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A role for calcium in the regulation of ATP-binding cassette, sub-family C, member 3 (ABCC3) gene expression in a model of epidermal growth factor-mediated breast cancer epithelial–mesenchymal transition

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ABSTRACT

Epithelial–mesenchymal transition (EMT), a process implicated in cancer metastasis, is associated with the transcriptional regulation of members of the ATP-binding cassette superfamily of efflux pumps, and drug resistance in breast cancer cells. Epidermal growth factor (EGF)-induced EMT in MDA-MB-468 breast cancer cells is calcium signal dependent. In this study induction of EMT was shown to result in the transcriptional up-regulation of ATP-binding cassette, subfamily C, member 3 (ABCC3), a member of the ABC transporter superfamily, which has a recognized role in multidrug resistance. Buffering of cytosolic free calcium inhibited EGF-mediated ABCC3 increases, indicating a calcium-dependent mode of regulation. Silencing of TRPM7 (an ion channel involved in EMT associated vimentin induction) did not inhibit ABCC3 up-regulation. Silencing of the store operated calcium entry (SOCE) pathway components ORAI1 and STIM1 also did not alter ABCC3 induction by EGF. However, the calcium permeable ion channel transient receptor potential cation channel, subfamily C, member 1 (TRPC1) appears to contribute to the regulation of both basal and EGF-induced ABCC3 mRNA. Improved understanding of the relationship between calcium signaling, EMT and the regulation of genes important in therapeutic resistance may help identify novel therapeutic targets for breast cancer.

1. Introduction

Metastasis, the process whereby cancer cells acquire the ability to escape the confines of the primary tumor, disseminate, and form tumors at distant sites in the body, is a leading cause of cancer related death [1]. Further contributing to poor outcome during the progression to metastatic disease is the association between increased tumor invasiveness, aggressiveness and drug resistance [2–4]. Epithelial–mesenchymal transition (EMT), a process first described in the context of embryonic development [5], is gaining increasing recognition for its role in cancer metastasis [6,7], and potential as a therapeutic target [8,9]. A number of EMT signaling pathways have been identified, involving various growth factors (e.g. transforming growth factor β (TGFβ) and epidermal growth factor (EGF)), transcription factors (e.g. Twist and Snail) and tumor microenvironment factors (e.g. hypoxia) [7,10,11]. Progression through EMT is associated with a loss of basal-apical polarity and

Abbreviations: ABC transporter, ATP-binding cassette transporter; ABCC3, ATP-binding cassette, subfamily C, member 3; BAPTA, AM, Glycine, N,N′-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[N-[2-[(acetyloxy)methoxy]-2-oxoethyl]]-, bis[(acetyloxy)methyl] ester; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMT, epithelial–mesenchymal transition; ER, estrogen receptor; FBS, fetal bovine serum; HER2, human epidermal growth factor 2 receptor; PR, progesterone receptor; PVDF, polyvinylidene difluoride; SOCE, store-operated calcium entry; TGFβ, transforming growth factor β; TRP, transient receptor potential; TRPC1, transient receptor potential cation channel, subfamily C, member 1.

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cell–cell adhesions, extracellular matrix degradation and acquisition of stem cell traits, resulting in cells that are more migratory, invasive, and resistant to cell death [7,10,11]. EMT is also a process important in breast cancer metastasis and the acquisition of therapeutic resistance [12–15]. Induction of EMT is linked to increased expression of specific members of the ATP-binding cassette (ABC) transporter superfamily [16], many of which have been shown to play a role in multidrug resistance [17]. TGFβ treatment and transient transfection with the transcription factor Twist in MCF-7 breast cancer cells is associated with a significant up-regulation of several ABC transporters reported to play a role in multidrug resistance [16]. EGF treatment also induces ABCC1, 3, 5 and 7 gene expression in MCF-7 cells, with a concomitant increase in cell migration and altered cell morphology (similar to that observed following an EMT) [18]. Collectively these studies suggest that the acquisition of therapeutic resistance concomitant with EMT is associated with the increased expression of specific ABC transporters.

The ABC transporter superfamily of energy-dependent efflux proteins consists of at least 48 members, some of which are recognized for their role in mediating multidrug resistance, by preventing intracellular drug accumulation, in different cancer types [17,19]. In addition to facilitating the transport of chemotherapeutics and other xenobiotics, specific members of the ABC transporter superfamily can also efflux various lipid and hormone substrates implicated in cancer biology [17,20,21], suggestive of a role beyond multidrug resistance in cancer progression, including migration, invasion and metastasis [17].

The calcium signal, which contributes to the regulation of a number of cellular processes important in invasion and metastasis [22–25], has recently been linked to both EGF and hypoxia-mediated EMT [26], in the MDA-MB-468 breast cancer cell line model [12,27,28]. In this model, the calcium permeable ion channel TRPM7 was identified as a regulator of specific signaling pathways and vimentin protein induction associated with EGF-mediated EMT.

In these studies we investigated the effects of EGF-mediated EMT on mRNA levels of ABCC1, ABCC3, and ABCC5 transporters in MDA-MB-468 breast cancer cells and the potential role of the calcium signal on such changes. We have also assessed the possible role of specific Ca^{2+} permeable ion channels on EGF-mediated Ca^{2+} sensitive up-regulation of ABCC3 levels in MDA-MB-468 breast cancer cells.

2. Materials and methods

2.1. Cell culture

MDA-MB-468 breast cancer cells [28] were routinely cultured in Dulbecco's Modified Eagle's Medium (Sigma Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich), and 4 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), and maintained in a humidified incubator at 37 °C with 5% CO_2. For induction of EMT, cells were cultured in serum-reduced media (0.5% fetal bovine serum) for 24 h, prior to treatment with epidermal growth factor (EGF) (50 ng/mL) (Sigma Aldrich) or acetic acid (vehicle control) for the indicated time, as previously described [26]. For studies assessing the effect of intracellular Ca^{2+} chelation on gene transcription, cells were spiked with 100 μM of the acetoxymethyl ester form of 1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetracetic acid (BAPTA, AM) (Invitrogen) for 1 h at 37 °C, after which time fresh media containing either EGF or acetic acid was added to each well, as previously described [26]. Cells were routinely tested for mycoplasma infection using the Mycoalert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

2.2. Immunoblotting

Total cellular protein was isolated using ice cold protein lysis buffer supplemented with protease and phosphatase inhibitors (Roche Applied Science, Penzberg, Germany), and gel electrophoresis and immunoblotting was performed as previously described [28]. PVDF membranes were incubated with anti-vimentin monoclonal antibody Clone V9 (Sigma–Aldrich), diluted 1:750, at 4 °C overnight. Monoclonal anti-β-actin antibody Clone AC-15 (Sigma–Aldrich), diluted 1:1,0 ,000, was used as a loading control. Goat-anti-mouse IgG HRP-conjugated secondary antibody (Bio-Rad) was used at a dilution of 1:10 000. Protein bands were visualized using SuperSignal West Dura Extended Duration Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA) and acquired using a VersaDoc Imaging System (Bio-Rad). Quantitative analysis was performed using Quantity One Software (v4.6.7 for Windows, Bio-Rad) as per the user guide, and protein density normalized to β-actin.

2.3. RNA isolation and real-time RT-PCR

Total cellular RNA was isolated and purified using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using Omniscript and Quantitect RT Kits (Qiagen), together with Random Primers, and Recombinant RNasin® Ribonuclease Inhibitor (used with Omniscript RT Kit only) (Promega, Madison, WI, USA), as previously described [26]. Gene amplification was performed using TaqMan FAST Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) together with the following Gene Expression Assays (Applied Biosystems): ABCC1 (Hs00219905_m1), ABCC3 (Hs00978473_m1), ABCC5 (Hs00981087_m1), E-cadherin (Hs00170423_m1), fibronectin (Hs01549976_m1), N-cadherin (Hs00983062_m1), ORAI1 (Hs00385627_m1), STIM1 (Hs00162394_m1), TRPC1 (Hs00608195_m1), TRPM7 (Hs00292383_m1), and vimentin (Hs00185584_m1). Reactions were run under universal cycling conditions using a StepOnePlus Real Time PCR System (Applied Biosystems). The comparative C_T method was used to quantify gene expression, with 18S ribosomal RNA serving as the internal reference gene [29].

2.4. siRNA transfection

Studies assessing the effect of siRNA-mediated gene silencing were performed using Dharmacon ON-TARGET*plus* SMARTpool siRNA (100 nM/well), together with DharmaFECT4 Transfection Reagent (0.2 μL/well) (Thermo Scientific) in 96 well plates, as previously described [28]. The following Dharmacon ON-TARGET*plus* SMARTpool siRNAs were used: Non-targeting (D-001810-10-05), ORAI1 (L-014998-00-0005), STIM1 (L-011785-00-0005), TRPC1 (L-004191-00-0005), and TRPM7 (L-005393-00-0005). Cells were cultured in the presence of siRNA-containing media for 48 h, after which time media was serum-reduced (0.5% FBS) for 24 h. Cells were then treated with fresh media containing either EGF or acetic acid (vehicle control) for a further 48 h prior to RNA isolation.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism (v6.04 for Windows). Details of statistical analyses used throughout this study are provided in the corresponding figure legends.

3. Results

3.1. Induction of EGF-mediated EMT is associated with an increase in ABCC3 mRNA

EMT is associated with the altered expression of a number of well-characterized markers. To confirm EGF-induced EMT in this study, we used real time RT-PCR to quantify expressional changes of various known EMT markers [7,8]. Treatment of MDA-MB-468 breast cancer cells with EGF for 24 h resulted in a significant decrease in the mRNA level of the epithelial marker E-cadherin, and significantly increased mRNA levels of the mesenchymal markers N-cadherin, fibronectin, and vimentin, relative to control-treated cells (Fig. 1A–D). Significant induction of vimentin expression at the protein level was also confirmed in EGF-treated relative to control-treated cells using immunoblotting (Fig. 1E and F). Characteristic of epithelial cells undergoing an EMT, EGF-treated (24 h) MDA-MB-468 cells showed signs of a morphological transformation from being 'cobblestone-like' in appearance with numerous cell–cell adhesions, to appearing more 'fibroblast-like' with fewer cell–cell adhesions [7] (Fig. 1G).

Increased expression of various members of the ABC superfamily of transporters has been reported in different EMT models in MCF-7 breast cancer cells [16]. Three ABC transporters shown to be significantly up-regulated following induction of EMT [16], and reported to be endogenously expressed in the MDA-MB-468 breast cancer cell line [30] were selected for this study. These were ABC, sub-family C, members 1, 3 and 5 (ABCC1, ABCC3, and ABCC5). Treatment of MDA-MB-468 cells with EGF for 24 h and 48 h had no effect on mRNA levels of ABCC1 (Fig. 2A), and modestly increased ABCC5 mRNA at 48 h (Fig. 2C). ABCC3 mRNA levels increased

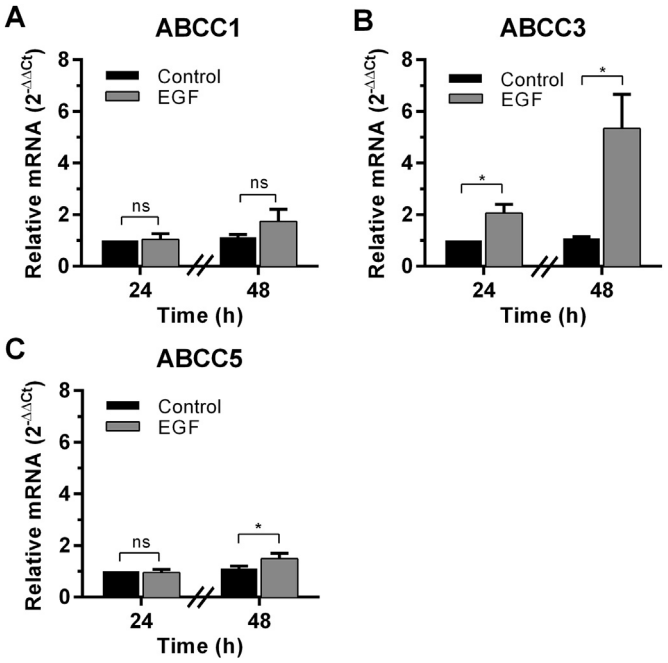

Fig. 2. Assessment of ABCC1, ABCC3, and ABCC5 mRNA levels in EGF-mediated EMT in MDA-MB-468 cells. Cells were serum-deprived (0.5% FBS) prior to treatment with EGF (50 ng/mL) or control (acetic acid) for 24 h and 48 h mRNA levels of (A) ABCC1, (B) ABCC3, and (C) ABCC5 were assessed using real time RT-PCR. Bar graphs represent mean ± SD for three independent experiments. Unpaired *t*-tests were performed to determine statistical significance. $^{*}P \leq 0.05$, (ns, $P > 0.05$).

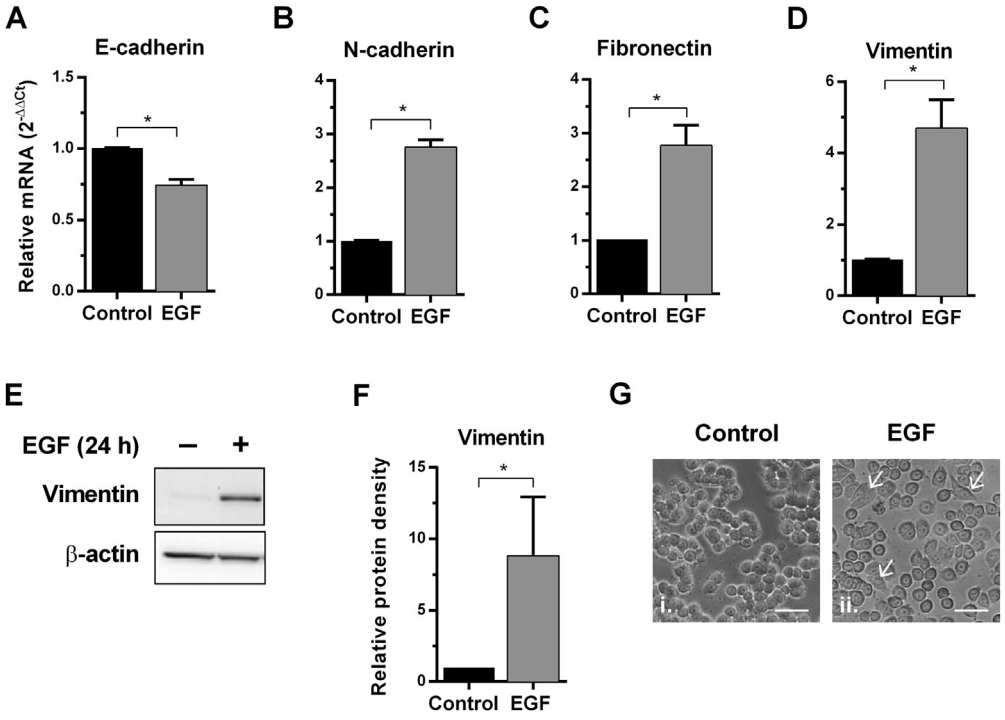

Fig. 1. EGF induction of an EMT-like phenotype in MDA-MB-468 cells. Cells were serum-deprived (0.5% FBS) prior to treatment with EGF (50 ng/mL) or control (acetic acid) for 24 h, resulting in (A) decreased mRNA levels of the epithelial marker E-cadherin, and (B) increased mRNA levels of the mesenchymal markers N-cadherin, (C) fibronectin, and (D) vimentin. (E) Representative immunoblot of EGF-induced vimentin protein expression, and (F) densitometric analysis (normalized to β-actin). Bar graphs represent mean ± SD for three independent experiments. Unpaired *t*-tests were performed to determine statistical significance. $^{*}P \leq 0.05$. (G) Phase contrast image of MDA-MB-468 cells at 20x magnification (i) in the absence, and (ii) presence of EGF (50 ng/mL, 24 h). Arrows indicate cells transitioning from an epithelial, 'cobblestone-like', to a mesenchymal, 'fibroblast-like', morphology. Scale bar = 50 μm.

significantly following both 24 h and 48 h EGF treatment, with approximately 2 and 5.3 fold increases, respectively (Fig. 2B).

3.2. EGF-induced ABCC3 mRNA increases are dependent on intracellular free Ca^{2+}

EGF-induced increases in vimentin mRNA and protein expression are dependent on intracellular free Ca^{2+} in the MDA-MB-468 breast cancer cell line [26]. Using the intracellular Ca^{2+} chelator BAPTA, AM to buffer against changes in $[Ca^{2+}]_i$, we confirmed the role of the calcium signal in EGF-induced vimentin mRNA increases in MDA-MB-468 cells (Fig. 3A). Using real time RT-PCR, we also demonstrated that pre-treatment of MDA-MB-468 cells with BAPTA, AM significantly inhibited EGF-induced increases in ABCC3 mRNA levels (Fig. 3B). This finding indicates that like vimentin, the calcium signal plays an important role in EGF-mediated ABCC3 mRNA induction in this model of EMT.

3.3. An siRNA-mediated investigation of potential regulators of ABCC3 mRNA expression

TRPM7 is a Ca^{2+} permeable ion channel, which when silenced significantly reduces vimentin protein induction associated with EGF-induced EMT in MDA-MB-468 breast cancer cells [26]. TRPM7 siRNA-mediated silencing (Fig. 4A) did not significantly attenuate ABCC3 mRNA induction with EGF treatment (48 h) relative to control-treated cells (Fig. 4E).

Based on our findings that EGF-mediated ABCC3 mRNA increases are inhibited by the buffering of intracellular free Ca^{2+}, but insensitive to TRPM7 silencing, we investigated the role of other proteins identified as regulators of intracellular Ca^{2+} levels in MDA-MB-468 cells [28]. We assessed the role of the calcium channel ORAI1, its activator STIM1, and the calcium permeable ion channel TRPC1, proteins important in SOCE and/or basal Ca^{2+} influx in MDA-MB-468 breast cancer cells [28].

Neither ORAI1, STIM1 nor TRPC1 silencing (Fig. 4B–D) attenuated the ability of EGF to increase ABCC3 mRNA. ORAI1 and STIM1 did not significantly alter ABCC3 mRNA in either non-EGF treated (control) or EGF-treated MDA-MB-468 cells (Fig. 4F,G). However, TRPC1 silencing resulted in a significant increase in ABCC3 mRNA in both non-EGF treated (control) and EGF-treated MDA-MB-468 cells (Fig. 4H).

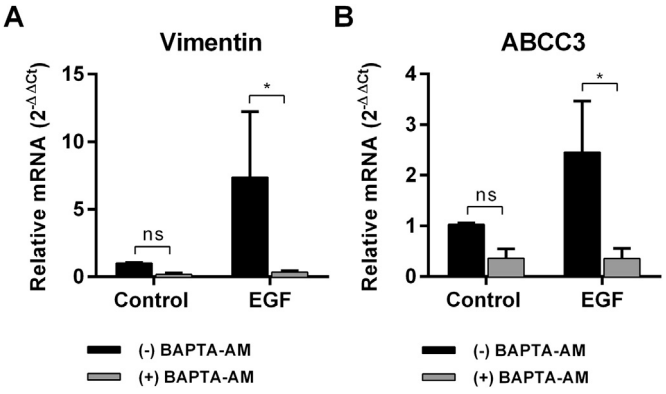

Fig. 3. EGF-induced ABCC3 mRNA increases are calcium-dependent. A role for calcium in the regulation of ABCC3 gene expression was assessed by pre-treating cells with the intracellular calcium chelator, BAPTA, AM (100 μM, 1 h). Calcium chelation significantly inhibited both (A) vimentin, and (B) ABCC3 mRNA increases in MDA-MB-468 cells treated with EGF (50 ng/mL) for 24 h. Bar graphs represent mean ± SD for three independent experiments. Two-way ANOVA, with Bonferroni's multiple comparisons, was performed to determine statistical significance. $^*P \leq 0.05$, (ns, $P > 0.05$).

4. Discussion

The progression from benign to invasive disease and its association with therapeutic resistance presents a major challenge to survival and treatment outcomes for patients diagnosed with cancer [1]. A role for EMT in breast cancer metastasis is now widely accepted, and there is increasing recognition of its association with therapeutic resistance; however, the regulation of the relationship between these two processes is still poorly understood. The EGF-mediated model of EMT in MDA-MB-468 breast cancer cells employed in the present study provides a useful tool for the *in vitro* study of processes important in breast cancer EMT [12,26,27]. Using this model, we assessed changes in the mRNA levels of three ABC transporters, ABCC1, 3 and 5, which are up-regulated following induction of EMT in MCF-7 cells. However, EGF-mediated EMT in MDA-MB-468 breast cancer cells was associated with pronounced up-regulation of only ABCC3 mRNA, with a modest but significant increase in ABCC5 only at 48 h after EGF treatment. There are a number of possible reasons for the differences in our findings including the method of EMT induction, of which a number of different models have been developed for the *in vitro* study of EMT in breast cancer cell lines [16,26,31]. An early study assessing the effects of EGF on the migratory behavior and multidrug resistance phenotype of MCF-7 cells reported transcriptional up-regulation of ABCC1, 3, 5 and 10 [18]. In addition to these transporters, the EGF signaling pathway is also implicated in the regulation of ABCG2 and ABCB1 [32]. Another variable potentially contributing to the differences in our findings is the nature of the cell lines used in each model of EMT. The MDA-MB-468 breast cancer cell line possesses features of the 'basal-like' breast cancer subtype, and is negative for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), while expressing high levels of the EGF receptor (EGFR) [33]. The MCF-7 cell line, which represents the 'luminal-like' breast cancer subtype, is positive for the ER and PR, and expresses low levels of the EGFR [33]. The method of EMT induction and cell line used in the different models of EMT appears to account for differences observed in ABC transporter expression between EMT induction in MDA-MB-468 and MCF-7 breast cancer cells.

Recent studies have identified a role for the calcium signal in the activation of specific proteins important in EGF-stimulated signal transduction pathways and the induction of some EMT markers in the MDA-MB-468 breast cancer cell line model [26]. In the present study we were able to demonstrate for the first time, that EGF-induced ABCC3 transcriptional up-regulation is also calcium signal dependent in MDA-MB-468 breast cancer cells. While siRNA-mediated silencing of the calcium permeable ion channel TRPM7 partially inhibited EGF-induced vimentin protein expression, we found no evidence of a role for TRPM7 in the regulation of ABCC3 mRNA, indicating that the calcium-dependent regulation of ABCC3 occurs via a TRPM7 independent pathway. It should be noted that TRPM7 silencing does not alter vimentin mRNA levels or the expression of another EMT marker, Twist, in MDA-MB-468 cells [26]. Moreover, attenuation of vimentin protein levels following TRPM7 silencing is only partial [26]. This suggests a role for other calcium signaling pathways in some elements of EGF-mediated EMT induction, including the induction of ABCC3.

Given that TRPM7 silencing did not alter ABCC3 mRNA levels, and in light of our finding that ABCC3 transcriptional regulation is calcium-dependent, we sought to investigate the potential contribution of calcium channels previously identified as regulators of calcium entry in the MDA-MB-468 model of EGF-induced EMT [28]. Silencing of ORAI1, and the calcium store sensor STIM1, which interacts directly with ORAI1 during SOCE, did not reduce EGF-mediated increases in ABCC3 mRNA. However, we did identify a

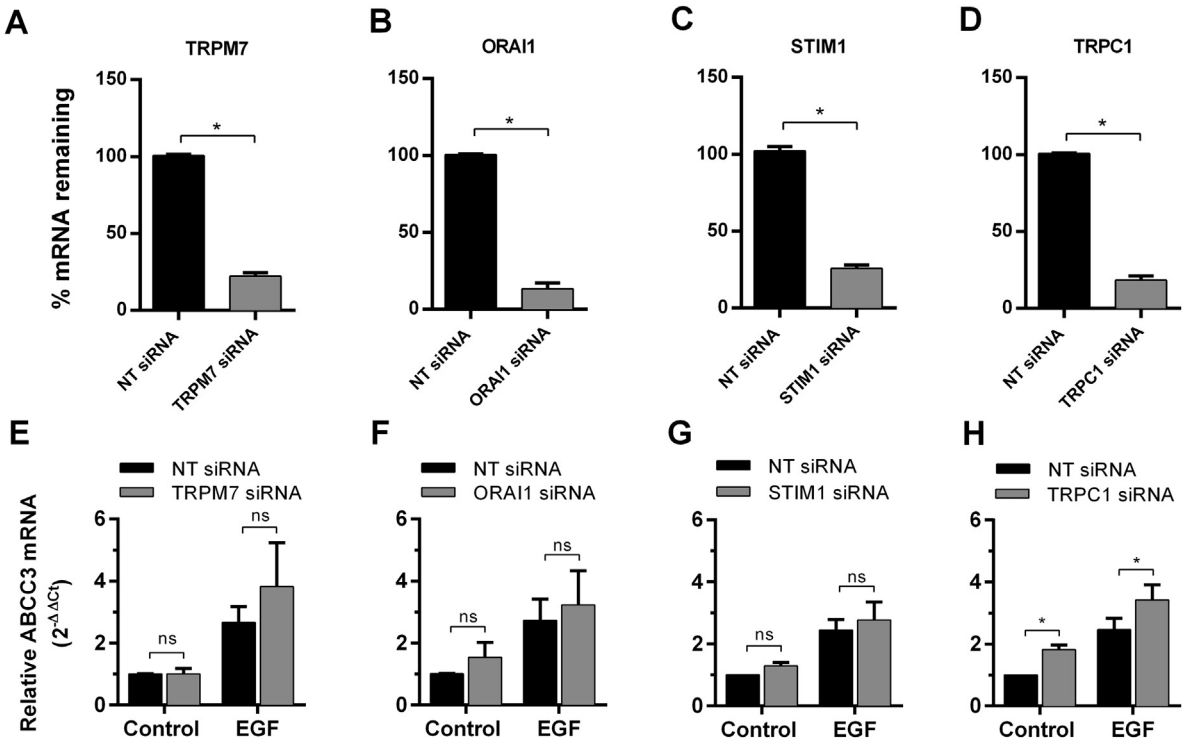

Fig. 4. siRNA-mediated silencing of TRPC1, but not TRPM7, ORAI1 or STIM1 results in significantly increased ABCC3 mRNA levels in MDA-MB-468 cells. Confirmation of (A) TRPM7, (B) ORAI1, (C) STIM1, and (D) TRPC1 gene silencing using Dharmacon ON-TARGET*plus* siRNA shown as percent mRNA remaining relative to NT siRNA control. Bar graphs represent mean ± SD from three independent experiments. An unpaired *t*-test was performed to determine statistical significance. $^{*}P \leq 0.05$. (E) TRPM7, (F) ORAI1, (G) STIM1, and (H) TRPC1 gene expression was silenced using Dharmacon ON-TARGET*plus* siRNA prior to treatment with EGF (50 ng/mL) or control for 48 h. Bar graphs show ABCC3 mRNA levels relative to non-targeting (NT) siRNA control and represent mean ± SD from three independent experiments. Two-way ANOVA, with Bonferroni's multiple comparisons, was performed to determine statistical significance. $^{*}P \leq 0.05$ (ns, $P > 0.05$).

role for the calcium channel TRPC1, with silencing of this Ca^{2+} permeable ion channel increasing both basal and EGF-induced levels of ABCC3. EGF-stimulated EGFR activation is linked to TRPC1-mediated calcium influx in non-small cell lung cancer cells, and a role for TRPC1 in the regulation of EGFR signaling has been proposed [34]. In addition to its role as a mechano-gated calcium influx channel, TRPC1 has been reported to participate in the store-operated calcium entry (SOCE) pathway in some cells [35,36]. Whilst TRPC1 does not appear to play a role in the SOCE pathway in MDA-MB-468 cells, its silencing decreases non-agonist stimulated calcium influx [28]. Determining the relationship between TRPC1 and ABCC3 transcriptional regulation is an area that requires further investigation.

The inability of an individual calcium channel (or sensor) to attenuate EGF-mediated increases in ABCC3 mRNA may indicate a degree of redundancy. EGF-mediated increases in intracellular calcium in MDA-MB-468 cells may involve several calcium signaling pathways, and hence silencing of one gene may be insufficient to abolish ABCC3 induction. Alternatively, other specific calcium channels and transporters may be important in the calcium dependent EGF-mediated induction of ABCC3 and could be the focus of future investigations.

Improved understanding of the mechanisms underlying the acquisition of an invasive cancer phenotype, and its association with therapeutic resistance may represent an opportunity for better patient outcomes. We have identified the calcium-dependent transcriptional up-regulation of the ABC transporter ABCC3 in a model of breast cancer EMT. Our studies have also identified the calcium channel TRPC1 as a regulator of ABCC3 levels in MDA-MB-468 breast cancer cells. Further studies are warranted to assess the expressional changes of ABCC3 in other models of breast cancer EMT and the calcium-dependent mechanism responsible for this up-regulation.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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