Quercetin enhances decidualization through AKT-ERK-p53 signaling and supports a role for senescence in endometriosis

Julia Delenko
Donald & Barbara Zucker School of Medicine at Hofstra/Northwell
Xiangying Xue
Northwell Health
Prodyot K Chatterjee
Northwell Health
Nathaniel Hyman
Northwell Health
Andrew J Shih
Northwell Health
Robert P Adelson
Northwell Health
Polona Safaric Tepes
Northwell Health
Peter K Gregersen
Northwell Health
Christine N Metz
cmetz@northwell.edu

Northwell Health

Research Article

Keywords: menstrual effluent, fertility, AKT signaling, p53 signaling, apoptosis, senescence

Posted Date: March 28th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4118076/v1

Additional Declarations: No competing interests reported.

Abstract

Background

Patients with endometriosis suffer with chronic pelvic pain and infertility, and from the lack of pharmacologic therapies that consistently halt disease progression. Differences in the endometrium of patients with endometriosis vs. unaffected controls are well-documented. Specifically, shed endometrial tissues (delivered to the pelvic cavity via retrograde menstruation) reveal that a subset of stromal cells exhibiting pro-inflammatory, pro-fibrotic, and pro-senescence-like phenotypes is enhanced in endometriosis patients compared to controls. Additionally, cultured biopsy-derived endometrial stromal cells from endometriosis patients exhibit impaired decidualization, a defined differentiation process required for human embryo implantation and pregnancy. Quercetin, a senolytic agent, shows therapeutic potential for pulmonary fibrosis, a disorder attributed to senescent pulmonary fibroblasts. In rodent models of endometriosis, quercetin shows promise, and quercetin improves decidualization in vitro. However, the exact mechanisms are not completely understood. Therefore, we investigated the effects of quercetin on menstrual effluent-derived endometrial stromal cells from endometriosis patients and unaffected controls to define the signaling pathways underlying quercetin's effects on endometrial stromal cells.

Methods

Menstrual effluent-derived endometrial stromal cells were collected and cultured from unaffected controls and endometriosis patients and then, low passage cells were treated with quercetin (25 μM) under basal or standard decidualization conditions. Decidualization responses were analyzed by measuring the production of IGFBP1 and PRL. Also, the effects of quercetin on intracellular cAMP levels and cellular oxidative stress responses were measured. Phosphokinase arrays, western blotting, and flow cytometry methods were performed to define the effects of quercetin on various signaling pathways and the potential mechanistic roles of quercetin.

Results

Quercetin significantly promotes decidualization of control- and endometriosis-endometrial stromal cells. Quercetin substantially reduces the phosphorylation of multiple signaling molecules in the AKT and ERK1/2 pathways, while enhancing the phosphorylation of p53 and total p53 levels. Furthermore, p53 inhibition blocks decidualization while p53 activation promotes decidualization. Finally, we provide evidence that quercetin increases apoptosis of endometrial stromal cells with a senescent-like phenotype.

Conclusions

These data provide insight into the mechanisms of action of quercetin on endometrial stromal cells and warrant future clinical trials to test quercetin and other senolytics for treating endometriosis.

Background

Senescence has received wide attention as a possible target for treating and/or preventing aging and chronic fibrotic disorders. For example, senolytic agents such as quercetin and dasatinib have shown promising results for pulmonary fibrosis in clinical trials (1, 2). In addition, these and other senolytic agents have been tested in numerous pre-clinical and pilot human studies and are reported to be effective in several disorders characterized by chronic inflammation and fibrosis (3). As a common dietary flavonoid, the biological activity of quercetin has been widely studied, with evidence for antioxidant, anti-inflammatory, immune modulatory, anti-bacterial, and anti-viral effects, as well as senolytic activities, among others. However, these effects vary among different cell types, and the exact signaling pathways have not been well-defined.

Reports in the literature support that quercetin may have therapeutic efficacy in animal models of endometriosis (4–6). Additionally, in combination with other natural agents, quercetin reduces pain in patients with endometriosis (7, 8). Endometriosis is a common, chronic inflammatory condition defined by endometrial-like lesions growing outside of the uterus, mainly in the pelvic cavity. It is characterized by chronic pelvic pain and often accompanied by dysmenorrhea, dyspareunia, dysuria, dyschezia, and infertility (9). The exact causes of endometriosis and associated infertility are not well understood. The delivery of menstrual tissues to the pelvic cavity is observed in nearly all menstruators (10) and, intriguingly, endometriosis is associated with altered endometrial tissues (that are shed as menstrual effluent, ME) (11–14). We have shown that shed endometrial tissues and cells of patients with endometriosis are different when compared to controls (15), which further supports studying menstrual effluent-tissues and cells as key biospecimens for investigating the pathogenesis of endometriosis. Accordingly, we have recently characterized ME-derived endometrial stromal cells (ME-eSCs) in patients with endometriosis vs. unaffected controls (16, 17). In patients with endometriosis, these cells exhibit dramatic defects in decidualization (16–19), a common and essential differentiation event that is required for embryo implantation and reproductive success.

Our recent single cell RNA-sequencing (scRNA-Seq) analysis of ME-eSCs (15) supports altered differentiation of eSCs in the setting of endometriosis, as well as their deviation toward a more pro-inflammatory, pro-fibrotic, and senescent-like phenotype that may promote endometriosis lesion formation and/or disease progression. While prior studies showed that quercetin promotes decidualization using biopsy-derived eSCs (20), this approach has not been applied to ME-eSCs from endometriosis cases and controls in a comprehensive manner. Additionally, the mechanisms and signaling pathways underlying quercetin's effects are not well understood. Thus, based on these findings and the lack of effective pharmacological therapies for treating endometriosis, we assessed the effects of quercetin on the decidualization process, with the hypothesis that quercetin would improve decidualization and reduce differentiation of ME-eSCs to cells with a pro-inflammatory and possibly

senescent-like phenotype. After confirming that quercetin significantly enhanced decidualization responses using ME-eSCs obtained from controls and endometriosis cases, we defined the signaling pathways and mechanisms involved in mediating quercetin's effect and explored the potential role of senescence, which was previously shown to inhibit decidualization (21).

Methods

Chemical and reagents

Quercetin was purchased from Alfa Aesar/Fisher Scientific (Waltham, MA, US). 8-bromoadenosine 3',5'-cyclic monophosphate sodium salt (cAMP), IBMX (3-isobutyl-1-methylxanthine), forskolin, hydrogen peroxide (H_2O_2), medroxyprogesterone acetate (MPA), and *N*-acetylcysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, US). Prostaglandin E2 (PGE2), Z-VAD-fmk (Z-VAD), pifithrin-α (PFT), nutlin-3a (Nut), and MK-2206 were purchased from MedChemExpress, LLC (Monmouth Junction, NJ, US).

Isolation and culture of menstrual effluent (ME)-derived endometrial stromal cells

Women of reproductive age (18 to 45 years old) who were not pregnant, not breastfeeding, and not on hormonal contraceptives, and who were menstruating and willing to provide menstrual effluent (ME) samples provided written informed consented through the IRB-approved ROSE study (IRB #13-376A). Patients with histologically confirmed endometriosis (documented in a pathology report following laparoscopic surgery and excision of lesions) were also recruited and enrolled as 'endometriosis' participants (cases). Control subjects (controls) who self-reported no history suggestive of a diagnosis of endometriosis were also recruited and enrolled through the ROSE study. No subjects reported having polycystic ovary syndrome (PCOS) or adenomyosis.

Endometrial stromal cells (eSCs) from ME collected from cases and controls were grown as previously described (17). Briefly, ME-eSCs were grown in DMEM containing 10% mesenchymal stem cell-fetal bovine serum (FBS), 1% penicillin-streptomycin-glutamine (PSQ) (maintenance media) (Gibco/Thermo-Fisher), and normocin (1:500) (Invivogen, San Diego, CA, US) (maintenance media) at 37°C with 5% CO_2; cells were split 1:6. Passage 1–3 eSCs were used for experiments (unless otherwise indicated) or were cryopreserved in 10% DMSO/90% FBS for later use. Formal sample size calculations were not performed, as the study of ME-eSCs does not involve ethical, time or cost issues which warrant sample size calculations (22). Sample sizes for functional cell-based assays were consistent with our prior studies (16, 17).

eSC proliferation assays

ME-eSCs were plated in 96-well plates (100 µL/well, $1.5X10^4$/mL) in maintenance media. The next day, media was aspirated and replaced with DMEM containing 2% FBS + 1% PSQ and normocin (1:500) (assay media) and treated with vehicle or quercetin (0–50µM) (N = 4–6 per condition) at 37°C with 5%

CO. After a 72 hr incubation, cells were washed once with cold PBS, aspirated, and frozen at -80°C until assayed for cell proliferation using the CyQUANT™ Cell Proliferation Assay, according to the manufacturer's directions (Thermo-Fisher, Waltham, MA, US). CyQUANT™ dye binds to DNA, and the fluorescence emitted by the dye is linearly proportional to the number of cells in the well. Data are shown as relative cell number for each subject's data point, with each group mean ± SD indicated for quercetin concentrations (0–50μM).

Decidualization assays using ME-eSCs

In the morning, confluent monolayers of ME-eSCs (passages 1–3) isolated/grown from controls and endometriosis cases were lifted with trypsin/EDTA, washed, resuspended in maintenance media (DMEM, 10% FBS, PSQ, and normocin), and plated in 96-well plates (100 μL/well, $2.5X10^5$/mL). The same evening, media was aspirated and replaced with decidualization assay media (DMEM, 2% FBS, PSQ, and normocin). After an overnight incubation, cells were treated with either vehicle or quercetin (25μM) for 4 hr (unless described otherwise) before the addition of cAMP (0.5 mM) ± MPA (10^{-7} M) to induce decidualization (N = 3–5 per condition). The dose and timing of quercetin addition were based on the literature and optimization studies using eSCs obtained from control subjects. After 48 hr (following cAMP ± MPA treatment), supernatants were collected and frozen at -80°C until assayed for insulin growth factor-binding protein 1 (IGFBP1) or prolactin (PRL) proteins by ELISA (R&D Systems, Minneapolis, MN), as previously described (17). Protein data for decidualization assays are shown as IGFBP1 or PRL protein (pg/mL) found in cell-free culture supernatants after 48 hr, unless otherwise specified.

In a subset of samples, decidualization was analyzed by measuring *IGFBP1* and *PRL* mRNA expression by quantitative reverse transcription PCR (qRT-PCR). Briefly, high quality RNA was isolated from cells using the RNeasy Universal Plus Mini Kit (Qiagen). RNA quality was analyzed using the NanoDrop spectrophotometer (Wilmington, DE, US) and the Bioanalyzer (Agilent Technologies Genomics, Santa Clara, CA, US); samples with OD260:280 and OD260:230 ratios > 1.9 were converted to double stranded cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, US). Real time qPCR (RT-qPCR) reactions using specific primers and the Roche Universal Probe Library (Supplementary Table 1) were performed in triplicate using the Eurogentec qPCR MasterMix Plus (AnaSpec, Inc, Fremont, CA, US) and the Roche LightCycler 480. Relative changes in gene expression were calculated as fold-changes using the comparative 2^(-ΔΔCt) method; based on optimization studies, human *HPRT* was used as the housekeeping gene for normalizing transcript levels, as previously described (17).

The effects of MK-2206 (AKT inhibitor), pifithrin-α (p53 inhibitor), nutlin-3a (p53 activator), and Z-VAD (pan-caspase inhibitor) on decidualization were examined as described above, except vehicle, MK-2206 (1μM), Z-VAD (40μM) or PFT (40μM) was added to eSC cultures with vehicle or quercetin (25μM); 4 hr later cells were treated with cAMP alone or cAMP ± MPA and processed as described above. Nutlin-3a (100nM) was added 24 hr following cAMP + MPA treatment. Doses were chosen based on the literature

and results of in-house optimization studies with eSCs where cytotoxicity (assessed by neutral red (23)) was not detected.

Immunofluorescent staining for IGFBP1 and assessment by confocal microscopy

Control-eSCs grown to confluence on glass cover slips in 24 well plates in maintenance media were switched to assay media (DMEM, 2% FBS, PSQ). The next day, cells were treated with vehicle or quercetin (25μM) for 4 hr prior to the addition of cAMP + MPA or vehicle, as described above for decidualization assays. After 36 hr, cells were treated with GolgiPlug (BD Biosciences, San Jose, CA) to block IGFBP1 release and then 12 hr later (48 hr after vehicle or cAMP + MPA), cells were washed and fixed with 4% formaldehyde (freshly prepared), permeabilized/blocked with 5% mouse serum/0.3% Triton X-100/1X PBS, and stained with anti-IGFBP1-AF647 antibody (Santa Cruz, sc-55474 AF647, 1:100) and then stained with Alexa488-phalloidin (Abcam, 1:5000) and washed and mounted with Antifade reagent with DAPI (Fisher Scientific). Cells were then examined under a confocal microscope (Zeiss LSM900) using the 20X objective.

Assessment of intracellular cAMP production by eSCs following forskolin or quercetin

Intracellular cAMP ($[cAMP]_i$) production by eSCs isolated from controls and endometriosis cases was analyzed using the cAMP ELISA kit (Cayman Chemical, Ann Arbor, MI, US) according to the manufacturer's guidelines. Briefly, eSCs were grown to confluence and then seeded into 6-well plates at $3x10^5$ cells/mL (2 mL/well) in maintenance media. Six hr later, maintenance media was aspirated and replaced with 2% FBS-containing assay media. After an overnight incubation, eSCs were treated with IBMX (a broad spectrum phosphodiesterase inhibitor, 0.1mM) for 30 min, followed by either vehicle, forskolin (an adenylate cyclase activator, 25μM), or quercetin (25μM, as indicated) and incubated at 37°C with 5% CO_2 using a method that was optimized in pilot studies. After 20 min, the media was aspirated and the cells were washed once with ice cold PBS and lysed with 0.25 mL 0.1N HCl for measuring $[cAMP]_i$, as per the manufacturer's directions. All samples were diluted 1:2 with ELISA diluent for the cAMP ELISA assay. Data are expressed for each subject's cells as cAMP pMol/mL with the median ± IQR (interquartile range) for the group.

Measurement of basal and H_2O_2-induced oxidative stress using eSCs

Control- and endometriosis-eSCs isolated from controls were plated in black 96-well plates with clear bottoms (100 μL/well, $2.5X10^5$/mL) in maintenance media. Media was aspirated and replaced with assay media and after an overnight incubation, cells were washed with HBSS, labeled with DCF-DA (20 μM) in HBSS for 30 min, and washed with HBSS 1% FBS. Phenol red-free DMEM containing 2% FBS + PSQ (100μL/well) was then added, and cells were treated with either vehicle or quercetin (25μM) (or NAC,

10mM) and incubated at 37°C with 5% CO_2 for 3 hr. Then cells were either treated with vehicle or H_2O_2 (500μM) and analyzed for oxidative stress 3 hr later in a quantitative manner using the VICTOR3 fluorescence plate reader (Perkin-Elmer) at an excitation wavelength of 485nm and emission wavelength of 535nm. This assay measures the conversion of non-fluorescent 2',7'-Dichlorofluorescein diacetate (DCFH-DA) into fluorescent dichlorofluorescein (DCF) by reactive oxygen species (ROS) intermediates (24). Data are presented for each subject's cells as percent vehicle-control oxidative stress, with the median ± IQR for each group (control vs. endometriosis case) according to condition (basal vs. induced).

Blocking the effect of H_2O_2-inhibition of decidualization

Testing the effect of quercetin on H_2O_2-impaired decidualization by eSCs was performed as described above, except eSCs in maintenance media were pre-treated with either vehicle or 250μM H_2O_2 for 2 hr and then washed. Maintenance media was replaced with 2% FBS-assay media. 48 hr later, eSCs were treated with vehicle or quercetin (25μM) for 4 hr and then stimulated with cAMP ± MPA to induce decidualization and processed as above.

Proteome profiler array

Confluent eSCs were plated in maintenance media in 100 mm plates and then switched to assay media 6 hr later. The next day, eSCs were treated with vehicle or quercetin (25μM) for 4 hr and then the cell lysates were processed and analyzed, as described by the Human Phospho-Kinase Array Panel Kit (ARY003C, R&D Systems). Image spots were quantified according to the manufacturer's protocol using ImageJ.

Western blotting

Control-eSCs or endometriosis-eSCs were plated in maintenance media in 15 mm plates and, when confluent, the media was replaced with 2% FBS-assay media. The next day eSCs were treated with vehicle or quercetin (25 μM) for 4 hr. Cell pellets were resuspended in lysis buffer containing protease and phosphatase inhibitors (Halt™ Protease and Phosphatase Inhibitor Cocktail, Thermo Fisher #PI78442) and incubated on ice for 25 min, followed by centrifugation at 13,000 rpm for 10 min at 4°C. Supernatants were collected and protein levels were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher, #23225); up to 65 μg/lane was run on 4–12% NuPAGE Bis-Tris 1.5 mm gels in NuPAGE MOPS running buffer (Invitrogen). Proteins were transferred onto Immobilon FL-PVDF membranes, which were blocked at room temperature for 1 hr in PBS 0.1% Tween-20 (PBST) and 5% nonfat milk powder (#9999 CST). Membranes were rinsed in PBST and incubated overnight at 4°C in primary antibody diluted in PBST containing 5% bovine serum albumin or 5% milk powder, depending on the manufacturer's recommendation (antibodies are listed in Supplementary Table 2). The next day, membranes were washed in PBST and incubated for 1 hr at RT in HRP-conjugated secondary antibodies diluted in PBST containing 5% nonfat milk. Membranes were washed again in PBST and imaged by the addition of Clarity ECL substrate (#1705060, BioRad) or SuperSignal West Dura Extended Substrate (#34075, Thermo Fisher) on a ChemiDoc system (BioRad). SeeBlue Plus2 Pre-stained molecular weight standard (#LC5925, Thermo Fisher) was used to estimate protein weight. Western blots were stripped

with ReBlot Plus (#2504MI, Millipore), according to the manufacturer's suggestions, and then re-blocked and probed as described above. Band densities were quantified using NIH ImageJ.

Assessment of senescence marker expression by western blotting

Briefly, eSCs were plated as described for western blotting above, except eSCs (p2-p3) in assay media were treated with vehicle or H_2O_2 (250 μM) for 2 hr. After the media was aspirated with replaced with assay media (DMEM 2% FBS, PSQ), eSCs were incubated for 2, 4, 6, or 8 days (as indicated) and then cell lysates were processed as described for western blotting using the Senescence Marker Antibody Sampler Kit (#56062, Cell Signaling Technology). For some experiments, eSCs were treated with vehicle or quercetin (Q, 25μM) for 4 hr post-H_2O_2 and analyzed for senescence markers at indicated times.

Assessment of ADCY1-10 gene expression by stromal cells in fresh menstrual effluent tissues by scRNA-Seq analysis

Single-cell RNA sequencing (scRNA-Seq) data from whole, fresh ME, as previously described (15), was re-analyzed to determine *ADCY1-10* expression by ME-eSCs from control subjects.

Assessment of apoptosis and senescence-like phenotype by flow cytometry

Apoptosis

Confluent cultures of control-eSCs (p3-p4) grown in maintenance media were switched to assay media and the following morning they were treated with vehicle or quercetin (25μM) for 24 hr or 48 hr (as indicated) prior to harvesting and processing for analysis of apoptosis by flow cytometry using FITC-Annexin V Apoptosis Detection Kit (BD Biosciences), according to the manufacturer's directions. Flow cytometry data (fold-change after quercetin treatment) was analyzed on a BD Symphony™ Flow Cytometer and using FlowJo™ v10 software.

Senescence-like phenotype

Confluent cultures of control-eSCs (p3-p4) were prepared as described above, except they were processed for fluorometric β-galactosidase staining using DDAO (9H-(1,3-dichloro-9,9-dimethylacridin-2-one), as previously described using the BD Symphony™ Flow Cytometer and FlowJo™ v10 software. The percentage of large senescent-like cells (as measured by forward scatter, FSC-A) among total viable cells per sample was calculated.

Statistics

Analyses and graphical presentations were performed using the GraphPad Prism 10 software. The results are presented as the data for each subject's cells with the mean ± standard deviation (SD) or median ±

interquartile range (IQR) shown for each group. ANOVA with appropriate post-tests were used for multiple comparisons. Unless indicated, two groups were compared using Wilcoxon matched-pairs signed-rank test (or by Student's t test for unpaired data, as indicated). P values of 0.05 or less were considered significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

Results

Quercetin significantly reduces the proliferation and enhances decidualization of ME-eSCs

As described for many cell types, including uterine biopsy-derived eSCs (5), quercetin dose-dependently (12–50μM) reduced the proliferation of ME-eSCs obtained from healthy controls (Fig. 1A). Similar results were observed with ME-eSCs obtained from patients with endometriosis, with clear inhibition detectable at 25μM quercetin (Supplementary Fig. 1).

The differentiation of eSCs into decidual cells is induced by progesterone (or its stable synthetic analogue, medroxyprogesterone acetate (MPA)) and the cAMP/protein kinase A signaling pathway, which can be replicated in vitro using cell-permeable cAMP alone or cAMP + MPA (25). Testing a range of quercetin doses with eSCs obtained from healthy controls revealed that quercetin dose-dependently enhanced both cAMP- and cAMP + MPA-induced decidualization, as quantified by IGFBP1 production, peaking at 25μM quercetin (Fig. 1B-C, respectively). IGFBP1 expression by control eSCs following cAMP + MPA-induced decidualization was confirmed by confocal microscopy (Fig. 1D and Supplementary Fig. 2). IGFBP1 was absent without deciduogenic stimulation, and as previously described (26) not all cells express IGFBP1 following stimulation. Furthermore, quercetin increased cAMP + MPA-induced IGFBP1 expression by eSCs as determined by confocal microscopy (Fig. 1D and Supplementary Fig. 2).

Using the optimal dose of quercetin (25μM), the most dramatic and consistent decidualization enhancing effects were observed when added 4 hr prior to cAMP or cAMP + MPA treatment (Fig. 1E), rather than at the same time (Fig. 1F) or following cAMP + MPA treatment (Fig. 1G). Thus, we employed the '4 hr pre-treatment' regimen with quercetin (25μM) for remaining experiments.

Endometriosis-eSCs exhibit impaired decidualization when compared to control-eSCs

As previously reported (17), eSCs from our cohort endometriosis cases exhibit reduced decidualization response following cAMP alone (Fig. 2A) and cAMP + MPA (Fig. 2B) when compared to eSCs obtained from healthy controls, regardless of the decidualization stimulus ($p < 0.005$). Note that eSCs from both cases and controls exhibit a range of decidualization responses (Fig. 2).

Quercetin promotes decidualization of eSCs from healthy controls and endometriosis cases

Using ME-eSCs isolated from healthy controls and endometriosis cases, quercetin treatment significantly enhanced both cAMP-induced (Fig. 3A-B, $p < 0.0001$) and cAMP + MPA-induced decidualization (as determined by IGFBP1 levels) (Fig. 3C-D, $p < 0.0001$) regardless of whether the donor was affected by endometriosis or not. Also, quercetin treatment of a subset of control-eSCs and endometriosis-eSCs significantly increased cAMP-induced and cAMP + MPA-induced production of PRL, another biomarker of decidualization (Fig. 3E-H). Similar results were obtained for *IGFBP1* and *PRL* mRNA expression when analyzed by qPCR (Supplementary Fig. 3). These data support that quercetin treatment 'corrects' the impaired decidualization response of endometriosis-eSCs to the level observed with control-eSCs (with vehicle-treatment).

It has been proposed that prostaglandin E2 (PGE2) and progesterone are the minimal ancestral deciduogenic signals that induce decidualization in eutherian mammals (27). As shown for cAMP- and cAMP + MPA-induced decidualization (Fig. 3), quercetin significantly elevates both PGE2- and PGE2 + MPA-induced decidualization by control-eSCs (Supplementary Fig. 4). Thus, quercetin enhances decidualization induced with multiple deciduogenic stimuli.

Quercetin does not generate intracellular cAMP production by eSCs

Because cAMP drives decidualization and quercetin enhances intracellular cAMP ($[cAMP]_i$) concentrations by the N1E-115 neuroblastoma cell line (28), we assessed the production of $[cAMP]_i$ by quercetin-treated eSCs. This approach was supported by scRNA-Seq analyses revealing multiple *ADCY* transcripts (that encode adenylyl cyclases required to synthesize 3',5'-cAMP) expressed by eSCs in fresh ME, including low expression of *ADCY1, 3, 6, 7*, and *9* transcripts (Supplementary Fig. 5). Forskolin induced $[cAMP]_i$ by control-eSCs (Fig. 4A). However, quercetin treatment did not induce $[cAMP]_i$ by eSCs (Fig. 4A-B) and therefore, likely does not enhance decidualization through this mechanism.

Quercetin does not exert antioxidant effects in eSCs

Measuring oxidative stress using the DCFDA assay, H_2O_2-treatment significantly induced oxidative stress in both control and endometriosis-eSCs (Fig. 5). While the well-known antioxidant, *N*-acetylcysteine (NAC), significantly reduced H_2O_2-induced oxidative stress by eSCs (Fig. 5A-D), quercetin did not reduce oxidative stress by eSCs when added after (Fig. 5A-B) or before H_2O_2 stimulation (Fig. 5C-D). These data suggest that quercetin does not enhance decidualization activity in eSCs through exerting antioxidant effects.

Quercetin treatment of eSCs alters numerous signaling pathways

Phospho-kinase arrays were used to investigate various signaling pathways altered by quercetin. Compared to vehicle treatment of control-eSCs, quercetin consistently reduced the expression of phospho-AKT (T308 and S473), phospho-ERK1/2 (multi), phospho-PRAS40 (T246), and phospho-WNK1

(T60), and significantly increased phospho-p53 (S46) expression, when assessed 4 hr after treatment (Fig. 6A-B, Supplementary Fig. 6).

We confirmed that quercetin decreased the expression of phospho-AKT (S473), phospho-ERK1/2 (multi), and phospho-PRAS40 (T246) when analyzed by western blotting 4 hr later using control-eSCs and endometriosis-eSCs without changing total unphosphorylated target protein expression (Fig. 6C-D). Variable expression levels of pAKT expression patterns were found among subjects. Time course analyses reveal that quercetin reduced phospho-AKT (S473), phospho-ERK1/2 (multi), and phospho-PRAS40 (T246) by control-eSCs within 30 min and this downregulation persisted for 4 hr (Fig. 6E). Accordingly, MK-2206, the highly selective inhibitor of AKT1/2/3, significantly enhanced both cAMP-induced IGFBP1 and PRL production (Fig. 6F and 6H, respectively) and cAMP + MPA-induced IGFBP1 and PRL production (Fig. 6G and 6I, respectively). The effect of MK-2206 on decidualization was similar to that achieved by quercetin and when MK-2206 was combined with quercetin, no additive effects were observed (Fig. 6F-I), supporting a similar or overlapping pathway. Together, these data support that quercetin modulates signaling via AKT and ERK pathways to effectively promote decidualization.

Quercetin increases p53 protein expression

Although quercetin-induced phospho-p53 (Ser45) expression was observed using the phospho-kinase array (Fig. 6A-B), it was not detected by standard western blotting methods likely due to the lower detection sensitivity for western blotting when compared to the array's capture method. Regardless, quercetin increased total p53 protein levels by control-eSCs and endometriosis-eSCs after 4 hr (Fig. 7A-B). Although higher p53 protein levels did not persist for 2 days (Fig. 7C-D), the increase in total p53 protein was detected as early as 2 hr post-quercetin addition (Fig. 7E). Consistent with these observations, pifithrin (PFT, 40μM), an inhibitor of p53, significantly reduced cAMP + MPA-decidualization when compared to vehicle (Fig. 7F) and caused a similar reduction even with quercetin-enhanced decidualization (Fig. 7G). By contrast, nutlin-3a, an inhibitor of MDM2 and promoter of p53, improved decidualization when added post-cAMP + MPA (Fig. 7H). Together, these data suggest the previously unrecognized effect of quercetin on p53 phosphorylation and total p53 protein expression by eSCs and support the potential role of p53 in decidualization.

eSCs show evidence of a senescence-like phenotype, which is reduced by quercetin

Our recent studies revealed numerous senescence-related gene markers that were consistently upregulated in vivo in eSCs found in fresh ME tissues obtained from endometriosis patients vs. controls (15). Under basal conditions, early passage eSCs (p2-p3) express SASP biomarkers IL-6 and MMP3 (Fig. 8A), and this SASP phenotype is reduced by quercetin treatment (Fig. 8A-C). Also under basal conditions, early passage control-eSCs (p2-p3) express senescence-related markers p21 and p16 (which increase with senescence), and lamin B1 (which decreases with senescence) (Fig. 8D). As expected, low dose H_2O_2, which induces a senescence-like phenotype in eSCs (29), further induced p16, p21, and MMP3

expression and reduced lamin B1 expression over time (Fig. 8D). Also, H_2O_2 treatment significantly reduced decidualization (Fig. 8E), while quercetin restored this inhibition of decidualization (Fig. 8E).

Inhibition of apoptosis blocks decidualization; quercetin induces apoptosis in a subset of eSCs

Based on the role of p53 (S46) in apoptosis and the increase in p53 expression by quercetin, we tested the effect of Z-VAD-fmk, a pan-caspase inhibitor that prevents apoptosis, on decidualization. Pretreating control-eSCs with Z-VAD-fmk (40 μM) significantly reduced decidualization and blocked quercetin-enhanced decidualization when compared to vehicle-treated eSCs (Fig. 9A-B). Accordingly, quercetin treatment significantly increased eSC apoptosis in a subset of eSCs when analyzed 24 and 48 hr later (Fig. 9C-D). In addition, quercetin treatment decreased the number of large beta-galactosidase$^+$ cells (Fig. 9E-F), which were likely senescent.

Discussion

Our data provide mechanistic insight as to how quercetin enhances the decidualization response of ME-eSCs. Decidualization refers to the differentiation of fibroblast-like eSCs into enlarged specialized decidual stromal cells that produce the growth factors needed to create a nutrient-rich uterine environment that is critical for implantation and successful pregnancy in humans (25). In mice and most other eutherian mammals this process occurs post-implantation. By contrast, in the 4% of mammals that menstruate, including humans, decidualization occurs spontaneously during each secretory phase of the menstrual cycle.

It is well-established that progesterone raises $[cAMP]_i$ levels to trigger decidualization in vivo and this process can be recapitulated with cultured eSCs using cell-permeable cAMP ± MPA (25). Using this model system, numerous studies report decidualization defects with uterine biopsy-derived eSCs (18, 19, 30) and ME-eSCs (16, 17) in the setting of endometriosis. Here, we report that quercetin enhanced the decidualization response regardless of whether ME-eSCs were obtained from endometriosis cases or unaffected healthy controls (Fig. 3) or if induced with cAMP ± MPA or PGE2, and quercetin treatment brought decidualization responses of endometriosis-eSCs to levels found in vehicle-treated control-eSCs. However, quercetin did not increase $[cAMP]_i$ levels (Fig. 4), nor did it appear to mediate decidualization through antioxidant activity (Fig. 5).

Previous studies have linked quercetin's effect on decidualization to its anti-proliferative effect (4, 5, 20). However, not all agents that inhibit eSC proliferation enhance decidualization responses, suggesting that this effect is likely mediated through specific signaling pathways. Our data show that there are variable expression levels of pAKT among subjects, even among controls. This may reflect their decidualization capacity – but requires future studies. Regardless, we now show that quercetin rapidly reduces the phosphorylation of AKT (S473/T308), as well as ERK1/2 (multi), PRAS40 (T246), and WNK1 (T60).

ERK1/2 and AKT pathways converge to inhibit p53. Accordingly, we found that quercetin increased phosphorylation of p53 (S46) and increased expression of total p53 protein.

These data are consistent with prior studies revealing AKT dephosphorylation during decidualization and increased phospho-AKT expression by stromal cells in the eutopic endometrium of endometriosis cases and endometriosis lesions (31–33). We propose that persistent phospho-AKT activity may explain decidualization defects observed in the setting of endometriosis that can be corrected with quercetin, since quercetin rapidly reduced AKT phosphorylation by eSCs (within 30 min, Fig. 6E). Likewise, the AKT inhibitor, MK-2206, significantly increased decidualization (Fig. 6F-I). AKT is a pro-survival factor with potent apoptosis-inhibiting activity whose function in regulating transcription factors and other proteins is mediated, in part, by its phosphorylation status. Thus, our findings are consistent with the 'AKT inhibitory action' of quercetin on eSCs (5) and other cells (34) and may explain the connection between inhibition of AKT signaling and increased decidualization and fertility (35, 36).

A prior study reported that quercetin reduced *TP53* mRNA expression by primary eSCs while promoting decidualization (20). By contrast, we observed that quercetin increased p53 phosphorylation (Fig. 6A-B) and total p53 protein levels (Fig. 7A-B). We did not assess *TP53* mRNA expression and *TP53* mRNA expression may not correlate with p53 protein expression and activation. Our data support that the effects of quercetin on p53 expression and activation are related to the convergence of the AKT and ERK1/2 pathways. AKT phosphorylates numerous proteins, including 'with no lysine kinase-1' or WNK1 (T60) (37) and 'proline-rich AKT substrate of 40 kDa' (PRAS40), which in turn enhances activation of PI3K/AKT signaling (38). Accordingly, we found that quercetin reduced the phosphorylation of PRAS40 and WNK1 and by eSCs within 30 min (Fig. 6E). Interestingly, PRAS40 also plays important roles in cellular senescence and p53 regulation (39). Specifically, phospho-PRAS40 decreases p53 expression level (39) and inhibits pro-apoptotic gene expression (40). Our data suggest that quercetin blocks signaling through this pathway and therefore may enhance p53 stability. Additionally, we found that quercetin-treated eSCs show a reduction in ERK1/2 phosphorylation (Fig. 6A-D). ERK1/2 is a parallel signaling pathway to the AKT pathway and downstream of receptor tyrosine kinase activation. Here again, inhibition of ERK phosphorylation would lead to a release of suppression of p53. These relationships are summarized in Fig. 10.

Quercetin, a member of a large family of flavonoids found in fruits, vegetables, tea, seeds, nuts, and medicinal botanicals, has been reported to have antioxidant, anti-inflammatory, and immunomodulatory activities throughout the body, including the female reproductive tract (41, 42). However, it is the senolytic property of quercetin that has garnered the most attention in recent years. Indeed, recent studies describe the inhibitory effects of eSC senescence and the senescence-associated secretory phenotype (SASP) on decidualization in vitro (43) and this provides a functional link between dysfunctional decidualization, senescence and reduced female fertility (20, 44). Therefore, we searched for evidence that quercetin induces apoptosis of senescent-like eSCs. As shown in Fig. 9, inhibition of apoptosis using Z-VAD, a pan-caspase inhibitor, inhibits decidualization. However, quercetin selectively induces apoptosis eSCs (Fig. 9C-D) and specifically eliminates senescent-like eSCs (Fig. 9E-F). This activity is accompanied by an

increase in decidualization and as previously reported, senescent-like eSCs inhibit decidualization (21). Thus, apoptotic elimination of senescent-like eSCs may be one mechanism by which quercetin enhances decidualization.

More direct proof of this hypothesis will require isolation and further experiments with purified senescent/senescent-like stromal cells. The hallmarks of cellular senescence are cell cycle arrest caused by increased expression of p16 and p21, changes in the nuclear membrane (loss of lamin B1), the production of SASP factors, and the expression of SA-β-gal. However, there are no global characteristics that identify senescent/senescent-like-like cells in all tissues, and that can be easily applied at the single cell level. This is a major challenge for studies of cellular senescence. For example, it appears that the 'senescence inducer' (e.g. replicative exhaustion, oxidative stress, ER or mitochondrial stress) influences the senescence phenotype and the 'senescence inducer' in the endometrium has not been defined. Also, markers of senescence tend to be different in various cell types and there is no standard gene or protein expression pattern that can definitively identify these cells across tissue types. However, increased p16 and p21 expression and reduction of lamin B1 protein are consistent with a senescence phenotype in a subset of our stromal cells as shown in Fig. 8D after exposure to H_20_2 (a common method to induce senescence) and the expression of SA-β-gal by larger eSCs shown in Fig. 9E-F.

In a larger context, the roles of senescence in aging, cell differentiation, as well as tissue injury and repair have been widely reported (45–47). Senolytic agents have been developed to treat aging-related and other conditions by selectively clearing senescent cells (i.e., growth arrested, viable cells that have undergone metabolic and gene expression changes) (3). In the context of reproduction, quercetin supplementation improves fecundity in young female mice (48) and increases pregnancy rates in a small cohort of patients with PCOS (49). Additionally, quercetin administration significantly reduces the growth of endometrial implants in rats (4) and mice (5, 6). However, in these studies there is no or limited direct evidence that the effects of quercetin are mediated by its senolytic activity.

Although it is relatively non-toxic, the main disadvantage of quercetin is its low bioavailability (50). The dose used herein is below ranges reported for many in vitro studies (reporting up to 120 μM (51–53)). Furthermore, it is unclear how to translate the in vitro dose used for cell culture studies to humans. Nonetheless, numerous efforts are underway to improve quercetin's solubility and bioavailability. One example is the food-grade lecithin-based formulation of quercetin, known as Quercetin Phytosome®, which exhibits enhanced solubility (54). When given orally and compared to non-modified quercetin, Quercetin Phytosome® facilitates the achievement of up to 20 times higher plasma levels of quercetin, without notable adverse effects (54). This form of quercetin and the development of new senolytics with higher bioavailability will permit future in vivo studies to test the effects of quercetin on decidualization capacity of ME-eSCs in controls or affected cases. Also, the use of non-invasive sampling methods for collecting ME-eSCs from various populations is critical for advancing our understanding of senolytics in the setting of endometriosis and other uterine health disorders.

Conclusions

Together, these findings warrant future studies to explore the role of senescence in endometriosis and the use of quercetin and other senolytic agents as potential therapies or adjunct treatments for endometriosis and associated infertility. Given the low risk of quercetin as a common dietary constituent, we propose that quercetin should also be tested in clinical trials for disease prevention in patients at risk for endometriosis, where decidualization defects and senescent-like phenotypes could be monitored through the longitudinal analysis of stromal cells in menstrual effluent.

Abbreviations

8-bromoadenosine 3',5'-cyclic monophosphate sodium salt (cAMP), dichlorofluorescein (DCF), 2',7'-Dichlorofluorescein diacetate (DCFH-DA), endometrial stromal cells (eSCs), fetal bovine serum (FBS), hydrogen peroxide (H_2O_2), IBMX (3-isobutyl-1-methylxanthine), insulin growth factor-binding protein 1 (IGFBP1), interquartile range (IQR), medroxyprogesterone acetate (MPA), menstrual effluent (ME), menstrual effluent-derived endometrial stromal cells (ME-eSCs), *N*-acetylcysteine (NAC), nutlin-3a (Nut), PBS 0.1% Tween-20 (PBST), penicillin-streptomycin-glutamine (PSQ), pifithrin-α (PFT), prolactin (PRL), prostaglandin E2 (PGE2), quantitative reverse transcription PCR (qRT-PCR), reactive oxygen species (ROS), standard deviation (SD), senescence-associated secretory phenotype (SASP), single cell RNA-sequencing (scRNA-Seq)

Declarations

Ethics Approval

This study was conducted with approval from the Institutional Review Board (IRB) of Northwell Health (IRB #13-376A). Written informed consent was obtained from all human subjects prior to their participation in this research.

Consent for publication

Only de-identified data is presented. Not applicable.

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding authors upon reasonable request. Materials described in the manuscript, with the exception of the primary menstrual effluent-derive endometrial stromal cells, are commercially available.

Competing Interests

All authors declare that they have no financial conflicts or non-financial interests to disclose.

Funding

This work was supported by the Feinstein Institutes, Northwell Health Innovations Award, and the Endometriosis Foundation of America

Author contributions

JD, CNM, PKG, and PST contributed to the study conceptualization and design. JD, XX, PKC, NH, AJS, RPA, and PST performed experimentation and data collection. XX and PKC maintained cells for ex vivo experiments. PKG, JD, CNM and AJS performed formal analyses. CNM and PKG secured and managed funding. JD, CNM and PKG wrote the original draft and JD, CNM, PKG, PKC, RPA, and AJS edited and finalized the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to thank the coordinators of the ROSE study – Margaret DeFranco, Kristine Sadicario, and Amber LaGuerre – for their help in recruiting and enrolling research subjects who donated menstrual effluent for the study. We would like to thank all research subjects who participated in the ROSE study, as well as Laurianna Frasson and Rixsi Herrera for processing ME and isolating/freezing endometrial stromal cells. In addition, we would like to thank Michael Ryan and the team in the Biorepository for maintaining and tracking cryopreserved endometrial stromal cells, as well as Amanda Chan, PhD, who assisted with the microscopy studies. Additionally, we would like to thank JD's thesis committee members: Drs. Kim Simpfendorfer, Philippe Marambaud, and Myoungsun Son for their research insights and advice over the past two years.

Authors' information

Dr. Peter K Gregersen and Dr. Christine N Metz launched the ROSE (Research OutSmarts Endometriosis) study over a decade ago to collect biological specimens (including menstrual effluent) and associated data from pat. The goals of the ROSE study are to improve our understanding of the pathogenesis of endometriosis to inform the development of non-invasive methods for detecting endometriosis and better treatments. The ROSE study team won the 2018 Northwell Health Innovation Challenge award.

References

1. Nambiar A, Kellogg D 3rd, Justice J, Goros M, Gelfond J, Pascual R, et al. Senolytics dasatinib and quercetin in idiopathic pulmonary fibrosis: results of a phase I, single-blind, single-center, randomized, placebo-controlled pilot trial on feasibility and tolerability. EBioMedicine. 2023;90:104481.
2. Justice JN, Nambiar AM, Tchkonia T, LeBrasseur NK, Pascual R, Hashmi SK, et al. Senolytics in idiopathic pulmonary fibrosis: Results from a first-in-human, open-label, pilot study. EBioMedicine. 2019;40:554–63.
3. Kirkland JL, Tchkonia T. Senolytic drugs: from discovery to translation. J Intern Med. 2020;288(5):518–36.

4. Jamali N, Zal F, Mostafavi-Pour Z, Samare-Najaf M, Poordast T, Dehghanian A. Ameliorative Effects of quercetin and metformin and their combination against experimental endometriosis in rats. Reprod Sci. 2021;28(3):683–92.
5. Park S, Lim W, Bazer FW, Whang KY, Song G. Quercetin inhibits proliferation of endometriosis regulating cyclin D1 and its target microRNAs in vitro and in vivo. J Nutr Biochem. 2019;63:87–100.
6. Zhang L, Mohankumar K, Martin G, Mariyam F, Park Y, Han SJ et al. Flavonoids Quercetin and kaempferol are NR4A1 antagonists and suppress endometriosis in female mice. Endocrinology. 2023;164(10).
7. Signorile PG, Viceconte R, Baldi A. Novel dietary supplement association reduces symptoms in endometriosis patients. J Cell Physiol. 2018;233(8):5920–5.
8. Fadin M, Nicoletti MC, Pellizzato M, Accardi M, Baietti MG, Fratter A. Effectiveness of the integration of quercetin, turmeric, and N-acetylcysteine in reducing inflammation and pain associated with endometriosis. In-vitro and in-vivo studies. Minerva Ginecol. 2020;72(5):285–91.
9. Zondervan KT, Becker CM, Missmer SA, Endometriosis. N Engl J Med. 2020;382(13):1244–56.
10. Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM. Retrograde menstruation in healthy women and in patients with endometriosis. Obstet Gynecol. 1984;64(2):151–4.
11. Brosens I, Brosens JJ, Benagiano G. The eutopic endometrium in endometriosis: are the changes of clinical significance? Reprod Biomed Online. 2012;24(5):496–502.
12. Bulun SE, Yilmaz BD, Sison C, Miyazaki K, Bernardi L, Liu S, et al. Endometr Endocr Rev. 2019;40(4):1048–79.
13. Vallve-Juanico J, Houshdaran S, Giudice LC. The endometrial immune environment of women with endometriosis. Hum Reprod Update. 2019;25(5):564–91.
14. Liu H, Lang JH. Is abnormal eutopic endometrium the cause of endometriosis? The role of eutopic endometrium in pathogenesis of endometriosis. Med Sci Monit. 2011;17(4):RA92–9.
15. Shih AJ, Adelson RP, Vashistha H, Khalili H, Nayyar A, Puran R, et al. Single-cell analysis of menstrual endometrial tissues defines phenotypes associated with endometriosis. BMC Med. 2022;20(1):315.
16. Warren LA, Shih A, Renteira SM, Seckin T, Blau B, Simpfendorfer K, et al. Analysis of menstrual effluent: diagnostic potential for endometriosis. Mol Med. 2018;24(1):1.
17. Nayyar A, Saleem MI, Yilmaz M, DeFranco M, Klein G, Elmaliki KM, et al. Menstrual effluent provides a novel diagnostic window on the pathogenesis of endometriosis. Front Reprod Health. 2020;2:3.
18. Barragan F, Irwin JC, Balayan S, Erikson DW, Chen JC, Houshdaran S, et al. Human endometrial fibroblasts derived from mesenchymal progenitors inherit progesterone resistance and acquire an inflammatory phenotype in the endometrial niche in endometriosis. Biol Reprod. 2016;94(5):118.
19. Klemmt PA, Carver JG, Kennedy SH, Koninckx PR, Mardon HJ. Stromal cells from endometriotic lesions and endometrium from women with endometriosis have reduced decidualization capacity. FertilSteril. 2006;85(3):564–72.

20. Kusama K, Yamauchi N, Yoshida K, Azumi M, Yoshie M, Tamura K. Senolytic treatment modulates decidualization in human endometrial stromal cells. Biochem Biophys Res Commun. 2021;571:174–80.
21. Deryabin P, Griukova A, Nikolsky N, Borodkina A. The link between endometrial stromal cell senescence and decidualization in female fertility: the art of balance. Cell Mol Life Sci. 2020;77(7):1357–70.
22. Faber J, Fonseca LM. How sample size influences research outcomes. Dent Press J Orthod. 2014;19(4):27–9.
23. Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc. 2008;3(7):1125–31.
24. Eruslanov E, Kusmartsev S. Identification of ROS using oxidized DCFDA and flow-cytometry. Methods in molecular biology. (Clifton NJ). 2010;594:57–72.
25. Gellersen B, Brosens JJ. Cyclic decidualization of the human endometrium in reproductive health and failure. EndocrRev. 2014;35(6):851–905.
26. Shi JW, Lai ZZ, Yang HL, Zhou WJ, Zhao XY, Xie F, et al. An IGF1-expressing endometrial stromal cell population is associated with human decidualization. BMC Biol. 2022;20(1):276.
27. Stadtmauer DJ, Wagner GP. Single-cell analysis of prostaglandin E2-induced human decidual cell in vitro differentiation: a minimal ancestral deciduogenic signaldagger. Biol Reprod. 2022;106(1):155–72.
28. Chen MM, Yin ZQ, Zhang LY, Liao H. Quercetin promotes neurite growth through enhancing intracellular cAMP level and GAP-43 expression. Chin J Nat Med. 2015;13(9):667–72.
29. Burova E, Borodkina A, Shatrova A, Nikolsky N. Sublethal oxidative stress induces the premature senescence of human mesenchymal stem cells derived from endometrium. Oxid Med Cell Longev. 2013;2013:474931.
30. Ticconi C, Di Simone N, Campagnolo L, Fazleabas A. Clinical consequences of defective decidualization. Tissue Cell. 2021;72:101586.
31. Cinar O, Seval Y, Uz YH, Cakmak H, Ulukus M, Kayisli UA, et al. Differential regulation of Akt phosphorylation in endometriosis. Reprod Biomed Online. 2009;19(6):864–71.
32. Madanes D, Bilotas MA, Baston JI, Singla JJ, Meresman GF, Baranao RI, et al. PI3K/AKT pathway is altered in the endometriosis patient's endometrium and presents differences according to severity stage. Gynecol Endocrinol. 2020;36(5):436–40.
33. Kim TH, Yu Y, Luo L, Lydon JP, Jeong JW, Kim JJ. Activated AKT pathway promotes establishment of endometriosis. Endocrinology. 2014;155(5):1921–30.
34. Lee YK, Park OJ. Regulation of mutual inhibitory activities between AMPK and Akt with quercetin in MCF-7 breast cancer cells. Oncol Rep. 2010;24(6):1493–7.
35. Yoshino O, Osuga Y, Hirota Y, Koga K, Yano T, Tsutsumi O, et al. Akt as a possible intracellular mediator for decidualization in human endometrial stromal cells. Mol Hum Reprod. 2003;9(5):265–9.

36. Fabi F, Grenier K, Parent S, Adam P, Tardif L, Leblanc V, et al. Regulation of the PI3K/Akt pathway during decidualization of endometrial stromal cells. PLoS ONE. 2017;12(5):e0177387.
37. Gallolu Kankanamalage S, Karra AS, Cobb MH. WNK pathways in cancer signaling networks. Cell Commun Signal. 2018;16(1):72.
38. Lv D, Guo L, Zhang T, Huang L. PRAS40 signaling in tumor. Oncotarget. 2017;8(40):69076–85.
39. Havel JJ, Li Z, Cheng D, Peng J, Fu H. Nuclear PRAS40 couples the Akt/mTORC1 signaling axis to the RPL11-HDM2-p53 nucleolar stress response pathway. Oncogene. 2015;34(12):1487–98.
40. Kim W, Youn H, Seong KM, Yang HJ, Yun YJ, Kwon T, et al. PIM1-activated PRAS40 regulates radioresistance in non-small cell lung cancer cells through interplay with FOXO3a, 14-3-3 and protein phosphatases. Radiat Res. 2011;176(5):539–52.
41. Anand David AV, Arulmoli R, Parasuraman S. Overviews of Biological Importance of Quercetin: A Bioactive Flavonoid. Pharmacogn Rev. 2016;10(20):84–9.
42. Shebeko SK, Popov IAZOS, Tarasenko OO, Shalamay AS. In: Zibadi S, editor. Effects of Quercetin and Its Combinations on Health. Second edition. R.R Watson VRP. London: Academic; 2018.
43. Deryabin PI, Borodkina AV. Stromal cell senescence contributes to impaired endometrial decidualization and defective interaction with trophoblast cells. Hum Reprod. 2022;37(7):1505–24.
44. Brighton PJ, Maruyama Y, Fishwick K, Vrljicak P, Tewary S, Fujihara R et al. Clearance of senescent decidual cells by uterine natural killer cells in cycling human endometrium. Elife. 2017;6.
45. Khosla S, Farr JN, Tchkonia T, Kirkland JL. The role of cellular senescence in ageing and endocrine disease. Nat Rev Endocrinol. 2020;16(5):263–75.
46. Wilkinson HN, Hardman MJ. Senescence in wound repair: emerging strategies to target chronic healing wounds. Front Cell Dev Biol. 2020;8:773.
47. Paramos-de-Carvalho D, Jacinto A, Saude L. The right time for senescence. Elife. 2021;10.
48. Beazley KE, Nurminskaya M. Effects of dietary quercetin on female fertility in mice: implication of transglutaminase 2. Reprod Fertil Dev. 2016;28(7):974–81.
49. Vaez S, Parivr K, Amidi F, Rudbari NH, Moini A, Amini N. Quercetin and polycystic ovary syndrome; inflammation, hormonal parameters and pregnancy outcome: A randomized clinical trial. Am J Reprod Immunol. 2023;89(3):e13644.
50. Salehi B, Machin L, Monzote L, Sharifi-Rad J, Ezzat SM, Salem MA, et al. Therapeutic potential of quercetin: New insights and perspectives for human health. ACS Omega. 2020;5(20):11849–72.
51. Bhatiya M, Pathak S, Jothimani G, Duttaroy AK, Banerjee A. A Comprehensive study on the anti-cancer effects of quercetin and its epigenetic modifications in arresting progression of colon cancer cell proliferation. Arch Immunol Ther Exp (Warsz). 2023;71(1):6.
52. Hashemzaei M, Delarami Far A, Yari A, Heravi RE, Tabrizian K, Taghdisi SM, et al. Anticancer and apoptosis–inducing effects of quercetin in vitro and in vivo. Oncol Rep. 2017;38(2):819–28.
53. Hisaka T, Sakai H, Sato T, Goto Y, Nomura Y, Fukutomi S, et al. Quercetin suppresses proliferation of liver cancer cell lines in vitro. Anticancer Res. 2020;40(8):4695–700.

54. Riva A, Ronchi M, Petrangolini G, Bosisio S, Allegrini P. Improved oral absorption of quercetin from Quercetin Phytosome(R), a new delivery system based on food grade lecithin. Eur J Drug Metab Pharmacokinet. 2019;44(2):169–77.

Figures

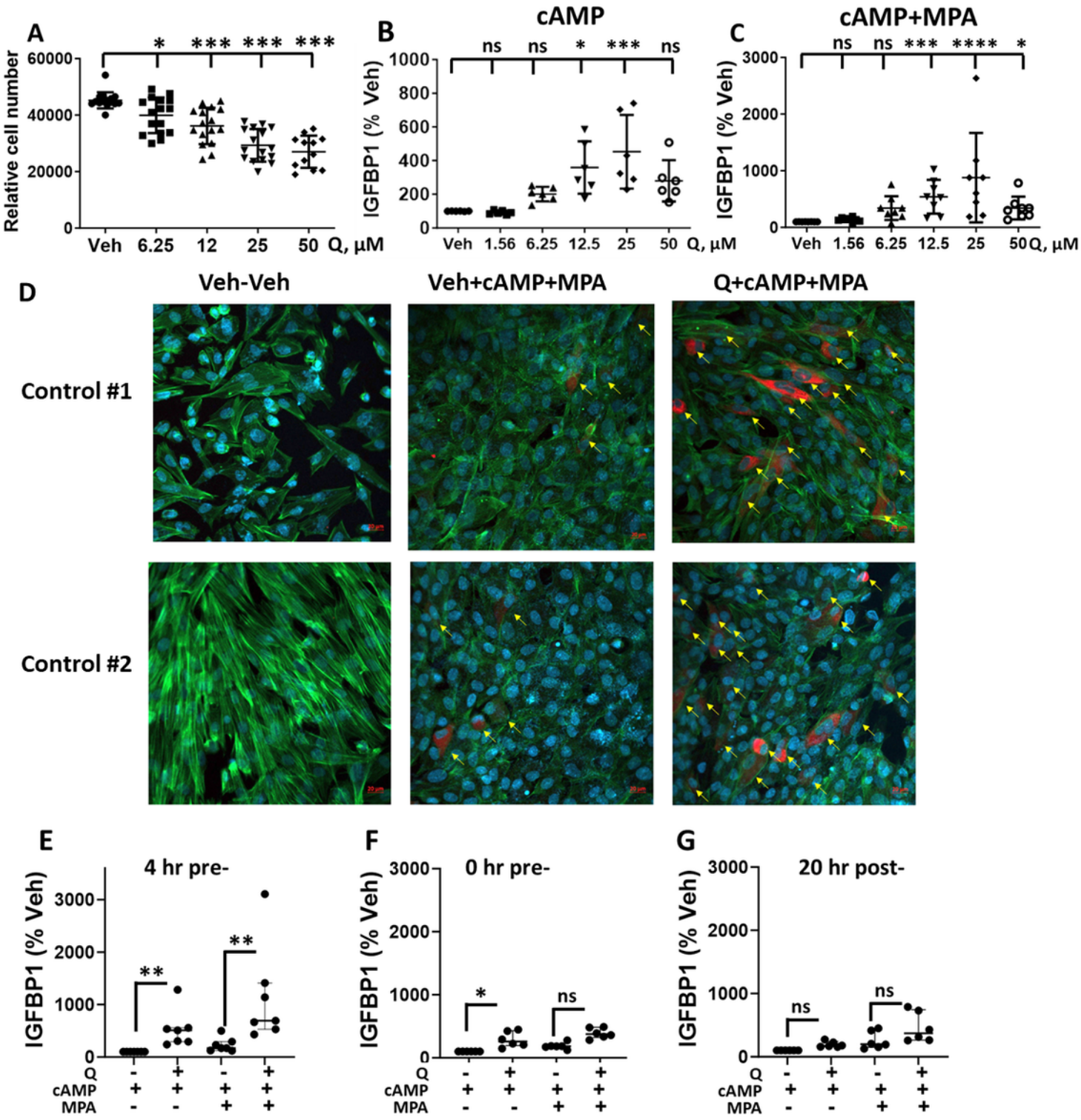

Figure 1

Quercetin inhibits eSC proliferation and enhances decidualization.

(A) Control endometrial stromal cells (eSCs) were treated with vehicle (Veh) or quercetin (Q, 6.25-50 μM) for 72 hr and the relative cell number was determined. Each point represents data from one individual's eSCs, with the group mean ± SD for vehicle and each Q dose. **(B-C)** Control eSCs were treated with vehicle (Veh) or quercetin (1.56-50 μM) for 4 hr prior to the addition of cAMP alone (B) or cAMP+MPA (C). After 48 hr, decidualization was analyzed by measuring IGFBP1 levels by ELISA. Each point represents data from one individual's eSCs, as IGFBP1 (percent Veh, where 100% = Veh+cAMP alone (IGFBP1) (B) or Veh+cAMP+MPA (IGFBP1) (C), with the group median ± interquartile range (IQR) for each Q dose. **(D)** eSCs from two control subjects were treated with vehicle (Veh) or quercetin (Q, 25μM) for 4 hr followed by vehicle or cAMP+MPA prior to immunofluorescent staining and confocal imaging; images show IGFBP1 (red), phalloidin (green), and DAPI (blue) staining (at 20x magnification). The scale bars (20 μm) are indicated. IGFBP1$^+$ cells are indicated by yellow arrows. Images showing individual channels are in Supplementary Fig. 2. **(E-G)** The effects of quercetin (Q, 25 μM) on decidualization when added 4 hr pre-cAMP±MPA (E) versus at the same time as (0 hr pre-) cAMP±MPA (F) or 20 hr post-cAMP±MPA stimulation (G). IGFBP1 levels were measured by ELISA 48 hr post cAMP±MPA stimulation. Data are shown as IGFBP1 (% Veh) where 100% = Veh+cAMP alone (IGFBP1). Each point represents data from one individual's eSCs, with the median ± IQR shown for each group comparing Veh vs. Q. *p<0.05, **p<0.01; ***p<0.001; ****p<0.0001; ns=non-significant.

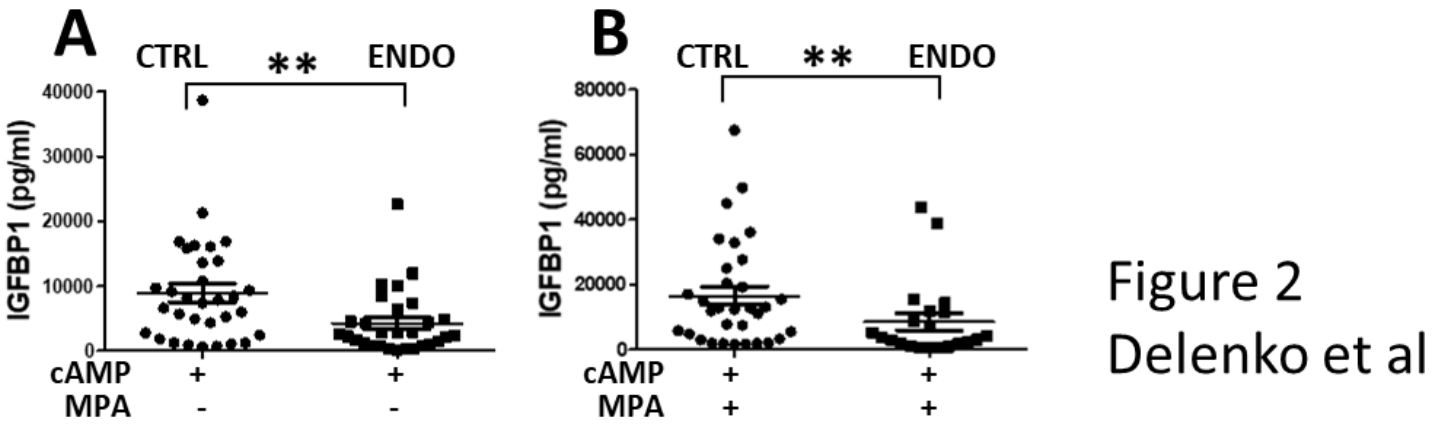

Figure 2

Endometriosis eSCs exhibit impaired decidualization. (A-B) Comparison of decidualization responses of eSCs from controls (CTRL) vs. endometriosis (ENDO) cases induced by cAMP alone (A) or cAMP+MPA (B), as determined by IGFBP1 levels measured 48 hr post cAMP±MPA by ELISA. Each point represents data from one individual's eSCs, with mean ± SD shown for each group. **p<0.001 CTRL vs. ENDO (Student's t test).

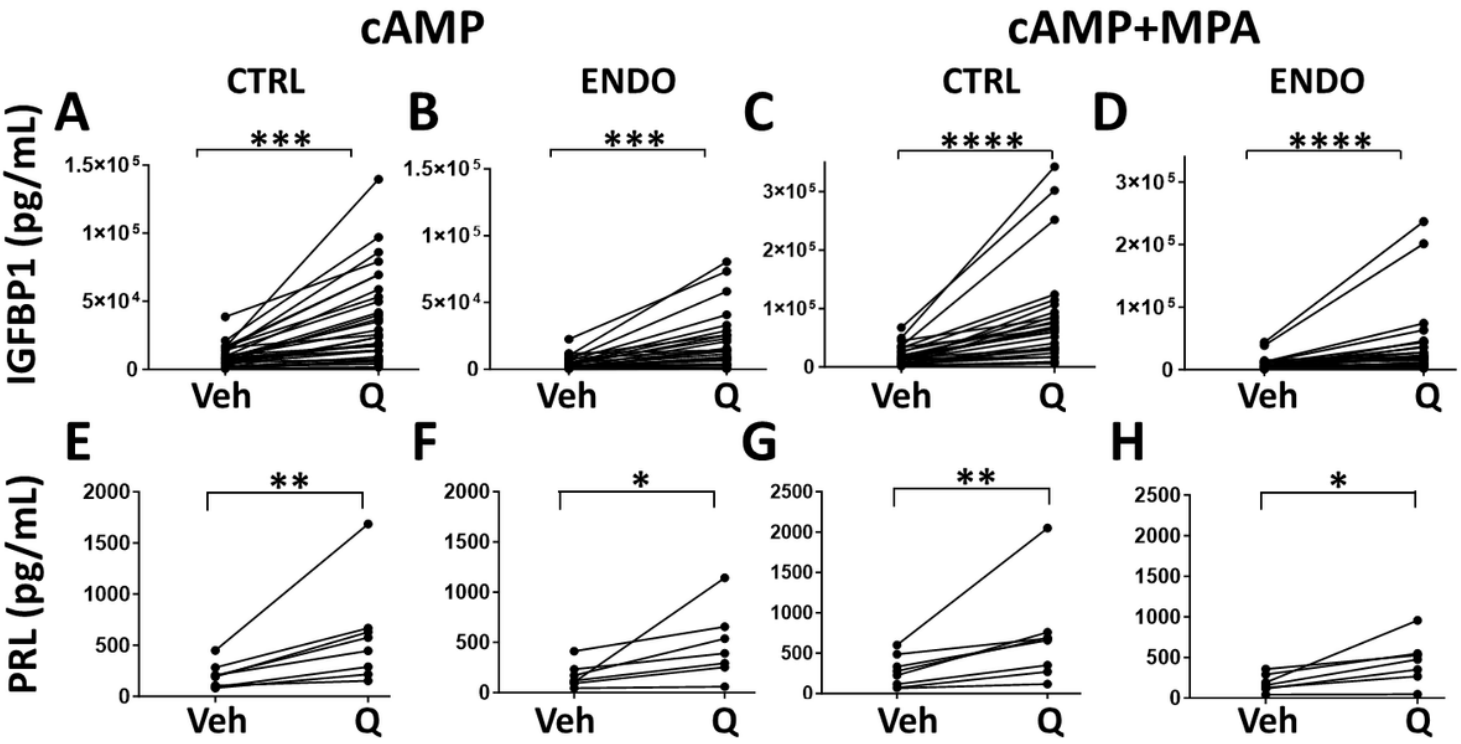

Figure 3

Quercetin enhances decidualization by control-eSCs and endometriosis-eSCs, as determined by IGFBP1 and PRL protein production. (A-H) Endometrial stromal cells (eSCs) from controls (A, C, E, and G, CTRL) and endometriosis cases (B, D, F, and H, ENDO) were treated with vehicle (Veh) or quercetin (Q, 25μM) for 4 hr prior to the addition of cAMP alone (A-B, E-F) or cAMP+MPA (C-D, G-H). After 48 hr, decidualization was analyzed by measuring IGFBP1 (A-D) or PRL (E-H) levels by ELISA. Data points connected by a line represent paired data points from one individual's eSCs (±Q). *p<0.05 Veh vs. Q-treated; **p<0.01 Veh vs. Q-treated; ***P<0.001 Veh vs. Q-treated; ****p<0.0001 Veh vs. Q-treated.

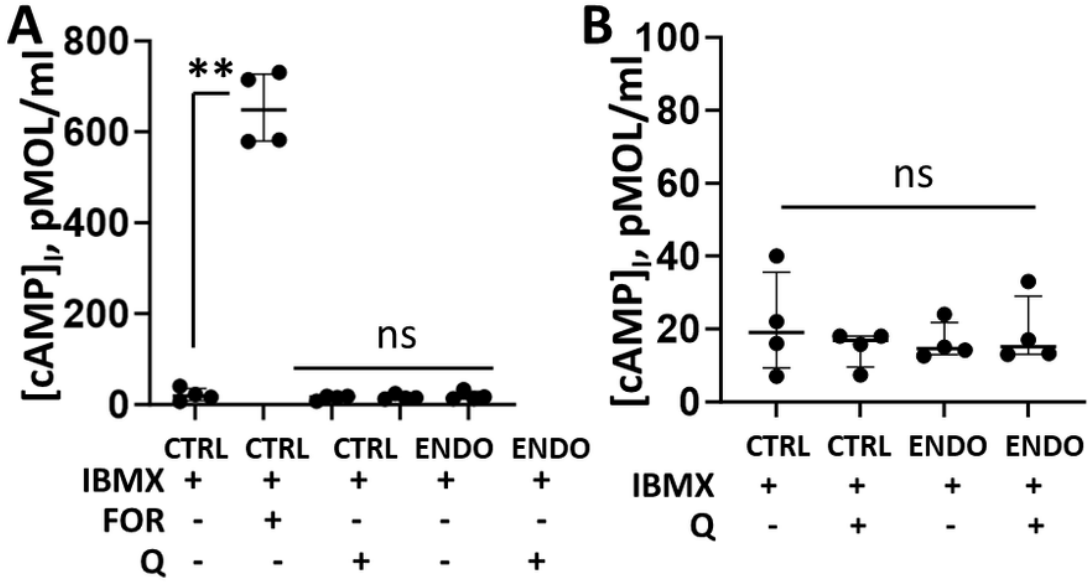

Figure 4
Delenko et al

Figure 4

Quercetin does not increase $[cAMP]_i$ concentrations in eSCs. (**A-B**) Endometrial stromal cells (eSCs) from controls (CTRL) or endometriosis cases (ENDO) were treated with IBMX (0.1mM, a phosphodiesterase inhibitor that blocks [cAMP]i degradation), followed by addition of either vehicle, forskolin (FOR, 25μM, an activator of adenylyl cyclases and hence, [cAMP]i) or quercetin (Q, 25μM). Lysates were analyzed by ELISA for [cAMP]i concentrations (A-B). For clarity, data without forskolin (FOR) treatment are also shown on a different scale (B). Each point represents data from one individual's eSCs, with median ± IQR shown for each group. **p<0.001 vs. vehicle (IBMX alone); ns=not significant.

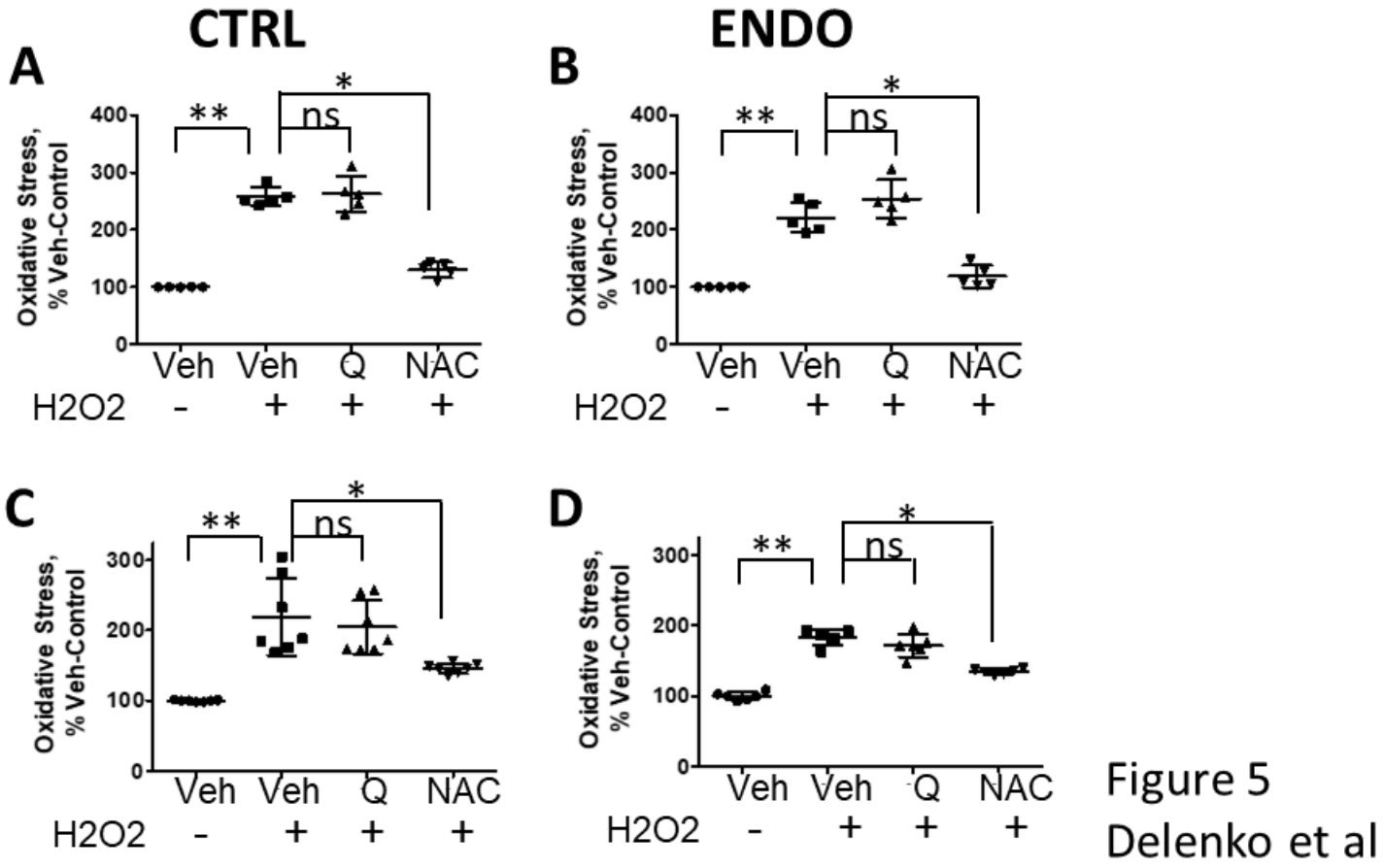

Figure 5
Delenko et al

Figure 5

Quercetin does not reduce oxidative stress in eSCs. (A-D) Endometrial stromal cells (eSCs) from controls (CTRL, A and C) or endometriosis cases (ENDO, B and D) were treated with either vehicle (Veh), quercetin (Q, 25μM), or N-acetylcysteine (NAC, 10mM) before H_2O_2 (500μM) (A and B) or after H_2O_2 (500μM) (C and D), and oxidative stress was measured 3 hr later using the DCF-DA assay. Data are shown as % Veh control (oxidative stress) where 100% = Veh-Veh-treated eSCs. Each point represents data from one individual's eSCs, with median ± IQR shown for each group. *p<0.05; **p<0.01; ns = not significant.

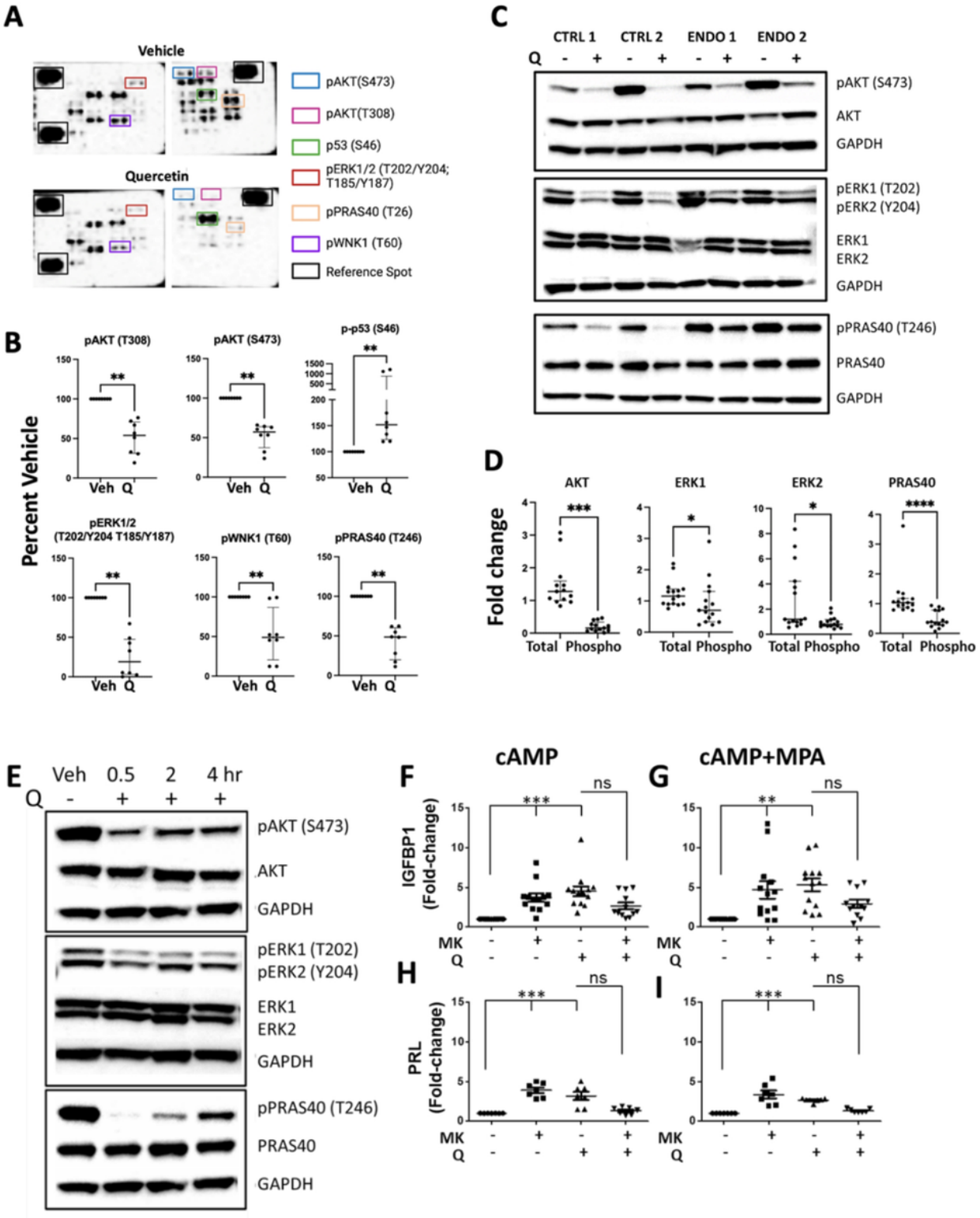

Figure 6

Quercetin inhibits AKT and ERK1/2 phosphorylation and signaling and promotes p53 (Ser46) phosphorylation. (A-B) Control endometrial stromal cells (eSCs) were treated with quercetin (Q, 25μM) for 4 hr before analyzing cell lysates using a phospho-kinase array panel. Representative arrays (A) and quantification of array analytes/spots (B) are shown. Data for differentially expressed targets are presented as specific spot density normalized to the reference spot comparing Veh- vs. Q-treated (B).

Each point represents the specific density of each duplicate analyte spot as a percentage of Veh-treated, with group median ± IQR shown for control- and endometriosis-eSCs. **p<0.01. The remaining arrays are in Supplementary Fig. 6. **(C-D)** Control (CTRL) and endometriosis (ENDO) eSCs were treated with Veh or Q (25μM) for 4 hr before western blotting for p-AKT, total AKT, p-ERK1/2, total ERK1/2, p-PRAS40, and total PRAS40. Representative blots and quantification of specific analytes are shown in (C) and (D), respectively. Band densities were normalized to GAPDH and shown as fold-change between Veh- vs. Q-treated eSCs in (D). Each point represents data from one individual's eSCs, with median ± IQR shown for each group. *p<0.05; ***p<0.001; ****p<0.0001. **(E)** Control-eSCs were treated with Veh or Q (25μM) for 0.5, 2, and 4 hr before western blotting for phospho-AKT, total AKT, phospho-ERK1/2, total ERK1, phospho-PRAS40, and total PRAS40. **(F-I)**Control-eSCs were treated with vehicle (Veh), AKT inhibitor MK-2206 (MK, 1μM), quercetin (Q, 25μM) or Q+MK for 4 hr prior to cAMP (F and H) or cAMP+MPA (G and I) stimulation; decidualization was assessed by measuring IGFBP1 (F and G) or PRL (H and I) by ELISA. Data are presented as fold-change in IGFBP1 or PRL over Veh-treated (Veh-treated=1). Each symbol represents data from one individual's eSCs, with median ± IQR shown for each group. *p<0.05; **p<0.01; ***p<0.001; ns=non-significant.

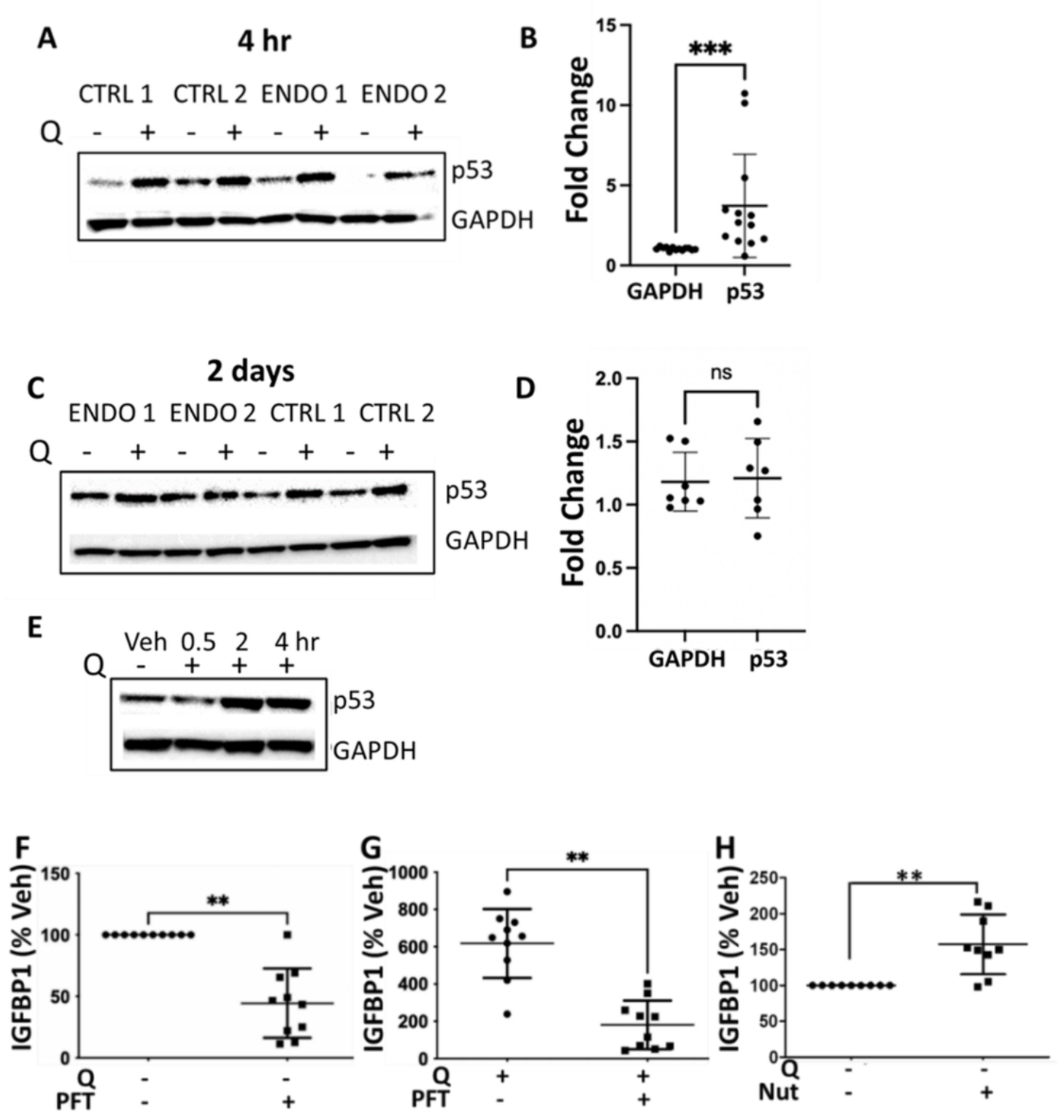

Figure 7

Quercetin induces p53, which contributes to decidualization. (A-D) Control (CTRL)- and endometriosis (ENDO)-endometrial stromal cells (eSCs) were treated with vehicle or quercetin (Q, 25 μM) for 4 hr (A, B) or 2 days (C, D) before western blot analysis of cell lysates for p53 and GAPDH. Representative blots are shown in (A) and (C). Band densities for control-eSCs were normalized to GAPDH and presented as fold-change after 4 hr (B) or 2 days (D) following quercetin treatment, with median ± IQR shown for each

group. ***p<0.001 comparing fold-change in GAPDH vs. p53. **(E)** Control-eSCs were treated with Veh or Q (25 μM) for 0.5, 2, and 4 hr before western blotting for p53 and GAPDH. (**F-G)** Control-eSCs were treated with vehicle or Pifithrin α(PFT, 40 μM) for 4 hr prior to cAMP+MPA stimulation (F) or PFT (40 μM) and Q (25μM) for 4 hr prior to cAMP+MPA stimulation (G). Decidualization was assessed by measuring IGFBP1 levels 48 hr later. **(H)** Control-eSCs were treated with Veh or nutlin-3a (Nut, 100nM) 24 hr after cAMP+MPA stimulation and decidualization was assessed by measuring IGFBP1 production 48 hr post-cAMP+MPA. For F-H, data are presented as IGFBP1 percent vehicle (Veh-treated cAMP=100%). Each point represents data from one individual's eSCs, with median ± IQR shown for each group. **p<0.01 vs. Veh-treatment (F and H) or Q (G).

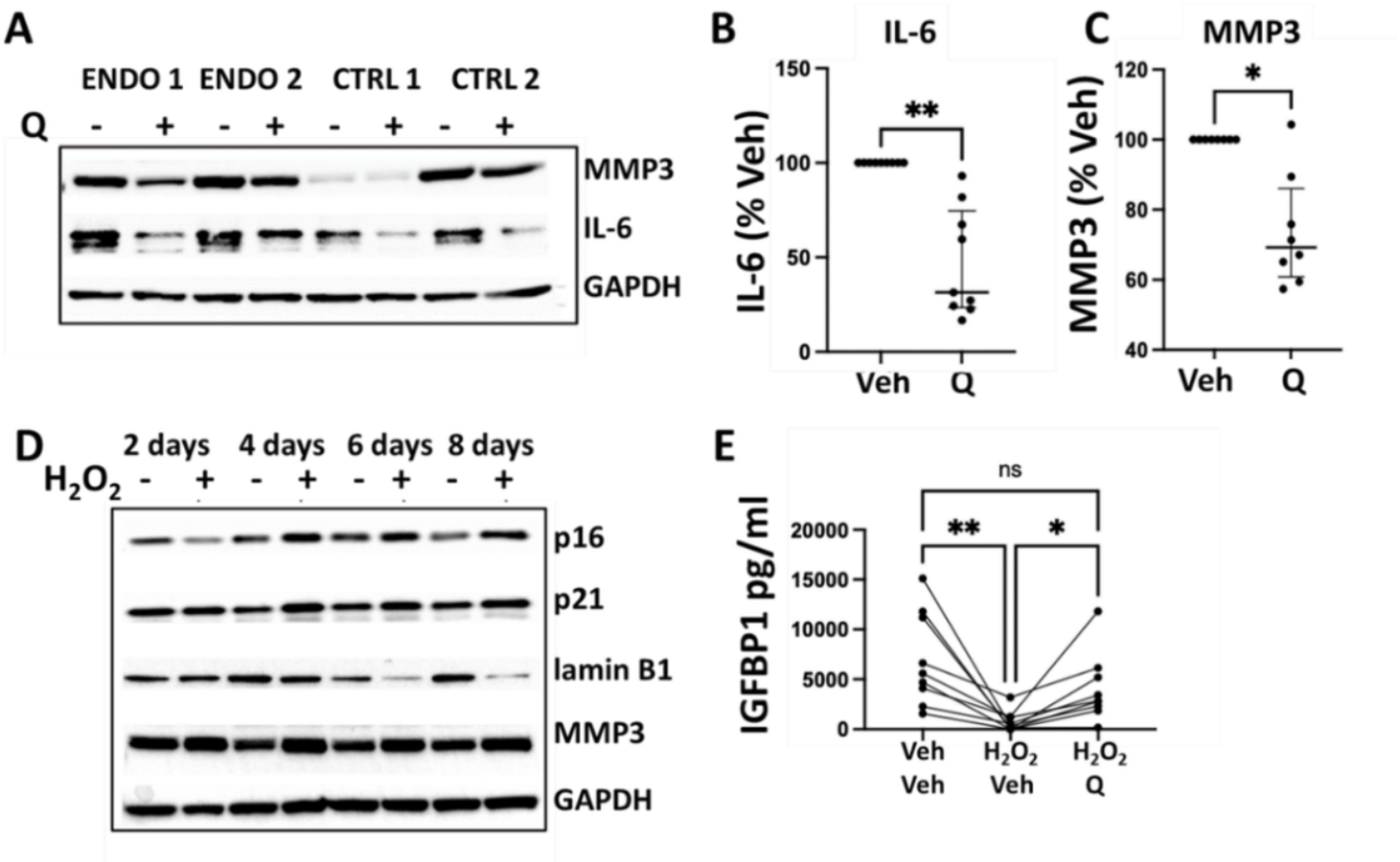

Figure 8

eSCs show evidence of senescence-like phenotype, which is reversed by quercetin.

(A) Endometriosis (ENDO)-endometrial stromal cells (eSCs) and control (CTRL)-eSCs were treated with vehicle or quercetin (Q, 25 μM) for 2 days before western blotting for SASPs, IL-6 and MMP3, or GAPDH. A representative blot is shown. **(B-C)** Quantification of blots (with band densities normalized to GAPDH) from control-eSCs treated with vehicle (Veh) or quercetin (Q, 25 μM) for 2 days for IL-6 and MMP3 are shown in (B) and (C), respectively. Data are presented as IL-6 percent vehicle or MMP3 percent vehicle (where Veh-treated=100%). Each point represents data from one individual's eSCs, with median ± IQR shown for each group *p<0.05; **p<0.01; ns=non-significant. **(D)** Control-eSCs were treated with vehicle or

250 μM H_2O_2 for 2 hr and then, eSCs were harvested at 2, 4, 6, and 8 days post-H_2O_2 exposure for western blotting analysis for p21, p16, lamin B1, MMP3, and GAPDH. **(E)** Control-eSCs were treated with vehicle (Veh) or H_2O_2 (250 μM) for 2 hr and then treated with vehicle (Veh) or quercetin (Q, 25 μM), as indicated, for 4 hr prior to cAMP+MPA-induced decidualization. After 48 hr, culture supernatants were assessed for IGFBP1 (pg/ml) by ELISA. Data points connected by lines represent paired data points from one individual's eSCs (comparing Veh vs H_2O_2 and H_2O_2-Q vs. H_2O_2+Q). *p<0.05; **p<0.01 Veh vs. Q-treated; ns=non-significant.

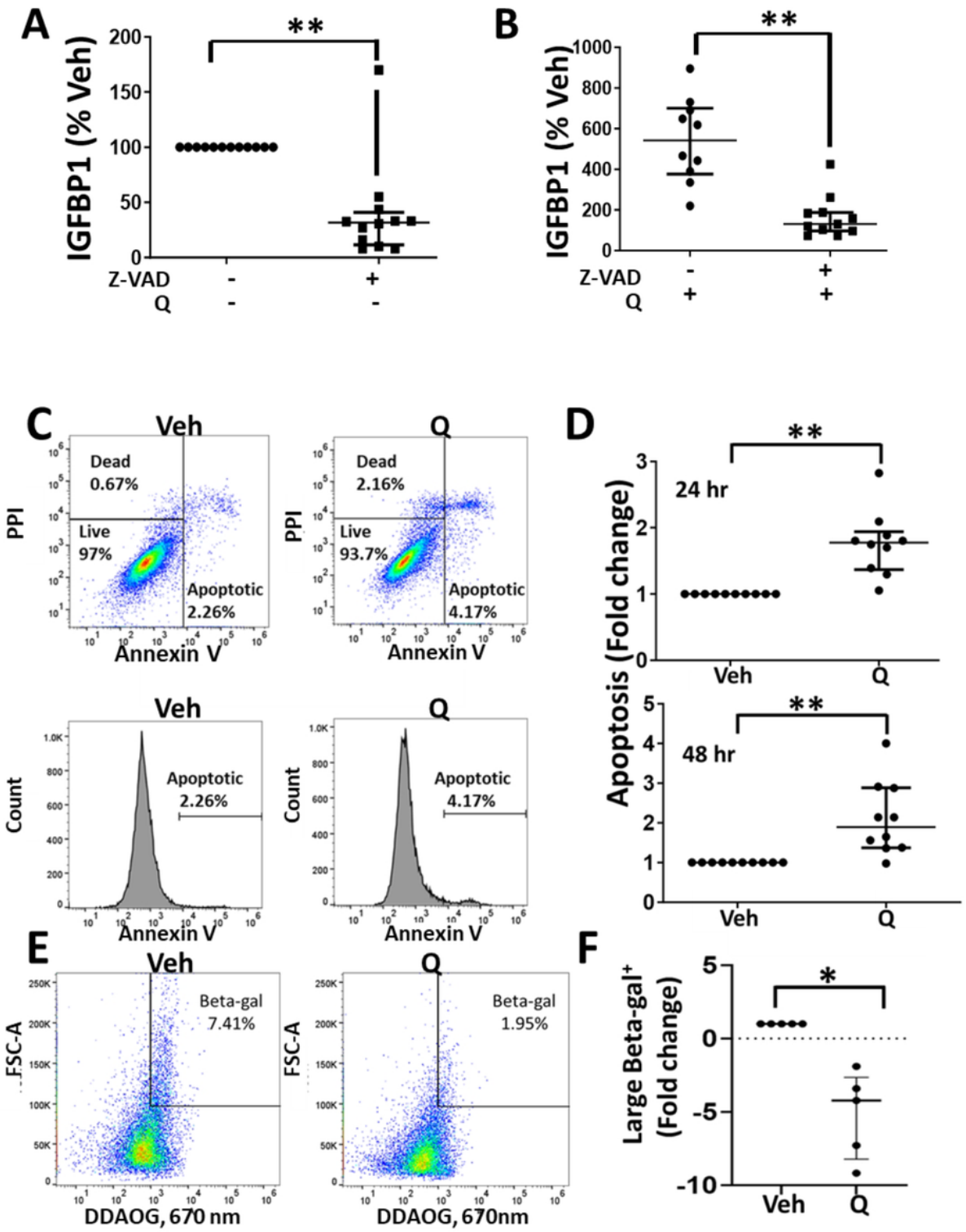

Figure 9

Inhibiting apoptosis blocks decidualization and quercetin induces apoptosis in a subset of eSCs.(A-B) Control-eSCs (p3) were treated with vehicle (Veh) or Z-VAD-fmk (0 vs. 40 μM), a pan-caspase inhibitor that blocks apoptosis, in the presence of vehicle (A) or quercetin (Q, 25 μM) (B) prior to inducing decidualization with cAMP+MPA. Decidualization was assessed 48 hr later by ELISA for IGFBP1. Each point represents data from one individual's eSCs, with the median ± IQR shown for each group. **p<0.01.

(C-D) Quercetin induces apoptosis in a subset of control-eSCs (passage 3-4), as determined by flow cytometry. Representative gating (upper panels) and histograms (lower panels) of Annexin V staining comparing vehicle-treated (Veh, left panels) vs. quercetin-treated (Q, 25 μM, right panels) eSCs 24 hr post-treatment (C). Quantification of quercetin-induced apoptosis (shown as fold-change) measured 24 hr and 48 hr post-vehicle (Veh) vs. post-quercetin (Q, 25 μM) treatment (D). Each point represents data from one individual's eSCs (fold-change in apoptosis), with median ± IQR shown for each group (±Q). **p<0.01. **(E-F)** Larger senescence-associated β-galactosidase (SA-βgal)+ control-eSCs are reduced following quercetin treatment. SA-βgal was determined using 9H-(1,3-Dichloro-9,9-Dimethylacridin-2-one) β-D-Galactopyranoside (DDAOG), a substrate of SA-βgal that yields a far-red fluorescent product detected by flow cytometry at 670nm. Y-axis (FSC-A, size) and X-axis (DDAOG, 670nm). Representative flow cytometry plots show the gating of larger DDAOG, 670nm/SA-βgal (Beta-gal)+ eSCs comparing vehicle-treated (Veh – left panel) vs. quercetin-treated (Q, 25 μM- right panel) eSCs (p3-4) at 48 hr post-treatment (E). The percentage of Beta-gal+/senescent cells (of total viable cells per eSC sample) is indicated on the representative plots in E. The fold-change in the number of larger Beta-gal+/senescent cells comparing vehicle (Veh) vs. quercetin (Q, 25 μM) is shown (F). Each point represents data from one individual's eSCs (fold-change in larger Beta-gal+ cells), with median ± IQR shown for each group (±Q). *p<0.05 comparing vehicle vs. Q.

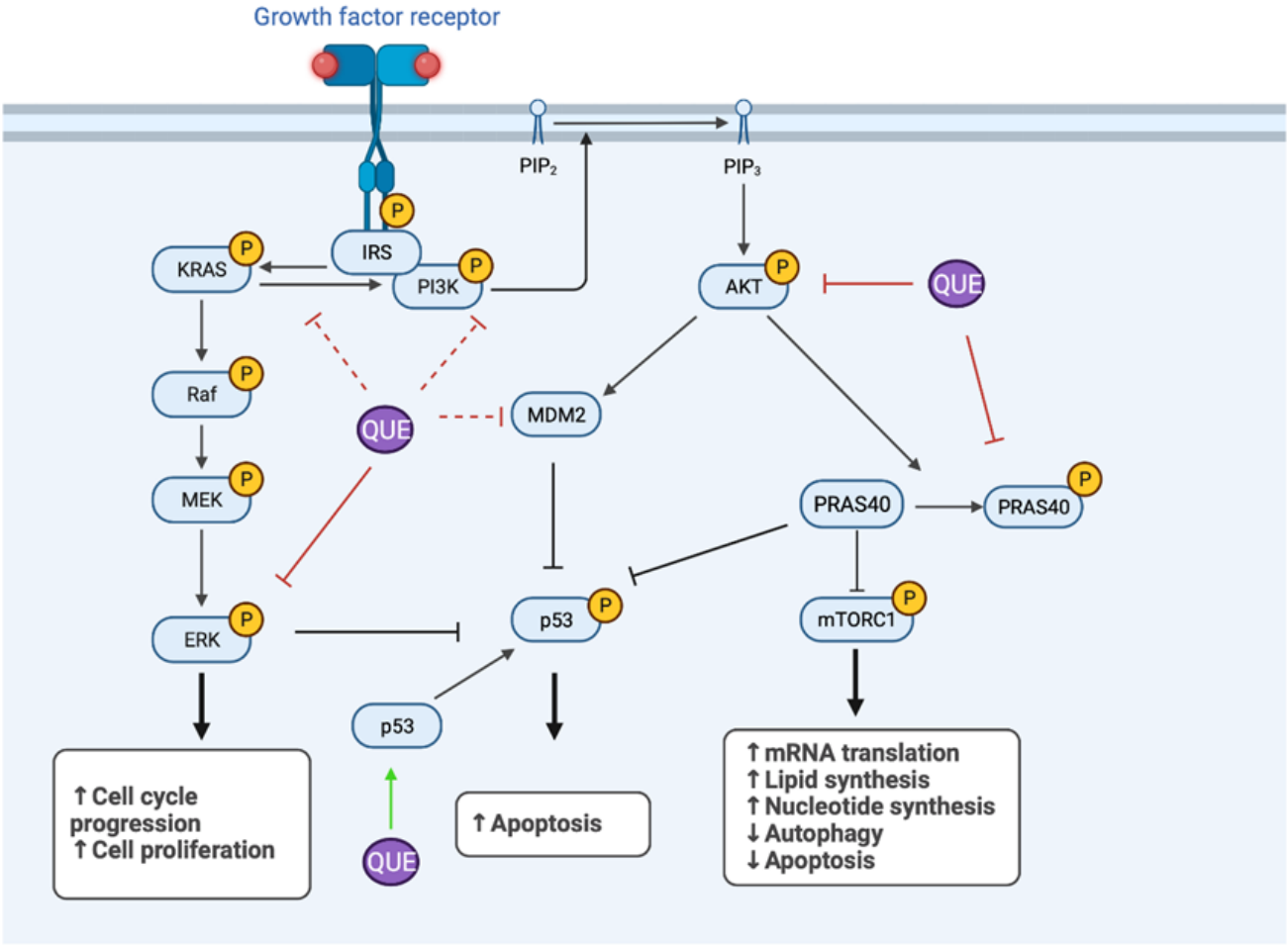

Figure 10

The AKT and ERK1/2 signaling pathways promote cell survival and proliferation and suppress apoptosis. Quercetin (QUE) promotes apoptosis in eSCs by inhibiting the AKT and ERK1/2 pathways and enhancing p53 stability and apoptosis. Green and red solid lines indicate positive and negative effects, respectively, based on our data. Dashed green and red lines indicate proposed positive and negative effects, respectively. Created with biorender.com.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 1202024WesternBlotsUncropped.pptx
- SUPPQDelenkoReproBioEndocrin0324.docx