

Original Article

Fisetin suppresses malignant proliferation in human oral squamous cell carcinoma through inhibition of Met/Src signaling pathways

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Abstract: Fisetin (3,7,3',4'-tetrahydroxyflavone) is a dietary flavonoid and has been indicated as a novel anti-cancer agent in several types of cancer cells. However, the mechanisms underlying the effect of fisetin in human oral squamous cell carcinoma (OSCC) remain unclear. Here, we report that fisetin significantly inhibits tumor cell proliferation and induces apoptosis in OSCC (UM-SCC-23 and Tca-8113) cancer cell lines. Further analysis demonstrates that fisetin also inhibits Met/Src signaling pathways using the PathScan[®] receptor tyrosine kinases (RTK) Signaling Antibody Array Kit. Fisetin resulted in decreased basal expression of Met and Src protein in UM-SCC-23 cancer cell lines, which validated by western blot. A student's *t*-test (two-tailed) was used to compare differences between groups. Furthermore, fisetin significantly inhibited the expression of a disintegrin and metalloproteinase 9 (ADAM9) protein in OSCC cells. Taken together, these results provide novel insights into the mechanism of fisetin and suggest potential therapeutic strategies for human OSCC by blocking the Met/Src signaling pathways.

Keywords: Fisetin, oral squamous cell carcinoma, receptor tyrosine kinases, Met/Src signaling pathways, ADAM9

Introduction

Oral squamous cell carcinoma (OSCC) is the 6th most common malignancy of the head and neck worldwide with an incidence exceeding 450,000 cases annually [1] in some Asian countries, it may account for more than 10% of all malignancies [2]. However, no effective strategy has been available for OSCC in several decades, though we previously noted that cetuximab was currently the only approved targeted therapeutic [3]. Thus, finding novel targets for therapeutic intervention and new biomarkers for OSCC is necessary and urgent.

Fisetin (3,7,3',4'-tetrahydroxyflavone) is a naturally occurring flavonoid that is widely distributed in plants, and it might be a potential thera-

peutic agent against human cancer cells due to its capacity to inhibit tumor cell proliferation, metastasis and induce apoptosis [4, 5]. Fisetin inhibits matrix metalloproteinases (MMP-14, MMP-1, MMP-3, MMP-7, and MMP-9) and reduces tumor cell invasiveness [6]. Fisetin inhibits migration and invasion by reversing the epithelial to mesenchymal transition (EMT) in melanoma and nasopharyngeal carcinoma [5, 7]. Fisetin suppresses ADAM9 (a disintegrin and metalloproteinase 9) expression, which is upregulated in various types of tumors, and associated with cancer cell invasion and migration [8]. Fisetin inhibits cancer cell proliferation and tumor growth by blocking the PI3K/AKT/mTOR pathway [9, 10]. Fisetin has therefore been shown to be a useful natural agent against various cancers with slight side effects.

Fisetin suppresses proliferation by targeting Met/Src

In this study, we report that fisetin significantly inhibits tumor cell proliferation and induces apoptosis in OSCC cancer cell lines. In addition, we discover that fisetin can significantly reduce the protein levels of Met/Src and ADAM9 in OSCC cells. These results could be attributed to the function of fisetin in regulating OSCC malignant proliferation via the Met/Src signaling pathways.

Materials and methods

Cell lines and materials

Human oral squamous cell carcinoma (UM-SCC-23 and Tca-8113) cells were used in our study. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life, Shanghai, China), supplemented with penicillin, streptomycin and fetal bovine serum (FBS, Gibco, Shanghai, China) to a final concentration of 10%. Fisetin was purchased from Sigma-Aldrich (Sigma-Aldrich, Shanghai, China). Stock solutions of fisetin were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C.

CCK8

UM-SCC-23 and Tca-8113 cells were plated in 96-well plates and cultured in DMEM medium containing 10% FBS. Cells were treated with the indicated concentrations of fisetin for 24 h. The cell Counting Kit-8 (CCK8, Dojindo Molecular Technologies, Inc., Japan) was used to evaluate cell proliferation. Generally, 10 µl CCK8 solution was added to each plate and cells were incubated for 1 h at 37°C. Cell viability was determined by absorbance, which was measured at 450 nm.

Apoptosis assays

The Annexin V assay was used to detect apoptotic and necrotic cells according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). This kit uses a dual-staining protocol in which apoptotic cells are stained with annexin-V, and the necrotic cells are stained with propidium iodide (PI). UM-SCC-23 cells were incubated with fisetin (20-80 µM) for 24 h. Cells cultured with DMSO were used as controls. Fluorescence was detected using FACSscan instrument (BD, Franklin Lakes, NJ, USA). The experiment was repeated in triplicate.

Cellular receptor tyrosine kinase (RTK) signaling assay

UM-SCC-23 cells were treated with 80 µM fisetin prior to using the PathScan® RTK Signaling Antibody Array kit with a chemiluminescent readout (cat. 7982; Cell Signaling Technology, Inc., Danvers, MA, USA) according to the manufacturer's instructions.

Immunoblotting

Cells were washed with cold PBS before being lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail (Roche, Basel, Switzerland). Proteins were separated with SDS-PAGE and transferred to a PVDF membrane. Membranes were probed with the specific primary antibodies and then with peroxidase-conjugated secondary antibodies. The bands were visualized by chemiluminescence (ECL, Amersham, Piscataway, NJ). The following antibodies were used: antibodies to Met (1:2,000, Cell signaling, #8198); Met [Tyr 1003] (1:1,000, Cell signaling, #3135); Src (1:2,000, Cell signaling, #2123); Src [Tyr 527] (1:1,000, Cell signaling, #2105); No-phospho-Src [Tyr 527] (1:1,000, Cell signaling, #2107); GAPDH (1:20,000, KangChen Bio-tech, KC-5G4); ADAM9 (1:1,000, Cell signaling, #2099s). The ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for densitometric analyses of western blots, and the quantification results were normalized to the loading control.

Bioinformatics analysis

Potential ADAM9 binding proteins were analyzed using the STRING program (<http://www.string-db.org/>). Potential fisetin targeting proteins were analyzed using the STITCH program (<http://stitch.embl.de/>).

Statistical analysis

All statistical analyses were performed by using the graphpad prism V6.0 (GraphPad Software, Inc. San Diego, CA, USA). Data were presented as the mean ± standard deviation from at least three separate experiments. The statistical analysis correlation of data between groups was checked for significance by Student's *t*-test (two-tailed).

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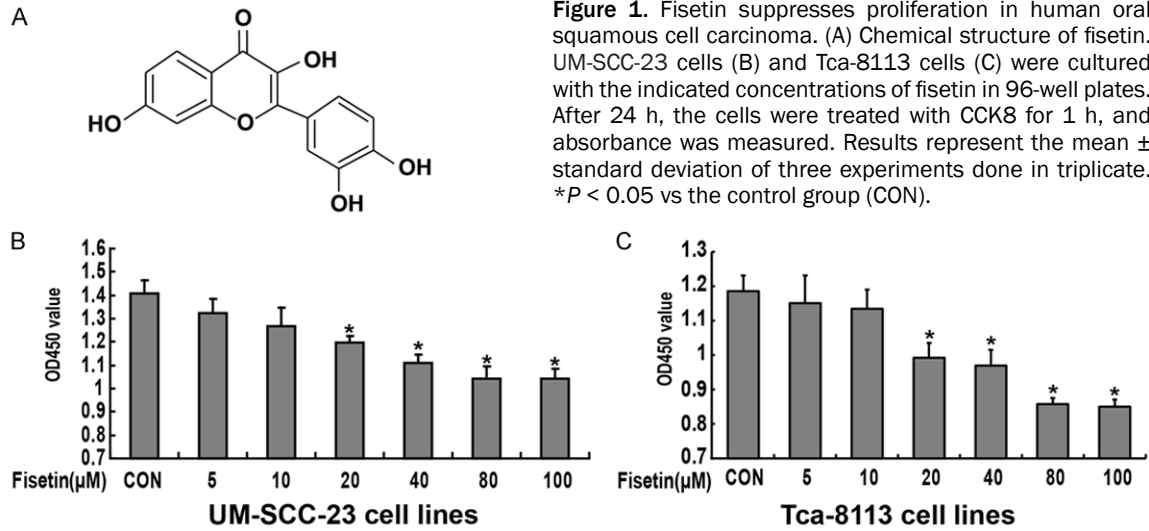


Figure 1. Fisetin suppresses proliferation in human oral squamous cell carcinoma. (A) Chemical structure of fisetin. UM-SCC-23 cells (B) and Tca-8113 cells (C) were cultured with the indicated concentrations of fisetin in 96-well plates. After 24 h, the cells were treated with CCK8 for 1 h, and absorbance was measured. Results represent the mean \pm standard deviation of three experiments done in triplicate. * $P < 0.05$ vs the control group (CON).

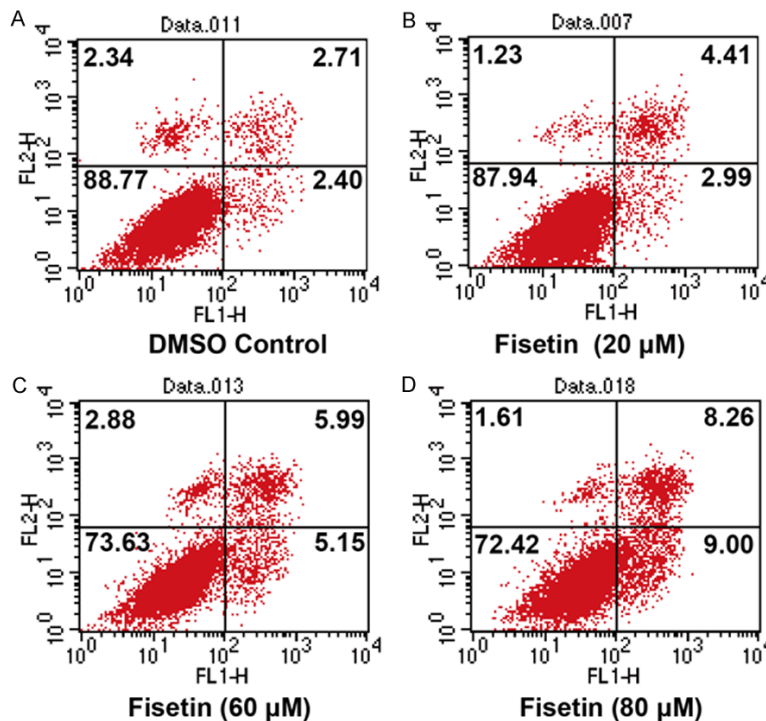


Figure 2. Fisetin promotes cell apoptosis in UM-SCC-23 cells. A-D. Cells were treated with either DMSO Control or 20-80 μ M fisetin for 24 h. At the end of treatments, cells were harvested and stained with annexin V and PI as detailed in Materials and Methods. Apoptotic cells were analyzed by flow cytometry as detailed in Materials and Methods.

Results

Fisetin reduces proliferation of OSCC cells

The chemical structure of fisetin is shown in **Figure 1A**. To investigate the effect of fisetin on the viability of OSCC cells, we conducted CCK8

assays using Tca-8113 and UM-SCC-23 cells. We found that fisetin significantly inhibited cell viability in a dose-dependent manner in OSCC cells (**Figure 1B, 1C**).

Fisetin induced apoptosis of OSCC cells

To explore whether the decrease in cell proliferation was correlated with the induction of apoptosis, Annexin V-FITC/PI staining was performed after fisetin treatment for 24 h. As shown in **Figure 2A-D**, UM-SCC-23 cells treated with different concentrations of fisetin for 24 h, and displayed significant levels of apoptosis in a concentration-dependent manner. The apoptotic rates of the UM-SCC-23 cells in the presence of 20, 60 and 80 μ M of fisetin for 24 h were 7.4, 11.14, and 17.26%, respectively.

Fisetin inhibits RTK signaling activation in OSCC cells

The inhibition of the RTK pathway in cancer cells is an important target for cancer therapy. Therefore, we investigated whether fisetin was capable of inhibiting RTK in UM-SCC-23 cells.

Fisetin suppresses proliferation by targeting Met/Src

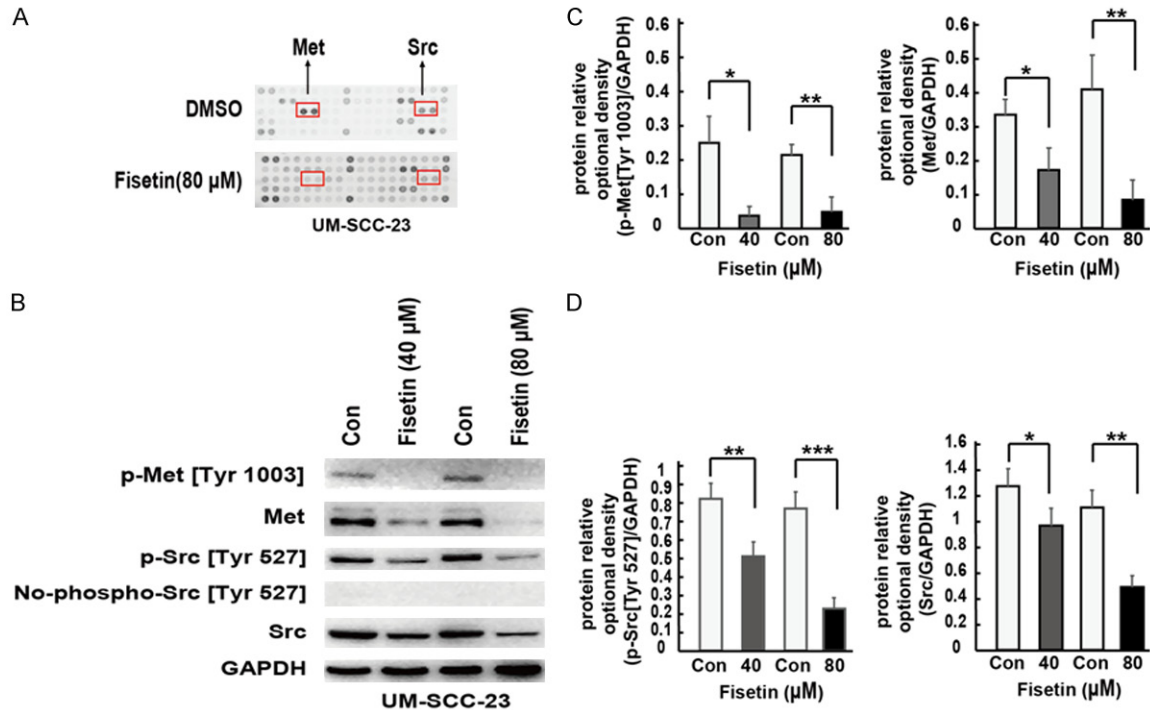


Figure 3. Effect of fisetin on receptor tyrosine kinase signaling proteins in human oral squamous cell carcinoma. A. UM-SCC-23 cells were treated with the indicated doses of fisetin for 24 h and chemiluminescent array images obtained using the PathScan® RTK Signaling Antibody Array Kit that involved in the regulation of the receptor tyrosine kinase. Images were captured following brief exposure of the slide to a standard chemiluminescent film. B. Cell lysates were analyzed by immunoblotting using specific primary antibodies, followed by detection with HRP-labeled appropriate secondary antibodies as mentioned in Materials and Methods. GAPDH was used as a loading control. C, D. Columns, mean of three independent treatments; bars, s.d. Data points are the means \pm s.d. of three experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. The P -value is determined by comparing each treatment with control group (CON).

For this purpose, UM-SCC-23 cells were treated with 80 μ M fisetin for 24 h using the PathScan® RTK Signaling Antibody Array Kit (Figure 3A, Supplementary Figure 1). Fisetin strongly suppressed the expression of Met and Src protein in UM-SCC-23 cells. Total Met and Src protein and phosphorylated Met (p-Met) and phosphorylated Src (p-Src) was evaluated using western blot analysis. As shown in Figure 3B-D, Supplementary Figure 2 total Met and Src proteins, p-Met and p-Src were significantly reduced by fisetin, suggesting that tyrosine phosphatases are involved in fisetin-mediated inhibition of the Met/Src signaling pathway.

Effects of fisetin on ADAM9 expression

Although previous results showed that fisetin suppresses ADAM9 expression in glioma cancer cells, whether this effect was associated with OSCC remained unclear. We found that ADAM9 may participate in several cancer related pathways including the ITGAV, UBC, and TNF

pathways (Figure 4A). The network shows that several proteins, including ADAM9, MTOR, AR and CDK6 are inhibited by fisetin (Figure 4B). Therefore, we used western blotting to examine the effect of fisetin on ADAM9 expression in Tca-8113 and UM-SCC-23 cells. We found that fisetin downregulated the expression of ADAM9 in OSCC cells (Figure 4C, 4D, Supplementary Figure 3). ADAM9 downregulation has been shown to suppress tumorigenesis and invasiveness of several kinds of cancer cells. Fisetin might be a potential therapeutic agent against human OSCC based on its capacity to inhibit ADAM9 expression.

Discussion

OSCC is one of the most common malignancies in the world, and has limited treatments with clinical efficacy [11]. We demonstrated that the dietary flavonoid fisetin has a growth inhibitory effect on two OSCC cancer cell lines in a dose-dependent manner. We also observed that fise-

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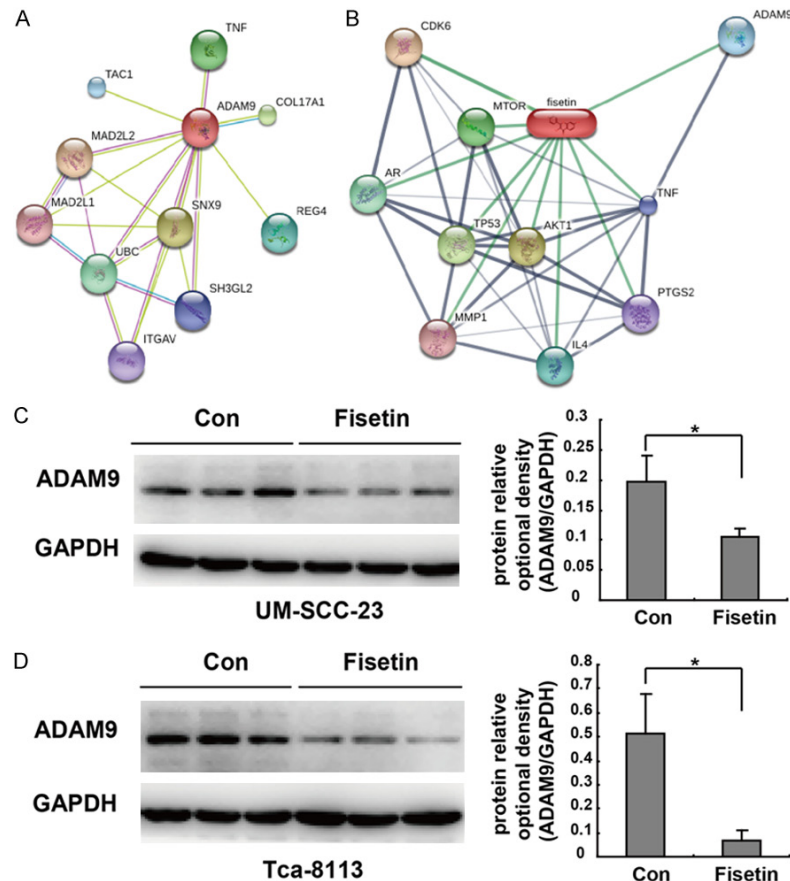


Figure 4. Fisetin reduced ADAM9 protein expression in UM-SCC-23 cells and Tca-8113 cells. A. Bioinformatics analysis of ADAM9 binding proteins. B. Bioinformatics analysis of fisetin targeting proteins. C, D. UM-SCC-23 cells and Tca-8113 cells were treated with fisetin (40 μ M) for 24 h. Western blotting was carried out to detect ADAM9 protein expression and GAPDH was used as a loading control (* versus CON, $P < 0.05$, Student's t -test, $n=3$).

tin treatment results in OSCC cell apoptosis. Furthermore, we showed a novel mechanism of fisetin in modulating receptor tyrosine kinases (including Met and Src) in a signaling pathway in OSCC cells. Fisetin could inhibit the invasive ability of glioma cancer cells, mainly through the inhibition of membrane-anchored metalloproteinase ADAM9 expression [8]. We confirmed that fisetin could inhibit ADAM9 expression in OSCC cells.

Met and Src are two tyrosine kinase receptors that are most commonly overexpressed in OSCC resulting in the increased proliferation and survival of OSCC cells [12-14]. Met and its ligand hepatocyte growth factor (HGF), are frequently implicated in cancer cellular proliferation, invasion, migration, and poor prognosis [15, 16]. Recently, the HGF/Met pathway has been identified as a promoter of tumorigen-

esis in head and neck squamous cell carcinoma (HNSCC) [17]. Inhibition of HGF/Met signaling may be a useful clinical therapeutic strategy for targeting OSCC cells [17, 18]. Western blot analyses demonstrated that fisetin decreased total Met protein levels and phosphorylated Met levels. Src activation is capable of modulating cell migration and invasion and other studies have also reported that Src was associated with OSCC cell migration [19]. These data suggest that Met/Src signaling is involved in cell tumorigenesis in OSCC and fisetin may be useful for the treatment of OSCC cancer cells.

In conclusion, fisetin is a naturally occurring compound that can be easily synthesized and has no toxicity to normal human cells [20]. All these properties may confer fisetin an advantage of being integrated into clinical cancer therapies. Although these

results need to be verified in experimental models *in vivo*, the present findings shed light on the mechanism of action of fisetin, which is critical for the design of targeted therapies against human OSCC.

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Disclosure of conflict of interest

None.

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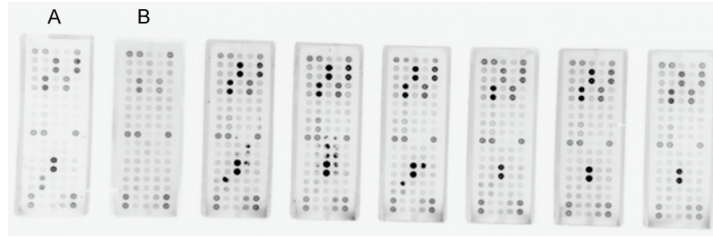
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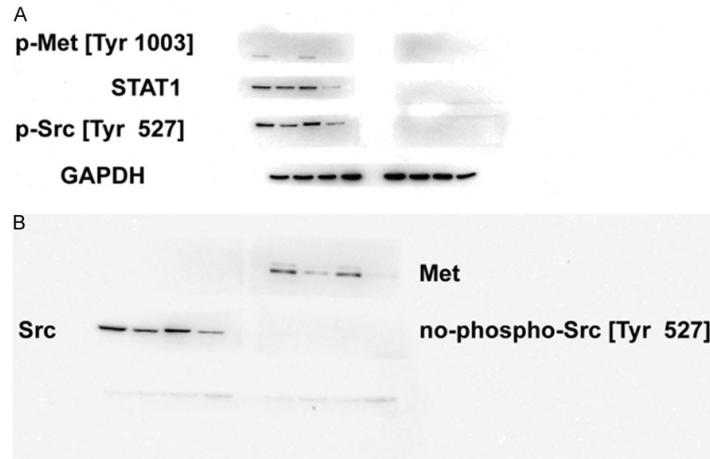
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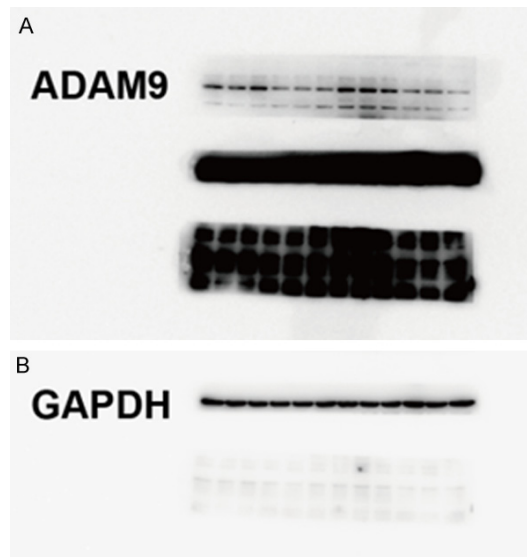
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Supplementary Figure 1. Figure 3A original western images. A. DMSO group. B. Fisetin (80 μ M) treatment group.



Supplementary Figure 2. Figure 3B original western images. A. Results of immunoblotting for p-Met [Tyr 1003], STAT1, p-Src [Tyr 527] and GAPDH. B. Results of immunoblotting for Met, Src, no-phospho-Src [Tyr 527].



Supplementary Figure 3. Figure 4C, 4D original western images. A. Results of immunoblotting for ADAM9. B. Results of immunoblotting for GAPDH.