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Osteoprotegerin Production by Breast Cancer Cells Is Suppressed by Dexamethasone and Confers Resistance Against TRAIL-Induced Apoptosis

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ABSTRACT

Osteoprotegerin (OPG) is a decoy receptor for receptor activator of NF-κB ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL). While RANKL is essential for osteoclastogenesis and facilitates breast cancer migration into bone, TRAIL promotes breast cancer apoptosis. We analyzed the expression of OPG and TRAIL and its modulation in estrogen receptor-positive MCF-7 cells and receptor-negative MDA-MB-231 cells. In both cells, OPG mRNA levels and protein secretion were dose- and time-dependently enhanced by interleukin (IL)-1β and suppressed by dexamethasone. In contrast to MCF-7 cells, MDA-MB-231 abundantly expressed TRAIL mRNA, which was enhanced by IL-1β and inhibited by dexamethasone. TRAIL activated pro-apoptotic caspase-3, -7, and poly-ADP-ribose polymerase and decreased cell numbers of MDA-MB-231, but had no effect on MCF-7 cells. Gene silencing siRNA directed against OPG resulted in a 31% higher apoptotic rate compared to non-target siRNA-treated MDA-MB-231 cells. Furthermore, TRAIL induced significantly less apoptosis in cells cultured in conditioned media (containing OPG) compared to cells exposed to TRAIL in fresh medium lacking OPG ($P < 0.01$) and these protective effects were reversed by blocking OPG with its specific ligand RANKL ($P < 0.05$). The association between cancer cell survival and OPG production by MDA-MB-231 cells was further supported by the finding, that modulation of OPG secretion using IL-1β or dexamethasone prior to TRAIL exposure resulted in decreased and increased rate of apoptosis, respectively ($P < 0.05$). Thus, OPG secretion by breast cancer cells is modulated by cytokines and dexamethasone, and may represent a critical resistance mechanism that protects against TRAIL-induced apoptosis. J. Cell. Biochem. 108: 106–116, 2009.

KEY WORDS: APOPTOSIS; BREAST CANCER; OSTEOPROTEGERIN; TRAIL

Despite advances in breast cancer therapy, treatment options for women suffering from breast cancer metastases to bone remain limited to palliative measures. TNF-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor superfamily, has the specific ability over other members of this family to predominantly kill tumor cells, while sparing non-transformed cells [Wiley et al., 1995; Almasan and Ashkenazi, 2003], a crucial requirement for the potential use as an anti-cancer drug [Zhang et al., 2000]. So far, a total of five TRAIL receptors have been identified [Griffith and Lynch, 1998]. Membrane-bound death receptor-4 (DR4 or TRAIL-R1) and death receptor-5 (DR5 or TRAIL-R2) contain death domains [Pan et al., 1997; Walczak et al., 1997], which when activated by TRAIL initiate apoptosis by signaling through the death-inducing signaling complex. Initial recruitment of the adaptor protein Fas-associated death domain is followed by activation of caspase-8. This leads to an activation of the extrinsic

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(via effector downstream caspases) or the intrinsic (mitochondrial-mediated) pathway, subsequently causing cell death [LeBlanc and Ashkenazi, 2003]. Decoy receptor-1 (DcR1 or TRAIL-R3) and decoy receptor-2 (DcR2 or TRAIL-R4) are expressed on the cell surface, but lack functional intracellular death domains to induce apoptosis and, thus, act as decoy receptors for TRAIL [LeBlanc and Ashkenazi, 2003].

Osteoprotegerin (OPG), a secreted glycoprotein and member of the TNF receptor superfamily represents another receptor for TRAIL [Emery et al., 1998]. OPG plays an essential role in regulating bone homeostasis by binding to and neutralizing receptor activator of NF-κB ligand (RANKL) [Simonet et al., 1997], an essential cytokine for osteoclast activation and differentiation. Some tumors appear to cause an imbalance of the OPG/RANKL ratio, with a shift towards RANKL, which has been demonstrated to promote osteoclast activity and to enhance the development of osteolytic lesions, a characteristic feature of advanced breast cancer [Mundy, 1997]. More recently, RANKL has also been implicated in breast and prostate cancer cell migration into bone [Jones et al., 2006; Armstrong et al., 2008].

The ability to bind TRAIL and RANKL places OPG in the unique position of regulating two different, yet fundamental aspects of tumor biology: apoptosis and migration. Earlier reports questioned the biological relevance of the OPG-TRAIL interaction [Truneh et al., 2000]. However, a recent study using native proteins and more physiological conditions showed that OPG has a similar affinity to RANKL and TRAIL [Vitovski et al., 2007]. These new findings are consistent with results obtained from complex cell-based in vitro experiments, showing that OPG derived from bone marrow stromal cells promotes survival of myeloma cells [Shipman and Croucher, 2003] and protects breast and prostate cancer cells from TRAIL-induced apoptosis [Neville-Webbe et al., 2004; Nyambo et al., 2004]. In addition, some breast and prostate cancer cell lines produced sufficient OPG levels to evade cell death from TRAIL [Holen et al., 2002; Holen et al., 2005]. In vivo studies found that a large number of breast cancers show a strong expression of OPG and reported a significant positive correlation between OPG and estrogen receptor expression [Van Poznak et al., 2006]. In addition, high OPG expression has been linked with tumor aggressiveness in gastric carcinoma [Ito et al., 2003]. The presence of TRAIL in tumors has most commonly been reported to originate from tumor-invading monocytes in response to interferon-α and -γ [Takeda et al., 2002]. Interestingly, two recent studies of primary breast cancer samples showed that 30–56% of tumor samples highly express TRAIL [Cross et al., 2006a; Van Poznak et al., 2006].

Since the role and regulation of endogenously expressed TRAIL in breast cancer remain unclear, we tested the hypothesis that modulating OPG expression in TRAIL-sensitive breast cancer cell lines may affect their capacity to evade TRAIL-induced apoptosis.

MATERIALS AND METHODS

MATERIALS

Recombinant human IL-1β and TRAIL were from Preprotech (Hamburg, Germany). Dexamethasone was obtained from Sigma–Aldrich (Munich, Germany) and OPG, RANKL, and OPG antibodies were from R&D Systems (Wiesbaden, Germany). Neutralizing monoclonal antibodies against TRAIL-R1 and TRAIL-R2 were from Alexis Biochemical (Lausen, Switzerland). Anti-poly-ADP-ribose polymerase (PARP) antibody, anti-cleaved caspase-3 (Asp175), and anti-caspase-7 antibody were from Cell Signaling Technology (Frankfurt, Germany), anti-β-actin antibody was from Santa Cruz (Heidelberg, Germany). The ECL detection reagents were from Amersham Bioscience (Little Chalfont, UK). Propidium iodide was from Sigma–Aldrich and the annexin V-FITC apoptosis detection kit and caspase-3 inhibitors II and V from Calbiochem (Schwalbach, Germany). The OPG assay was from Immundiagnostik (Bensheim, Germany). Primers were from biomers.net (Ulm, Germany).

CELL CULTURES

Estrogen receptor (ER)-negative MDA-MB-231 cells were maintained in McCoy's medium (Bio West, Nuaillé, France), and ER-positive MCF-7 cells were maintained in RPMI 1640 medium (Bio West). Media were supplemented with 10% fetal bovine serum (PAN Biotech, Aidenbach, Germany) and 1% penicillin/streptomycin (Biochrom AG, Berlin, Germany). Cells were passaged after reaching 70% confluence using trypsin, and were kept at 37°C in a humidified atmosphere (5% CO_2). For analysis of OPG and TRAIL expression, cells were starved in medium containing 0.1% BSA 12 h prior to stimulation. For MTT assays, FACS, and Western blot analysis, cells were not serum-starved, but media were replaced prior to stimulation, unless otherwise stated.

MTT ASSAY

To analyze cell viability, cells were seeded and grown in 6-well plates. After reaching 70% confluence, cells were challenged with varying concentrations of TRAIL for a constant time period of 24 h. For analysis, 30 μl of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) substance was added to each well and incubated for 1 h at 37°C. Supernatant was then carefully removed and 400 μl of dimethyl sulfoxide was added to release incorporated MTT substance. After gently shaking the 6-well plates for 15 min, 100 μl of each probe was analyzed, and duplicate photometrical analysis at 570 nm was performed.

CELL DEATH DETECTION ASSAY

Apoptosis of both breast cancer cell lines was studied by the detection and quantification of cytoplasmic histone-associated DNA fragments (mono- and oligonucleasomes) using a photometric enzyme immunoassay (Cell Death Detection $ELISA_{PLUS}$, Roche Diagnostics, Indianapolis, IN).

ANNEXIN V/PROPIDIUM IODIDE STAINING

Apoptotic cell death was analyzed by annexin V-FITC staining using an annexin V kit (BenderMedSystems, Vienna, Austria). For assessment of apoptosis, 5×10^4 cells were cultured in 6-well plates and treated with 50 ng/ml of TRAIL after pretreatment with IL-1β or dexamethasone. After 24 h, cells were washed with cold PBS, and 1×10^5 cells were resuspended in 195 μl cold 1× binding buffer plus 5 μl annexin V followed by 10 min incubation at room temperature. Cells were washed again and resuspended with 190 μl

binding buffer. Ten microliters of propidium iodide from Sigma–Aldrich at a concentration of 20 μg/ml were added immediately before analysis. Apoptosis was assessed by dual color flow cytometry on a FACScan cytofluorometer from Becton Dickinson (Mountain View, CA) using Cell Quest software package (Becton Dickinson). Annexin V-positive cells were considered early apoptotic, annexin- and propidium iodide-positive cells late apoptotic/necrotic. Unstained cells were classified as viable cells.

CELL CYCLE ANALYSIS

Cell cycle analysis was performed by flow cytometry. Gene silencing of OPG was performed using small interfering RNA (siRNA) as described below. Twenty-four hours after the second transfection, cells were trypsinized and washed once with PBS, fixed in 70% ethanol, and stored at 4°C for subsequent cell cycle analysis. Fixed cells were washed with PBS and centrifuged to remove ethanol. Cells were incubated in the presence of 20 μg/ml RNase, DNase-free water (Roche Molecular Biochemicals, Mannheim, Germany) with 50 μg/ml propidium iodide for 3 h at room temperature in the dark. The DNA content of 1×10^6 stained cells was analyzed on a Becton Dickinson FACS Calibur flow cytometer. The fractions of cells in the G0/G1, S, and G2/M phases were calculated using Cell Quest software (Becton Dickinson, Topsham, ME).

RNA ISOLATION

Total RNA was isolated using the RNeasy kit from Qiagen (Hilden, Germany). Ten micrograms of total RNA were used for Northern blot analysis of OPG and TRAIL mRNA. RNA was quantified using a BioPhotometer (Eppendorf, Hamburg, Germany).

RT–PCR

The Omniscript RT kit from Qiagen was used for the reverse transcription of 2 μg of RNA. RT-PCR was performed on a Genius thermocycler (Labtech International, Burkhardtsdorf, Germany) with 30 cycles. Primer sequences were: TRAIL sense 5′-AAAGAG-CAACAATCCATCTCT-3′, TRAIL anti-sense 5′-TGGTGCCTC-TTCT-CTCTTTTG-3′. Samples were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

REAL-TIME PCR (qRT-PCR)

Real-time PCR was performed using a 7500 Fast-Real-Time-PCR-System from Applied Biosystems (Foster City, CA) and a standard protocol. Cyclophilin was used as housekeeping gene. Primer sequences were chosen using PrimerExpress 3.0 (Applied Biosystems) and were as follows: OPG sense 5′-GCTAACCTCACCTTCGAG-3′, OPG anti-sense 5′-TGATTGGACCTGGTTACC-3′, TRAIL sense 5′-CTTCACAGTGCTCCTGCAGT-3′, TRAIL anti-sense 5′-TTAGC-CAACT-AAAAAGGCCCC-3′, TRAIL-R1 sense 5′-CAAGACCTTCAA-GTTTGTCGTCGTC-3′, TRAIL-R1 anti-sense 5′-TGCTGCACTTCC-GGCACATCTC-3′, TRAIL-R2 sense 5′-GGTTTTGTTGTTTAGTTACA-TTAGGCTTT-3′, TRAIL-R2 anti-sense 5′-ACACCAA-CCACGAGT-GACACA-3′, cyclophilin sense 5′-CCCTCCACCCATTTGCT-3′, and cyclophilin anti-sense 5′-CAATCCAGCTATGGGA-3′.

NORTHERN BLOT ANALYSIS

After separation on a denaturing 1.5% (w/v) agarose gel containing 2.2 M of formaldehyde, RNA was transferred onto a nylon membrane by capillary blotting. The human OPG, TRAIL, and β-actin cDNA insert were radiolabeled with 5 μl [α-^{32}P]dCTP using random primer DNA labeling [Hofbauer et al., 1999a]. All experiments were repeated at least twice, and representative blots are shown. Control hybridization with human β-actin cDNA verified equal RNA loading.

OPG PROTEIN ASSAY

Conditioned medium was harvested from cultured cells before RNA analysis and centrifuged to remove debris. Samples were stored at −20°C until analysis. OPG protein content was determined in triplicate measurements with an immunoassay from Immundiagnostik as described elsewhere [Viereck et al., 2003].

WESTERN BLOT ANALYSIS

Breast cancer cells were lysed in ice-cold buffer (1% Triton X-100, 10% glycerol, 50 mM HEPES pH 7.4, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 5 mM sodium vanadate, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 1.5 mg/ml benzamidine, and 34 μg/ml phenylmethylsulfonyl fluoride) and sonicated. Insoluble cell debris was removed by centrifugation at 10,000 rpm for 5 min. Protein content was quantified by the Bradford method, and 5 μg were separated by 12% SDS–PAGE for PARP and 15% SDS–PAGE for cleaved caspase-3 and -7 and transferred onto nitrocellulose membranes. After blocking in 5% non-fat dry milk for 1 h, membranes were incubated overnight at 4°C with antibodies to PARP and cleaved caspase-3 and -7. Signals were detected using horseradish peroxidase-conjugated anti-rabbit IgG antibody and chemoluminescent reaction with ECL detection reagent.

TIME-LAPSE MICROSCOPY

For time-lapse experiments, cells were seeded at 5×10^4 cells/well and cultured in 6-well plates in the presence of TRAIL and/or OPG, with the cells kept in serum. Phase-contrast photographs were taken every 30 min by a live cell imaging system with an Axiovert inverted microscope (Carl Zeiss, Jena, Germany).

SMALL INTERFERING RNA (siRNA)

Pre-designed siRNA for OPG (Hs_TNFRSF11B_2 HP and Hs_TNFRSF11B_4 HP siRNA) was purchased from Qiagen, Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transfection. Cells were transfected twice using 50 pmol of siRNA following a supplied protocol, and 24 h after the second transfection cells were harvested for further analysis. Controls were transfected with non-target siRNA.

STATISTICAL ANALYSIS

Each experiment was reproduced at least twice. Values for protein measurement are expressed as the mean ± SD of triplicate measurements unless otherwise stated. Student's paired *t*-test was used to analyze differences between the sample of interest and its control. Time course and dose responses were compared by multiple

measurements ANOVA and corrected by Student–Newman–Keul's test for differences between groups. A P-value of less than 0.05 was considered statistically significant.

RESULTS

To analyze effects of IL-1β on ER-negative MDA-MB-231 and ER-positive MCF-7 cells, we performed dose-response experiments with exposure of IL-1β at concentrations ranging from 0 to 10 ng/ml for 6 h in serum-starved cells. In addition, time-dependent effects were analyzed, using 10 ng/ml of IL-1β for 0–48 h. OPG mRNA steady state levels were assessed by Northern blot analysis (Fig. 1A). Both cell lines increased OPG mRNA expression following IL-1β exposure in a dose- and time-dependent manner. These findings were confirmed by real-time PCR. After 6 h, MCF-7 cells showed 9-fold ($P < 0.01$) and MDA-MB-231 cells 2-fold ($P < 0.05$) higher OPG mRNA level compared to controls. In agreement with these findings, OPG protein concentrations secreted by MCF-7 and MDA-MB-231 cells were elevated by 2.5-fold ($P < 0.0001$) and 1.5-fold ($P < 0.01$) after 48 h of stimulation (Fig. 1A). Similar experiments were conducted with the glucocorticoid dexamethasone. When stimulated with increasing concentrations (0–10^{-7} M) and increasing duration (0–24 h), we observed a dose- and time-dependent decrease of OPG mRNA levels in both cell lines as assessed by Northern blot analysis (Fig. 1B). At the highest dexamethasone concentration of 10^{-7} M, OPG mRNA levels were suppressed below the lower limit of detection, and real-time PCR revealed a decrease of OPG mRNA levels in MCF-7 and MDA-MB-231 cells to 31% ($P < 0.0001$) and 50% ($P < 0.01$) of baseline levels, respectively. Analysis of OPG protein levels in the supernatant showed that OPG secretion was suppressed by dexamethasone by 79% in MCF-7 and by 53% in MDA-MB-231 cells, respectively (Fig. 1B).

Since recent reports showed that 30% of primary breast cancers express TRAIL, we next analyzed the expression of TRAIL gene expression in MCF-7 cells and MDA-MB-231 cells by PCR. While MDA-MB-231 cells showed a strong expression of TRAIL mRNA steady state levels, TRAIL mRNA levels were undetectable in MCF-7 cells. The osteosarcoma cell line SaOS-2 was used as a positive control and showed robust TRAIL expression (Fig. 2A). Having established that MDA-MB-231 cells, but not MCF-7 cells express TRAIL, we analyzed if IL-1β or dexamethasone regulated its expression. MDA-MB-231 cells were stimulated with either IL-1β (10 ng/ml) or dexamethasone (10^{-7} M) for 6 h, and TRAIL mRNA levels were analyzed by real-time PCR. IL-1β induced TRAIL mRNA levels by 2.5-fold ($P < 0.01$), while dexamethasone decreased TRAIL mRNA expression by 70% ($P < 0.001$) (Fig. 2B). Furthermore, Northern blot analysis demonstrated a dose-dependent increase of TRAIL mRNA levels following treatment with IL-1β and showed an inhibition after dexamethasone exposure at a dose of 10^{-7} M. TRAIL supplementation did not upregulate OPG expression, nor was endogenous TRAIL expression downregulated (data not shown). To

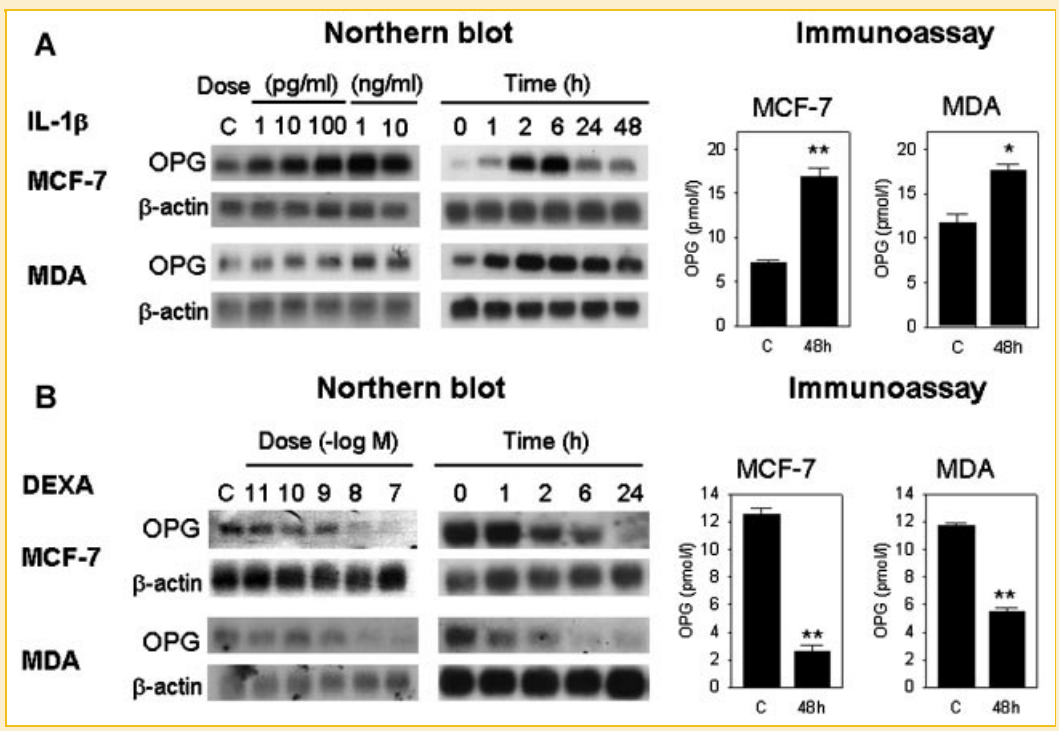

Fig. 1. Interleukin-1β and dexamethasone modulate OPG secretion in MCF-7 and MDA-MB-231. A: OPG mRNA levels and protein secretion were determined following stimulation with IL-1β. For time kinetics, IL-1β was used at a concentration of 10 ng/ml, dose responses were analyzed after 6 h (mRNA level) or 48 h (protein secretion). B: OPG mRNA levels and protein secretion following stimulation with dexamethasone. For time kinetics, dexamethasone was used at a concentration of 10^{-7} M. For dose response studies, the cells were stimulated for 6 h. OPG and β-actin were analyzed by Northern blot. The values represent the mean ± SD from triplicate measurements. $^{*}P < 0.01$, $^{**}P < 0.001$ by Student's t-test. C, control (untreated cells); DEXA, dexamethasone; IL, interleukin; OPG, osteoprotegerin.

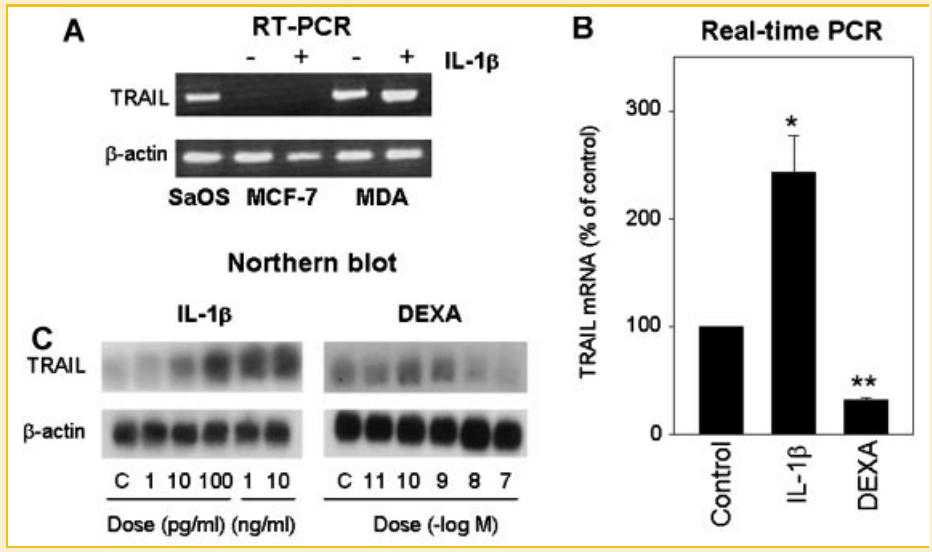

Fig. 2. Interleukin-1β enhances and dexamethasone suppresses TRAIL mRNA expression in MDA-MB-231 cells. A: RT-PCR for TRAIL mRNA expression. Basal and IL-1β stimulated expression (10 ng/ml for 6 h) were studied. Cells were serum-starved and SaOS cells were used as a positive control. β-Actin was used as house-keeping gene. B: Real-time PCR to analyze TRAIL expression of MDA-MB-231 following IL-1β and dexamethasone stimulation. Cells were serum-starved and stimulated with IL-1β (10 ng/ml) or dexamethasone (10^{-7} M) for 6 h. Expression is shown relative to control and normalized to cyclophilin. Data are given as the mean ± SD values from 2 individual experiments. $^{*}P < 0.01$, $^{**}P < 0.001$. C: Northern blot analysis of TRAIL expression. Serum-starved cells were dose-dependently stimulated with IL-1β (2 h) or dexamethasone (6 h). Representative blots are shown. C, control (untreated cells); DEXA, dexamethasone; IL, interleukin; OPG, osteoprotegerin; TRAIL, TNF-related apoptosis-inducing ligand.

study the effects of TRAIL on breast cancer cells, both cell lines were challenged for 24 h with increasing concentrations of TRAIL, ranging from 0 to 100 ng/ml. Cell viability was assessed using an MTT assay. While MDA-MB-231 cells showed a significant dose-dependent decrease in viability ($P < 0.01$ by ANOVA), MCF-7 cells remained unaffected by the presence of TRAIL (Fig. 3A). To extend these findings, cells were challenged with 50 ng/ml of TRAIL for up to 6 h. Activation of the caspase cascade was then assessed by Western blot analysis. In agreement with our initial findings, we detected cleaved forms of caspase-3 and -7 in MDA-MB-231 cells after TRAIL exposure for 2 h. Furthermore, PARP cleavage was observed after 4 h, clearly indicating an activation of intracellular signaling pathways of apoptosis. However, no cleavage of caspase-3, -7, or PARP was detected in MCF-7 cells (Fig. 3B). As reported, MCF-7 cells lack caspase-3 due to a 47-base pair deletion within exon 3 of the CASP-3 gene, which results in impaired translation of CASP-3 mRNA [Jänicke et al., 1998; Jänicke, 2009]. Inhibition of caspase-3 in MDA-MB-231 cells using two different specific caspase-3 inhibitors, TRAIL-induced apoptosis decreased from 18.6% to 8.0% and 9.8%, respectively (Fig. 3C). We next analyzed TRAIL-R1 and TRAIL-R2 mRNA expression. MDA-MB-231 showed a 5-fold stronger mRNA expression of both TRAIL-R1 and TRAIL-R2 compared to MCF-7 cells, with TRAIL-R2 being the predominantly expressed receptor (Fig. 4A). Of note, concurrent incubation of TRAIL (100 ng/ml) with a neutralizing antibody against TRAIL-R2 (500 ng/ml) resulted in reversal of the TRAIL-induced effects on cell viability after TRAIL-R2 neutralization (from 28% cell loss to 12%, respectively), whereas neutralization of the TRAIL-R1 (500 ng/ml) had only a minor effect (Fig. 4B). These findings indicate that TRAIL-R2 is the functionally more relevant death-receptor in mediating breast cancer apoptosis. Of note, immunoclearance of OPG did not increase TRAIL sensitivity of MCF-7 cells (2.8% apoptotic cells in control vs. 2.6% apoptotic cells immuno-cleared of OPG) as assessed by FACS analysis, indicating that factors other than OPG account for their resistance to TRAIL. Due to the observed resistance of MCF-7 cells towards TRAIL, further experiments were conducted with TRAIL-sensitive MDA-MB-231 cells.

Detailed analysis of TRAIL effects on MDA-MB-231 cells using annexin V/propidium iodide staining revealed a dose-dependent increase of apoptosis. Maximum effects after 24 h of stimulation were obtained with concentrations ranging between 100 and 150 ng/ml. We next analyzed the ability of OPG to reverse the pro-apoptotic TRAIL effects. Time-lapse microscopy showed that the effects of TRAIL (50 ng/ml) on cell morphology and cell number could be largely rescued by adding similar concentrations of OPG (Fig. 5A). When OPG was added to cells that had been exposed to TRAIL (50 ng/ml), OPG dose-dependently increased cell survival (Fig. 5B). An OPG concentration of 50 ng/ml was sufficient to significantly ($P < 0.05$) prevent the pro-apoptotic effects initiated by similar concentrations of TRAIL (Fig. 5C).

To investigate the physiological relevance of our findings, we conditioned medium for 3 d, to test if the amount of secreted OPG from MDA-MB-231 cells was sufficient to protect cells against TRAIL-induced apoptosis. Thereafter, culture medium was removed from the cells and diluted in one-third of fresh medium, which was subsequently transferred onto freshly seeded cells. FACS analysis revealed that cells challenged with 50 ng/ml TRAIL showed a significantly lower increase in annexin V-positive apoptotic cells when kept in conditioned medium (1.3-fold) compared to those kept in fresh medium (4.1-fold, $P < 0.01$). Addition of a 20-fold higher concentration of RANKL, the cognate ligand for OPG, reversed the anti-apoptotic effect of medium conditioned for 72 h from a

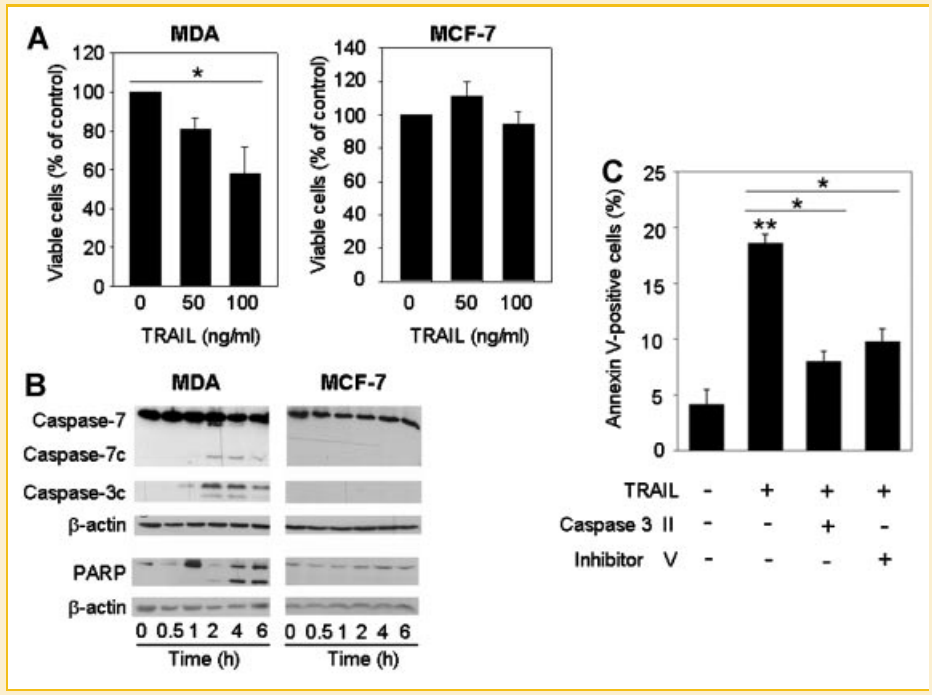

Fig. 3. MDA-MB-231 but not MCF-7 cells are sensitive to TRAIL-induced apoptosis. A: MTT assay to assess cell viability following exposure of 0–100 ng/ml TRAIL for 24 h. Data are shown as percentage of viable cells compared to control and represent the mean ± SD of 3 individual experiments. $^{*}P < 0.01$ by ANOVA. B: Western blot analysis of caspase-3, -7, and PARP cleavage in MDA-MB-231 and MCF-7 cells. Caspase activation was analyzed after exposure of 50 ng/ml TRAIL for various time points as indicated. C: Inhibition of caspase-3 in MDA-MB-231 cells using two different specific caspase-3 inhibitors. Two hours before administration of TRAIL at 50 ng/ml, caspase 3 inhibitor II (50 μM) and V (25 μM) were added. Apoptosis was assessed using annexin V staining after 24 h. Data are shown as the mean percentage ± SD of three individual experiments. $^{*}P < 0.01$, $^{**}P < 0.001$ by Student's *t*-test. Caspase-3c and -7c, cleaved caspase-3 and -7; IL, interleukin; OPG, osteoprotegerin; PARP, poly-ADP-ribose polymerase; TRAIL, TNF-related apoptosis-inducing ligand.

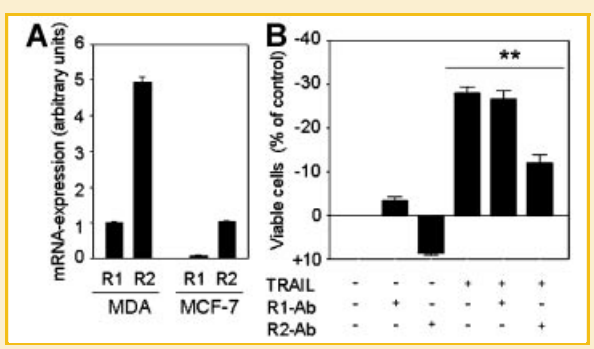

Fig. 4. TRAIL-R2 is the more relevant TRAIL receptor. A: Real-time PCR to compare TRAIL-R1 and TRAIL-R2 expression in MDA-MB-231 and MCF-7 cells. Cells were serum-starved and expression is shown normalized to cyclophilin. RNA was obtained and analyzed from two individual experiments. B: Specific blocking of TRAIL-R1 and TRAIL-R2 with a neutralizing antibody (500 ng/ml). Two hours before administration of TRAIL at 100 ng/ml, TRAIL-R1 and TRAIL-R2 antibodies were added, and cell viability was analyzed after further 24 h. Data are shown as the mean percentage ± SD of viable cells from triplicate measurement compared to control. $^{**}P < 0.001$ by Student's *t*-test. R1, TRAIL receptor-1; R2, TRAIL receptor-2; TRAIL, TNF-related apoptosis-inducing ligand.

1.3- to 2.4-fold increase of apoptosis ($P < 0.05$) (Fig. 6A). Next, we repeatedly stimulated cells every 24 h with either IL-1β (10 ng/ml) or dexamethasone (10^{-8} M). After 3 days the amount of secreted OPG was determined from the supernatant (Fig. 6B). As expected, cells treated with IL-1β released significantly more OPG, while dexamethasone-treated cells showed lower OPG secretion ($P < 0.001$). The supernatant was then supplemented with TRAIL (50 ng/ml), and cell viability was analyzed 24 h later. We observed an inverse correlation between OPG concentrations and apoptosis. Cells that had been stimulated with IL-1β and secreted the highest OPG concentrations showed significantly higher survival than cells that had been stimulated with dexamethasone, when challenged with TRAIL ($P < 0.05$). These effects became even more apparent, when cell survival and apoptosis were directly compared to their controls (Fig. 5C). IL-1β-treated cells showed a lower percentage of non-viable (annexin V- and/or propidium iodide-positive) cells after TRAIL addition compared to untreated cells ($P < 0.05$). Interestingly, these protective effects were mainly mediated by reducing the necrotic/late apoptotic (double positive) rather than the apoptotic (annexin V-positive) fraction. By contrast, cells that had been stimulated with dexamethasone showed a significantly higher vulnerability to TRAIL exposure than untreated controls ($P < 0.05$).

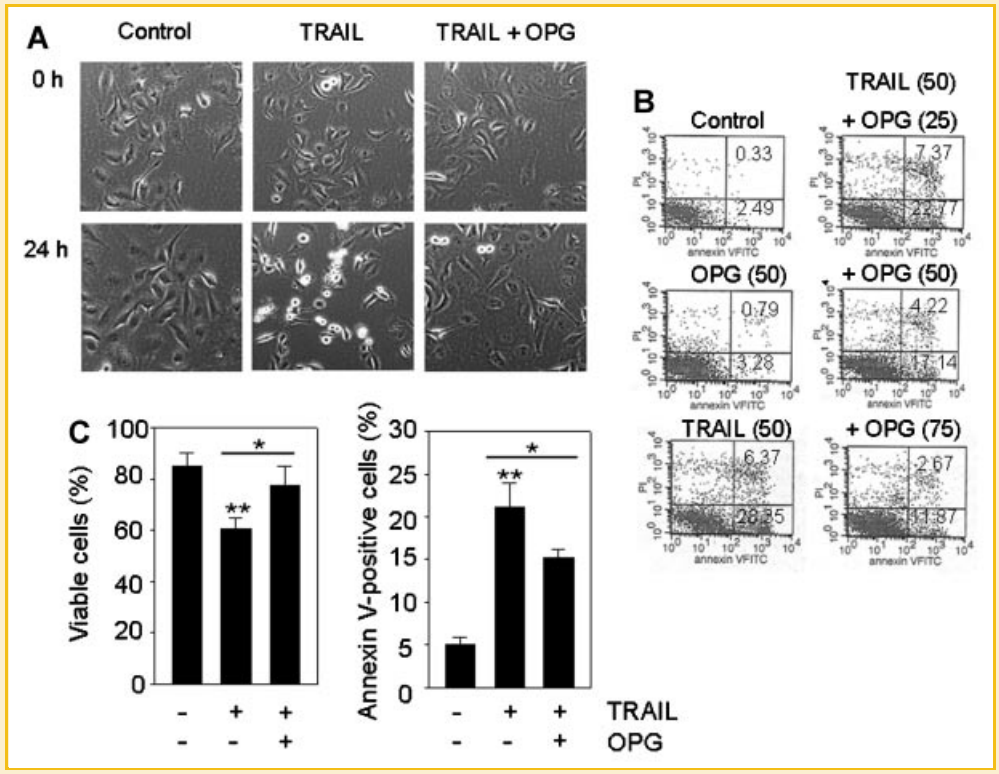

Fig. 5. Exogenous OPG dose-dependently inhibits apoptotic effects of TRAIL. A: Time-lapse microscopy following TRAIL exposure. MDA-MB-231 cells show clear changes in cell morphology and decreased cell number following TRAIL exposure (50 ng/ml). These effects were reversed by exogenous OPG at 50 ng/ml. B: FACS analysis of MDA-MB-231 cells following 24 h of TRAIL and/or OPG exposure. Concentrations are given in ng/ml and representative results are shown. C: Percentage of viable (non-stained) and annexin V-positive cells following exposure to TRAIL (50 ng/ml) and a combination of TRAIL and OPG (each at 50 ng/ml). Data are given as the mean ± SD from two individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. IL, interleukin; OPG, osteoprotegerin; TRAIL, TNF-related apoptosis-inducing ligand.

Having established a correlation between secreted OPG levels and survival from supplemented TRAIL, we investigated the role of OPG as a survival factor in more detail. We used siRNA to transiently knock-down OPG expression and secretion in MDA-MB-231 and MCF-7 cells. Successful knock-down was verified by real-time PCR and ELISA (Fig. 7A,B). MTT assays were performed 24 h after transfection. OPG knock-down resulted in a decreased number of viable MDA-MB-231 cells, while it did not affect the cell number of TRAIL-insensitive MCF-7 cells (Fig. 7C). To further characterize the mechanism(s) of decreased cell number, cell cycle and apoptosis were analyzed in MDA-MB-231 cells. While the cell cycle remained largely unchanged (data not shown), the rate of apoptosis was significantly higher in cells that were depleted of OPG than in non-target siRNA-treated controls (Fig. 7D).

DISCUSSION

The notion that some breast cancer cells secrete sufficient levels of OPG to evade TRAIL-induced apoptosis [Holen et al., 2005] prompted us to investigate if modulating OPG secretion affects breast cancer apoptosis. OPG is known to be regulated by a variety of cytokines, hormones, and drugs in bone and other cells [Hofbauer et al., 1999a,b; Collin-Osdoby et al., 2001], but the local regulators of OPG expression in breast cancer cells have not been characterized in detail. IL-1β has previously been reported to increase OPG production by breast cancer cell lines [Kapoor et al., 2008], and we have recently shown that estrogens suppress OPG expression in ER-α-positive MCF-7 cells [Rachner et al., 2008].

The concept that substances that downregulate OPG may improve TRAIL susceptibility formed our concept to analyze effects of dexamethasone on OPG expression in ER-α-positive MCF-7 and ER-α-negative MDA-MB-231 cells. While estrogens downregulate OPG in MCF-7 cells [Rachner et al., 2008], a recent study showed that ER-positive cell lines are largely TRAIL-resistant [Rahman et al., 2009]. Thus, OPG-lowering strategies would most likely not affect cell survival and might even promote skeletal metastasis, since breast cancer migration is promoted by RANKL [Jones et al., 2006], which in turn is inhibited by OPG. Dexamethasone has been unambiguously shown to decrease OPG production in bone cells [Hofbauer et al., 1999a; Kondo et al., 2008], and glucocorticoids are applied as co-treatment with chemotherapy in breast cancer. The glucocorticoid receptor may regulate breast cancer biology [Moutsatsou and Papavassiliou, 2008]. For instance, dexamethasone increased drug efficacy by downregulating the breast cancer resistance protein ABCG2 in vitro [Honorat et al., 2008] and induced sodium iodine symporter expression in breast cancer cells in vivo [Willhauck et al.,

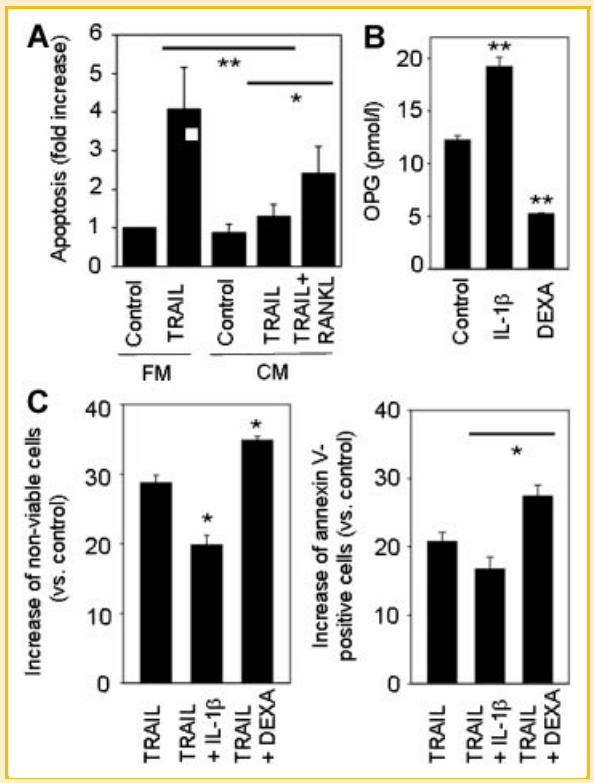

Fig. 6. TRAIL mediated apoptosis is inversely correlated with OPG secretion. A: Medium of MDA-MB-231 cells was conditioned for 3 days. Subsequently, cells were challenged with TRAIL (50 ng/ml). Significantly less cells stained annexin V-positive in conditioned medium (CM) compared to fresh medium (FM), which could be reversed by a 20-fold higher concentration of RANKL. Data are given as a fold increase compared to control and represent the mean ± SD from four individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. B: OPG levels measured in the supernatant following IL-1β (10 ng/ml) or dexamethasone (10^{-7} M) stimulation. $^{**}P < 0.001$. C: Cells were treated with IL-1β or dexamethasone for 3 d and then challenged with TRAIL for another 24 h. Cell viability was analyzed using annexin V/propidium iodide staining. Data are given as mean ± SD values from two individual experiments, compared to similarly pre-treated cells without TRAIL. $^{*}P < 0.05$ by Student's *t*-test. IL, interleukin; OPG, osteoprotegerin; TRAIL, TNF-related apoptosis-inducing ligand.

2008], which would facilitate radioiodine therapy. Here, we found that dexamethasone suppressed OPG production in a dose- and time-dependent fashion, at a substantial degree, and irrespective of the ER status. By contrast, the pro-inflammatory cytokine IL-1β enhanced OPG production time- and dose-dependently. Maximum effects were observed after 6 h, but effects could be detected as long as 48 h after stimulation. These findings indicate that OPG production by breast cancer cells is subject to systemic regulators of bone and tumor cell biology, such as cytokines and hormones. While the effects of IL-1β and dexamethasone occurred irrespective of the ER status, effects on OPG expression were consistently stronger in the more differentiated ER-positive MCF-7 cells.

In accordance with a study by Rahman et al. [2009], MCF-7 cells were not affected by TRAIL, whereas TRAIL dose-dependently increased the rate of apoptosis in MDA-MB-231 cells. Of interest, TRAIL effects were fully reversed by similar concentrations of exogenous OPG, suggesting that the affinity of OPG to TRAIL may be higher than previously expected, and physiologically relevant [Vitovski et al., 2007]. Furthermore, we found a correlation between OPG secretion and breast cancer survival. In keeping with these findings, breast cancer cells with suppressed OPG secretion induced by dexamethasone were more susceptible to TRAIL-induced apoptosis compared to untreated cells. Vice versa, IL-1β-treated cells that upregulated OPG production were most resistant. While previous studies suggested that local sources of OPG are irrelevant to protect cells from TRAIL [Shipman and Croucher, 2003; Neville-Webbe et al., 2004; Nyambo et al., 2004], our in vitro data indicate a survival advantage for breast tumors that secrete high levels of OPG or are located in areas with high OPG production such as bone. Clearly, this concept needs to be validated by further in vivo studies.

Most studies have focused on the ability of OPG to protect the cells against exogenous TRAIL. The role of endogenous TRAIL expression has not been investigated in detail, although a considerable number of primary breast cancers express TRAIL [Van Poznak et al., 2006]. We show that TRAIL-sensitive MDA-MB-231 cells, unlike MCF-7, express high endogenous levels of TRAIL mRNA that is regulated by IL-1β and dexamethasone. Intriguingly, deletion of endogenous OPG enhanced the TRAIL/OPG ratio and caused an increased rate of apoptosis. A recent study performed in osteoclasts confirmed the functional antagonism of endogenous TRAIL and exogenous OPG in regulating apoptosis [Chamoux et al., 2008]. In general, benign breast tumors and normal breast tissue do not express OPG [Cross et al., 2006b], indicating that OPG expression is acquired during tumorigenesis and may contribute to tumor progression by blunting the TRAIL response. Of interest, mammary epithelial cells are able to produce TRAIL, possibly as an intrinsic defense mechanism against malignant transformation [Sohn et al., 2001]. It still remains unclear why and at what stage of progression some breast cancer cells gain TRAIL sensitivity while others do not.

Although TRAIL safety has been shown by in vivo studies [Walczak et al., 1999], the complexity of a cytokine system comprised of five receptors may result in unpredictable pharmacological interactions, which has led to the development of specific agonistic antibodies for TRAIL-R1 and TRAIL-R2 [Ichikawa et al., 2001, 2003]. Constitutive and acquired TRAIL resistance still remains a problem, if low concentrations are applied [Li et al., 2006]. Combination of TRAIL with anti-cancer drugs may reverse acquired TRAIL resistance and could therefore potentiate its anti-tumor effects [Gliniak and Le, 1999; Keane et al., 1999; Matsuzaki et al., 2001].

Especially triple negative tumors (lacking estrogen and progesterone receptors as well as HER-2 amplification) which offer limited treatment options may benefit from TRAIL treatment or selective TRAIL-R1/TRAIL-R2 stimulation, if the in vitro findings demonstrating TRAIL-sensitivity [Rahman et al., 2009] can be corroborated in primary cancer cells. TRAIL susceptibility of breast cancer cells may be augmented by agents that suppress OPG expression. In this regard, dexamethasone may be a useful substance to combine with TRAIL, since it reduces OPG expression in a wide range of different

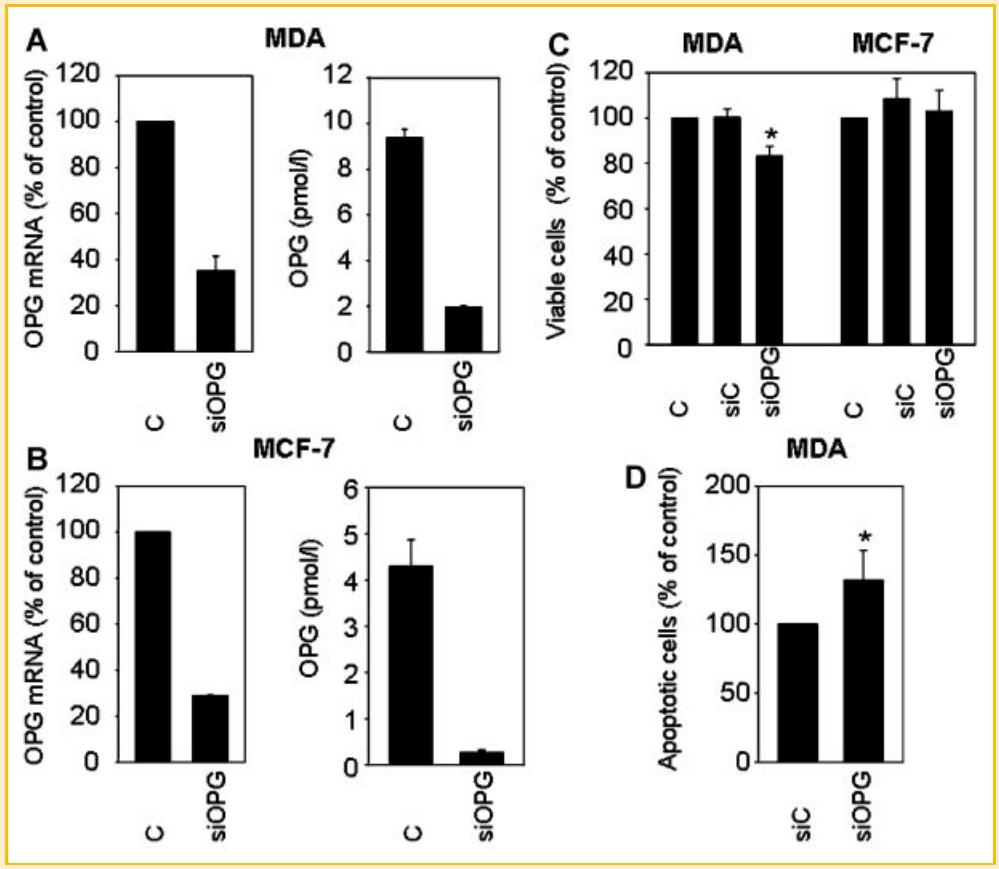

Fig. 7. OPG gene-silencing decreases cell viability in MDA-MB-231 by promoting apoptosis. A,B: Transient knock-down of OPG in MDA-MB-231 and MCF-7 cells using siRNA. Real-time PCR analysis was performed to verify inhibition of OPG mRNA levels. Supernatant was analyzed to show suppressed OPG protein secretion. C: MTT assay to analyze cell viability after OPG depletion. MDA-MB-231 cells showed decreased cell viability, whereas MCF-7 cells were not affected. Data are given as the mean ± SD from three individual experiments. $^{*}P < 0.05$. D: Apoptosis was analyzed using a cell death detection ELISA (see Materials and Methods Section), following OPG knock-down in MDA-MB-231 cells. Data are given as the mean ± SD from five individual transfections and are normalized to control. $^{*}P < 0.05$. OPG, osteoprotegerin; siC, non-target siRNA; siOPG, siRNA for osteoprotegerin.

cells, while the TRAIL/OPG ratio remains unchanged. Further in vivo studies are required to evaluate the efficacy and safety of such combination therapy.

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