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Sorafenib Inhibits Proliferation, Migration and Invasion of Breast Cancer Cells

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Keywords
Breast cancer · Metastasis · Epithelial-to-mesenchymal transition · Cancer stem cells · ERK1/2

Abstract
Introduction: Metastatic breast cancer has poor prognosis due to limited therapeutic options. Protein kinase dysregulations have a major role in breast cancer progression and metastasis. In this study, we investigated the anti-cancer activity of sorafenib, a multikinase inhibitor, which targets receptor tyrosine kinases in breast cancer. Although treatment with sorafenib has increased the patient survival and inhibited metastatic migration in hepatocellular carcinoma, its role in breast cancer migration, metastasis, and intracellular signaling modulation is unknown. ***Methods:*** Breast cancer cell lines MCF7 and MDA-MB-231 were treated with sorafenib and its effect on proliferation, migration, invasion and gene expression was analyzed. ***Results:*** We found that sorafenib has an anti-proliferative and cytotoxic effect on breast cancer cells. Importantly, sorafenib inhibited the migration and invasion of breast cancer cells in vitro. Mechanistically, sorafenib increased mitochondrial superoxide production, suppressed breast cancer stem cell self-renewal, inhibited epithelial mesenchymal transition and ERK signaling. ***Conclusion:*** Thus, sorafenib has anti-cancer activity against breast cancer cells and could improve the survival of breast cancer patients by inhibiting their invasive and metastatic properties.

Introduction

Breast cancer accounts for 30% of the newly diagnosed cases of cancer in women [1–3], with most cancer-related deaths occurring as a result of metastasis to distal organs [4]. Breast cancer metastasis is a sequential process driven by several factors and it begins with local invasion in the host tissue [4–6]. Dysregulation of cell adhesion molecules [7] and integrins [8], overexpression of metalloproteinases (MMPs) [9] and epithelial-to-mesenchymal transition promote invasive outgrowth of metastatic breast cancer cells. Breast cancer stem cells contribute toward the development of metastasis because of their inherent resistance to chemotherapeutic drugs. Activation of intracellular signaling pathways such as ERK1/2 and p38 MAPK have been shown to advance tumor progression [10, 11].

Sorafenib is an FDA-approved multikinase inhibitor for hepatocellular carcinoma (HCC) and renal cell cancer

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[12–14]. It is a potent inhibitor of cell surface receptor tyrosine kinases such as VEGF and PDGF receptor kinases, Flt-3, and c-kit [13]. In addition, sorafenib also inhibits intracellular serine/threonine kinases such as Raf-1 [12]. In HCC cells, sorafenib treatment inhibited cell adhesion, cell cycle and cell proliferation-related genes and resulted in an overexpression of apoptosis-related genes [15]. Anti-tumor activity of sorafenib in HCC was attributed to inhibition of angiogenesis and downregulation of RAF/MEK/ERK pathway [16].

Sorafenib has been tested in breast cancer clinical trials as a monotherapeutic agent as well as in combination with other cytotoxic drugs (taxanes, capecitabine, etc.), and endocrine therapy, in which some of the combinations were found to be promising [17]. However, molecular effects of sorafenib on breast cancer cells were not studied and hence not well understood. We studied the effect of sorafenib on breast cancer cell proliferation, breast cancer stem cells, migration and local invasion. Treatment with sorafenib led to decreased cell proliferation in breast cancer cells with an arrest in the G0/G1 phase. Breast cancer cell migration and local invasion capacity were adversely affected by sorafenib treatment, along with an increase in E-cadherin and a reduction in MMP-9 gene expression. Sorafenib treatment led to a reduction in $CD44^{+}/CD24^{-/lo}$ cells in MDA-MB-231 metastatic breast cancer cells. We studied the effect of sorafenib on intracellular signaling pathways and found that phosphorylation of ERK1/2, p38 MAPK and STAT5 were downregulated. Hence, we report important inhibitory effects of sorafenib treatment on the invasive and metastatic potential of breast cancer cells.

Materials and Methods

Reagents and Cell Lines

DMEM, collagen, protease inhibitor cocktail, propidium iodide, phalloidin-TRITC and DAPI (4′,6-diamidino-2-phenylindole) were purchased from Sigma-Aldrich (India). Fetal bovine serum, recombinant human epidermal growth factor (EGF), phosphatase inhibitor cocktail, anti-human antibodies against phospho-ERK1/2, E-cadherin, GAPDH, fluorescent dye conjugated antibodies against EpCAM, CD24, and HRP conjugated secondary antibodies were from Thermo Fisher Scientific. Fluorescent dye conjugated antibodies against CD44, the phosphorylated form of p38 MAPK, and STAT5 were obtained from BD Biosciences. Sorafenib was purchased from Natco Pharma Ltd. (India). Breast cancer cell lines MCF7 and MDA-MB-231 were obtained from National Center for Cell Sciences, Pune (India). Cell culture dishes, flasks and plates were obtained from Eppendorf India. Coverslip bottom imaging dishes were purchased from ibidi GmbH (Germany).

Cell Cycle Analysis

Cell cycle analysis was done as previously described [18]. Briefly, the cells were fixed and permeabilized with 70% ice-cold ethanol for 30 min at 4°C. The cells were treated with RNAse A and stained with propidium iodide to stain the cellular DNA. Cell cycle was analyzed by flow cytometry.

Mitochondrial Superoxide Analysis

Superoxide production in the treated cells was determined using the Mitosox red mitochondrial superoxide indicator kit (Life Technologies) according to the manufacturer's instructions. Briefly, the Mitosox reagent was added to the cells and incubated at 37°C for 10 min and Mitosox fluorescence was analyzed by flow cytometry.

Phospho-Protein Flow Cytometry

Identification of phosphorylated forms of proteins was done through flow cytometry as previously described [19–21]. Briefly, the cells were fixed with formaldehyde (2%), permeabilized with ice-cold methanol (100%) and stained with fluorescent dye conjugated anti-phospho-protein specific antibody for 1 h at room temperature. Phospho-protein expression levels were analyzed through flow cytometry.

Gene Expression Analysis

For gene expression analysis, total RNA was extracted from the cells using Purelink RNA mini kit (Thermo Fisher Scientific) following the manufacturer's instructions. The RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and OligodT primers following the manufacturer's instructions. Gene expression at mRNA levels was analyzed by real-time PCR using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) with 7500 Real-time PCR system (Thermo Fisher Scientific). The expression level was calculated using the $\Delta\Delta$Ct method.

F-Actin and E-Cadherin Staining

Cells were cultured in coverslip bottom dishes coated with fibronectin (10 ng/mL) or poly-l-lysine (50 ng/mL). Cells were fixed with paraformaldehyde (4%), permeabilized with Triton X-100 (0.1%) and blocked with FBS (2%) in PBS. Cells were incubated with either F-actin or anti E-cadherin antibody overnight at 4°C. For E-cadherin staining, cells were further stained with a fluorescent dye conjugated secondary antibody. Nucleus was stained with DAPI and the cells were imaged with Zeiss Axio Observer and CCD camera (Zeiss).

Wound Healing Migration Assay

Cells were seeded at a density of 20,000 cells/cm^2 and allowed to attach for 24–36 h or until they reached confluency. A scratch was made in the cell monolayer, cell migration was monitored and documented microscopically at regular intervals. The migration speed of the cells was calculated by measuring the distance covered by the cells at each time point. The cells were serum starved for 12 h prior to the migration assay to negate the effect of cell proliferation during migration.

3D Spheroid Invasion Assay

Breast cancer cell spheroids were prepared by seeding cells at a density of $0.5–2 \times 10^4$ cells/mL in agar-coated 96-well plates. After 4 days of seeding, the spheroids were transferred to collagen (50 μg/mL)-coated 96-well plates. Test materials were added to the

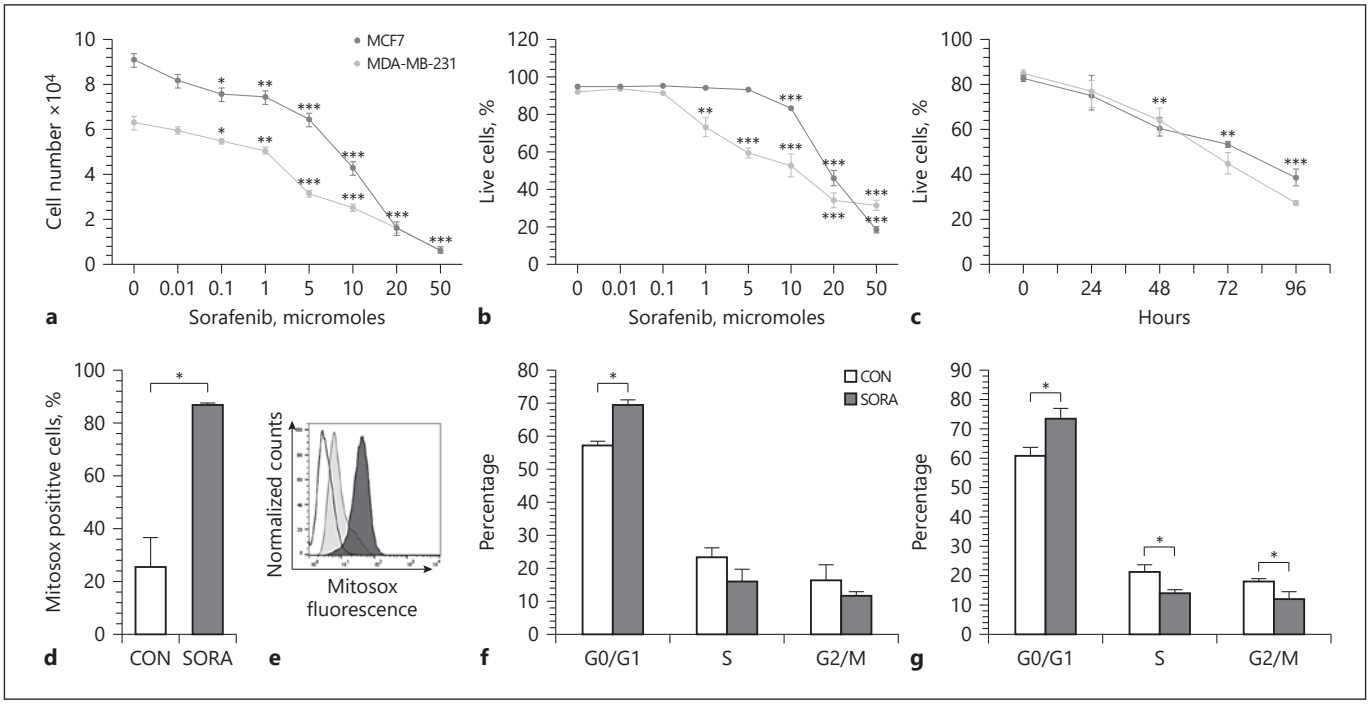


Fig. 1. Effect of sorafenib on proliferation and survival of breast cancer cells. **a**, **b** MCF7 and MDA-MB-231 breast cancer cells were treated with different concentrations of sorafenib as indicated and its effect on proliferation was determined by cell counting (**a**) and survival (**b**) was determined by propidium iodide (PI) staining through flow cytometry. **c** MCF7 and MDA-MB-231 cells were treated with sorafenib (10 μM) for the indicated time points and live cell percentage was determined by PI staining through flow cytometry. **d** Percentage of cells containing mitochondrial superoxide in sorafenib (SORA, 10 μM) treated and untreated (CON) MDA-MB-231 cells was assessed by staining the cells with Mitosox and quantified by flow cytometry. **e** Representative flow cytometry histogram showing the Mitosox-positive cells in control (gray histogram), and sorafenib-treated cells (black histogram). Black line represents the unstained cells. MDA-MB-231 (**f**) and MCF7 (**g**) cells were left untreated (CON) or were treated with EGF (10 ng/mL) or sorafenib (SORA, 10 μM) for 48 h and the cell cycle profile was analyzed by flow cytometry. Values are mean ± SD, $n = 3$ independent experiments, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

wells in DMEM with 2% FBS and spheroids were allowed to migrate. Invasion of the cells from the spheroid into the collagen matrix was monitored and documented microscopically.

Colony Formation Assay

One hundred cells were plated in each well of a 6-well plate and treated with test materials for 48 h. Fresh media was added after treatment and colonies were stained with 0.1% crystal violet after 7–10 days. The number of colonies were counted microscopically.

Protein Expression and Western Blotting

Total protein was isolated with RIPA buffer (25 mm Tris-HCl [pH 7.4], 150 mm NaCl, 1 mm EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1× protease inhibitor cocktail, 1× phosphate inhibitor cocktail) and protein concentration was determined using Precision red advanced protein assay reagent (Cytoskeleton Inc., USA) according to the manufacturer's instructions. 20–30 μg of protein was loaded in each well and proteins were resolved on 12% SDS-PAGE and transferred onto a nitrocellulose membrane using a semi-dry electroblotting apparatus (Bio-Rad Laboratories). Membranes were probed with the indicated antibodies to detect the protein expression.

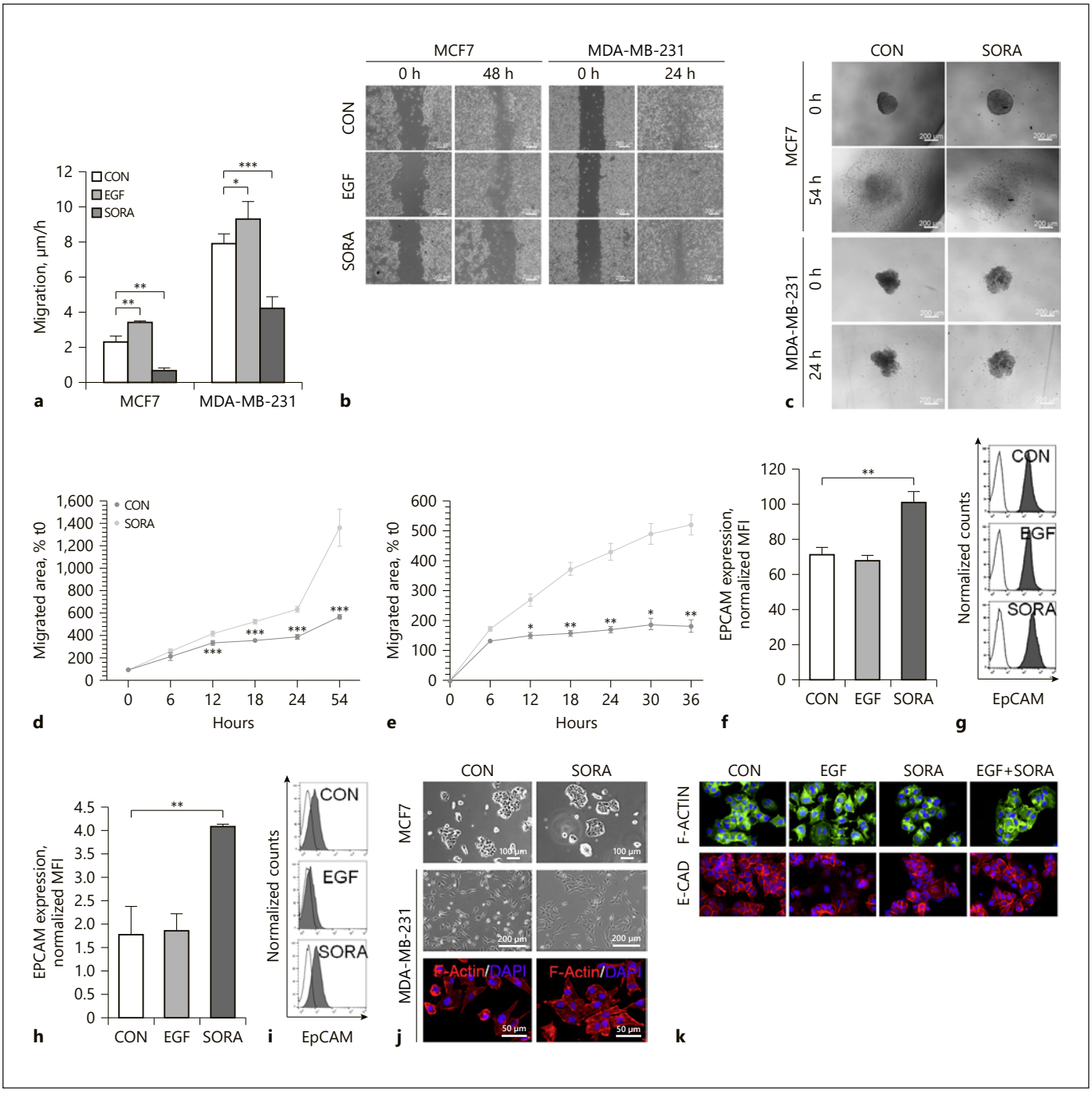
Data Analysis

Statistical analysis was performed using SPSS software and Student's *t* test was used to compare treated versus untreated groups. *p* values <0.05 were considered statistically significant. Cell migration in the wound healing assay was analyzed by TScratch software and 3D spheroid migration was analyzed using ImageJ software. Flow cytometric data were analyzed using FlowJo software (FlowJo, LLC). During flow cytometry analysis, geometric mean fluorescence intensity was calculated to detect the changes in the expression of cell surface receptors or phospho-protein levels. The expression levels were normalized to isotype controls.

Results

To evaluate the effect of sorafenib on breast cancer cells, we treated breast cancer cell lines MCF7 and MDA-MB-231 with increasing concentrations of sorafenib. We found a concentration-dependent inhibition of cell proliferation in MCF7 and MDA-MB-231 cells (Fig. 1a). The

cell viability of breast cancer cells was also affected in a concentration-dependent manner, with MCF7 cells being more sensitive to sorafenib treatment. In MCF7 cells, we found a significant decrease in cell survival at 5 μm, whereas the IC50 value for MDA-MB-231 was approximately 20 μm (Fig. 1b). Considering the proliferation and cell survival effects observed with various concentrations of sorafenib, the rest of the study was carried out with a sorafenib concentration of 10 μm for both MCF7 and MDA-MB-231 cells. Further, we observed a time-dependent decrease in cell survival, with a significant reduction seen at 48 h of treatment (Fig. 1c). Similarly, sorafenib treatment resulted in a significant increase in the mitochondrial superoxide levels, confirming the role of sorafenib in inducing cell death (Fig. 1d, e). As expected, we found a significant increase in the percentage of cells



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at G0/G1 stage of the cell cycle in both MCF7 and MDA-MB-231 following sorafenib treatment (Fig. 1f, g), correlating with its inhibitory effect on cell proliferation.

Next, we evaluated the effect of sorafenib on migration and invasion, to test whether sorafenib can inhibit metastasis of breast cancer cells. While treatment with EGF significantly increased the migration, sorafenib treatment resulted in a remarkable reduction in the migration of both MCF7 (~3.5-fold) and MDA-MB-231 (~2-fold) cells (Fig. 2a, b). Further, we performed, 3D spheroid invasion assay, an assay that closely mimics the migration in in vivo conditions with sorafenib treatment. While MCF7 cells showed a significantly reduced invasion after sorafenib treatment, there was a drastic inhibition in invasion of the metastatic MDA-MB-231 cells on collagen matrix in the presence of sorafenib (Fig. 2c–e). The reduced migration was accompanied with increased EpCAM expression (Fig. 2f–i), a marker that represents loss of migration potential in breast cancer cells [22]. Further, sorafenib treatment increased the cell-cell contact and reduced actin projections in MCF7 and MDA-MB-231 cells confirming its anti-migratory effect (Fig. 2j). In MCF7 cells, filopodia formation and loss of E-cadherin expression induced by EGF treatment was reversed when sorafenib was added along with EGF (Fig. 2k).

The proliferation, progression and metastatic properties of cancer cells is mediated by cancer stem cells in several cancer types including the breast cancer [23]; therefore, we explored the effect of sorafenib on breast cancer stem cells and self-renewal properties. We found a significant decrease in the self-renewal ability of both MCF7 and MDA-MB-231 cells after sorafenib treatment as determined by colony formation assay (Fig. 3a, b). The cancer stem cells in breast cancer is defined by $CD44^{+}/CD24^{-/lo}$ and CD49F expressing cells [23, 24]. On treatment with sorafenib, we found a significant increase in CD24 expression in MDA-MB-231 cells indicating a reduction in the breast cancer stem cell population (Fig. 3c, d). Although small, yet a significant decrease in the expression of CD49F was observed in sorafenib-treated MDA-MB-231 cells (Fig. 3e). Similarly, gene expression analysis showed a significant increase in CD24 transcript levels during sorafenib treatment, whereas CD44 or CD24 expression was unaffected during EGF treatment in both MDA-MB-231 and MCF7 cells (Fig. 3f, g). No changes in ALDH1A3 expression was detected with either EGF or sorafenib treatment compared to the control cells and a significant decrease in CD49F (ITGA6) transcript level was observed after sorafenib treatment in MDA-MB231 cells (Fig. 3f). Furthermore, gene expression analysis showed that sorafenib treatment significantly induced E-cadherin, EpCAM (Fig. 4a), TIMP2, TIMP3 and TIMP4 expression (Fig. 4b) and diminished the expression MMP1 and MMP9 in MDA-MB-231 cells (Fig. 4c). Expression of vimentin (VIM) was unaffected; however, sorafenib significantly reduced the expression of migration-related marker EREG (Fig. 4a). By analyzing the signaling pathways disrupted by sorafenib treatment in breast cancer cells, we found that, while EGF treatment increased the phospho-ERK1/2 levels as expected, sorafenib treatment reduced ERK1/2 phosphorylation significantly in MDA-MB-231 cells (Fig. 4d, e). Similarly, sorafenib treatment resulted in significant reduction of phosphorylated protein levels of p38 MAPK and STAT5 in MDA-MB-231 cells (Fig. 4f). Taken together, sorafenib treatment significantly suppressed the proliferation, survival, migration, invasion and reduced the expression of several migration-related genes and inhibited key intracellular signaling pathways in breast cancer cells.

Fig. 2. Inhibition of migration and invasion after sorafenib treatment. **a**, **b** MCF7 and MDA-MB-231 cells were subjected to wound healing assay during treatment with EGF or sorafenib (SORA), migration speed was calculated and compared with untreated control (CON) cells. **b** Representative images of wound healing migration assay. Values are mean ± SD, $n = 3–6$. **c** 3D spheroid invasion was performed with MCF7 and MDA-MB-231 cells on collagen (50 µg/mL) coated wells during treatment with sorafenib (SORA) or in control (CON) untreated conditions. Migration of the cells out of spheroid was documented at regular intervals and the migrated area was normalized to the spheroid area at t = 0. The migration area at different time points for MCF7 (**d**) and MDA-MB-231 (**e**) are shown. Values are mean ± SD, $n = 4–7$. EpCAM expression in MCF7 (**f**, **g**) or MDA-MB-231 (**h**, **i**) cells treated with EGF or sorafenib (SORA) for 48 h, compared with control (CON) cells. Representative flow cytometric histograms showing EpCAM expression under different treatment conditions for MCF7 (**g**) and MDA-MB-231 (**i**) cells. Isotype control (black line) and antibody-stained cells (grey histogram). Values are mean ± SD, $n = 3–4$. **j** Phase contrast microscopic images of MCF7 and MDA-MB-231 cells treated with sorafenib (SORA) or left untreated (CON). The bottom panel shows fluorescent microscopic images of MDA-MB-231 stained with F-actin and DAPI after treatment with sorafenib. **k** Fluorescent microscopic images of MCF7 control (CON) cells or treated with EGF, sorafenib (SORA) or a combination of EGF and SORA (EFG + SORA). The top panel shows F-actin staining and the bottom panel shows E-cadherin (E-CAD) staining for the treated cells. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

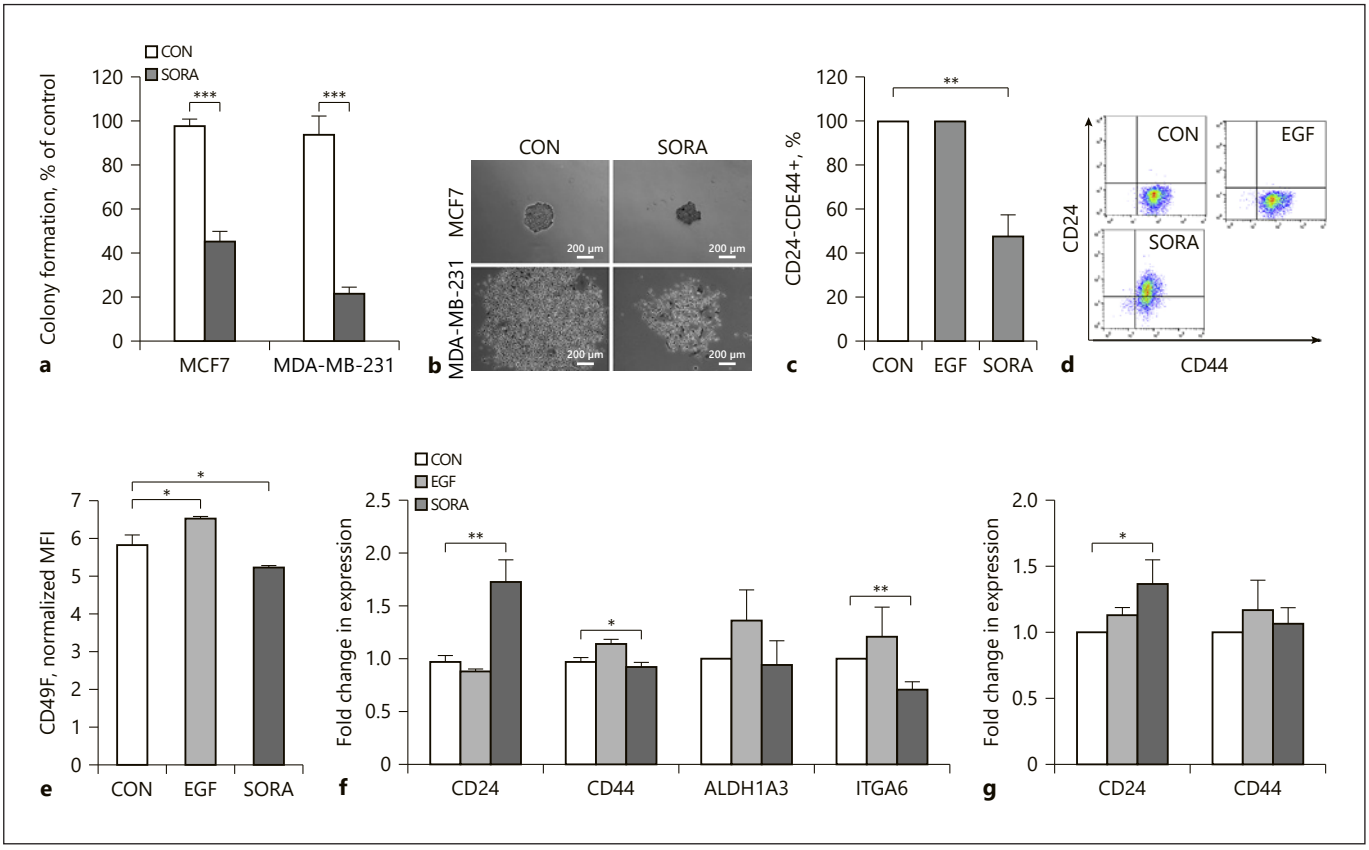

Fig. 3. Effect of sorafenib treatment on breast cancer stem cells. **a** Clonogenic assay was performed for sorafenib (SORA) and untreated (CON) MCF7 and MDA-MB-231 cells and the percentage of colonies obtained after sorafenib treatment is shown. **b** Representative images showing colonies after sorafenib treatment of MCF7 and MDA-MB-231 cells. **c** Percentage of MDA-MB-231 cells with the phenotype $CD44^{+}CD24^{-}$ in untreated (CON), EGF (10 ng/mL) or sorafenib (SORA, 10 μM) treated cells. **d** Representative flow cytometric dot blot showing CD44, CD24 expression pattern in MDA-MB-231 under different treatment conditions. **e** Graph showing CD49F expression in control (CON), EGF or sorafenib (SORA) treated MDA-MB-231 cells. **f** Gene expression analysis by real-time PCR showing expression levels of CD24, CD44, ALDH1A3 and ITGA6 in control (CON), EGF and sorafenib (SORA) treated MDA-MB-231 cells. **g** Gene expression analysis by real-time PCR showing expression levels of CD24 and CD44 control (CON), EGF and sorafenib (SORA) treated MCF7 cells. Values are mean ± SD, $n > 3$ independent experiments, *$p < 0.05$, **$p < 0.005$, *** $p < 0.0005$.

Discussion

In this study, we present the effect of the multikinase inhibitor sorafenib on proliferation, cell cycle, migration, invasion and gene expression of breast cancer cells. The effect of sorafenib on gene expression and various intracellular signaling molecules was analyzed to understand the potential use of this drug in metastatic breast cancer patients. There was a significant decrease in cell proliferation of metastatic triple negative breast cancer cell line MDA-MB-231 and MCF7 breast cancer cells upon treatment with sorafenib as reported in other cancers [16, 25]. As reported in hepatocellular carcinoma [16], sorafenib treatment resulted in the accumulation of cells in the G1 phase of cell cycle and increased cell death.

Metastasis is one of the major reasons for therapy failure in breast cancer [26]. Sorafenib treatment significantly inhibited the metastatic feature of breast cancer cells as identified through the migration and invasion assay. In a 3D invasion assay, sorafenib significantly inhibited the migration of cells out of the spheroid into the collagen matrix. The reduction in migration was accompanied by upregulation of EpCAM expression, where downregulation leads to metastatic phenotype and results in poor clinical outcome [22]. Moreover, MDA-MB-231 cells induced to express high EPCAM were found to have reduced invasion phenotype [27]. Ha et al. [28] and Yoshi-

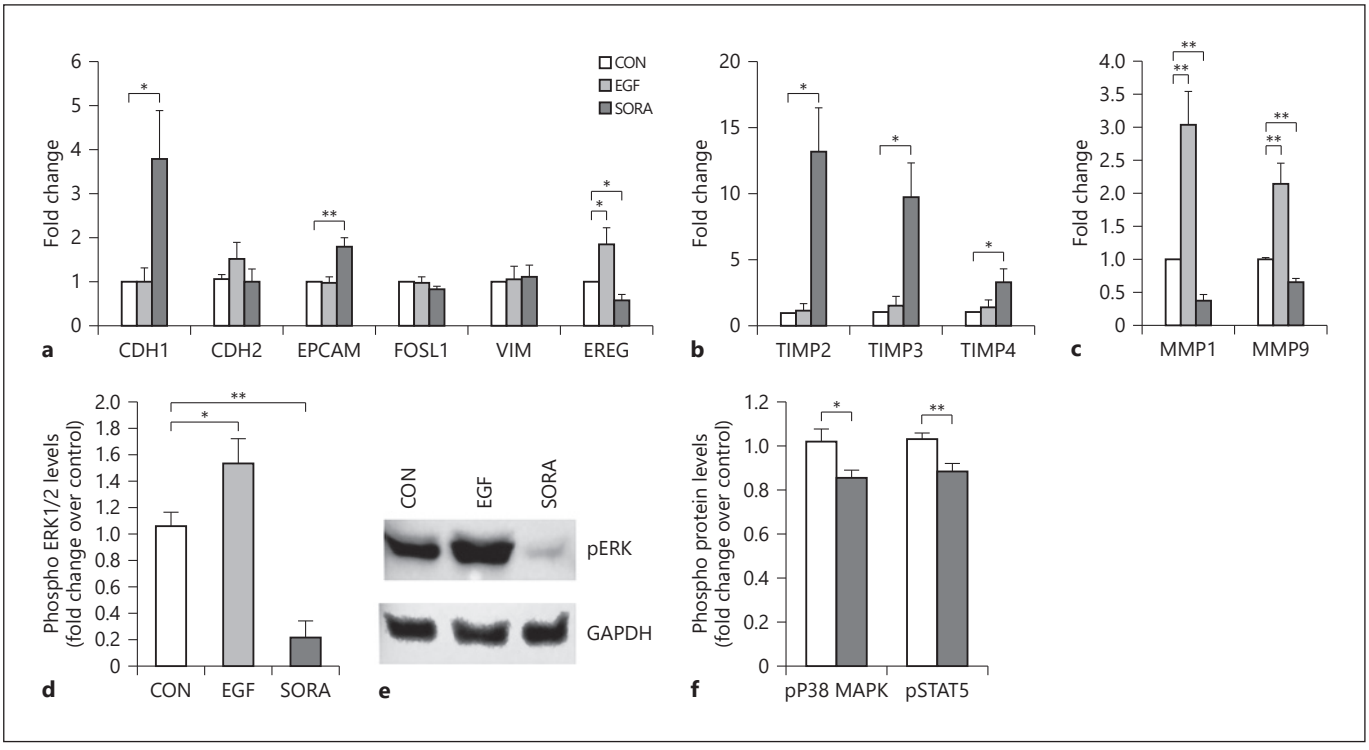

Fig. 4. Gene expression and signaling pathway changes due to sorafenib treatment. **a–c** Expression levels of represented genes were determined through real-time PCR in MDA-MB-231 cells left untreated (CON) or treated with EGF and sorafenib (SORA) for 24 h. Expression levels were normalized to the respective GAPDH levels and fold change with respect to the control untreated cells were determined. Values are mean ± SE, $n = 3$, $^*p < 0.05$, $^{**}p < 0.005$. **d**, **e** Phospho-ERK1/ERK2 (Thr185, Tyr187) levels in MDA-MB-231 cells left untreated (CON) or after treatment with EGF or sorafenib (SORA) for 24 h was determined by Western blotting. Expression was normalized to GAPDH expression levels in each sample. Values are mean ± SD, $n = 3$, $^*p < 0.05$, $^{**}p < 0.005$. **f** Phospho-p38 MAPK (pT180/pY182) and phospho-STAT5 (pY694) levels in control untreated (CON) or sorafenib (SORA) treated MDA-MB-231 cells after 24 h of treatment was determined by phospho-protein flow cytometry. Values are mean ± SD, $n = 3$, $^*p < 0.05$, $^{**}p < 0.005$.

da et al. [29] reported similar inhibition of migration and invasion in hepatocellular carcinoma cells after sorafenib treatment. In association with reduced migration and invasion, there was a significant upregulation of E-cadherin (CDH1) and TIMPs but a downregulation of MMPs, MMP1 and MMP9.

Breast cancer stem cells, responsible for cancer initiation, progression, chemoresistance and recurrence were identified as $CD44^+/CD24^{-/lo}$ along with other markers such ALDH and CD49F [23, 24, 30]. $CD44^+/CD24^{-/lo}$ phenotype is enriched in basal like breast cancer and the majority of MDA-MB-231 cells have $CD44^+/CD24^{-/lo}$ phenotype. CD24 expression, which inhibits stemness in breast cancer cells [31], was upregulated, whereas CD44, which promotes stemness, was downregulated upon sorafenib treatment. However, sorafenib did not affect the expression of ALDH1A3, a marker that in combination with CD44 and CD24 expression identifies breast cancer stem cells [23]. Nevertheless, CD49F, a breast cancer stem cell marker [32] and an indicator of metastasis [32], was downregulated on sorafenib treatment, again, suggesting its anti-metastatic effect.

ERK signaling was found to have an important role in metastatic signaling [33] and high ERK levels correlate with poor prognosis in triple-negative breast cancer patients [10]. In our study, we found that sorafenib treatment drastically reduced phospho-ERK1/2 levels, thereby abrogating the ERK signaling. As reported earlier in other cell types, sorafenib downregulated phosphorylated levels of p38MAPK [25, 34] and STAT5 [35, 36] in MDA-MB-231 cells. Activation of p38MAPK has been found to transduce metastatic signaling and proliferation [11], whereas STAT5 signaling promotes tumor growth and metastasis in breast cancer cells [37, 38].

Thus, sorafenib treatment effectively inhibited proliferation, migration, invasion of breast cancer cells, modi-

fied gene expression, signaling and could be used in combination with other drugs to inhibit metastasis in breast cancer patients, which can be studied further.

Statement of Ethics

Statement of Ethics is not applicable since no animal or human samples were used for the study.

Disclosure Statement

The authors declare no competing conflict of interest.

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Author Contributions

S.D. and R.S. performed the experiments. S.D., A.K. and B.G.J. analyzed the data. A.K. and B.G.J. wrote the manuscript. B.G.J. conceived and designed the study.

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