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ZnR/GPR39 upregulation of K^+/Cl^--cotransporter 3 in tamoxifen resistant breast cancer cells

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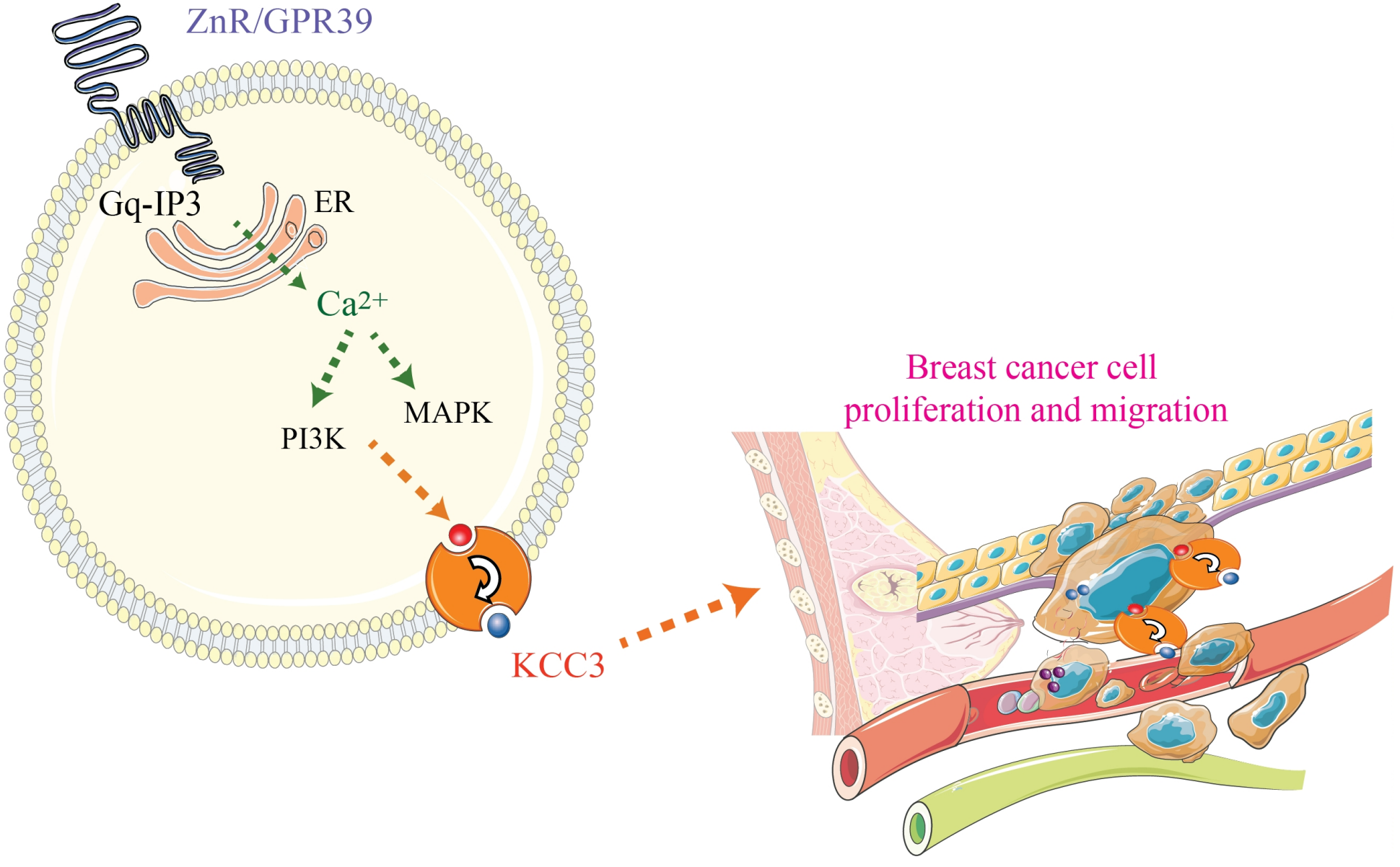
Highlights:

- ZnR/GPR39 activates KCC3 in tamoxifen resistant breast cancer cells
- Zn^{2+} does not activate Ca^{2+} signaling or KCC3 in estrogen-dependent MCF-7 cells
- ZnR/GPR39-dependent KCC3 activation enhances cell proliferation and migration


ZnR/GPR39
Gq-IP3
ER
Ca2+
MAPK
PI3K
KCC3
Breast cancer cell
proliferation and migration

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Abstract

Expression of the zinc receptor, ZnR/GPR39, is increased in higher grade breast cancer tumors and cells. Zinc, its ligand, is accumulated at larger concentrations in the tumor tissue and can therefore activate ZnR/GPR39-dependent Ca^{2+} signaling leading to tumor progression. The K^+/Cl^- co-transporters (KCC), activated by intracellular signaling, enhance breast cancer cell migration and invasion. We asked if ZnR/GPR39 enhances breast cancer cell malignancy by activating KCC. Activation of ZnR/GPR39 by Zn^{2+} upregulated K^+/Cl^- co-transport activity, measured using NH_4^+ as a surrogate to K^+ while monitoring intracellular pH. Upregulation NH_4^+ transport was monitored in tamoxifen resistant cells with functional ZnR/GPR39-dependent Ca^{2+} signaling but not in MCF-7 cells lacking this response. The NH_4^+- transport was Na^+-independent, and we therefore focused on KCC family members. Silencing of KCC3, but not KCC4, expression abolished Zn^{2+}-dependent K^+/Cl^- co-transport, suggesting that KCC3 is mediating upregulated NH_4^+- transport. The ZnR/GPR39-dependent KCC3 activation accelerated scratch closure rate, which was abolished by inhibiting KCC transport with [(DihydroIndenyl) Oxy] Alkanoic acid (DIOA). Importantly, silencing of either ZnR/GPR39 or KCC3 attenuated Zn^{2+}-dependent scratch closure. Thus, a novel link between KCC3 and Zn^{2+}, via ZnR/GPR39, promotes breast cancer cell migration and proliferation.

Keywords: ZnR/GPR39; zinc signaling; breast cancer; K^+/Cl^- co-transport; KCC3; MAPK; PI3K.

Abbreviations: Estrogen receptor, ER; K^+/Cl^- co-transporter 3, KCC3; Tamoxifen resistant cells TAMR; Mitogen activated kinase, MAPK; phosphatidylinositol 3-kinase, AKT/PI3K

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1. Introduction

Estrogen receptor (ER) alpha, which triggers breast cancer cell proliferation, is expressed in most breast tumors and thus anti-estrogen drugs, *e.g*. tamoxifen, became a preferred treatment approach [1]. However, relapse occurs in about one third of the patients when anti-estrogen-resistance develops and tumor subpopulations emerge [2-4]. Mechanisms underlying this resistance involve loss of the ER and activation of downstream signaling pathways, such as the mitogen activated kinase (MAPK) and phosphatidylinositol 3-kinase (AKT/PI3K), independent of estrogen [5, 6]. Notable, expression of a Zn^{2+}-sensing G-protein coupled receptor, ZnR/GPR39, is increased in higher stage ER-negative breast cancer tissue and in ER-negative breast cancer cell lines, compared to ER expressing cells [7]. Activation of ZnR/GPR39, by Zn^{2+}, triggered upregulation of MAPK and AKT/PI3K signaling pathways in ZnR/GPR39 expressing breast cancer cells. In agreement, unbiased screens identified a correlation between upregulation of *GPR39* and the aggressiveness of breast cancer cell lines and tissues [7, 8]. Changes in Zn^{2+} levels and in the expression of Zn^{2+} transporters are also monitored in breast cancer tissues [9-12], this dys-homeostasis may trigger release of Zn^{2+} and subsequent activation of ZnR/GPR39. Moreover, injury or cell death of epithelial cells results in liberation of Zn^{2+} from intracellular stores, which is sufficient to activate ZnR/GPR39 [13]. In breast cancer cells, ZnR/GPR39 activation compensates for loss of ER signaling and induces estrogen-independent activation of MAPK and AKT/PI3K. Thus, ZnR/GPR39 may lead to tumor progression via a poorly understood mechanism.

Increased expression and transport activity of members of the K^+/Cl^- cotransporters (KCC1-4) family enhanced tumor progression and invasiveness [14-16]. Under physiological conditions, the KCC cotransporters constitute major efflux pathways for K^+ and Cl^-, and are responsible for cell volume regulation, trans-epithelial ion transport and salt absorption. However, KCC transporters are also associated with enhanced breast cancer cell proliferation. For example, KCC inhibition induced cell cycle arrest, by modulating expression of p21, cyclin D1 and cyclin E2 [17]. In addition, upregulation of KCC3 and KCC4 following activation of MAPK and AKT/PI3K pathways enhanced breast cancer cell proliferation and migration [16]. Overexpression of KCC was also monitored in ovarian and cervical cancer cells and tissues [18, 19], and activation of MAPK and AKT/PI3K pathways increased KCC membrane expression followed by enhanced proliferation and invasiveness [20-22]. Although ZnR/GPR39 is a major regulator of MAPK and AKT/PI3K pathways, it is not known whether this pathway upregulates KCC in breast cancer cells.

In neurons and colonocytes, ZnR/GPR39 is modulating ion transport by upregulating KCC2 and KCC1 activity, respectively [23, 24]. Activation of ZnR/GPR39 by synaptically

released Zn^{2+} enhances neuronal KCC2 activity and the Cl^- inward gradient. Thus, ZnR/GPR39 hyperpolarizes the reversal potential of $GABA_A$–mediated Cl^- currents and reduces susceptibility to seizures [25]. In colonocytes, KCC1 is expressed on the basolateral membrane, and ZnR/GPR39-dependent upregulation of KCC1 enhances Cl^- absorption and decreases water loss [24]. Activation of ZnR/GPR39 and subsequently KCC1 reduces cholera toxin induced diarrhea, providing a mechanism for the established role of Zn^{2+} in reducing diarrhea [24]. We therefore reasoned that ZnR/GPR39 may activate KCC and thereby promote breast cancer progression. Here, we show that ZnR/GPR39 activation in tamoxifen-resistant breast cancer cells upregulates KCC3 activity and enhances cell proliferation and migration.

2. Materials and Methods

Cell culture: MCF-7 cells, a human breast cancer cell line, were cultured in phenol-red RPMI medium supplemented with 5% fetal calf serum, and penicillin-streptomycin (10 IU/ml 10 g/ml). TAMR cells [26], MCF-7 cells resistant to 4-hydroxytamoxifen (4-OH-TAM), were cultured in phenol-red-free RPMI medium supplemented with 5% charcoal-stripped steroid-depleted fetal calf serum plus penicillin-streptomycin, 4 mM L-glutamine and 4-OH-TAM (10^{-7} M in ethanol) (Gibco). For imaging measurements, 1,000,000 cells were seeded onto 10 mm glass coverslips and imaged after 48 h as described below. MCF-7 cells that were seeded for imaging measurements were grown in phenol-red-free RPMI medium.

Silencing: For gene-silencing experiments, cells were transfected with siGPR39/KCC3/ KCC4 or a scrambled (si-control) siRNA construct (40nM, Sigma-Aldrich) in 60 mm plates, using the transfection reagent Dharmafect as described by the manufacturer (Thermo-scientific). Cells were imaged or used for qPCR analysis 48 h after transfection. The target sequence of the GPR39 for siRNA was: sense 5' CCATGGAGTTCTACAGCATtt, anti-sense: 5' ATGCTGTAGAACTCCATGGtt. The target sequence of the si-control for siRNA was: sense 5' GCCCAGATCCCTGTACGTtt, anti-sense 5' ACGTACAGGGATCTGGGCtt. The target sequence of the KCC3 for siRNA was: sense 5' CGGACATAAGAAAGCTCGAtt, anti-sense 5' TCGAGCTTTCTTATCTCCGtt. The target sequence of the KCC4 for siRNA was: sense 5' GGTGGAACAAGAGAGCTTCtt, anti-sense 5' GAAGCTCTCTTG TTCCACCtt. PCR was used to estimate the presence of KCC family members in MCF-7 and TAMR cells. Cells were seeded on 60 mm plates, after 48 hours cells were trypsinized with 0.05% trypsin (Biological Industries). Cell lysates were homogenized using QIAshredder, as described by the manufacturer (QIAGEN). Total RNA was purified using RNeasy Mini Kit as described by the manufacturer (QIAGEN). Purified RNA (1μg) was converted to cDNA using synthesis Kit (Life Technologies). The cDNA was used for PCR analysis with the Red load TaqMaster

(Larova) and primers supplied by Sigma-Aldrich: KCC1 (recognizes all variants): forward: TGGGACCATTTTCCTGACC reverse: CATGCTTCTCCACGATGTCAC; KCC3 (recognizes all variants): forward: GCCCGAAACACAAAATCACT reverse: GGATACCTG GGGGAAGATGT; KCC4: forward: GACTCGTTTCCGCAAAACC reverse: AGAGTG CCGTGATGCTGTTGG. The same cDNA was diluted at 1:5 and subjected to real time PCR procedure (Taqmen, Applied Biosystems), which was done with ABsolute Blue QPCR kit (Thermo Scientific). Required primers and probes were predesigned and supplied by Solaris: KCC1 (recognizes 1,2,4,5,6 variants): forward: CTGGCACTGTTTGAGGAAGA reverse: GCTTTC CCAGAAGAGACGAT probe: TCCGCCCAAAGGTATCG; KCC3 (recognizes all variants): forward: CCTAATGGCTGGCGTCAA reverse: ATGGGCAGCAGTTGT CACTC probe: CTT TTATTGGCACAGTTCG; KCC4 forward: TACCTGGACAAGCA CATGGA reverse: GCT CATTAGGGACCGTAT probe: GCGGGCCGAGGAGAACATAC.

Ion transport experiments: Cells grown on coverslips were loaded at room temperature with 1μM 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM, 10 min, Tef Labs) or 5μM Fura-2 AM (20 min, Tef Labs) in Ringer's solution composed of (in mM): 120 NaCl; 5.4 KCl; 1.8 CaCl2; 0.8 MgCl2; 10 HEPES; 10 glucose; pH 7.4, containing 0.1% bovine serum albumin. Cells were then washed twice in Ringer's solution containing 0.1% bovine serum albumin and incubated for an additional 20 min at room temperature. Treatment with extracellular Zn^{2+} and inhibitors was done immediately prior to imaging, as indicated for each experiment. Coverslips were then mounted in a perfusion chamber of a Zeiss Axiovert 200 microscope and images were acquired every 3s (Indec Imaging Workbench 5) using a 10× objective with 4 × 4 binning of the image (Sensi-Cam, PCO), as done previously [23]. BCECF was excited at 440 nm and 470 nm while Fura-2 was excited at 340 nm and 380 nm, with polychrome monochromator (TILL Photonics), both were imaged with a 510-nm long-pass filter. The fluorescent signal is presented as the ratio between the two wavelengths (R=F440/F470 or R=F340/F380). All results shown are traces of the averaged responses of ~60 cells in each experimental paradigm, for each condition at least 3 independent experiments. As marked in the graphs, n represents the number of slides used for each treatment.

NH_4Cl paradigm: Cells were perfused with K^+-free Ringer's solution to obtain baseline fluorescence and then NH_4Cl was added (30mM to TAMR cells and 15mM to MCF-7 cells) while monitoring BCECF fluorescence. In a set of experiments, N-methyl-d-glucamine (NMDG, 120mM) replaced NaCl in the Ringer's solution yielding a Na^+-free solution. Application of NH_4Cl, due to the resting equilibrium with NH_4^+, induces diffusion of NH_3 into the cells and alkalinization of the cells. The inward gradient of NH_4^+, acting as a surrogate to K^+, activates ion transporters and intracellular NH_4^+ induces cellular acidification. The rate of NH_4^+ influx (that is the rate of acidification), determined using a linear fit of the decrease in

fluorescent signal during a 60s period following the peak, represents transporter activity, as previously described [23, 27].

In-Vitro Scratch Assay: Cells were seeded at 4×10^4 cells/ well in 96-well plates, grown to confluency and then deprived of serum for 24h. A scratch was performed using 200µl plastic pipette tip (~2 mm in width), as previously described [13], in 37°C Ringer's solution with Ethylenediaminetetraacetic acid (EDTA) (100µM), a concentration that does not affect Ca^{2+} levels but chelate any residual Zn^{2+} that may activate ZnR/GPR39. Cells were then treated with Zn^{2+} (200µM) or EDTA (100µM) for 10 min and subsequently washed extensively. The Ringer's solution was then replaced back to phenol-red-free RPMI medium supplemented with 1% fetal calf serum plus penicillin-streptomycin, 4 mM L-glutamine. The Gq inhibitor YM254890 (1µM, 30 min) or KCC inhibitor DIOA (20µM, 30 min) were applied 30 min prior to scratching (DMSO at the same concertation was used as control). siRNA transfection was done 48h prior scratching. The percentage of scratch closure was determined by acquiring bright field images immediately after performing the scratch, and after 24h. Area measurements of the cell-free region were performed using ImageJ software, and the ratio of denuded areas at these times are presented.

Statistical analysis: Each bar graph represents an average±S.E.M of at least three independent experiments. Statistical analysis was performed using paired Student's t-test, comparing each treatment to Zn^{2+} treatment unless otherwise mentioned. * $p < 0.05$; ** $p < 0.01$; *** $p<0.001$.

3. Results

Since both ZnR/GPR39 and KCC enhance breast cancer cell proliferation, we asked if ZnR/GPR39 regulates KCC ion transport activity. Activation of ZnR/GPR39 primarily mediates Zn^{2+}-dependent Ca^{2+} release via the Gq-IP3 pathway [28-30]. Using the Ca^{2+}-sensitive fluorescent dye, Fura-2, we monitored Zn^{2+}-dependent Ca^{2+} release in tamoxifen resistant TAMR cells (Fig. 1A). The response was triggered by a brief exposure to 200µM Zn^{2+}, which activates ZnR/GPR39 in breast cancers cells but does not induce intracellular Zn^{2+} rise [7]. Importantly, such transient rises likely imitate Zn^{2+} release from cellular vesicles found in the mammary gland cells or Zn^{2+} liberation following cell death [13, 31, 32]. But Zn^{2+}-dependent Ca^{2+} responses were absent in MCF-7 breast cancer cells (Fig. 1B), from which the TAMR cells were derived [26, 33]. To compare whether this signaling induced Zn^{2+}-dependent transport activity in TAMR or MCF-7 cells, we used the well-established NH_4Cl transport paradigm in which NH_4^+ serves as a surrogate for K^+ and its transport affects intracellular pH that is monitored with BCECF [23, 24]. Application of Zn^{2+}, at concentrations that activated

ZnR/GPR39 in TAMR cells, was followed by enhanced NH_4^+ transport compared to non-treated cells (Fig. 1C, F). In contrast, application of Zn^{2+} to MCF-7 cells failed to enhance rates of ion transport (Fig. 1D, F). To determine the specific role of ZnR/GPR39, we silenced this receptor using siRNA constructs (siGPR39, Fig. 1E) in TAMR cells. Control cells transfected with a scrambled construct (si-control) showed Zn^{2+}-dependent Ca^{2+} signaling, but transfection of TAMR cells with siGPR39 resulted in loss of this signaling (Fig. 1E inserts). Purinergic response to ATP, triggered a similar intracellular Ca^{2+} release in si-control and siGPR39 cells indicating that the Gq-IP3 pathway was intact. We then monitored NH_4^+ transport in cells treated with or without Zn^{2+}. The Zn^{2+}-dependent enhancement of NH_4^+ transport was maintained in si-control cells (Fig. 1E middle panel), but in siGPR39 transfected cell Zn^{2+} failed to upregulate ion transport rates (Fig. 1E right panel). Thus, ZnR/GPR39 is essential for Zn^{2+}-dependent activation of NH_4^+ transport in breast cancer cells (Fig. 1F).

Since NH_4^+ transport may be mediated by Na^+-dependent NKCC transporters or Na^+-independent KCCs [34], we asked if the presence of Na^+ is essential for ZnR/GPR39 upregulation of ion transport. When NMDG replaced Na^+ in the perfusing solution, application of Zn^{2+} increased ion transport rates to a similar level as monitored in the presence of Na^+ (Fig. 2A-B). This result suggests that the Zn^{2+}-dependent increase in ion transport is mediated by a Na^+-independent KCC. Similarly, an inhibitor of NKCC subfamily, bumetanide (1μM, a concentration that does not affect KCC-dependent transport) did not attenuate the Zn^{2+}-dependent upregulation of ion transport in TAMR cells (Fig. 2C). In contrast, the KCC inhibitor [(DihydroIndenyl) Oxy] Alkanoic acid (DIOA, 20μM or 100μM) abolished Zn^{2+}-dependent upregulation of NH_4^+ transport in TAMR cells (Fig. 2D-E). This set of results suggests that KCC activity is upregulated by ZnR/GPR39 (Fig. 2F).

We next asked which of the KCC family members is regulated by Zn^{2+} in TAMR cells. The Zn^{2+}-dependent Ca^{2+} signaling found in TAMR cells, correlated with increased ZnR/GPR39 mRNA expression in the TAMR cells compared to MCF-7 cells [7]. We then asked KCC mRNA expression is also different between these cells. We found higher mRNA expression of KCC3 and KCC4, but not KCC1, in TAMR compared to MCF-7 cells (Fig. 3A). We therefore focused on KCC3 and KCC4 asking if they are regulated by ZnR/GPR39. First, we monitored NH_4^+ transport in BCECF loaded TAMR cells that were transfected with a scrambled siRNA construct (si-control). Upregulation of NH_4^+ transport by Zn^{2+} was maintained in si-control cells compared to transport rates in cells not treated with Zn^{2+} (Fig. 3B). In contrast, silencing of KCC3 expression attenuated Zn^{2+}-dependent increase of NH_4^+ transport rate (siKCC3,Fig. 3C, E). Upregulation of transport by Zn^{2+} was maintained in siKCC4 transfected cells (Fig. 3D, E). Altogether this set of experiments indicates that silencing

of KCC3, but not KCC4, reverses the Zn^{2+}-dependent upregulation of NH_4^+ transport in TAMR cells (Fig. 3E), suggesting that Zn^{2+} upregulates KCC3-dependent transport.

Finally, we asked whether Zn^{2+} via ZnR/GPR39-dependent upregulation of KCC3 enhances breast cancer cell proliferation and migration by using a scratch assay. First, we compared scratch closure between TAMR cells, expressing a functional ZnR/GPR39, and MCF-7 cells, deficient in ZnR/GPR39. Application of Zn^{2+}, at a concertation that selectively activates ZnR/GPR39, enhanced rates of scratch closure in TAMR cells (Fig. 4A, F) but not in MCF-7 cells (Fig. 4B, F). To determine the role of ZnR/GPR39 downstream signaling, we treated TAMR cells with a Gq inhibitor (YM254890, 1μM) that inhibits ZnR/GPR39-dependent Ca^{2+} signaling [13]. Note that Zn^{2+} failed to enhance scratch closure when cell were treated with this Gq inhibitor (Fig. 4C, F). To specifically determine the role of ZnR/GPR39, cells were transfected with siGPR39 or si-control. We found that scratch closure rates were enhanced by Zn^{2+}-treatment in si-control cells (Fig. 4D, F), but not in siGPR39 transfected cells (Fig. 4E, F). Altogether these results suggest that Zn^{2+}-dependent cell growth is mediated by ZnR/GPR39. Next, we tested whether accelerated scratch closure rates were mediated by KCC activity. In the presence of DIOA, Zn^{2+} treatment failed to accelerate rates of scratch closure, (Fig. 5A, D). Since KCC3 activity was upregulated by Zn^{2+} (Fig. 3), we then transfected cells with siKCC3 and determined its direct role in cell migration and proliferation. Zn^{2+} did not enhance rates of scratch closure in cells transfected with siKCC3 but was effective in cells transfected with si-control (Fig. 5B-C, D). Thus, we suggest that Zn^{2+} via ZnR/GPR39 activates KCC3 and thereby enhances rates of scratch closure in ZnR/GPR39-expressing TAMR cells.

4. Discussion

The ZnR/GPR39 pathway was previously shown to induce Ca^{2+} release and downstream activation of ion transport, which was essential for regulating ion gradients, absorption and cell growth [13, 23, 24, 35, 36]. We now provide evidence for a role of ZnR/GPR39 in upregulating KCC3-dependent ion transport and leading to enhanced breast cancer cell proliferation and migration. The Zn^{2+}-dependent Ca^{2+} release triggered by ZnR/GPR39 was essential for upregulation of ion transport activity in TAMR cells, as NH_4^+ transport was diminished following silencing of ZnR/GPR39. In agreement, MCF-7 cells lacking ZnR/GPR39 do not show Zn^{2+}-dependent upregulation of transport activity. Finally, activation of both ZnR/GPR39 and KCC3 was essential to enhance scratch closure in TAMR breast cancer cells.

Activation of ZnR/GPR39 signaling cascade by Zn^{2+} enhanced scratch closure in keratinocytes, skin cells [13, 37], and was associated with a well-known role of zinc in enhancing wound healing. Similarly, enhanced proliferation was induced by ZnR/GPR39 in

colonocytes and prostate cancer cells [38-41]. Activation of ZnR/GPR39 requires transient changes in extracellular Zn^{2+}, and release of this ion from injured cells was sufficient to activate ZnR/GPR39 signaling [13]. Malignant tumors *in-vivo* are characterized by regions of cell death due to lack of oxygen, glucose and/or other nutrients [42, 43]. Such massive cell death can trigger extracellular Zn^{2+} transients and activate ZnR/GPR39 in neighboring breast cancer cells. In addition, breast epithelial cells accumulate large amounts of Zn^{2+} in lysosomes [44-46], and express multiple Zn^{2+} transporters that maintain the distribution of this ion in cellular compartments [47]. As Zn^{2+} transporters expression is modulated in breast tumor tissue [5, 9, 48], aberrant Zn^{2+} transport can induce release of Zn^{2+} and also trigger activation of ZnR/GPR39. Finally, release of lysosomal ATP from breast cancer cells following TRPML1 activation [49] may also induce release of Zn^{2+} that is selectively stored in this organelle. Thus, numerous sources for transient Zn^{2+} release in the tumor microenvironment can activate ZnR/GPR39 and subsequent KCC3 upregulation that will promote tumor progression.

Several studies associated ZnR/GPR39 with cancer and tumor growth, but the role of Zn^{2+}-dependent signaling was not specifically addressed. For example, levels of ZnR/GPR39 were higher in breast cancer tumors compared to benign biopsies [7, 8, 50]. Similar increase in ZnR/GPR39 was monitored during epidermal wound healing, at a stage of maximal growth when the epidermis was thickest [37]. In esophageal squamous cell carcinoma (ESCC), ZnR/GPR39 was found to contribute to cell growth, foci formation and metastasis via activation of cyclin D1 [51]. In addition, in HT29 cells, a colorectal adenocarcinoma cell line, silencing ZnR/GPR39 attenuated cell growth while activation of ZnR/GPR39 reduced butyrate-induced apoptosis [40]. In breast cancer cells, ZnR/GPR39-dependent activation of MAPK and AKT/PI3K enhanced proliferation and migration [7]. Here, we show that Zn^{2+}-dependent enhancement of proliferation and migration of TAMR breast cancer cells requires activation of both ZnR/GPR39 and the K^+/Cl^- cotransporter, KCC3.

Solute and ion secretion by epithelial cells controls cell volume, which also regulates the activity of KCC1, KCC3 and KCC4 [34, 52]. Regulation of cell volume involves re-organization of the actin cytoskeleton. Indeed, the guanine nucleotide exchange factor, Vav2, which is responsible for cell spreading, interacts with KCC3 via a proline rich domain and this interaction localizes KCC3 to actin rich membrane protrusions [53]. Similar changes in the actin cytoskeleton are required for lamellipodia formation and cell migration, processes that play an important role in cancer metastasis. Our data indicate that ZnR/GPR39 upregulation of KCC3 activity is required to enhance cell migration. This is based on reduced scratch closure following either: silencing of ZnR/GPR39, inhibition of its downstream signaling or silencing of KCC3 (Fig. 4-5). It would be interesting to determine in future studies if ZnR/GPR39 upregulation of KCC3 activity affects actin cytoskeleton and thereby cell migration.

Activation of KCC3 and KCC4 by IGF-1 and downstream MAPK pathway enhanced MCF-7 breast cancer cell proliferation, epithelial mesenchymal transition and migration of these cancer cells [16, 20, 54]. Activation of MAPK was essential to mediate ZnR/GPR39-dependent activation of KCC2 in neurons [23]. Upregulation ZnR/GPR39 was observed in estrogen receptor deficient breast cancer cells, and involved activation of MAPK and AKT/PI3K pathways in TAMR cells [7]. Our results indicate that even though both KCC3 and KCC4 mRNA were increased in TAMR cells compared to MCF-7, the Zn^{2+}-dependent ZnR/GPR39 signaling pathway upregulated KCC3 activity, but not KCC4. Moreover, silencing of KCC3 reduced ZnR/GPR39-dependent scratch closure, suggesting that in this model KCC3 is the major mediator of Zn^{2+} and ZnR/GPR39 dependent cell proliferation. Activity of KCC3 was also essential for the ZnR/GPR39-dependent growth effect, as it was abolished by application of DIOA, an inhibitor of the transport itself. Similarly, activation of KCC3-dependent transport was required for proliferation of cervical cancer cells and KCC3 specific localization in lamellipodia was associated with their migration [53, 55]. Our data show that ZnR/GPR39 activates KCC3-dependent ion transport to enhance breast cancer proliferation and migration.

In this study, we provided evidence that activation of Zn^{2+}-dependent ZnR/GPR39 signaling cascade results in breast cancer growth mediated by KCC3. Both ion transport and GPCRs are targeted in cancer treatment [56-58], developing treatment that will target ZnR/GPR39-dependent KCC3 activation in breast cancer tissue could effectively attenuate tumor progression.

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Figure Legends:

Figure 1: **Zn^{2+}-dependent up regulation of ion transport is mediated by ZnR/GPR39.** (A) TAMR or (B) MCF-7 cells were loaded with Fura-2 and fluorescence was monitored in the respective cells following application of Zn^{2+} (200μM) to trigger ZnR/GPR39 response, or ATP to activate purinergic signaling, as control. (C) TAMR or (D) MCF-7 cells loaded with BCECF (0.5μM) were superfused with K^+ free Ringer's solution. At the indicated time, NH_4Cl was added (30 mM for TAMR cells and 15 mM for MCF-7 cells) and changes in BCECF fluorescence were monitored (see Methods). To activate ZnR/GPR39, prior to imaging, cells were pre-treated with Zn^{2+} (200μM, 2 min, grey trace) or without it, as control (black trace). (N=14 or 21, in control or Zn^{2+} treated respectively) (E) TAMR cells were transfected with scrambled siRNA sequence (si-control) or with siRNA sequence aimed to silence ZnR/GPR39 (siGPR39). Left panel shows mRNA expression level of ZnR/GPR39 following transfection. The NH_4^+ paradigm was applied to si-control (middle panels) or siGPR39 (right panels) transfected cells that were pre-treated with or without Zn^{2+}, as in B (N=10 slides for si-control or siGPR39). Insets in middle and right panels show Fura-2 fluorescence monitored in si-control or siGPR39, respectively. (F) Averaged rates of NH_4^+ -dependent acidification as monitored in C-E (N= number of slides in 4 independent experiments for each paradigm, *, $p<0.05$, N.S. is non-significant; between Zn^{2+} treated and control cells for each independent paradigm).

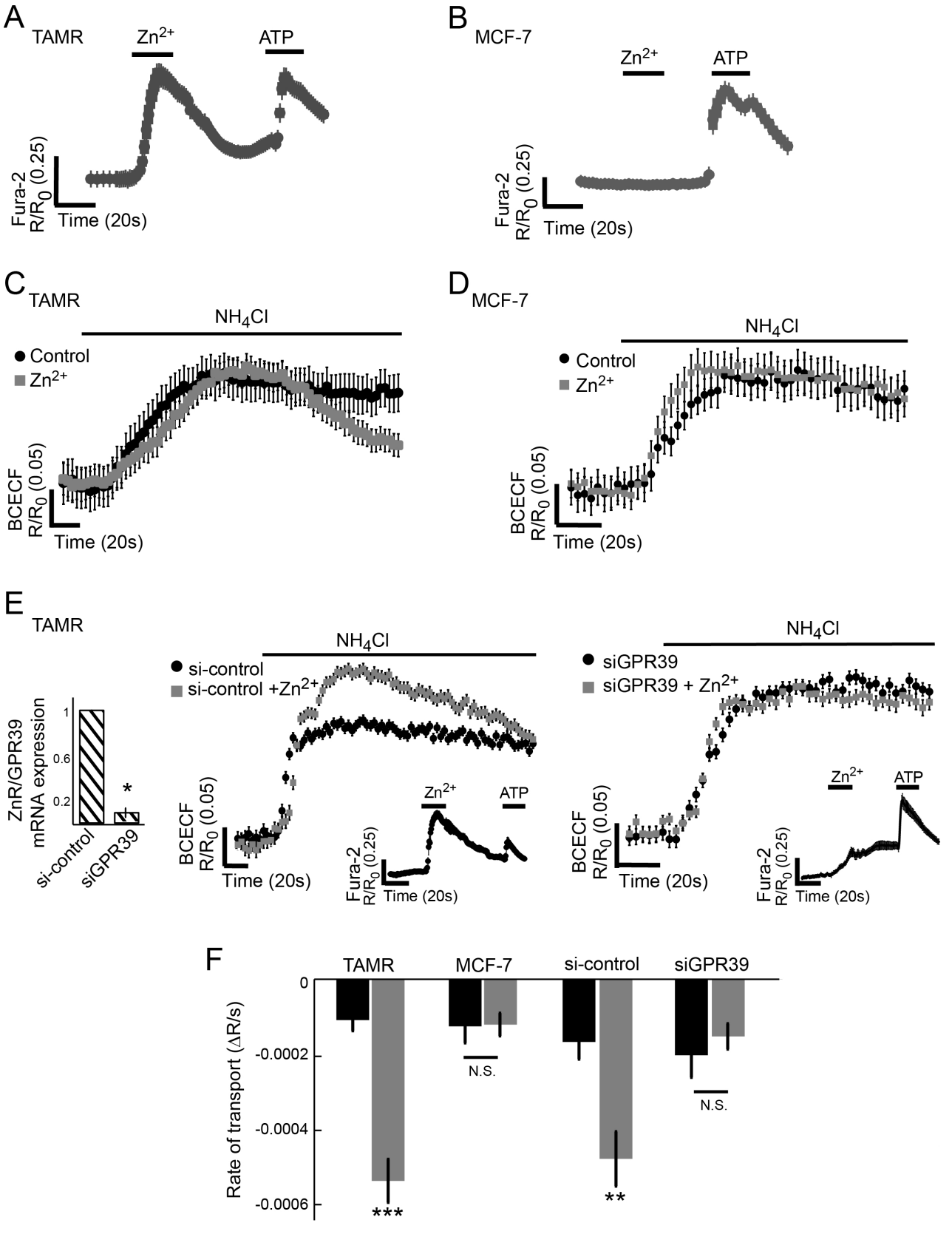
Figure 2: **ZnR/GPR39-dependent transport in TAMR cells is mediated by a KCC.** (A) TAMR cells were pre-treated with Zn^{2+} (200μM, grey traces) or without it (black traces) to trigger ZnR/GPR39-dependent ion transport as measured using the NH_4^+ paradigm (see Fig. 1). (B) TAMR cells were superfused with NMDG containing Ringer's solution (replacing Na^+) and pre-treated with or without Zn^{2+} as in A. (C) The NKCC blocker, bumetanide (1μM) was applied prior to the NH_4^+ paradigm (application of 30mM NH_4Cl to BCECF loaded cells, see Methods). Cells were pre-treated with or without Zn^{2+} and ion transport measured as in A. (D) Cells were incubated with the KCC inhibitor, DIOA (100μM), and the NH_4^+ paradigm was applied with or without Zn^{2+} treatment. (E) Cells were incubated with DIOA (20μM). (F) Averaged rates of NH_4^+ transport in TAMR cells as monitored in A-E (N=12 or 15 slides, in control or Zn^{2+} treated respectively, in 3 independent experiments for each paradigm, *, $p<0.05$, N.S. is non-significant; between Zn^{2+} treated and control cells for each independent paradigm).

Figure 3: **ZnR/GPR39-dependent transport in TAMR cells is mediated by KCC3**. (A) mRNA expression levels of KCC family members and ZnR/GPR39 were measured using qPCR, in MCF-7 and TAMR cells. mRNA levels were normalized to the expression in MCF-7 cells (N=3). (B) The NH_4^+ paradigm was applied to TAMR cells (application of 30mM NH_4Cl to BCECF loaded cells, see Methods) that were transfected with scrambled (si-control) siRNA constructs and cells were treated with or without Zn^{2+} as in Fig. 1. (C) TAMR cells were transfected with siKCC3 or (D) siKCC4 and the NH_4^+ paradigm was performed (30mM NH_4Cl to BCECF loaded cells) in cells treated with or without Zn^{2+} as in Fig. 1. Insets in C-D indicate the mRNA expression levels of KCC after transfection with the indicated siRNA construct. (E) Averaged rates of NH_4^+ transport in TAMR cells as monitored in B-D (N=16 or 18 slides, in control or Zn^{2+} treated respectively, in 3 independent experiments for each paradigm, *, $p<0.05$, N.S. is non-significant; between Zn^{2+} treated and control cells for each independent paradigm).

Figure 4: **Zn^{2+}, via ZnR/GPR39, enhances scratch closure rates in TAMR cells.** TAMR cells (A) and MCF-7 cells (B) were grown to confluence, and a scratch was performed in the presence of EDTA (100μM) to chelate residual Zn^{2+}. Cells were treated with or without Zn^{2+} (200μM, 10min, N=41-69 wells for each treatment). (C) TAMR cells were pre-treated with the

Gq inhibitor, YM254890 (1μM, N=21 wells for each treatment) and the scratch assay was performed as in A. (D) TAMR cells were transfected with si-control or (E) siGPR39, and the scratch assay was performed as in A (N=14 wells for each treatment). (A-E) For each condition representative images are shown, of cell cultures immediately after scratch (top panels, 0h) and at 24h (bottom panels), and with Zn^{2+} treatment (right panels) or without it (left panels). (F) The average of void area closure (difference between area of void at 0h and at 24h) normalized to the initial void area, representing the percentage of scratch closure over 24h for cells treated with Zn^{2+} (grey bars) or without it (black bars, control). (N=number of wells in 3 independent experiments for each condition; *, $p<0.05$, N.S. is non-significant; between Zn^{2+} treated and control cells for each independent paradigm).

Figure 5: **ZnR/GPR39 enhances scratch closure rates via KCC3 in TAMR breast cancer cells.** TAMR cells were used for the scratch assay as in Figure 4. (A) Cells were treated with DIOA (20μM) and then with or without Zn^{2+} (200μM, N=37-41 wells for each treatment) prior to performing the scratch. (B) TAMR cells were transfected with si-control or (C) siKCC3 and the scratch assay was applied (N=20 wells for each treatment). For each condition representative images are shown, of cell cultures immediately after scratch (top panels, 0h) and at 24h (bottom panels), and with Zn^{2+} treatment (right panels) or without it (left panels). (D) The average of void area closure (difference between area of void at 0h and at 24h) normalized to the initial void area, representing the percentage of scratch closure over 24h for cells treated with Zn^{2+} (grey bars) or without it (black bars, control). (N=number of wells in 3 independent experiments for each condition; *, $p<0.05$, N.S. is non-significant; between Zn^{2+} treated and control cells for each independent paradigm).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B
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Zn2+
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Time (20s)
C
TAMR
NH4Cl
Control
Zn2+
BCECF R/R0 (0.05)
Time (20s)
D
MCF-7
NH4Cl
Control
Zn2+
BCECF R/R0 (0.05)
Time (20s)
E
TAMR
ZnR/GPR39 mRNA expression
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0.6
0.2
*
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siGPR39
NH4Cl
si-control
si-control +Zn2+
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Time (20s)
Zn2+
ATP
Fura-2 R/R0 (0.25)
Time (20s)
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BCECF R/R0 (0.05)
Time (20s)
Zn2+
ATP
Fura-2 R/R0 (0.25)
Time (20s)
F
TAMR
MCF-7
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N.S.
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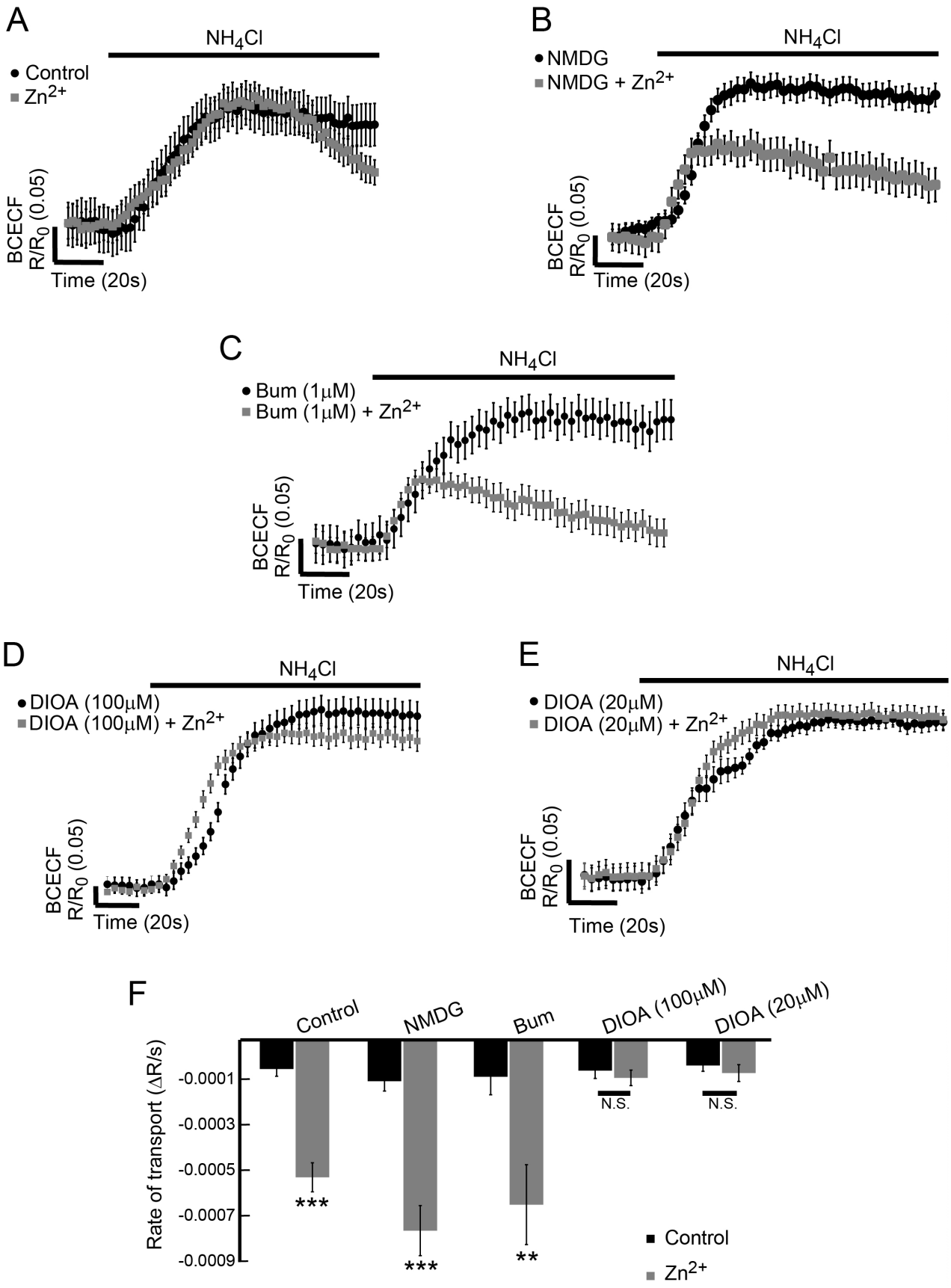
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Time (20s)
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Bum (1μM) + Zn2+
BCECF R/R0 (0.05)
Time (20s)
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DIOA (100μM) + Zn2+
BCECF R/R0 (0.05)
Time (20s)
E
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DIOA (20μM)
DIOA (20μM) + Zn2+
BCECF R/R0 (0.05)
Time (20s)
F
Control
NMDG
Bum
DIOA (100μM)
DIOA (20μM)
Rate of transport (ΔR/s)
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N.S.
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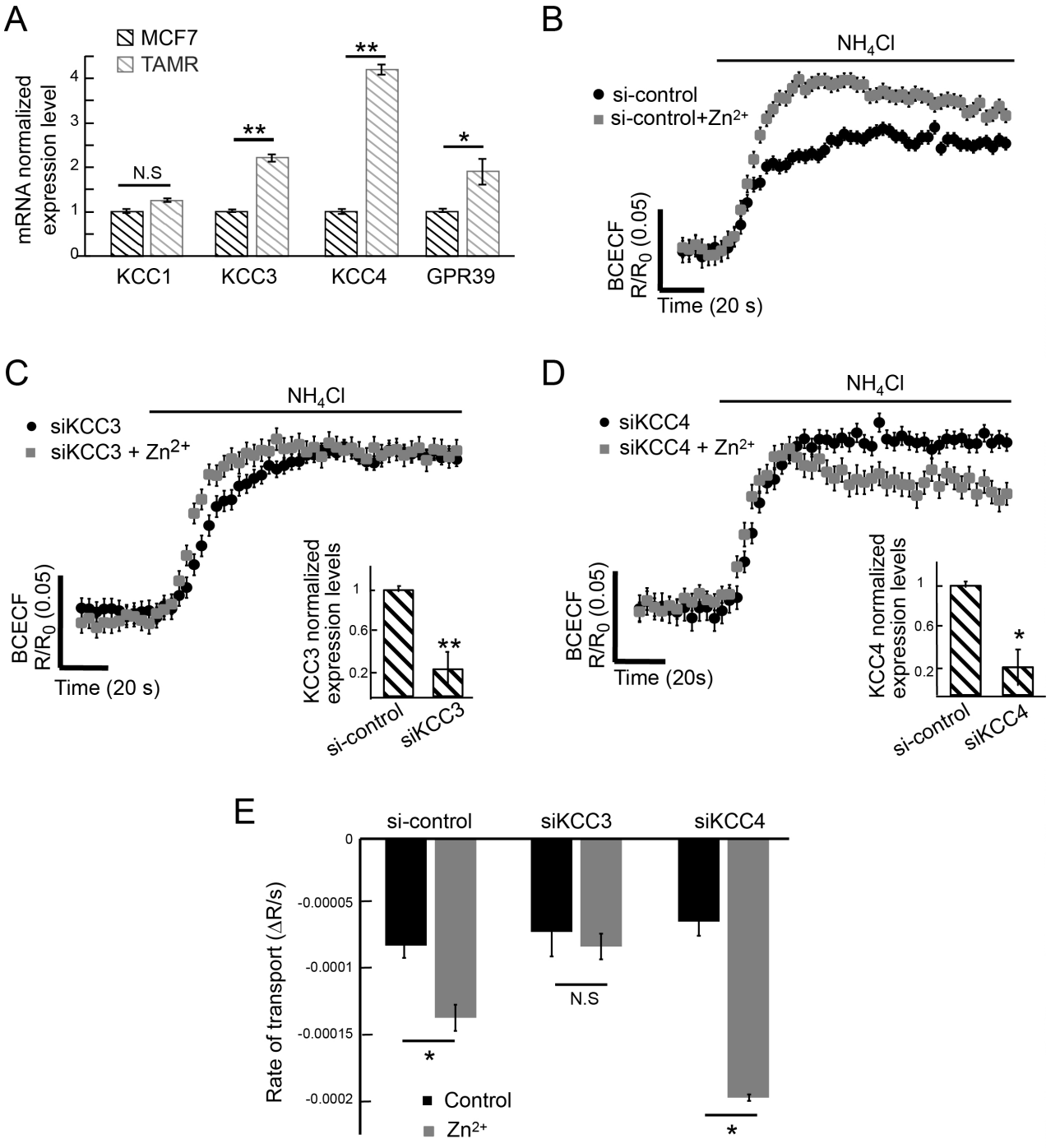
Control
Zn2+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KCC4
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si-control+Zn2+
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KCC3 normalized expression levels
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D
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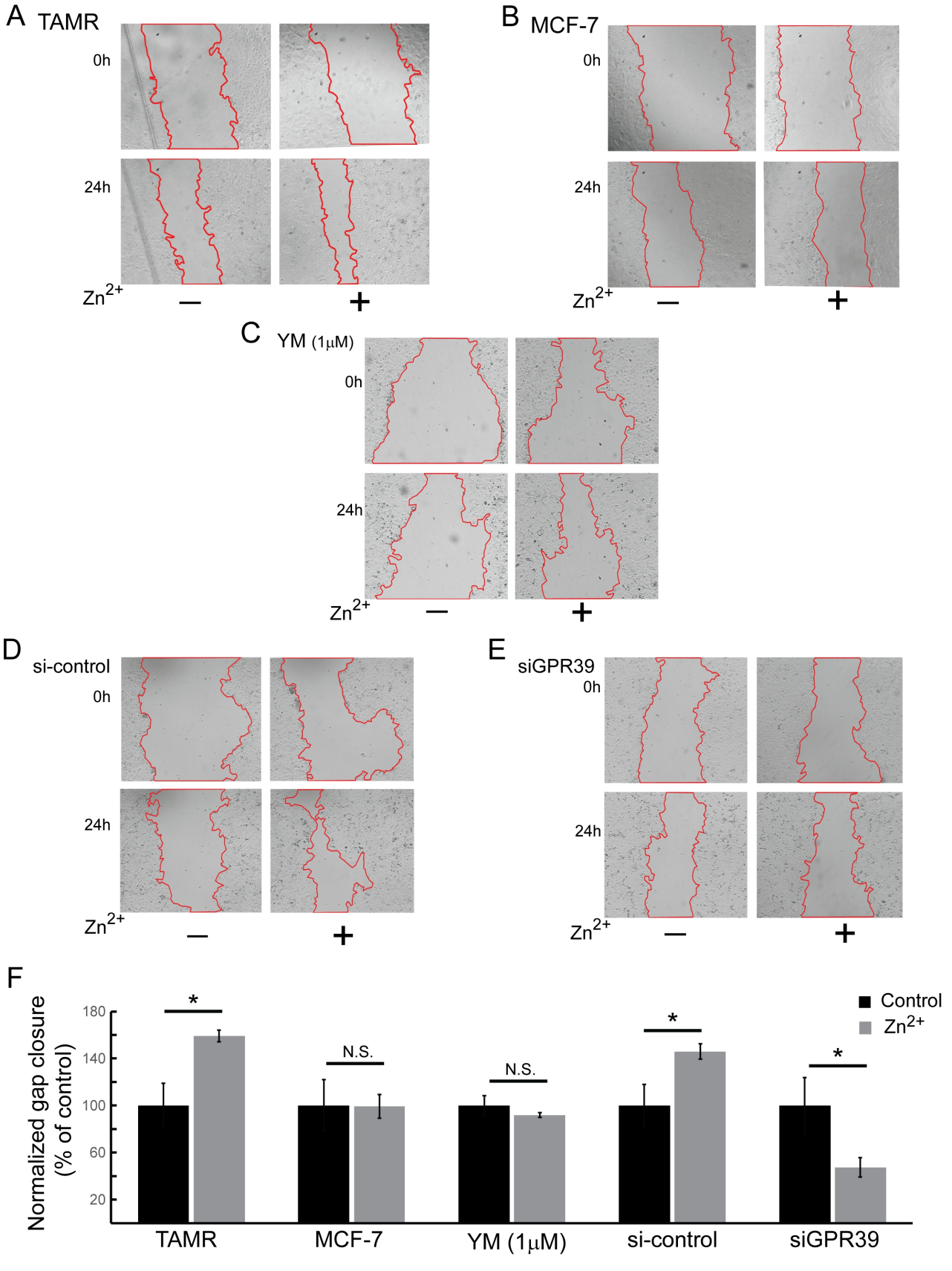
Rate of transport (ΔR/s)
N.S
Control
Zn2+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D
si-control
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24h
Zn2+
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+
E
siGPR39
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24h
Zn2+
—
+
F
Normalized gap closure
(% of control)
180
140
100
60
20
*
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N.S.
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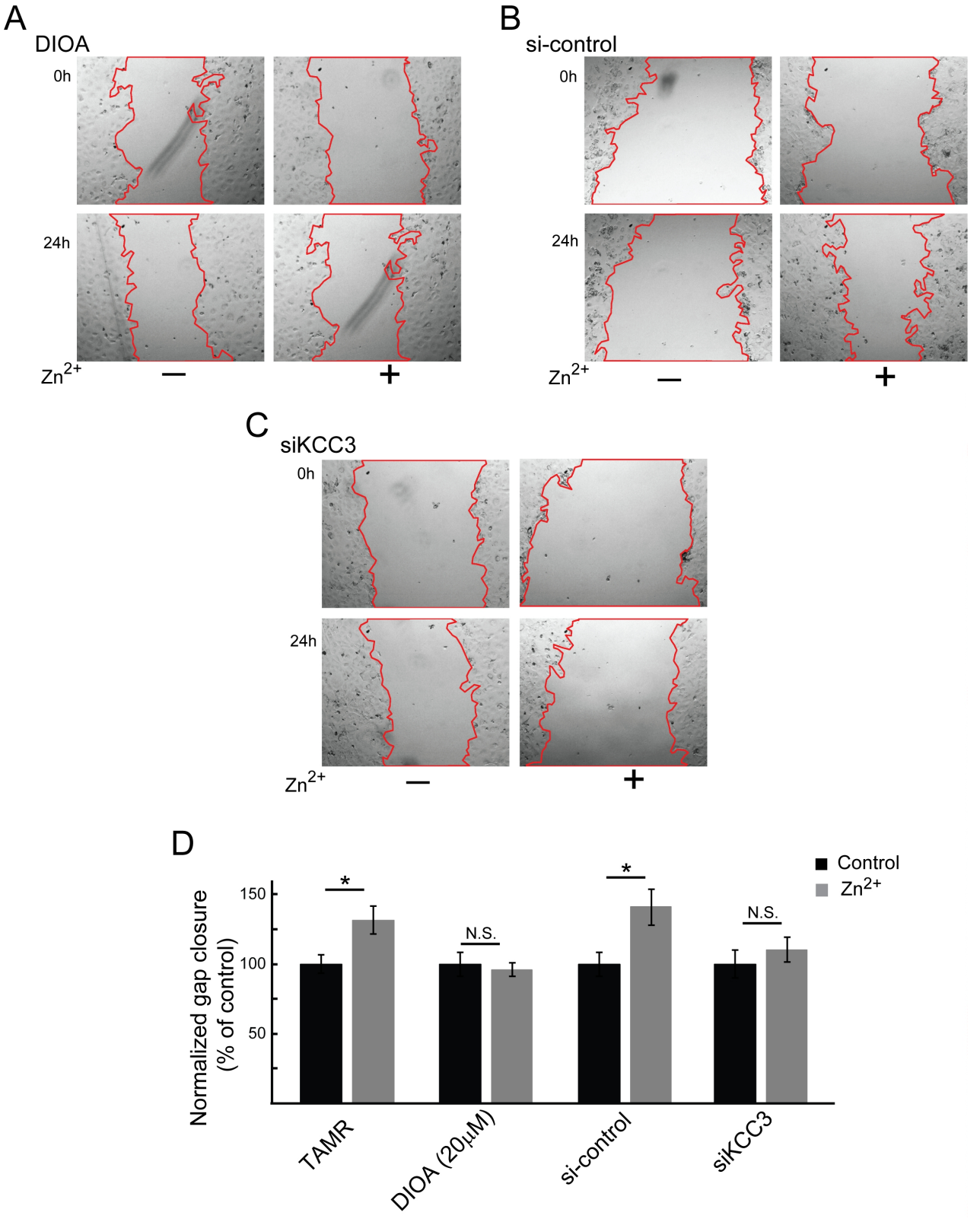
Control
Zn2+
TAMR
MCF-7
YM (1μM)
si-control
siGPR39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0h
24h
Zn2+
—
+
D
Normalized gap closure
(% of control)
150
100
50
*
N.S.
*
N.S.
Control
Zn2+
TAMR
DIOA (20μM)
si-control
siKCC3

Conflict of Interests statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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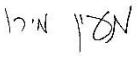
Maayan Mero 

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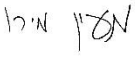
Maayan Mero 

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