRNA level of p21 (Figure 5G). Hesperetin increased the mRNA level of p53, ESR1, NOTCH1, HES1 and PPARG in monolayer cells (Figure 5H), and increased the mRNA level of p53, HES1 and PPARG in mammospheres (Figure 5I). This result is supported by a previous study which showed that the activation of Notch signaling mediates G1/S cell cycle progression.46 However, hesperetin treatment did not affect the mRNA level of CMYC in either monolayer cells or mammospheres.

P53 is a tumor suppressor gene and a transcription factor that regulates the cell cycle, apoptosis, and stemness.57 The results of this study showed the downregulation of p53 in mammospheres (Figure 5C), which is supported by a review article which showed that p53 is the barrier to cancer stem cell formation.48 Hesperetin treatment significantly increased the mRNA levels of p53 in monolayer cells (Figure 5H) and mammospheres (Figure 5I). p53 plays an important role in stemness properties, which is downregulated or loses function in stem cells of various cancers.49 A review article showed that p53 is important in cell cycle arrest induction by hesperetin.49 Collectively, the future study of the role of p53 in hesperetin treatment in BCSCs is warranted.

KEGG-pathway enrichment analysis revealed that the Notch and PPAR signaling pathways are regulated by hesperetin (Table S5). Notch1 protein, a member of the Notch family of receptors, plays a crucial role in the biological processes of cell proliferation, cell fate, differentiation, and cell death.51 Activation of the Notch signaling pathway plays a pivotal role in cancer development and maintenance of CSCs properties.52,53 Signaling by Notch is initiated upon ligand binding to the Notch receptor, followed by proteolytic cleavage by ADAM-family proteases and gamma-secretase and release of the Notch intracellular domain and translocation of Notch intracellular domain into the nucleus to bind the repressor of Notch target gene activator.54 Notch signaling induces transcription of target genes, including HES1, CYMC, p21, p53, and CyclinD1.55 Overexpression of Notch1 leads to constitutive activation of Notch signaling and apoptosis in human fibroblast cells.56 The results of this study showed that hesperetin increased the mRNA level of NOTCH1, HES1, and p53 (Figure 5H and 5I). Previous studies showed that hesperetin suppresses proliferation and induces apoptosis by inducing NOTCH1 expression in human gastrointestinal cancer cells.57 In addition, hesperetin activated the Notch1 signaling pathway and suppressed the proliferation of HTh7 anaplastic thyroid cancer cells.58 Taken together, the molecular mechanism of hesperetin in inhibition of BCSCs through Notch signaling needs to be explored further.

Peroxisome proliferator-activated receptor-gamma (PPAR-γ) or PPARG is a nuclear receptor that regulates the biological process of lipid metabolism.59 Recent studies highlight the emerging role of PPARG in cancer biology, including regulating cell-cycle arrest, apoptosis, angiogenesis, invasion, and cell migration.60 Activation of PPARG inhibits stemness in glioblastoma and colorectal cancer cells.61,62 Activation of PPARG inhibits metastasis by blocking β-catenin signaling and inhibiting MMP9 expression and activity.63 Activators of PPARG inhibit MMP9 expression via inhibition of NFkB activation.64 Activation of PPARG by a PPARG agonist inhibits migration and invasion in hepatocellular carcinoma.65 The results of this present study showed inhibition of migration, upregulation of PPARG and downregulation of MMP9 upon hesperetin treatment (Figures 4E, 5E, and 5I), which is supported by previous studies. Hesperidin is a glycoside form of hesperetin that showed cardioprotective activity in diabetic rats via activation of PPARG.66 Hesperetin activates PPARG in adipocyte differentiation.67 A review article discussed the possibility that PPARG agonists might inhibit cell proliferation by regulating several pathways that are hallmarks of cancer, for instance, PI3K/mTOR, MAPK, and Wnt/β-catenin.68 Moreover, a bioinformatics study demonstrated the potential of hesperetin as PPARG agonist for anticancer drugs.69 Taken together, these results suggest that PPARG plays an important role in hesperetin activity against BCSCs. However, the molecular mechanisms involved need to be clarified further.

Crosstalk between p53, PPARG and Notch signaling is also an interesting topic for future study. Activation of PPARG induces cell cycle arrest and apoptosis in MCF-7 cells via crosstalk between PPARG and p53.70 This study showed mRNA upregulation of PPARG and downregulation of β-catenin upon hesperetin treatment in mammospheres (Figure 5E and 5I). This result is supported by a previous study that demonstrated that a PPARG agonist induces β-catenin inhibition in type-2 diabetes and colon cancer.71 A study by Yun et al showed that PPARG promotes tumor suppressor activity by inhibition of cell proliferation via increasing p21 and downregulation of β-catenin and stem-cell-mediated eradication of MMP2 and MMP9 activity.72 Notch signaling also appears to be an important regulator of PPARγ.73 Taken together, these results suggest that p53, PPARG, and Notch signaling are potential targets of hesperetin in inhibition of BCSCs, but the molecular mechanisms involved need further investigation.

Conclusion
In this study, using a bioinformatics approach, we identified P53, PPARG, and Notch signaling as potential targets of hesperetin in the inhibition of BCSCs. Moreover, an in vitro study showed that hesperetin exhibits cytotoxicity on mammospheres, inhibits mammosphere and colony formation, and inhibits migration. Moreover, hesperetin
treatment modulates the expression of p53, PPARG, and NOTCH1. Taken together, these results suggest that hesperetin has potential for BCSC treatment by targeting p53, PPARG and Notch signaling. Further investigation of the molecular mechanisms involved is required for the development of hesperetin as a BCSC-targeted drug.

Ethical Issues
Not applicable.

Conflict of Interest
The authors declare no conflict of interest.

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Supplementary Materials
Supplementary file 1 contains Tables S1-S6.

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