Hydroxytyrosol inhibits cancer stem cells and the metastatic capacity of triple-negative breast cancer cell lines by the simultaneous targeting of epithelial-to-mesenchymal transition, Wnt/β-catenin and TGFβ signaling pathways

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Abstract

Purpose This study was aimed to determine the impact of hydroxytyrosol (HT), a minor compound found in olive oil, on breast cancer stem cells (BCSCs) and the migration capacity of triple-negative breast cancer (TNBC) cell lines through the alteration of epithelial-to-mesenchymal transition (EMT) and embryonic signaling pathways.

Methods BCSCs self-renewal was determined by the mammosphere-forming efficiency in SUM159PT, BT549, MDA-MB-231 and Hs578T TNBC cell lines. Flow cytometric analysis of $CD44^{+}/CD24^{-/low}$ and aldehyde dehydrogenase positive ($ALDH^{+}$) subpopulations, migration by the "wound healing assay", invasion and western blot of EMT markers and TGFβ signaling were investigated in SUM159PT, BT549 and MDA-MB-231 cell lines. Wnt/β-catenin signaling was assessed by western blot in BT549 cells expressing WNT1 and MDA-MB-231 cells. Changes in TGFβ activity was determined by SMAD Binding Element (SBE) reporter assay.

Results HT reduced BCSCs self-renewal, $ALDH^{+}$ (aldehyde dehydrogenase) and $CD44^{+}/CD24^{-/low}$ subpopulations, tumor cell migration and invasion. Consistently, HT suppressed Wnt/β-catenin signaling by decreasing p-LRP6, LRP6, β-catenin and cyclin D1 protein expression and the EMT markers SLUG, ZEB1, SNAIL and VIMENTIN. Finally, HT inhibited p-SMAD2/3 and SMAD2/3 in SUM159PT, BT549 and MDA-MB-231 cells, what was correlated with a less TGFβ activity.

Conclusion In conclusion, we report for the first time the inhibitory role of HT on BCSCs and tumor cell migration by targeting EMT, Wnt/β-catenin and TGFβ signaling pathways. Our findings highlight the importance of the chemopreventive compound HT as a novel candidate to be investigated as an alternative targeted therapy for TNBC.

Keywords: hydroxytyrosol; olive oil; triple-negative breast cancer; cancer stem cells; epithelial-to-mesenchymal transition

Introduction

Breast cancer is commonly associated with high incidence and death rates in women. Based on the hormone-receptor status, estrogen (ER), progesterone (PR) and HER2 (human epidermal growth factor receptor 2) receptors, breast cancer can be classified into different types: luminal A/B (ER^+, $PR^{+/-}$, $HER2^{+/-}$), normal-like, HER2 (ER^-, PR^-, $HER2^+$), and basal, which includes triple-negative breast cancer (TNBC) (ER^-, PR^-, $HER2^-$). TNBC is a very aggressive form of breast cancer, which is characterized by a poor survival rate, high proliferation, heterogeneity, metastases, drug resistance, incidence of relapse and lack of approved targeted therapies for its treatment [1, 2].

Aggressiveness and poor clinical outcome of TNBC have been attributed, at least in part, to the enrichment in breast cancer stem cells (BCSCs) [3–5], which can be detected in breast tumors by the positiveness in Aldehyde dehydrogenase 1 (ALDH1) activity ($ALDH^+$) or by the surface markers $CD44^+/CD24^{-/low}$ [6, 7]. Like their normal counterparts, BCSCs exhibit self-renewal and differentiation capacities, leading to tumor growth re-initiation, relapses, metastases, and a heterogenous progeny of differentiated cancer cells [7–9]. TNBC tumors display signaling pathways (Wnt/β-catenin or TGFβ, among other) that are observed in BCSCs [3]. Activation of these pathways modify epithelial BCSCs into a more aggressive and metastatic mesenchymal-like phenotype through the activation of epithelial-to-mesenchymal transition (EMT). EMT is evoked during tumor invasion and metastasis, is induced by transcription factors such as SLUG, SNAIL or ZEB1 and shows mesenchymal markers such as VIMENTIN [10, 11]. EMT-induced BCSCs exhibit increased self-renewal and tumor-initiating capabilities, less proliferative behavior and enhanced resistance to apoptosis and chemotherapy [12]. Indeed, it has recently been reported that EMT is not required for lung metastasis but it contributes to chemoresistance

and recurrent lung metastasis formation after chemotherapy in breast carcinoma [13]. Accordingly, targeting BCSCs and EMT would reduce TNBC aggressiveness and increase patient survival.

It has been proposed that many of the extra-virgin olive oil (EVOO) health benefits are due to the minor compounds (phenolic compounds, flavonoids, lignans or secoiridoids) present in this edible oil [14, 15]. Within the phenolic fraction, hydroxytyrosol (HT) is the most representative component, which is associated with a plethora of effects, including antiatherogenic, antimicrobial and antiviral, iron chelator, hypolipidemic, anti-inflammatory, antithrombotic, hypoglycemic, or cardioprotective abilities. In terms of anticancer properties, HT inhibits the proliferation of different cancer cell lines (breast, prostate, colon, or leukemia), due to a cell cycle arrest in G2/M and G0/G1, and increases tumor cell death by apoptotic events [14, 16, 17]. In a previous work in a rat model of breast cancer, we were the first group to report that HT inhibited tumor growth and cell proliferation by the modulation of genes associated with cell proliferation, apoptosis, motility, oncogenesis and developmental processes, among others. Importantly, HT caused a marked induction of the Wnt/β-catenin signaling inhibitor *SFRP4* (Secreted Frizzled Related Protein 4) [18], suggesting that HT may exert an inhibitory role on Wnt/β-catenin signaling.

Based on these premises, here we investigated whether HT can inhibit BCSCs and metastasis of TNBC cells through the modulation of EMT and signaling pathways such as Wnt/β-catenin or TGFβ.

Materials and methods

Reagents

Hydroxytyrosol (HT) (3,4-dihydroxyphenyl ethanol) (Sigma) was resuspended in absolute ethanol at a stock concentration of 50 mg/ml. Working dilutions were made in PBS (1X, pH 7.4). Human recombinant Transforming Growth Factor-β1 (TGFβ1) was obtained from Peprotech.

Cell culture

TNBC cell lines, MDA-MB-231, BT549 and Hs578T were purchased from the American Type Culture Collection, while SUM159PT cells were obtained from Asterand Bioscience. SBE (SMAD binding element) reporter-HEK293 (SBE-HEK293) cell line was purchased from BPS Bioscience. BT549 cells expressing exogenous WNT1 or DKK1 (BT549-WNT1 or BT549-DKK1) were generated, as published previously [19], by infection with the retrovirus vector LNCX containing WNT1 or DKK1 cDNA, followed by selection of pooled colonies in geneticin (Santa Cruz Biotechnology). Control cells were infected in parallel with the empty vector (BT549-LNCX). All cells were maintained in DMEM medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% antibiotic-antimycotic (Gibco) (growth medium). SBE-HEK293 cells were cultured under geneticin selection, following the manufacturer's instructions.

Mammosphere-forming efficiency (MSFE)

Cells were seeded at a density of 5,000 cells/well in 24-well ultra-low attachment plates in MammoCult medium (StemCell Technologies) supplemented with 10% proliferation

supplements, 4µg/ml heparin, and 0.48µg/ml hydrocortisone and treated with HT at 0, 0.5, 1, 5, 10, 25, 50, 75 and 100 µM for 72 h. HT was replenished every 24 h. Then, primary mammospheres were harvested, dissociated with trypsin and single cells were re-plated in 24-well ultra-low attachment plates at a density of 500cells/cm^2 in MammoCult medium and 0.5% methylcellulose (MethoCult. StemCell Technologies), to minimize clumping as published previously [2], with no additional treatment with HT. The secondary mammospheres, with a diameter greater than 50 µm, were counted after 72 h using GelCount colony counter (Oxford Optronix). Mammosphere-forming efficiency was calculated by dividing the number of secondary mammospheres by the number of cells seeded (secondary MSFE). Diameter (µm) of mammospheres was also assessed. The assays were conducted in 6 replicates from two independent experiments.

Migration assay

Migration index was analyzed by the wound healing assay in SUM159PT, MDA-MB-231 and BT549 cells treated with HT (0, 10, 25, 50, 75, 100 µM) for 72 h in DMEM supplemented with 0.1% FBS. HT was replenished every 24 h. After treatment, a wound was done in the cell monolayer with a 100µl pipette tip and images were taken at 0, 14 or 24h. The assay was conducted by duplicate in three independent experiments.

Boyden chamber assay

Invasion was assayed with the CultreCoat Medium BME Cell Invasion Assay (Trevigen) as we described with modifications [2]. Cells were treated with HT (75 µM) for 72 h in DMEM supplemented with 0.5% FBS. HT was replenished every 24 h. Cells were seeded onto a 1X-

BME-coated transwell chamber (50,000 cells). Growth medium was added in the bottom well and cells were incubated for 16 h. The number of invading cells, by triplicates from two independent experiments, was quantified with a standard curve with Calcein AM at 485 nm excitation and 520 nm emission wavelength.

Flow cytometry

ALDH1 activity changes were analyzed with the Aldefluor (ALDF) assay (StemCell Technologies) as described previously [2, 20]. SUM159PT and BT549 cells were treated with HT (0, 25 and 50 μM) for 96h. MDA-MB-231 cells were treated with HT (0, 50, 75 μM) for 6 days. HT was replenished every 48 h. Treated cells ($2x10^5$) were incubated with the ALDF substrate for 60 min at 37ºC. Negative controls were treated with the ALDF inhibitor diethylaminobenzaldehyde (DEAB). $CD44^+/CD24^{-/low}$ subpopulation was assayed, as published previously [21], in SUM159PT and MDA-MB-231 cells treated with HT (0, 25 μM) as described above. $ALDF^+$ ($ALDH^+$) and $CD44^+/CD24^{-/low}$ subpopulations were analyzed in a FACSVerse (BD Biosciences) flow cytometer.

Western blotting

Western blotting was performed as described previously [22]. Wnt/β-catenin signaling was assessed in BT549-WNT1, BT549-DKK1 (control of Wnt inhibition), BT549-LNCX (negative control) and MDA-MB-231 cells after HT treatment (0, 0.5, 1, 5, 10, 25, 50, 75, 100 μM) for 48h. EMT markers were determined in SUM159PT, MDA-MB-231 and BT549 cells treated with HT (0, 25, 50, 75 μM) for 24h. SMAD2/3 activity was tested in the three cell lines treated with HT (0, 25, 75 μM) for 0, 0.5, 1, 2, 4 and 6 h. Protein bands were detected with the

ImageQuantLAS4000 digital imager and analyzed in triplicate by densitometry with the Image J software. β-actin was used to normalize the arbitrary densitometric units.

Primary antibodies to phospho-LRP6 (S1490), LRP6, β-catenin, ZEB1 (D80D3), SNAIL (C15D3), SLUG (C19G7), VIMENTIN (D21H3), phospho-SMAD2 (Ser465/467)/3(Ser423/425) (D27F4), SMAD2/3, ZO-1, and β-Actin were from Cell Signaling. Cyclin D1 was purchased from Santa Cruz Biotechnology and Millipore. All primary antibodies were used at a 1:1000 dilution.

SBE-reporter assay

SBE-HEK293 cells were treated with HT (0, 25, 50, 75μM) with or without TGFβ1 (10ng/mL) for 24 h. SBE activity was analyzed in 6 replicates from two experiments with the ONE-Step Luciferase Assay System (BPS Bioscience), according to the manufacturer's instructions.

Statistical analysis

All data were analyzed using GraphPad Prism. Data are presented as mean ± SEM. Differences between vehicle and each concentration of HT were analyzed by two-tailed Student's *t*-test. MSFE and migration results are normalized to vehicle group (100%). A *P*-value <0.05 was considered significant.

Results

HT reduces BCSC subpopulation and self-renewal capacity by the modulation of Wnt signaling and EMT

BCSC self-renewal can be assessed by generating secondary mammospheres from disaggregated primary mammospheres [23]. Treatment of primary mammospheres with HT led to a dose-dependent reduction of non-treated secondary mammospheres in SUM159PT, BT549 and MDA-MB-231 cell lines. In Hs578T cells, HT treatment reduced secondary MSFE, but it was not dose-dependent. Interestingly, even the smallest HT concentration (0.5 μM) caused a marked reduction of secondary MSFE in SUM159PT, BT549, MDA-MB-231 and Hs578T compared to the control group (41, 55, 49 and 50 % decrease, respectively). Diameter of mammospheres was also lower in SUM159PT, BT549 and MDA-MB-231, however, in Hs578T cells, although the MSFE was reduced by HT, the size of the spheres was enhanced by HT (Fig. 1A – 1D). Our results suggest that HT decreased BCSC self-renewal in TNBC cell lines.

It was reported that BCSCs can transition between two different phenotypes: a more quiescent and invasive mesenchymal-like type (expressing $CD44^{+}/CD24^{-/low}$) and a more proliferative epithelial-like type ($ALDH^{+}$), that reflect basal and luminal normal stem cells in the breast [24]. We investigated whether those BCSC subpopulations are affected by HT. We found that a 4-days treatment with HT reduced the $ALDH^{+}$ population in SUM159PT cells at 25 (0.84 %) and 50 μM (0.45 %) compared with vehicle (1%) (Fig. 2A). In BT549 cells, the percentage of $ALDH^{+}$ was reduced by HT treatment for 4 days at 25 (0.55 %) and 50 μM (0.99 %) compared with the non-treated cells (5.86 %) (Fig. 2B). Treatment of MDA-MB-231 cells with HT for 6 days resulted in less percentage of $ALDH^{+}$ at 75 μM (0.22%) compared with 0 μM (0.7%) (Fig. 2C). The mesenchymal-like $CD44^{+}/CD24^{-/low}$ BCSC subpopulation was also diminished by a 4-days treatment with HT at 25 μM (52%) compared to vehicle group (70%) in SUM159 cells (Fig. 2D). In MDA-MB-231 cells, we found a reduction of $CD44^{+}/CD24^{-/low}$ by treatment with HT at 25 μM (56%) for 6 days compared to vehicle group (97%) (Fig. 2E).

Wnt/β-catenin signaling pathway plays a significant role in the regulation of BCSC self-renewal and differentiation in breast cancer [9], and is frequently found in invasive TNBC, as well as the overexpression of its essential co-receptor LRP6 (low-density lipoprotein receptor-related protein 6) [25, 26]. We tested whether the effect of HT on BCSC self-renewal and subpopulations was due to a modulation of this signaling pathway in BT549-WNT1 and MDA-MB-231 cells. Our results showed a dose-dependent reduction of phosphorylated (p)-LRP6, LRP6 and β-catenin protein levels, what correlated with an inhibition of the Wnt/β-catenin target gene cyclin D1, in both cell lines. Decreased β-catenin and cyclin D1 were seen in BT549-DKK1 cells, that were used as control of Wnt/β-catenin signaling inhibition (Fig. 3A).

It is accepted that EMT promotes BCSC numbers [10, 27]. We asked whether HT (0, 25, 50, 75 μM) could decrease BCSC numbers by inhibiting EMT. We found a reduction of the EMT-related transcription factors ZEB1 and SLUG in SUM159PT, MDA-MB-231 and BT549 cells. SNAIL levels were diminished after HT treatment in MDA-MB-231 and BT549 cells. Noteworthy, SLUG was inhibited markedly by HT in all cell lines. Inhibition of these transcription factors was correlated with a less protein expression of the mesenchymal marker VIMENTIN in the three cell lines (Fig. 3B). Levels of the epithelial marker ZO-1 were also enhanced by HT treatment in SUM159PT and BT549 cells, but no significant changes were detected in MDA-MB-231 cells (Fig. 3C).

Overall, our data demonstrate that HT inhibits BCSC self-renewal and number by blocking Wnt/β-catenin signaling pathway and EMT in TNBC cell lines.

The metastatic potential of TNBC cell lines is ablated by HT

TNBC is characterized by a high metastatic capacity and ability to recur in distant organs, lungs and brain mainly [28]. Given that HT was able to inhibit BCSCs and EMT, which are involved in metastatic events [9, 10], we sought to determine whether HT could decrease the metastatic potential of TNBC cell lines. Our results demonstrated that HT reduced the migration ability of SUM159PT, BT549 and MDA-MB-231 cells in a dose-dependent manner. However, the wound healing capacity of SUM159PT cells was less affected by this phenolic compound than in the other two cell lines. A concentration of 100 μM in BT549 caused a marked cell death and the migration index could not be assessed (Fig. 4A – 4C). Accordingly, we found that HT diminished the number of invading cells (Fig. 4D – 4F), what confirmed the ablating effects of HT on the metastatic potential of tumor cells.

HT inhibits SMAD2/3-dependent TGFβ signaling

TGFβ signaling pathway is a well-known inducer of EMT, BCSC and the metastatic properties of tumor cells [10, 29], and is expressed commonly in TNBC [3]. We investigated the impact of HT treatment on TGFβ signaling by treating the three TNBC cell lines with 25 and 75 μM (Fig. 5A and 5B) of HT for 0, 0.5, 1, 2, 4 and 6 h. p-SMAD2/3 and total SMAD2/3 protein levels were assessed as indicator of active TGFβ signaling. In SUM159PT cells, 25 and 75 μM of HT caused a decrease in the p-SMAD2/3-SMAD2/3 ratio in the first 4 hours after treatment. In BT549 cells, we observed a time-dependent decrease in the p-SMAD2/3-SMAD2/3 ratio with the minimum activity at 6 h at both concentrations. However, in MDA-MB-231 cells, the minimum p-SMAD2/3-SMAD2/3 ratio by HT at 25 μM was found after 1 h treatment (Fig. 5A). On the contrary, a time-dependent decrease was seen with 75 μM of HT (Fig. 5B).

To verify that TGFβ signaling activity is inhibited by HT, SBE activity was measured in SBE-HEK293 cells treated with 0, 25, 50 and 75 μM of HT with and without TGFβ1 for 24 h (Fig. 5C). Our results demonstrated a dose-dependent inhibition of SBE activity in basal conditions compared with vehicle (29, 42 and 63% reduction, respectively). TGFβ1-enhanced SBE activity (3-fold increase compared to non-treated control) was blocked by 25, 50 and 75 μM of HT (57, 73 and 68 %, respectively).

Collectively, our data suggest that HT can block the TGFβ signaling activity through the inhibition of SMAD2/3 activation.

Discussion

Treatment failure and metastatic relapses are the leading causes of death in TNBC patients, who have the highest recurrence and worse survival rates within 3 years after the therapeutic approach [1, 28, 30]. It has been postulated that breast tumor relapse is driven by a subpopulation of BCSCs, which are intrinsically resistant to chemo- and radiotherapy [31, 32]. Consistently, these treatments enrich residual breast tumors for cells with EMT- and BCSCs-like characteristics [33], which is associated with a high rate of metastatic recurrences and shorter survival of TNBC patients [34]. Gene expression profiling of human TNBC tumors identified six subtypes, what depicts its heterogeneity. Among these subtypes, mesenchymal and mesenchymal stem-like TNBC exhibit gene expression profiles involving pathways associated with BCSCs, EMT and cell migration (TGFβ, Rac1/Rho, Wnt/β-catenin, mTOR, among other) [3]. Therefore, a dual targeting of BCSCs and EMT through the inhibition of TGFβ and/or Wnt/β-catenin pathways represents an attractive therapeutic approach to reduce tumor growth, recurrence, metastasis, and treatment resistance in TNBC.

Naturally-occurring dietary compounds are gaining interest as chemopreventive agents in cancer due to their low or no toxicity and availability [35]. Growing evidences demonstrate the inhibitory effects of plant-derived natural compounds, such as curcumin, piperine, sulforaphane, resveratrol, honokiol or diallyl trisulfide, on CSCs, EMT and metastasis in breast cancer [36–45], what reinforces their powerful potential in cancer therapeutics. In the present study, we investigated the role of hydroxytyrosol, which is a naturally-occurring compound present in EVOO, on CSCs, EMT and the metastatic properties of TNBC cells. HT has emerged as a good plant-derived chemopreventive agent in cancer, not only because its antitumor properties, but also because it has high availability in the human diet and has demonstrated no toxic effects in clinical studies with healthy volunteers and toxicity studies in animals [14, 46–48].

Our findings show for the first time that HT inhibits efficiently BCSC self-renewal, as seen by a reduction of secondary MSFE and size of spheroids, and the $ALDH^+$ and $CD44^+/CD24^{-/low}$ BCSC subpopulations. It has recently been reported that the EVOO-derived secoiridoid decarboxymethyl oleuropein aglycone (DOA) can target BCSCs through the inhibition of mTOR and DNA methyltransferases (DNMTs) [49]. Based on our results, and because oleuropein aglycone (OA) is the precursor of HT [14], we suggest that the effects of this secoiridoid on BCSCs could be maintained after the natural hydrolysis into HT, what could be, at least partly, responsible for the activity of DOA. In this regard, the bioactive features of HT and OA have been attributed to the ortho-dihydroxy (catechol) moiety [16], which is also present in other dietary compounds that inhibit CSCs, EMT and tumor cell migration such as quercetin, caffeic acid, salvianolic acid B or luteolin [50–55]. Whether the inhibitory effects of OA, DOA or HT on BCSCs are due to the presence of the catechol group remains unknown. Similar to what was described for those other natural compounds, and consistent with an inhibition of BCSCs, we

found decreased migration rates and number of invading cells following HT treatment, as well as the protein levels of the EMT markers ZEB1, SLUG, SNAIL and VIMENTIN, and the enhancement of the epithelial marker ZO-1.

In a previous work, we reported in a rat model of breast cancer that HT treatment promoted the expression of *SFRP4* [18], however, whether HT inhibited Wnt/β-catenin was not investigated. It has recently been found that the natural compound diosgenin inhibits BCSCs through the SFRP4-mediated inhibition of Wnt/β-catenin [56]. Our results demonstrate that HT causes a dose-dependent inhibition of p-LRP6 and LRP6 protein levels, resulting in decreased β-catenin and cyclin D1 expression, in BT549 cells expressing exogenous WNT1 ligand and MDA-MB-231 cells, what indicates that HT attenuates the Wnt/β-catenin signaling [25, 26, 57]. It is known that existence of an interplay between Wnt/β-catenin and TGFβ/SMAD signaling pathways to mediate processes of EMT, migration and invasion [58–61]. Here, we show that p-SMAD2/3-SMAD2/3 ratio was inhibited by HT within a 6-hours treatment in SUM159PT, BT549 and MDA-MB-231 TNBC cell lines. These results were validated by an SBE assay following treatment with HT, which diminished SBE in basal conditions and upon addition of the TGFβ1 ligand. Further studies should be carried out to determine whether the effects of HT on Wnt/β-catenin signaling depend on the inhibition of TGFβ/SMAD activity or whether blockade of both pathways is due to independent mechanisms. Overall, our findings suggest that HT is a dual suppressor of Wnt/β-catenin and TGFβ/SMAD signaling activity, which are common pathways targeted by natural compounds and define their inhibitory role on CSCs, EMT and metastasis [35, 42, 43, 50, 52–54].

It is accepted that chemotherapy not only enriches tumors for CSCs, but also contributes to the development of EMT-derived metastatic relapses. Given that non-EMT tumor cells, which are

sensitive to chemotherapy, are the major contributors to generate macrometastatic lesions, combination therapies of chemotherapy and anti-EMT/BCSCs agents may comprise a very attractive approach to eliminate relapse and metastatic recurrences [13]. Our previous investigations demonstrate that HT is able to enhance the antitumor activity of paclitaxel in an animal model of breast cancer [62]. Overall, our work shows for the first time that HT, found in EVOO, inhibits BCSC self-renewal, EMT, epithelial and mesenchymal-like BCSC subpopulations and tumor cell migration and invasion through the dual inhibition of Wnt/β-catenin and TGFβ signaling pathways. These findings outline the relevance of HT, not only as a potent chemopreventive agent that can be easily supplied by consumption of olive oil and/or olives [14], but also as a potential targeted therapy that may be an appropriate partner in combination treatments to eliminate chemoresistance, metastatic recurrence and, therefore TNBC aggressiveness, without affecting the effectiveness of chemotherapeutic drugs (Fig. 6).

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Compliance with ethical standards

Conflict of interest

The authors declare no potential conflict of interests.

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595 **Figure Legends**

596

FIGURE 1

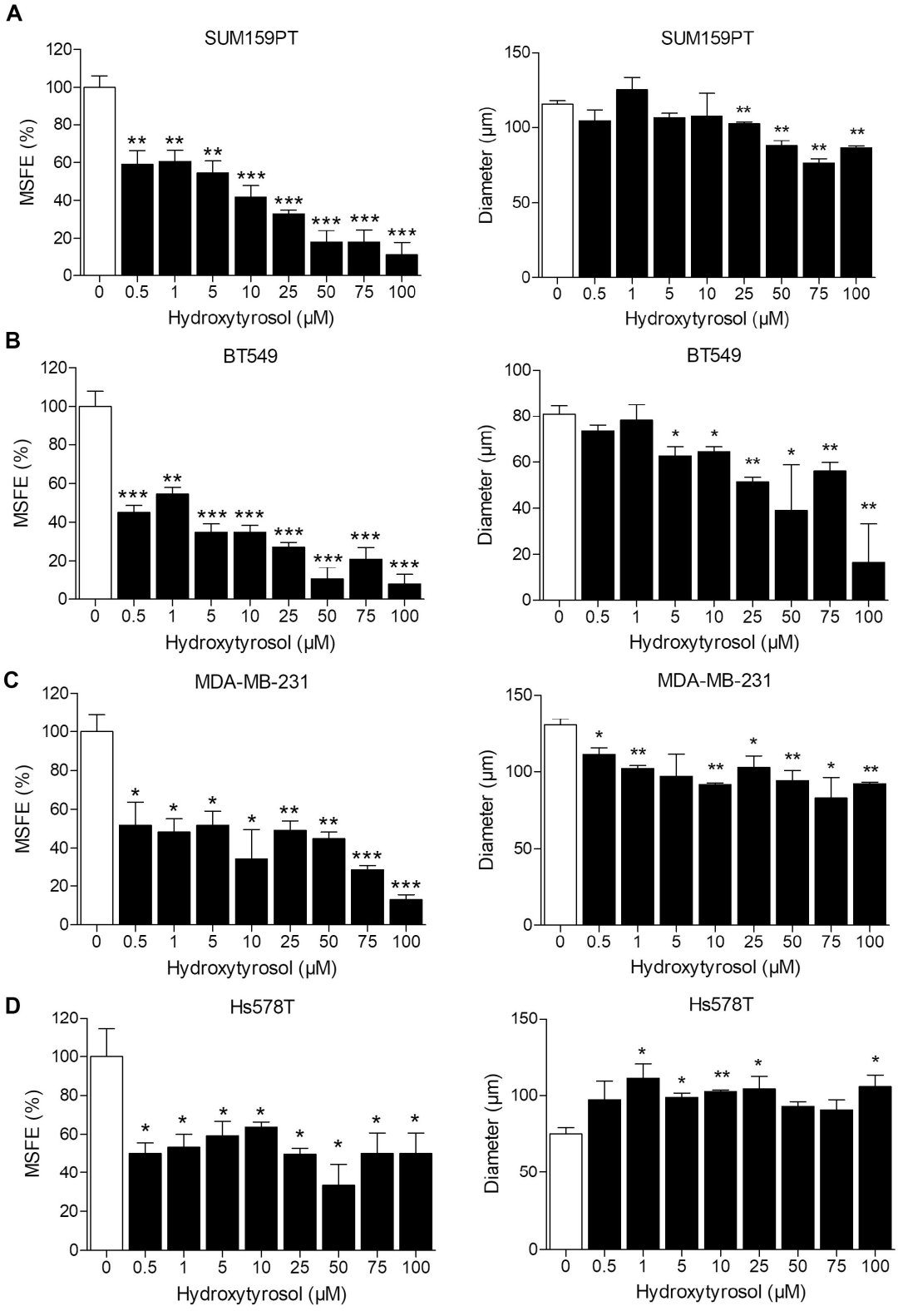
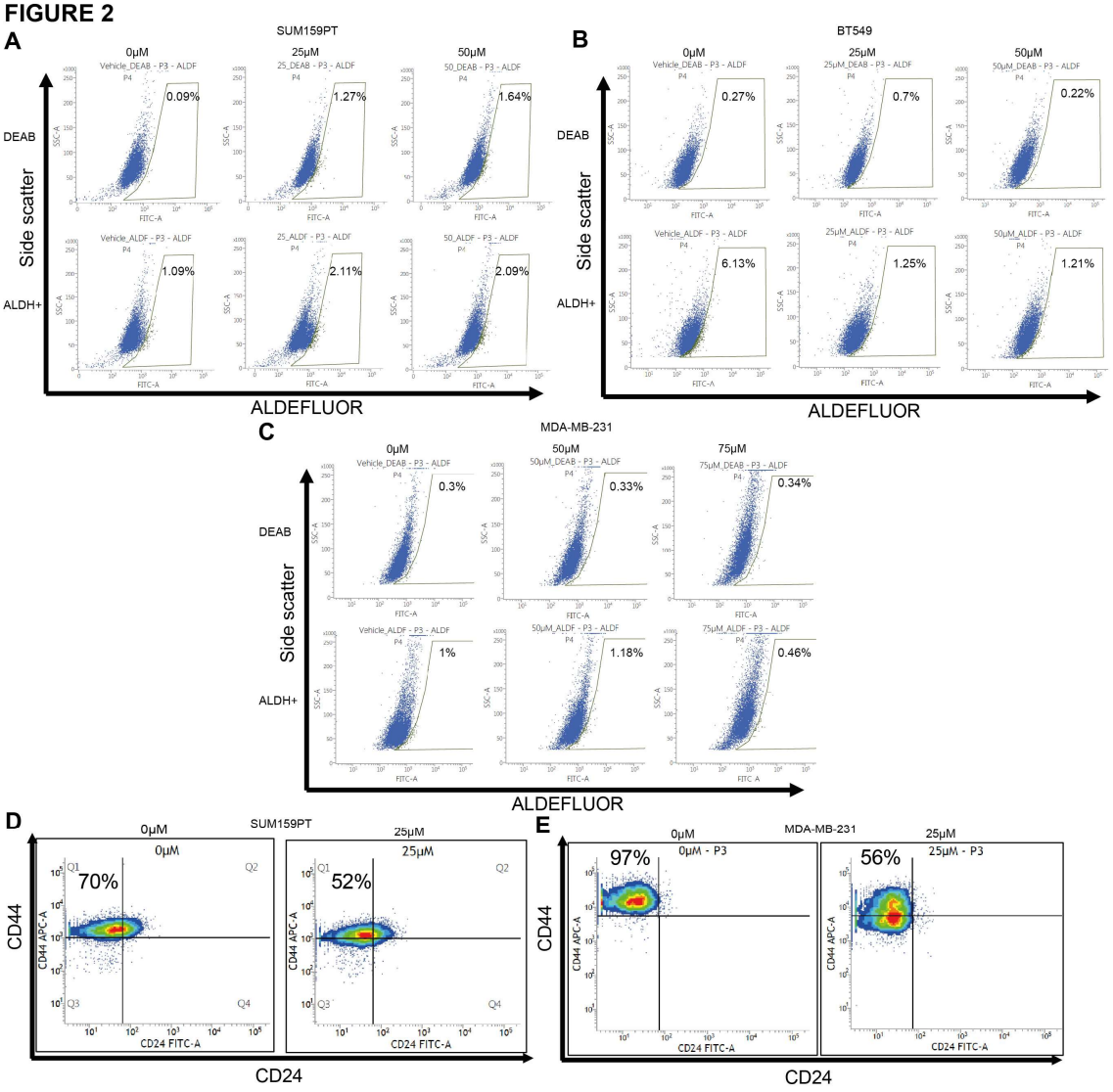


Fig. 1. Hydroxytyrosol diminishes BCSC self-renewal. Mammosphere-forming efficiency (MSFE) and diameter of the second generation of mammospheres after treatment with HT (0, 0.5, 1, 5, 10, 25, 50, 75 and 100 μM) for 72 h in **(A)** SUM159PT, **(B)** BT549, **(C)** MDA-MB-231 and **(D)** Hs578T cell lines. Results were normalized to the vehicle group (0 μM). Data are presented as mean ± SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

620

FIGURE 2



621

622 **Fig. 2. BCSC subpopulation is reduced by hydroxytyrosol treatment. (A)** Aldefluor assay
623 showing the $ALDH^+$ subpopulation in SUM159PT, **(B)** BT549, **(C)** MDA-MB-231 cell lines
624 treated with HT at the concentrations indicated for 4 **(A and B)** or 6 days **(C). (D)** Flow
625 cytometric analysis of $CD44^+/CD24^{-/low}$ cell population in SUM159PT and **(E)** MDA-MB-231
626 cells treated with HT (25 μM) for 4 and 6 days, respectively. Representative analyses of
627 duplicates from two independent experiments are shown.

FIGURE 3

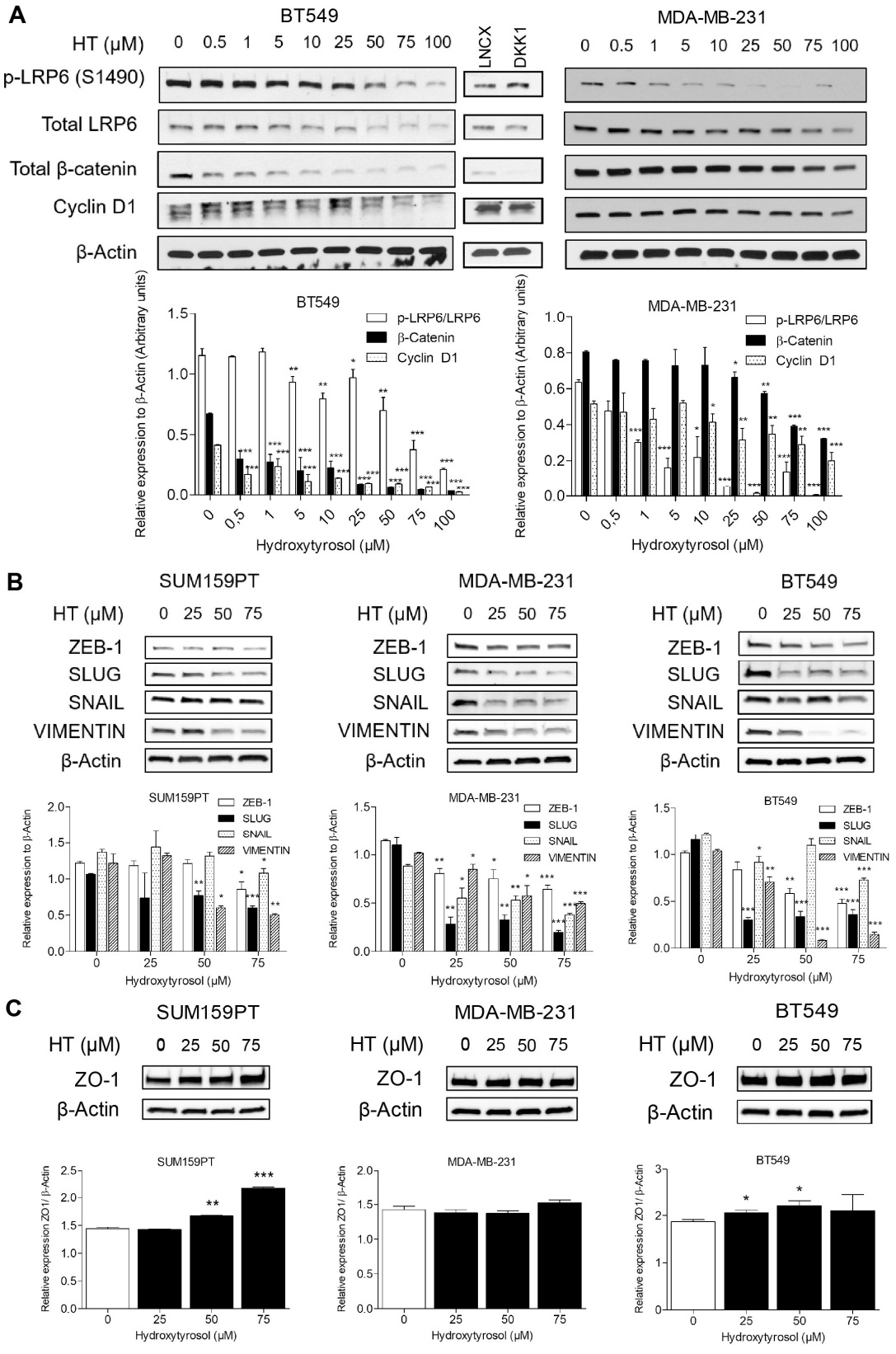

A
BT549
MDA-MB-231
HT (μM) 0 0.5 1 5 10 25 50 75 100
LNCX
DKK1
p-LRP6 (S1490)
Total LRP6
Total β-catenin
Cyclin D1
β-Actin
Relative expression to β-Actin (Arbitrary units)
p-LRP6/LRP6
β-Catenin
Cyclin D1
Hydroxytyrosol (μM)
B
SUM159PT
MDA-MB-231
BT549
HT (μM) 0 25 50 75
ZEB-1
SLUG
SNAIL
VIMENTIN
β-Actin
Relative expression to β-Actin
Hydroxytyrosol (μM)
C
SUM159PT
MDA-MB-231
BT549
HT (μM) 0 25 50 75
ZO-1
β-Actin
Relative expression ZO1/ β-Actin
Hydroxytyrosol (μM)

Fig. 3. Hydroxytyrosol inhibits Wnt/β-catenin signaling and EMT. (A) Western blot of p-LRP6, total LRP6, β-catenin and cyclin D1 after HT treatment (0, 0.5, 1, 5, 10, 25, 50, 75 and 100 μM) for 48 h in BT549-WNT1, BT549-DKK1 (positive control of Wnt inhibition), BT549-LNCX (negative control) and MDA-MB-231 cell lines. **(B)** Changes in protein expression of EMT markers (ZEB1, SLUG, SNAIL, VIMENTIN) in SUM159PT, MDA-MB-231 and BT549 cells after treatment with HT (0, 25, 50 and 75 μM) for 24 h. **(C)** Protein levels of the epithelial marker ZO-1 in the three cell lines treated with HT (0, 25, 50 and 75 μM) for 48 h. Densitometric analysis of each blot is shown. Data are presented as mean ± SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

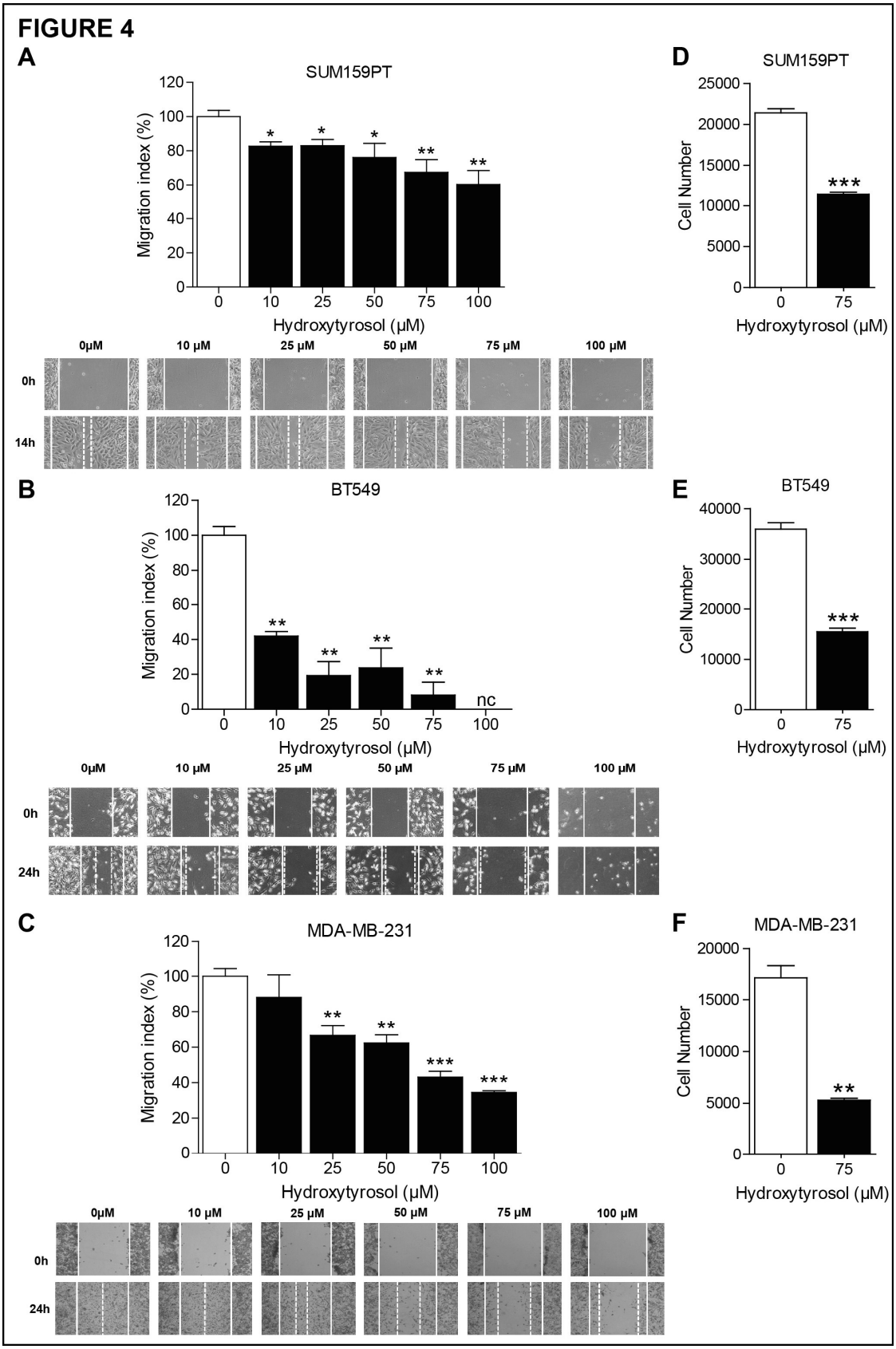
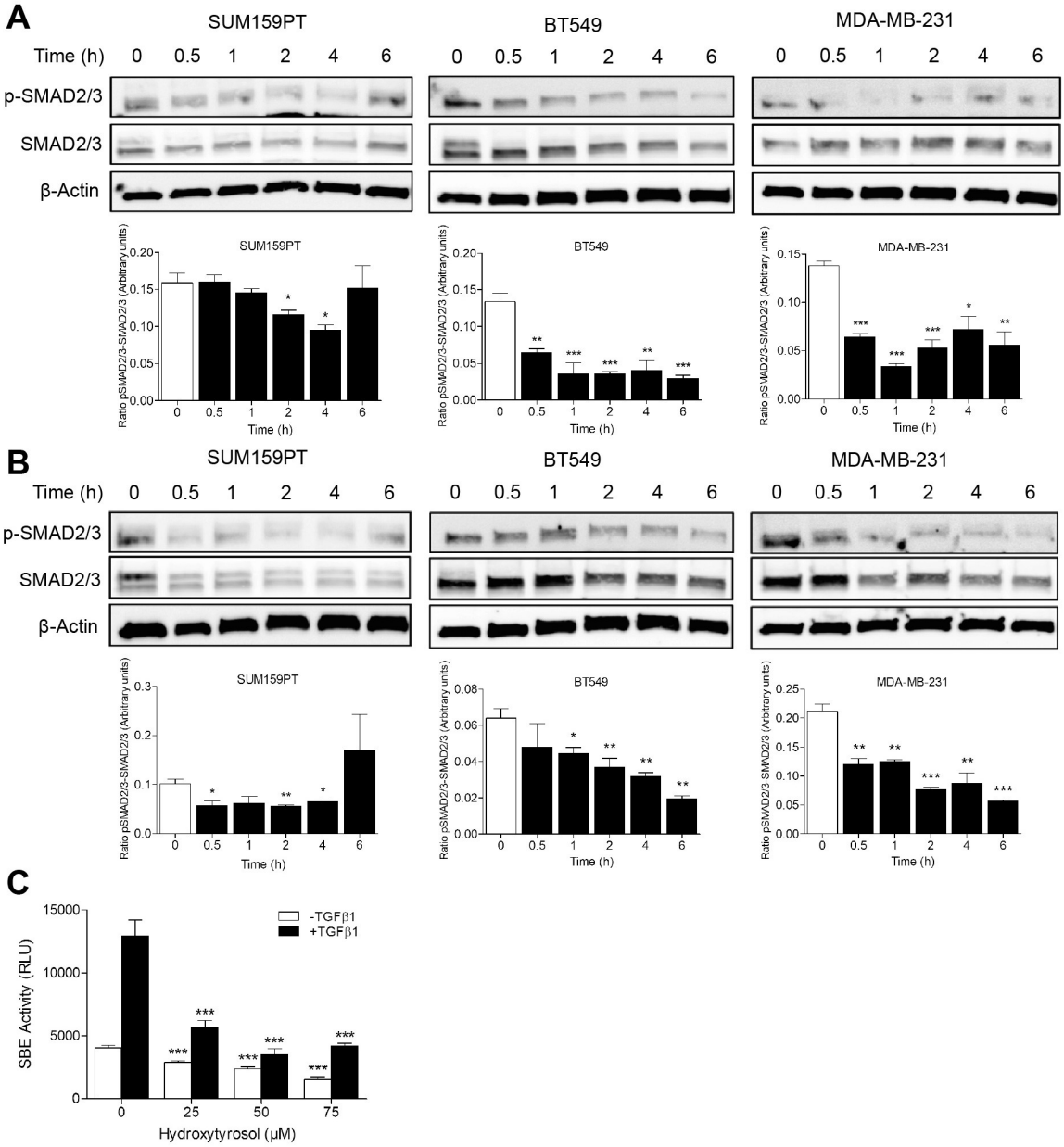


FIGURE 4
A
SUM159PT
Migration index (%)
Hydroxytyrosol (μM)
0μM
10 μM
25 μM
50 μM
75 μM
100 μM
0h
14h
D
SUM159PT
Cell Number
Hydroxytyrosol (μM)
B
BT549
Migration index (%)
Hydroxytyrosol (μM)
nc
0h
24h
E
BT549
Cell Number
Hydroxytyrosol (μM)
C
MDA-MB-231
Migration index (%)
Hydroxytyrosol (μM)
0h
24h
F
MDA-MB-231
Cell Number
Hydroxytyrosol (μM)

Fig. 4. Effects of hydroxytyrosol on the metastatic capacity of TNBC cells. (A) Migration index and representative images of the wound healing assay in SUM159PT (10X optical focus), **(B)** BT549 (10X optical focus) and **(C)** MDA-MB-231 (4X optical focus) cells treated with HT (0, 10, 25, 50 and 75 μM) for 72 h. Results were normalized to the vehicle group (0 μM). **(D)** Invasion of SUM159PT, **(E)** BT549 and **(F)** MDA-MB-231 cells treated with HT (75 μM) for 72 h. Data are presented as mean ± SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

720

FIGURE 5

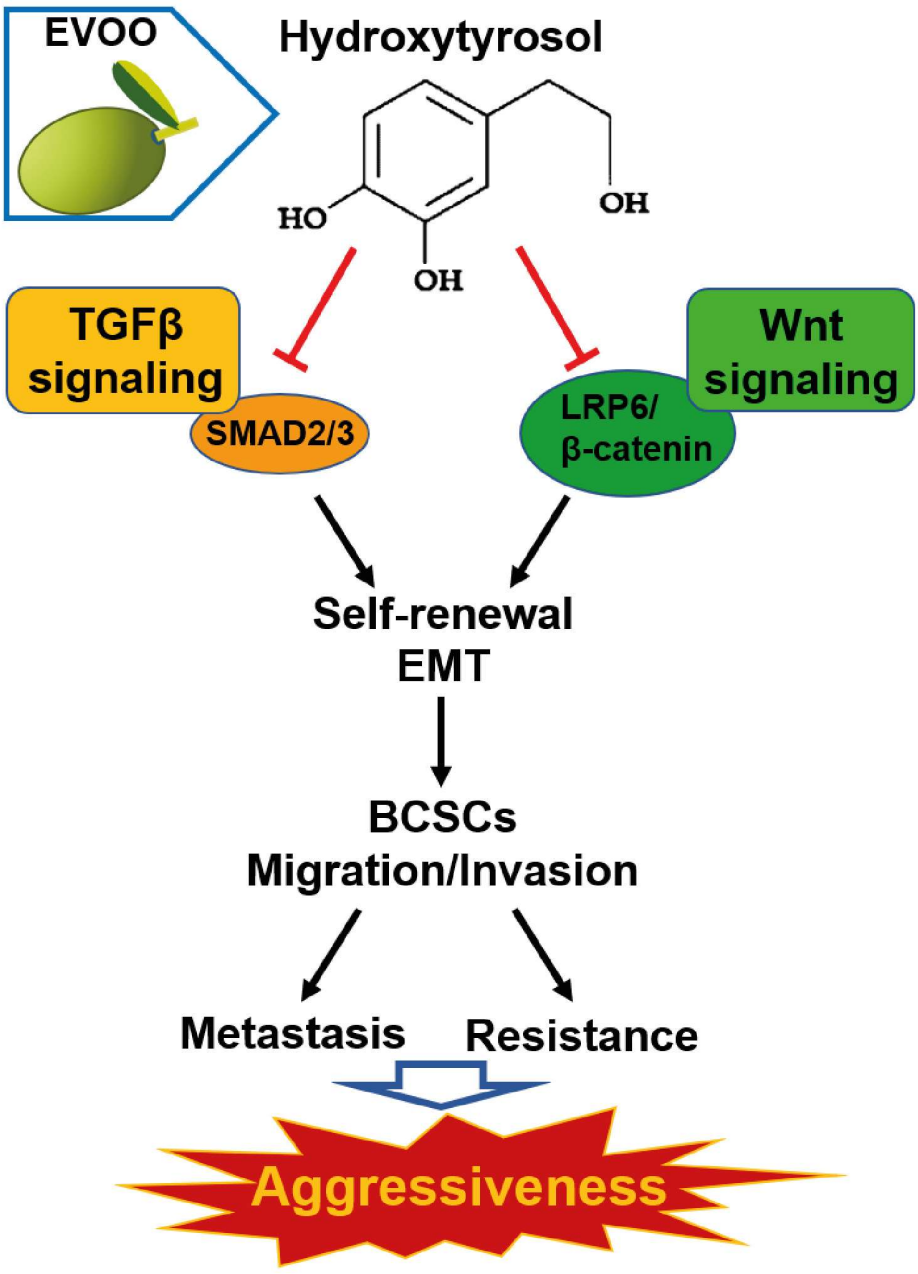


721 **Fig. 5. Inhibition of TGFβ/SMAD2/3 signaling by HT.** Western blot of p-SMAD2/3 and total
722 SMAD2/3 protein levels, and densitometric analysis of the ratio p-SMAD2/3- SMAD2/3, in
723 SUM159PT, BT549 and MDA-MB-231 cell lines treated with **(A)** 25 μM and **(B)** 75 μM of HT
724 for 0, 0.5, 1, 2, 4 and 6 h. **(C)** SBE reporter assay in SBE-HEK293 cells after HT treatment (0,

25, 50 and 75 μM) with/without TGFβ1 for 24 h. RLU: Relative Light Units. Data are presented as mean ± SEM. *** $P < 0.001$.

748

FIGURE 6



768

769 **Fig. 6. Schematic diagram summarizing the study results.** Hydroxytyrosol (HT), a small

770 molecule present in extra-virgin olive oil (EVOO), decreases the epithelial and mesenchymal-

like BCSC subpopulations, self-renewal, epithelial-to-mesenchymal transition (EMT) and tumor cell migration through the dual inhibition of Wnt/β-catenin and TGFβ signaling pathways. These effects would reduce TNBC metastasis and resistance, and therefore, its aggressiveness.