The Antiasthma Medication Ciclesonide Suppresses Breast Cancer Stem Cells through Inhibition of the Glucocorticoid Receptor Signaling-dependent YAP Pathway

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Research article

Keywords: CSCs, Ciclesonide, Glucocorticoid receptor, YAP, Glucocorticoid

Posted Date: February 28th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-15474/v1

Abstract

Background Ciclesonide is an inhaled corticosteroid used to treat mild-to-severe asthma. However, whether it has anticancer and anti-cancer stem cell (CSC) effects is unknown. This study focused on studying the effect on breast cancer and breast CSCs by ciclesonide and determining its molecular mechanism.

Methods The sensitivity of breast cancer by ciclesonide was determined by cell apoptosis, migration, colony formation, and xenograft. Effect of ciclesonide on CSC formation through GR/YAP pathway was determined by SiRNA of GR and YAP, GR degradation assay, GR antagonist, nuclear localization of GR and YAP, and YAP inhibitor. CSC subpopulation was determined by mammosphere culture, CD44+/CD24-, and aldefluor assay.

Results Here, we showed that ciclesonide inhibits breast cancer and CSC growth. Similar glucocorticoids (GC), dexamethasone (DEX) and prednisone, did not suppress CSC formation. Ciclesonide-induced glucocorticoid receptor (GR) degradation was dependent on ubiquitination. We showed via GR small interfering RNA (siRNA) that GR plays a crucial role in CSC formation. We showed via western blot and immunofluorescence assays that ciclesonide reduces the nuclear level of GR. The GR antagonist RU-486 also inhibited CSC formation. Ciclesonide reduced the protein level of the Hippo transducer Yes-associated protein (YAP). GR siRNA induced a decrease in YAP protein expression and inhibited mammosphere formation. The YAP inhibitor verteporfin inhibited CSC formation and transcription of the connective tissue growth factor and cysteine-rich protein 61 genes. The GR/YAP1 pathway regulated breast CSC formation.

Conclusions We describe that ciclesonide inhibition CSCs through GR and YAP signaling pathway. These findings suggest that the GR/YAP signaling pathway regulates breast CSC formation and revealed a new approach for targeting GR and YAP to inhibit CSC formation.

Introduction

The Hippo pathway, discovered in the fruit fly Drosophila melanogaster, is a tumor suppressor signaling pathway that controls cell growth, tissue homeostasis, and organ size through the regulation of cell proliferation and apoptosis [1]. The main kinases in the Hippo signaling pathway are the serine/threonine kinase Mst1/2 and the tumor suppressor LATS1/2 [2]. The LATS1/2 kinase phosphorylates and inhibits the Hippo pathway effectors Yes-associated protein (YAP) and TAZ. Phosphorylated YAP/TAZ was degraded in cytosol and dephosphorylated YAP/TAZ are localized in the nucleus [3]. YAP/TAZ, transcriptional coactivators and TEAD, a transcription factor complex, induce the expression of genes mediating cell growth, proliferation, and survival [3, 4]. Aberrant activation of YAP has been found in metastatic breast cancer and increases the self-renewal of cancer stem cells (CSCs) [4–6]. Aberrant activation of YAP/TAZ induces CSC traits, anoikis resistance, epithelial-mesenchymal transition (EMT), drug resistance, and metastasis [2].

Steroid hormones regulate a physiological process through their binding to transcription factors, the estrogen receptor (ER), the progesterone receptor (PR) and the glucocorticoid receptor (GR) [7]. GR is divided into five splice variants, GR-α, GR-β, GR-γ, GR-P, and GR-A. The GR-α is responsible for GC-mediated transcriptional activity. GR is a mediator of GCs related to stress, and stress contributes to cancer progression [8]. The activation of GR is important for CSCs formation and chemo-resistance of breast cancer cells and GR signaling affects the mechanical properties of the tumor through deposition of fibronectin and YAP activation [1]. GCs regulate apoptosis and proliferation of breast cancer [9]. An increase in stress hormones during breast cancer progression results in GR activation at distant metastatic sites and reduces survival [10]. GR activation induces heterogeneity and metastasis, and GCs promote breast cancer metastasis. Doctors must be cautious in the use of GCs to treat breast cancer patients who have developed cancer-related complications [10]. Radiation-induced GR expression increases the CSC population in prostate cancer through SGK1-Wnt/β-catenin signaling, thus limiting the efficacy of radiotherapy for prostate cancer treatment [11].

The antiasthma medication ciclesonide is a synthetic GC drug for treating mild-to-severe asthma. This inhaled ciclesonide is effective for asthma and reduces airway inflammation [12]. The liganding-binding affinity of ciclesonide to the GR is very higher than DEX [13]. We showed that ciclesonide inhibited breast cancer growth and CSC formation by suppressing the GR signaling-dependent YAP signaling pathway. The GR/YAP axis is thus a therapeutic target for controlling breast CSCs in breast cancer.

Materials And Methods

Cell and mammosphere Culture

Two breast cancer cell lines, MCF-7 and MDA-MB-231, were obtained from the American Type Culture Collection (Rockville, MD, USA). All human breast cancer cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, CA, USA) and 1% penicillin/streptomycin (HyClone, Thermo Fisher Scientific). Cancer cells ($3.5x10^4$ or $0.5x10^4$) were incubated in an ultralow attachment 6-well plate in MammoCult culture media (STEMCELL Technologies, Vancouver, BC, Canada). Breast cancer cells were cultured in an incubator containing 5% CO_2 under 37°C. The formed mammospheres were counted by using the NICE program [14]. Ability of mammosphere formation was estimated by determining the mammosphere formation efficiency (MFE) (%) as in a previous study [15].

Antibodies and small interfering RNAs (siRNAs)

Antibody-specific against YAP, pYAP, and GR proteins were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-ubiquitin, anti-β-actin, and anti-Lamin b antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-CD44 FITC and anti-CD24 PE antibodies were purchased from Abcam (Cambridge, MA, USA). The GR- and YAP-specific siRNAs were obtained from BIONEER (Daejeon, Korea).

Cell proliferation

Cell proliferation assay was used as in a previous study [16]. Breast cancer was incubated in a 96-well plate in the presence of ciclesonide for 1 day. Cell proliferation was tested using a CellTiter 96® Aqueous One Solution cell kit (Promega, Madison, WI, USA), and the optical density of 490 nm was analyzed using a 96-well plate reader (SpectraMax, San Jose, CA, USA).

Colony formation and migration assays

MDA-MB-231 cancer cells (1,000 cells/well) were incubated with ciclesonide for 7 days in DMEM containing 10% FBS and 1% penicillin/streptomycin. The colonies were incubated and counted. For the migration assay, breast cancer cells were incubated in a 24-well plate, and a scratched line in a cell monolayer was made using a pipette tip [17].

Annexin V/PI staining and cell apoptosis

For the Annexin V/PI assay, breast cancer cells were incubated with ciclesonide (20 μM) in 6-well plates. Apoptotic cells were analyzed by FITC-Annexin V/PI staining according to the manufacturer's protocol (BD, San Jose, CA, USA). The stained cells were detected by flow cytometry. For Hoechst 33258 staining, cancer cells were cultured with 20 μM ciclesonide for 24 h, and were stained with Hoechst 33258 (10 mg/ml) solution for 30 min at 37°C. The stained cells were detected under a fluorescence microscope (Lionheart FX, BioTek, Winooski, VT, USA).

Flow cytometric analysis and aldehyde dehydrogenase (ALDH1) activity

Flow cytometric analysis was used in a previous study [17]. Cancer cells ($1x10^6$) were incubated with anti-CD44-FITC and anti-CD24-PE antibodies (Abcam, Cambridge, MA, USA) for 20 min. The cells were centrifuged and washed three times with 1x FACS buffer and analyzed using an Accuri C6 (BD, San Jose, CA, USA). ALDH activity was assayed using an ALDEFUOR^{TM} assay kit (STEMCELL Technologies) used in a previous study [17]. Cancer cells were incubated in ALDH assay buffer at 37°C for 30 min. ALDH-positive population was examined by using flow cytometer.

Gene expression analysis

Total RNA from cancer cells and mammosphere were extracted and purified, and real-time reverse transcription-quantitative PCR (RT-qPCR) was tested using a one-step RT-qPCR kit (Enzynomics, Daejeon, Korea) used as in a previous study [16]. The specific primers are shown in online resource, supplementary Table S1.

Immunoblot analysis

Breast cancer cells and mammospheres were lysed with RIPA buffer containing protease inhibitors and total proteins were quantified with BCA protein quantitation kit. The protein samples were electrophoresed on 12% SDS gel. The gel with separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Burlington, MA, USA). Membranes were incubated first in Odyssey blocking

buffer for 1 h and then overnight with primary antibodies. The anti-GR, anti-YAP, anti-pYAP, anti-lamin B, and anti-β-actin antibodies were used. After washing and drying, membranes were incubated with IRDye 680RD- and 800W-conjugated secondary antibodies, and images were acquired using an ODYSSEY CLx (LI-COR, Lincoln, NE, USA).

Caspase-3/7 assay

Caspase-3/7 assay was used in a previous study [18]. Cancer cells were cultured with ciclesonide. Caspase-3/7 activity was measured using a Caspase-Glo 3/7 kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Then, 100 μl of caspase reagent was added to 96-well microplates and incubated, and the activity was assayed in a GloMax® luminometer (Promega, Madison, WI, USA).

SiRNA of GR and YAP

To examine the inhibitory function of GR and YAP on mammosphere formation, breast cancer cells were transfected with siRNAs targeting human GR and YAP (Bioneer, Daejeon, South Korea). The GR and YAP siRNAs (NM_181651.1) and a scrambled siRNA were purchased from Bioneer (Daejeon Cor., South Korea). For siRNA transfection, cancer cells were cultured and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The protein levels of GR and YAP were determined via immunoblot analysis.

Immunoprecipitation (IP)

Mammospheres were washed with 1x PBS and resuspended in lysis buffer. The lysates were incubated with an anti-ubiquitin antibody for 16 h at 4°C. Protein A/G agarose (Thermo Scientific, IL, USA) was added to the mixtures. The mixtures were centrifuged, and the precipitates were washed with lysis buffer 5 times, run on SDS-PAGE gels and subjected to immunoblotting.

Immunofluorescence (IF)

Cancer cells were fixed with 3.8 % paraformaldehyde for 40 min, permeabilized with 0.5% Triton X-100 for 15 min, blocked with 3% bovine serum albumin (BSA) for 1 hour and labeled with an anti-GR primary antibody followed by an Alexa Fluor 488-conjugated anti-mouse secondary antibody. We used nonspecific signal conditions to confirm the specificity of the primary antibodies for immunofluorescence. The nuclei were stained with DAPI, and stained GR was visualized with a fluorescence microscope (Lionheart, BioTek, VT, USA).

In vivo mice experiments

Twelve female nude mice were injected with three million MDA-MB-231 cells and after one week, injected with/without ciclesonide (10 mg/kg). Tumor volumes were examined for 45 days using the following formula: $(\text{width}^2 \times \text{length})/2$. The mouse experiments were used as in a previous study [19]. Animal experimentation was performed in accordance with protocols approved by the Institutional Animal Care

and Use Committee (IACUC) of Jeju National University (JNU-IACUC; Approval Number 2017-003). Female nude mice (5 weeks old) were obtained from OrientBio (Seongnamsi, South Korea) and maintained in mouse facility for 1 week.

Statistical analysis

Our all data were processed using GraphPad Prism 7.0 software (GraphPad Prism Inc., San Diego, CA, USA). Our all data values are presented as the means ± standard deviations (SDs). Our data were analyzed with one-way ANOVA. *P*-values of less than 0.05 or 0.01 were considered to indicate significance.

Results

Ciclesonide induces growth inhibition and apoptosis of breast cancer cell lines

We analyzed the inhibitory effect of ciclesonide on growth of cancer cells and found that ciclesonide showed growth inhibition of cancer cells (Fig. 1a, b and supplementary Fig. S3a). Ciclesonide induced increase of the apoptotic breast cancer cells (Fig. 1c). Ciclesonide induced caspase 3/7 activity in a ciclesonide concentration-dependent manner (Fig. 1d). After treatment with ciclesonide, breast cancer cells exhibited apoptotic body formation (Fig. 1e). In addition, ciclesonide induced the inhibition of migration and colony formation (Fig. 1f, g). Our results show ciclesonide effectively suppresses the cancer hallmarks of cell migration, proliferation, and colony formation of breast cancer.

Ciclesonide inhibits tumor growth

As ciclesonide has anti-proliferative effect on breast cancer, we used an in vivo mouse model to examine whether it reduces tumor growth. The body weights of control and ciclesonide-treated mice did not change (Fig. 2a). The tumor weights from ciclesonide-injected nude mouse were lower than those of tumors from control nude mice (Fig. 2b). The tumor volumes from ciclesonide-injected mouse were smaller than those of tumors from control mice (Fig. 2c). Our results indicated that ciclesonide effectively reduced tumor growth.

Effect of ciclesonide and synthetic GCs (prednisone and dexamethasone) on breast CSCs

We treated mammospheres with ciclesonide concentrations (0, 5, and 10 μM). Ciclesonide suppressed breast mammosphere formation (Fig. 3a and supplementary Fig. S3b). We assessed the mammosphere formation using the most commonly used synthetic GCs, prednisone and dexamethasone. Neither of these two GCs affected mammosphere formation (Fig. 3b and supplementary Fig. S3c). The $CD44^+/CD24^-$ population of breast cancer cells was assayed under ciclesonide treatment. Ciclesonide reduced the $CD44^+/CD24^-$ cell proportion from 80.9% to 71.2% (Fig. 3c). We also tested the inhibitory function of ciclesonide on ALDH-positive cell population. Ciclesonide induced reduction of the ALDH-

positive cell proportion from 1.0% to 0.4% (Fig. 3d). Our data indicate that ciclesonide inhibits mammosphere formation.

Ciclesonide attenuates mammosphere formation via GR inhibition

To examine the molecular function of ciclesonide in tumorsphere formation, the level of GR protein was examined in mammospheres, as ciclesonide binds GR. GR levels were decreased significantly after ciclesonide treatment but not dexamethasone treatment (Fig. 4a). However, the transcription level of the GR gene was not changed under treatment with either ciclesonide or dexamethasone (Fig. 4b). Treatment with the proteasome inhibitor MG132 protected GR from ciclesonide-induced degradation, suggesting that ciclesonide increased the proteasomal degradation of GR (Fig. 4c). We performed immunoprecipitation with as anti-ubiquitin antibody and western blotting with an anti-GR antibody in ciclesonide-treated cells treated. The level of ubiquitinated GR was increased under ciclesonide treatment (Fig. 4d). These data suggest that ciclesonide induces the degradation of the GR protein via an ubiquitin-dependent pathway (Fig 4c, d).

The GR signaling pathway is an important signaling pathway for breast CSC formation

To determine whether GR is essential for CSCs, we examined CSC formation in response to siRNA-mediated silencing of GR. Breast cancer cells with siRNA-mediated specific knockdown of GR indicated a 50% decrease in mammosphere formation (Fig. 4e). Our data showed that nuclear GR levels were significantly reduced under ciclesonide treatment (Fig. 4f), and our immunofluorescence data showed that ciclesonide-treated cells showed reduction of nuclear GR proteins than untreated cancer cells (Fig. 4g). Treatment with RU486, a GR antagonist, inhibited the formation of mammospheres (Fig. 4h). Our data suggest that GR signaling are essential for mammosphere formation.

Ciclesonide regulates the GR/YAP signaling axis

We examined whether ciclesonide regulates the GR/YAP signaling axis. YAP levels were significantly reduced under ciclesonide treatment. In addition, the transcription level of the YAP gene was changed under ciclesonide treatment (Fig. 5a). The nuclear YAP level was significantly reduced under ciclesonide treatment (Fig. 5b). Cancer cells with YAP downregulation via siRNA of YAP showed a 50% decrease in mammosphere formation (Fig. 5d). To check the specificity of ciclesonide, we check the level of YAP and pYAP under ciclesonide at lung cancer A549 cell. Cicesonide did not reduce the level of YAP and pYAP in A549 lung cancer (Online Resource, Supplementary Fig. S2).

Cells with GR downregulation via siRNA of GR showed a reduction in the YAP protein level (Fig. 5c). To examine whether ciclesonide regulates the YAP signaling axis, we used verteporfin, a YAP inhibitor that inhibits the physical YAP-TEAD interaction [20]. Verteporfin dramatically reduced breast CSC formation (Fig. 5e). We examined the transcription levels of YAP target genes (*Ctgf* and *Cyr65*) in mammospheres derived from breast cancer cells and found that the mRNA levels of CTGF and CYR61 were reduced under verteporfin treatment (Fig. 5f). We examined the correlation between GR (*NR3C1*) and YAP CTGF, and

CYR61 three gene expression in breast cancer patients from a publicly available data from The Cancer Genome Atlas (TCGA). GR (*NR3C1*) gene expression showed a significant positive correlation with YAP1, CTGF, and CYR61 gene expression in breast invasive carcinoma (Fig. 5g). These results show that ciclesonide regulates the GR/YAP1 signaling axis to promote breast CSC formation.

Ciclesonide inhibits the expression of CSC marker genes and mammosphere growth

To examine inhibitory ciclesonide on CSC marker gene expression, we evaluated the transcriptional levels of CSC marker gene expression. Ciclesonide inhibited gene transcriptions such as Sox2, Nanog, Oct4, and CD44 in breast CSCs (Fig. 6a). To examine inhibitory ciclesonide on mammosphere growth, mammosphere was incubated with ciclesonide for 2 days and isolated as single cell and the cancer cells were counted. Ciclesonide increased cell death derived from mammosphere (Fig. 6b). These data indicate that ciclesonide induces a reduction in mammosphere growth and inhibits breast CSC through deregulation of the GR/YAP1 signaling (Fig. 6c).

Discussion

Although dramatic advances have been made in cancer research, breast cancer remains an important health problem. Breast cancer is the most common cancer affecting women, and recently, researchers have focused on young breast cancer patients aged < 45 years [21]. This cancer is heterogeneous, complex, and aggressive. Breast CSCs are a key factor in tumor heterogeneity and cause chemoresistance and metastasis [22]. Breast CSCs can survive in target organs and generate metastasis up to two decades after diagnosis [22].

In this report, our data indicated that ciclesonide inhibits breast cancer cell and CSC growth. Similar GCs, DEX and prednisone, did not suppress CSCs (Fig. 1, 3, and Online Resource, Supplementary Fig. S3). Clinically, DEX have been used to cancer patients to reduce the side effects of chemotherapy and nausea [23]. Clinical data indicates that DEX induces chemotherapeutic resistance and results in poor prognosis in cancers patients [24, 25]. The budesonide, also inhibits breast cancer cell proliferation and CSC growth (data not shown). The molecular structures of budesonide and ciclesonide are distinguished by their hydrophobic groups [26]. Thus, we thought that ciclesonide and DEX show different effects on breast CSC because of their different hydrophobic structure. GCs regulates Hedgehog pathway activity [26]. Prednisone promotes Smoothened (SMO) accumulation and induces Hedgehog signal, but both ciclesonide and budesonide inhibit SMO and Hedgehog signal [26]. Cyclopamine inhibits breast cancer independent of Smoothened (SMO) and have unique secondary molecular targets [27].

Our data indicated via GR siRNA that ciclesonide-induced ubiquitin-dependent GR degradation and GR play a crucial role in CSC formation. The GR antagonist RU-486 also inhibited CSC formation. Our data indicated that ciclesonide inhibits breast CSC formation through GR signaling deregulation. GCs induce chemoresistance and tumor relapse, as evidenced by the unexpected expansion of cancers that are

chemo-resistant and highly metastatic [1, 10, 28]. Ciclesonide has an anticancer effect because of its different hydrophobic structure.

CSCs induced cancer recurrence and metastasis, which are important reasons for cancer mortality [29]. Induction of CSCs is found in a wide range of human cancers with induced YAP activation [2]. YAP acts as a major inducer of CSC formation by upregulating SOX2 and SOX9 [30, 31]. The YAP protein plays a role in CSC maintenance. Ciclesonide reduced the protein level of the Hippo transducer YAP, and GR siRNA induced decreases in the YAP protein level and inhibited mammosphere formation. The YAP inhibitor verteporfin inhibited CSC formation, along with CTGF and CYR61 gene transcription. The ciclesonide-regulated GR/YAP pathway in turn regulated breast CSCs survival.

Ciclesonide, a synthetic GC, decreases airway inflammation and controls asthma [12]. Ciclesonide binds efflux transporters, p-glycoprotein and breast cancer resistance protein (BCRP) and inhibits efflux [32]. For the first time, we suggested that ciclesonide inhibits the function of the GR and YAP proteins. In addition, we showed that the GR/YAP1 signal regulates breast CSC survival. Our results showed that the GR/YAP signal regulates breast CSC formation and revealed a novel approach for targeting GR and YAP to inhibit CSC formation. Ciclesonide may extend knowledge about new concepts in anticancer therapy and inflammation.

Conclusion

In our study, ciclesonide inhibits growth of breast cancer cell and CSCs growth. Ciclesonide induced glucocorticoid receptor (GR) degradation dependent on ubiquitination. We show GR gives an important role to CSCs formation by using Si-RNA of GR. GR antagonist, RU-486 also inhibits CSC formation. Ciclesoinde reduced Hippo transducer YAP protein (Yes-associated protein). Si-RNA of GR induced decrease of YAP protein and inhibition of mammosphere formation. YAP inhibitor, verteporfin inhibit CSC formation, CTGF and CYR61 gene. Ciclesonide inhibits function of GR and YAP protein. GR/YAP pathway regulate breast CSCs formation. Our results show GR/YAP1 signaling pathway regulate breast CSCs formation and open the new way to target GR and YAP to inhibit CSCs formation.

Abbreviations

CSC: Cancer stem cell; GR: Glucocorticoid receptor; siRNA: Small interfering RNA; YAP: Yes-associated protein; EMT: Epithelial-mesenchymal transition; ER: Estrogen receptor; PR; Progesterone receptor; GC: Glucocorticoid; DEX: Dexamethasone; FBS: Fetal bovine serum; IF: Immunofluorescence; IP: Immunoprecipitation; ALDH1; Aldehyde Dehydrogenase; PVDF: Polyvinylidene fluoride; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction

Declarations

Ethics Approval and Consent to participate

Not applicable

Consent for publication

All authors have given consent for publication

Availability of supporting data

All remaining data and materials are available from the authors upon reasonable request.

Competing interests

The authors declare that they have no competing interest.

Funding

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2016R1A6A1A03012862) and by the project for Cooperative Research Program for Agriculture Science and Technology Development (PJ01316701) Rural Development Administration, Republic of Korea

Authors' contributions

HS Choi and JH Kim designed and performed all the experiments, and HS Choi and DS Lee wrote the manuscript. SL Kim helped to design and perform the experiments. DS Lee supervised the study. All authors read and approved the final manuscript.

Acknowledgements

Not applicable

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Supplementary Information

Supplementary information accompanies this paper at http://doi.org/

Addition file1: Table S1. Real-time RT-qPCR primer sequences of human *CD44, Nanog, Sox2, Oxt4, GR, YAP, CTGF, CYR61, and β-actin* genes.

Addition file2: Figure S1. Ciclesonide reduced the levels of cytosolic and nuclear GR protein (green) in MDA-MB-231 cells, as evidenced by immunofluorescence. Nuclei were stained with DAPI (red), and GR was labeled with an anti-GR antibody (green). Magnification, x100. Total and nuclear fluorescence with/without ciclesonide were determined by using Gen5 cell imaging program of Lionheart FX machine.

Addition file3: Figure S2. Effect of ciclesonide on expression levels of YAP1 and phosphoYAP1 in lung cancer, A549. (A) Western blot analysis of YAP protein under ciclesonide (CIC) and knockdown of YAP using Si-RNA in A549. (B) Western blot analysis of p-YAP protein under ciclesonide (CIC) in A549.

Addition file4: Figure S3. Ciclesonide reduced the proliferation of MCF-7 cells. (A) MCF-7 cells were cultured in a plate under the ciclesonide. The growth of cancer cells was assessed with MTS reagent. (B) Mammospheres were cultured for 7 days in MammoCult media. Treatment with ciclesonide (5 and 10 μM) decreased mammosphere formation to 5% under control conditions. *$p<0.05$ vs. the control. (C) Treatment with prednisone or DEX (40 and 80 μM) did not decrease the MFE.

Figures

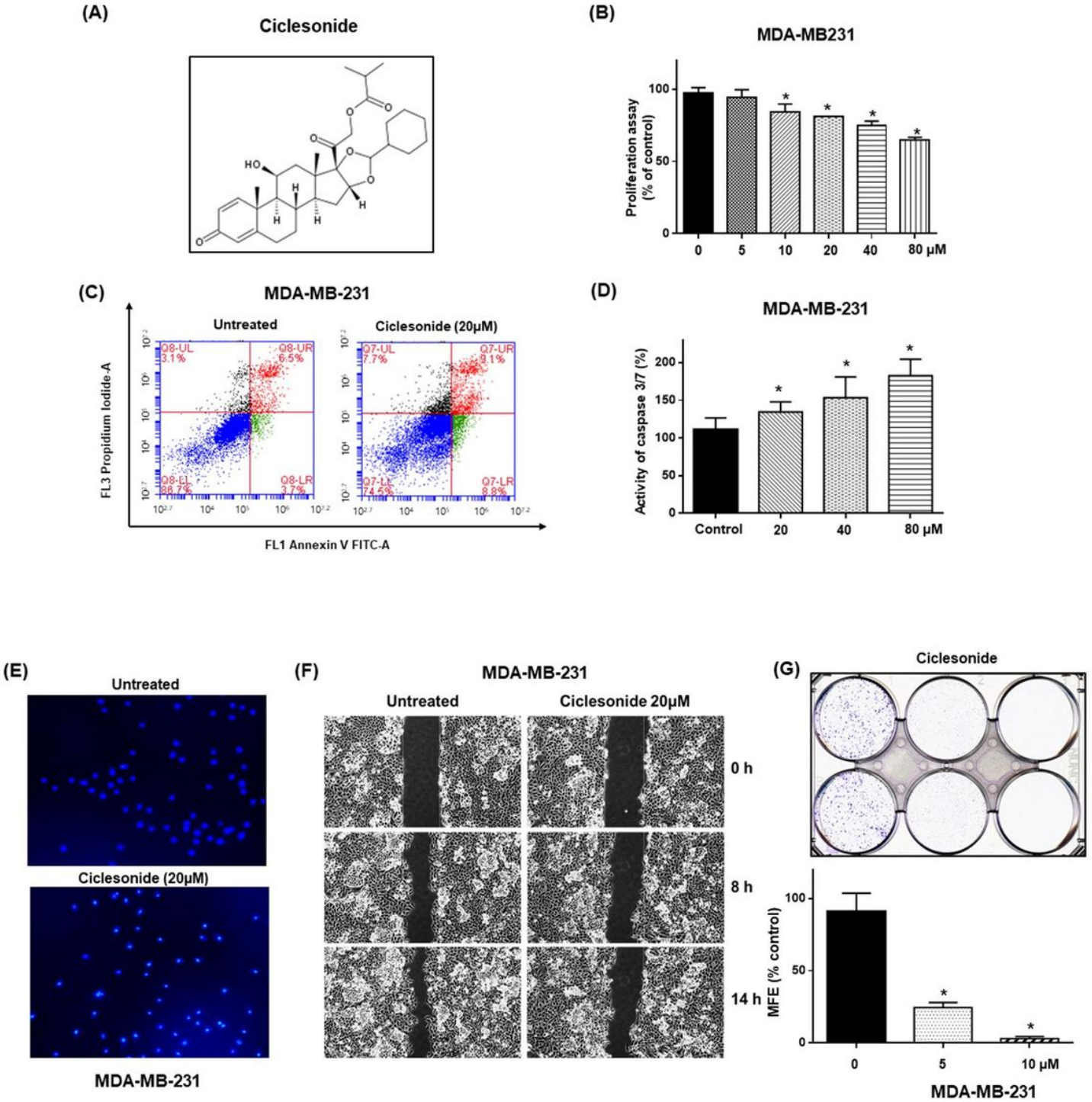

Figure 1

Ciclesonide reduces the proliferation of MDA-MB-231 cells. a The chemical structure of ciclesonide. b MDA-MB-231 cells were incubated with the ciclesonide. The growth of breast cancer was assessed with MTS reagent. c Ciclesonide (20 μM) increased the apoptosis of MDA-MB-231 cells. Apoptotic cells were

analyzed using Annexin V/PI staining. d Caspase 3/7 activity was determined with a Caspase-glo 3/7 assay kit (Promega, Madison, WI, USA). Ciclesonide increased caspase3/7 activity in a concentration-dependent manner. e Ciclesonide (20 μM) induced the formation of apoptotic bodies, as evidenced by staining with Hoechst 33342 dye (magnification, 40x). f Ciclesonide (20 μM) decreased cell migration, as evaluated by a scratch assay. g The effect of ciclesonide on colony formation of breast cancer cell. A 1000 cancer cells were cultured with 5 and 10 μM ciclesonide. Data from experiments are shown as the means± SDs; *p < 0.05 vs. the control

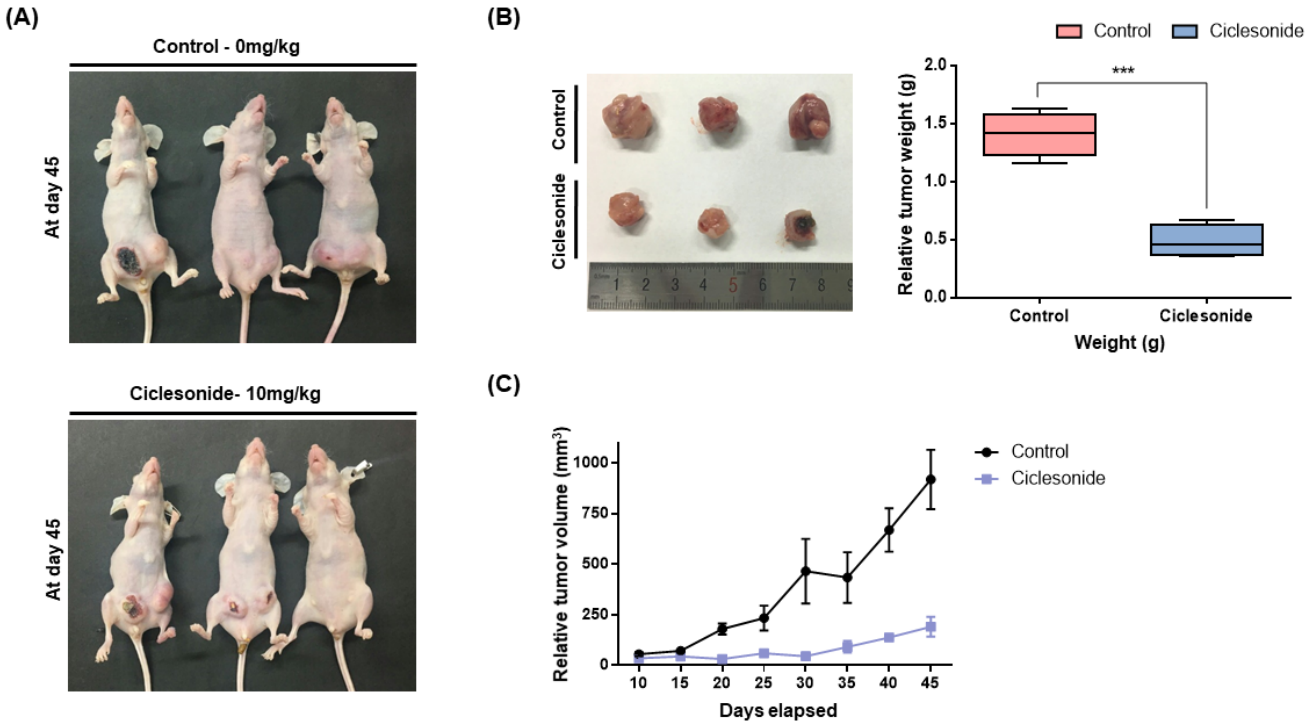

Figure 2

Ciclesonide decreases tumor growth in a mouse model. The 2x106 of breast cancer cells were injected into the mammary fat pads at nude mice. a The inhibitory effect of ciclesonide on tumor growth in MDA-MB-231 tumor-bearing nude mice. b Mice were treated with ciclesonide at 10 mg/kg. After 45 days, images were acquired. Inhibitory effect of ciclesonide on the tumor weight. The nude mice were sacrificed on day 45, and tumors were weighed. Data from triplicate experiments are shown as the means± SDs; ***p < 0.05 vs. the control. c The volumes of tumors from nude mice during the 45-day experimental period were comparable with those of tumors from control and ciclesonide-injected mic. Tumor volumes were estimated as follows: (width2 x length)/2.

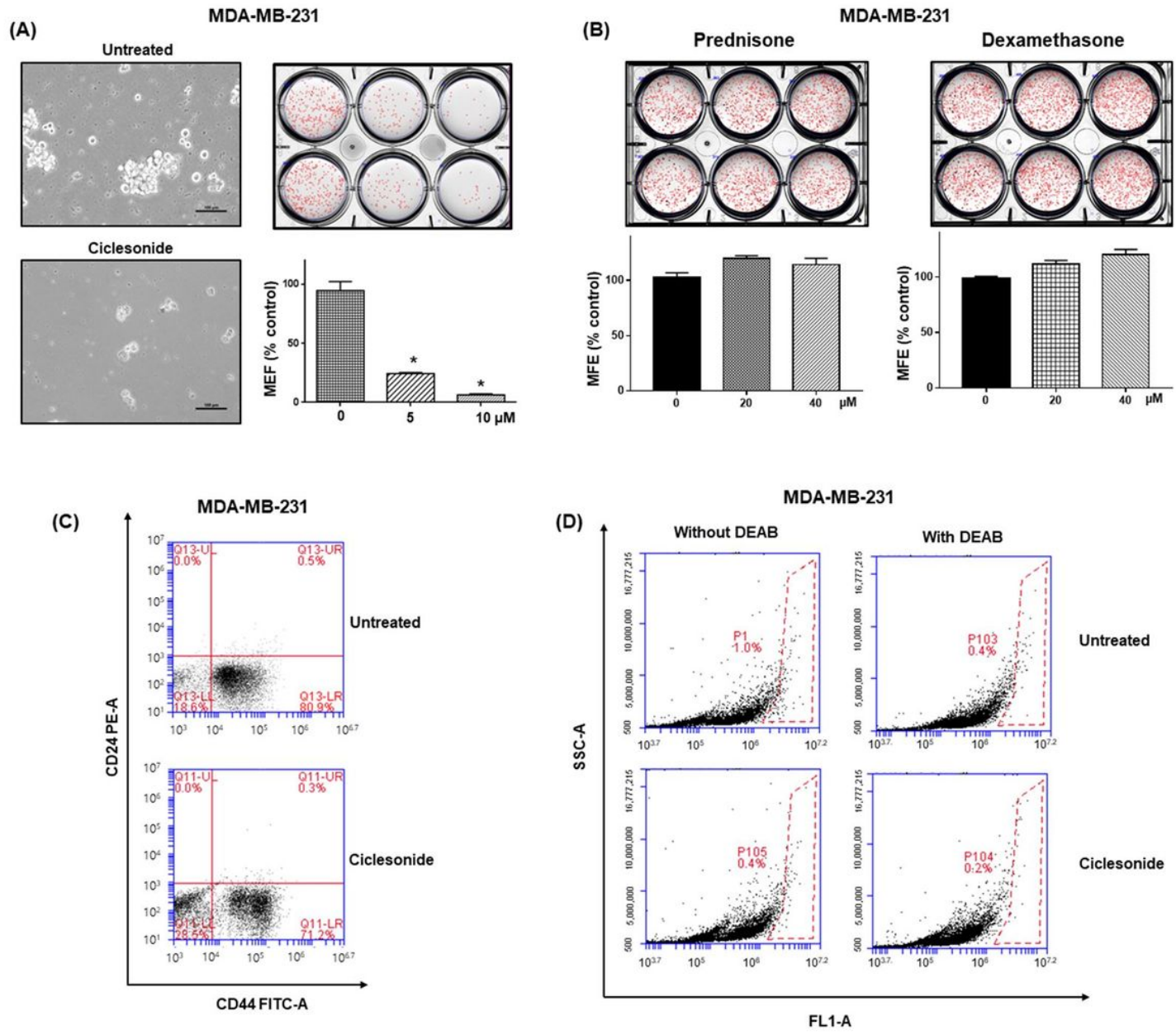

Figure 3

Ciclesonide reduces the formation of mammospheres from breast cancer cells. a Mammospheres were cultured for 7 days in MammoCult media. Treatment with ciclesonide (5 and 10 μM) decreased mammosphere formation to 5% that under control conditions. *p<0.05 vs. the control. bTreatment with prednisone or DEX (20 and 40 μM) did not decrease the MFE. c The proportion of CD44high/CD24low cells was quantified with anti-CD44-FITC and anti-CD24 PE antibodies. The CD44high/CD24low cell proportion was decreased after ciclesonide treatment. d The ALDH1-positive cell population was estimated by an ALDEFLUOR kit. The proportion of ALDH-positive cells was decreased after ciclesonide treatment. The right panel shows red-dot plots of negative control cells which were treated with the ALDH inhibitor, DEAB. The left panel shows ALDH-positive cells not treated with DEAB.

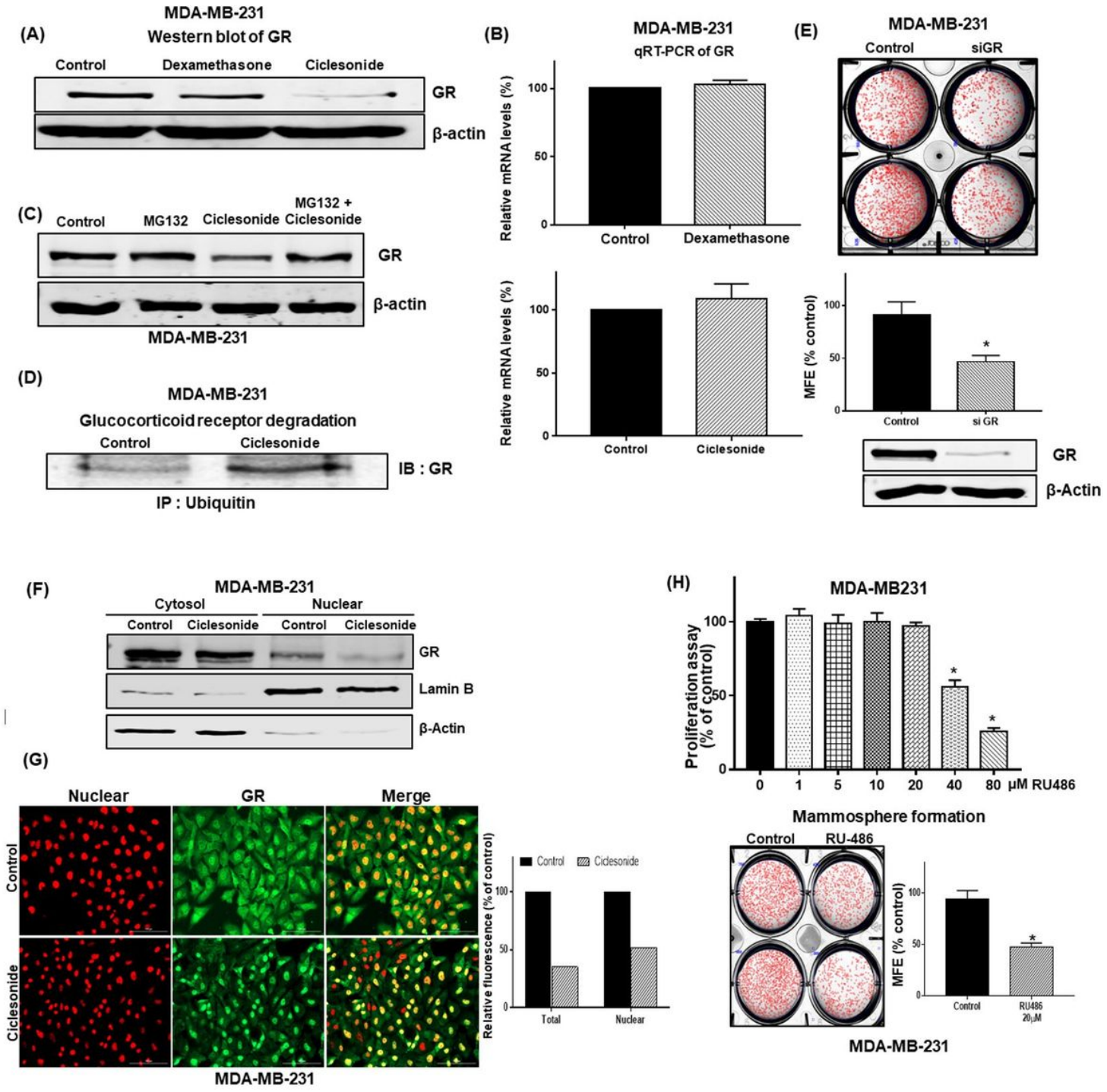

Figure 4

Ciclesonide blocksactivation of GR signaling pathway through the downregulation of GR. Mammospheres were cultured for 7 days and incubated with ciclesonide for 2 days. After treatment with ciclesonide (10 μM), the expression level of GR was measured with an anti-GR antibody. a Ciclesonide treatment decreased GR expression in MDA-MB-231-derived mammospheres. b After treatment with ciclesonide and dexamethasone, the transcription level of GR was assessed by real-time RT-qPCR using specific primers. We used β-actin as the control. c Mammospheres were cultured with proteasome inhibitor, MG-132 and ciclesonide (10 μM) for 1 day and lysed for immunoblot using an anti-GR antibody

and β-actin as the control. d Mammospheres were cultured with ciclesonide (10 μM) for 24 h and lysed for immunoprecipitation with an anti-ubiquitin antibody and immunoblot analysis with an anti-GR antibody. e The effect of GR protein on mammosphere formation was assessed using GR siRNA. Mammospheres derived from GR siRNA-treated cells were cultured for 7 days in complete MammoCult medium. The MFE was determined. Data from triplicate experiments are shown as the means± SDs; *p < 0.05 vs. the control. f Mammospheres were treated with ciclesonide (10 μM) for 1 day. Cytosolic and nuclear proteins were separated on a 10% SDS-PAGE gel and subjected to immuno blotting with an anti-GR antibody. β-Actin and Lamin B were used as the loading controls for the cytosolic and nuclear protein extracts, respectively. g Ciclesonide reduced the levels of cytosolic and nuclear GR protein (green) in MDA-MB-231 cells, as evidenced by immunofluorescence. Nuclei were stained with DAPI (red), and GR was labeled with an anti-GR antibody (green). Magnification, x100. Relative fluorescence of total and nuclear GR were estimated by Gen5 cell imaging program (Online Resource, Supplementary Fig. S1). h Effect of RU486, a GR antagonist, on mammosphere formation. Cancer cells were incubated in a plate with the ciclesonide. Cell growth was evaluated with MTS reagent. Mammosphere formation was examined by determining the MFE with RU486 (scale bar = 100 μm). Data from triplicate experiments are presented as the means± SDs; *p < 0.05 vs. the control.

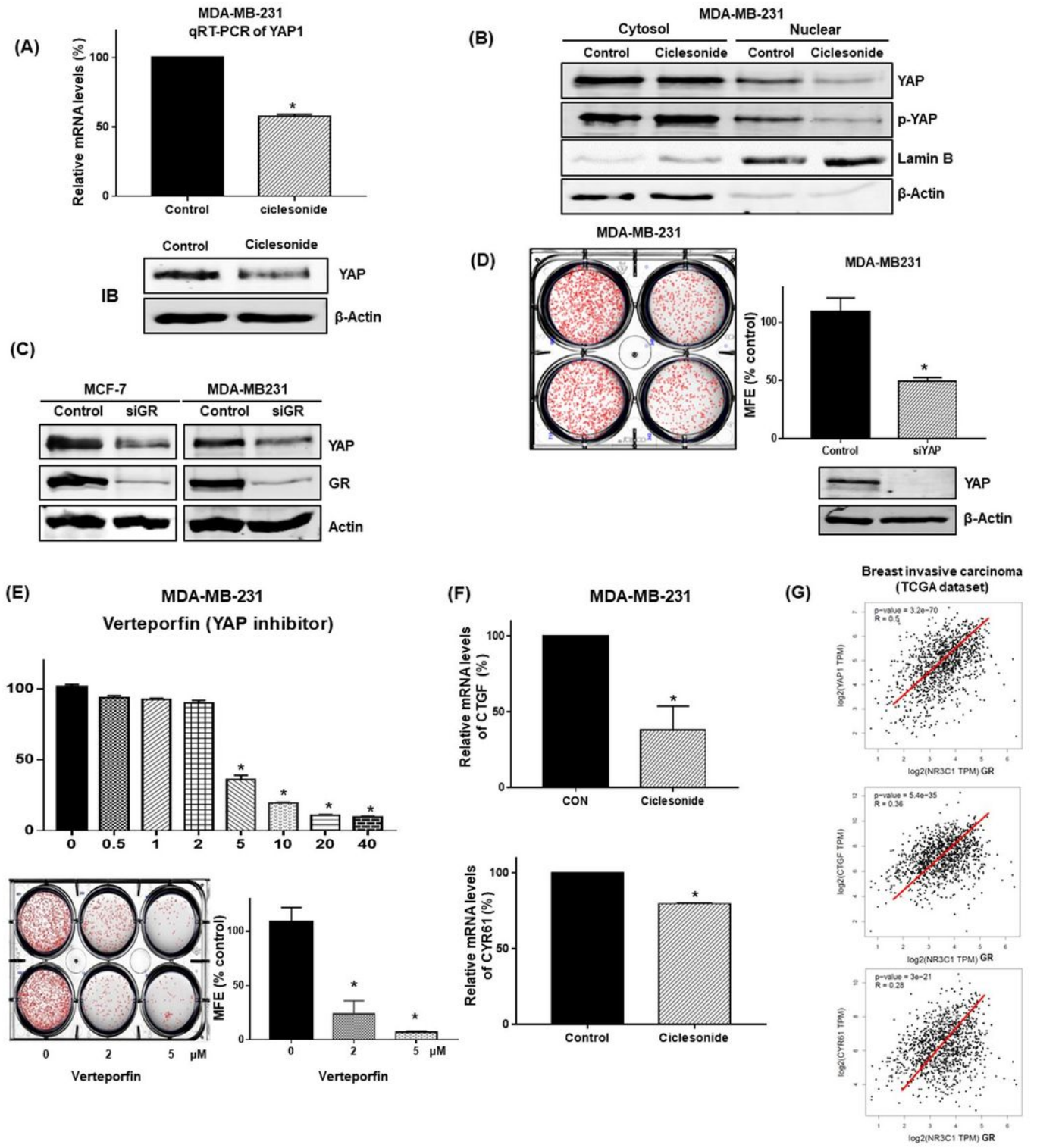

Figure 5

Ciclesonide reduced YAP nuclear localization and regulated breast CSC formation through YAP signaling. a After treatment with ciclesonide (10 μM), the transcripts and protein levels of YAP1 were measured by real-time RT-qPCR using specific primers and an anti-YAP1 antibody, respectively. We used β-actin as the control. b Mammospheres were treated with ciclesonide (10 μM) for 1 day. After separation on 10 % PAGE gels, cytosolic and nuclear protein extracts were transferred to membranes, followed by western blotting

with anti-YAP and anti-p-YAP1 antibodies. β-Actin and Lamin B were used as loading controls for the cytosolic and nuclear protein extracts, respectively. c siRNA-mediated silencing of GR decreased the expression of YAP and GR proteins in MCF-7 and MDA-MB-231 cells. GR protein expression was downregulated via GR siRNA, and the protein levels of YAP1 and GR were measured with anti-GR and anti-YAP1 antibodies. d siRNA-mediated silencing of YAP1 induced a significant decrease in the MFE in MDA-MB-231 cells. YAP1 was downregulated in MDA-MB-231 cells by YAP1 siRNA, and a mammosphere formation assay was performed with these cells. e Treatment with verteporfin, a suppressor of YAP-TEAD complex formation, resulted in a decrease in the MFE in MDA-MB-231 cells. The cancer cells were cultured in a 96-well plate with the indicated concentration of verteporfin. The growth of the cells was measured with MTS reagent. Data from triplicate experiments are shown as the means± SDs; *p < 0.05 vs. the control. Mammospheres were cultured for 7 days in MammoCult medium. After treatment with verteporfin (2 and 5 μM), mammosphere formation was evaluated. *p<0.05 vs. the control. f After treatment with ciclesonide, RT-qPCR of mammospheres was performed using CTGF- and CYR61-specific primers. Data from triplicate experiments are shown as the means± SDs; *p < 0.05 vs. the control. g Correlation analysis of YAP1/GR (NR3C1 gene), CTGF/GR, and CYR61/GR in breast cancer patients from a public TCGA dataset. Analysis of the breast invasive carcinoma dataset showed that the correlation coefficient of YAP1/GR (NR3C1 gene), CTGF/GR, and CYR61/GR were 0.5, 0.36, and 0.27.

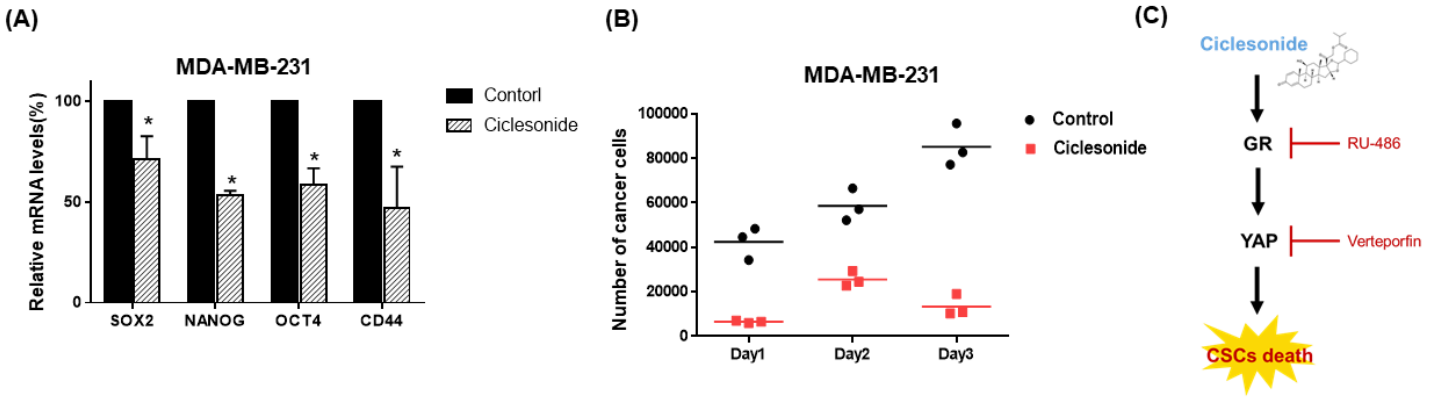

Figure 6

The Inhibitory effect of the antiasthma medication ciclesonide on CSCs levels in breast cancers. a The mRNA levels of SOX2, Nanog, Oct4, and CD44 were assessed in ciclesonide-treated mammospheres using specific primers. β-Actin was used as the loading control. *p<0.05 vs. the control. b Ciclesonide (10

μM) inhibited mammosphere growth. Ciclesonide-treated mammospheres were dissociated into single cells, and equal numbers of cells were cultured in 6 mm dishes. One day after plating, the cells were counted. After 2 and 3 days, the cells were counted in triplicate, and the mean value was plotted. c The proposed schematic suggests that ciclesonide induced GR degradation, YAP downregulation, and CSCs inhibition. GR and YAP activity contributes to CSC and inhibition of GR and YAP expression via siRNA suppresses CSCs growth (C).

Supplementary Files

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

- additionalfile4FigureS3.pdf
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- Additionalfile1TableS1.pdf
- Addiitonalfile3FigureS2.pdf