TRPM7 Is Required for Breast Tumor Cell Metastasis

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Abstract

TRPM7 encodes a Ca2+-permeable nonselective cation channel with kinase activity. TRPM7 has been implicated in control of cell adhesion and migration, but whether TRPM7 activity contributes to cancer progression has not been established. Here we report that high levels of TRPM7 expression independently predict poor outcome in breast cancer patients and that it is functionally required for metastasis formation in a mouse xenograft model of human breast cancer. Mechanistic investigation revealed that TRPM7 regulated myosin II–based cellular tension, thereby modifying focal adhesion number, cell–cell adhesion and polarized cell movement. Our findings therefore suggest that TRPM7 is part of a mechanosensory complex adopted by cancer cells to drive metastasis formation. Cancer Res; 72(16); 4250–61. ©2012 AACR.

Introduction

Metastasis formation is a complicated multistep process involving tumor cell dissemination from the primary tumor, matrix invasion, entry into the circulatory system, extravasation through capillary endothelium, and, finally, the outgrowth of secondary tumors in distant organs. Each of these events requires extensive and continuous Ca2+-dependent remodeling of the actomyosin cytoskeleton as well as close interactions with the surroundings of a cell, mediated by dynamic adhesive structures, such as focal adhesions and adherens junctions. These specialized cell adhesion sites convey mechanical cues across the plasma membrane, affecting both the physical properties of their surroundings as well as intracellular cytoskeletal dynamics. As the formation and maturation of focal adhesions and adherens junctions is dependent on the applied mechanical forces, these structures are considered to function as mechanosensors that integrate mechanical cues from inside and outside the cell (1–5). The complex protein–protein interactions within these adhesion sites significantly contribute to tumor progression and metastasis formation. Hence, proteins that regulate adhesion formation or turnover represent interesting therapeutic targets to limit the metastatic potential of cancer cells (6).

Members of the mammalian transient receptor potential (TRP) cation channel family are considered key players in mechanosensory signaling (7–11). TRP channels organize into large macromolecular complexes linked to the actomyosin cytoskeleton, which may serve to localize signal transduction pathways and/or enhance the rate of signal transmission (7, 12, 13). Tethered to the cytoskeleton, their ion conducting properties can be modulated by different stimuli, including mechanical cues, resulting in a variety of cellular responses. In earlier work, we and others identified TRPM7, a Ca2+-permeable nonselective cation channel with kinase activity, as a regulator of actomyosin contractility, cell adhesion, and directed cell migration (14–16). However, a role for this bifunctional channel in cancer progression has not been examined. Here we show that high TRPM7 expression, at the time of diagnosis, predicts poor therapy outcome in a large cohort of breast cancer patients. Moreover, TRPM7 is a critical determinant of breast cancer cell migration in vitro and metastasis formation in vivo.

Materials and Methods

TRPM7 protein expression in primary breast cancer tissue samples detected by immunohistochemistry

Formalin-fixed, paraffin-embedded breast tumor tissue, derived from the tumor bank of the Department of Laboratory Medicine of the Radboud University Medical Centre, was probed for TRPM7 (1:400; Cayman Chemical Company), followed by biotin-conjugated donkey anti-mouse IgG and DAB and counterstained with hematoxylin.
TRPM7 expression measurements in patient samples

Our discovery cohort consisted of 368 early-stage breast cancer samples, described in a previous study (17). The validation cohort consisted of 144 patients with unilateral breast cancer who had undergone resection of their primary tumor between November 1987 and December 1997 (18). TRPM7 expression levels in tumor samples derived from the discovery cohort were determined by microarray analysis using Affymetrix U133B Genechips (Affymetrix). Raw data are available at the Gene Expression Omnibus repository database (GEO accession number: GSE65332). TRPM7 expression levels in the validation cohort were determined by quantitative PCR reactions on cDNA samples derived from primary tumors, using power SYBR-green reagent (Applied Biosystems) in combination with TRPM7-specific primers (forward: TACGGTCTACGCCAGG; reverse: GCATCTTCTCCTAGATTGGC) according to manufacturer’s recommendations. TRPM7 gene expression levels were normalized to the HPRT housekeeping gene (forward: GGTCTTCTTCCAGACCAAGCT; reverse: TGA-CACGTGCAAAACAATGCA) and calculated according to the cycling threshold method (19). Statistical analyses were carried out using SPSS software (version 16.0; SPSS). Discovery and validation cohorts were dichotomized using median TRPM7 expression as cut-off. Survival curves were visualized by Kaplan–Meier plots, using recurrence-free and distant metastasis-free survival as endpoints and compared using log-rank tests. HRs were estimated by univariate Cox regression analysis and validated using log-rank tests. The independent prognostic value of TRPM7 was assessed by univariate and multivariate Cox regression analysis on combined discovery and validation cohorts.

Generation and validation of cell lines

Human TRPM7 short hairpin RNAs (shRNA; #1: 5-GGCAGGCAGAGCCGCA-3; #2: 5-CAGCACGACCCTAC-3) were introduced in MDA-MB-231 [HTB-26, American Type Culture Collection (ATCC)] and human MCF7 TRPM7 shRNA #1 was introduced in MCF7 human breast cancer cells (HTB-22, ATCC), using the pLKO lentiviral expression vector according to manufacturer’s instructions (Sigma Aldrich). A nonfunctional shRNA (5-GTCAGAAAGAACAAAATCT-3) was used as negative control. Transduced cells were selected with 1 μg/mL puromycin. For bioluminescent imaging, control and TRPM7 knockdown MDA-MB-231 cells were cotransduced with a retroviral pLZS luciferase reporter construct and selected with 0.5 mg/mL Zeocin. For rescue of TRPM7 expression levels, HA-tagged mouse TRPM7, containing one mismatch with respect to the human-specific shRNA (14), was introduced in MDA-MB-231 TRPM7 shRNA cells. Transduced cells were selected with 1 mg/mL G418. TRPM7 mRNA expression levels were determined by quantitative reverse transcriptase PCR with the additional use of mouse-specific TRPM7 primers (forward: TAGGCCTTTACGCCAGGACC; reverse: GATCTTCTCCTAGATTGGCAG). TRPM7 protein levels were determined by radioactively labeling the TRPM7 kinase domain as described previously (14).

Cell viability and proliferation measurements

The effect of TRPM7 knockdown on cell viability and proliferation was assessed by MTS assays according to manufacturer’s instructions (Promega). Cell-cycle distribution of the different cell lines was determined by fluorescence-activated cell sorting (FACS) analysis on cells stained with propidium iodide. Cells were harvested and the cell pellet was incubated in staining solution (1 mg/mL sodium citrate, 0.1 mg/mL RNaseA, 20 μg/mL propidium iodide, and 0.1% Triton X-100). Cells were washed and subjected to FACS analysis. Cell-cycle distribution was quantified using FlowJo analysis software.

Mouse xenograph experiments

All animal work was carried out in accordance with protocols approved by the Animal Welfare Committee (DEC-NKI-10.034). Immunodeficient Rag2−/−IL2rg−/− mice were used for metastasis experiments. MDA-MB-231-Luc control and TRPM7 knockdown cells were collected and washed with PBS. Subsequently, 0.2 mL PBS containing 5 × 10^6 cells was injected into a tail vein. Tumor growth was monitored by bioluminescence imaging from day 7 onwards. Beetle luciferin (Promega) was dissolved at 15 mg/mL in PBS and stored at −20°C. Animals were anaesthetized with 2% to 3% isoflurane. Luciferin solution was injected intraperitoneally (0.01 mL/g body weight). Light emission was measured 15 minutes later, using a cooled CCD camera (IVIS; Xenogen), coupled to Living Image acquisition and analysis software over an integration time of 1 minute. Signal intensity was expressed as flux (photons/sec) integrated over the lung region. Lung tissue was collected at day 30 after injection. Tissues were fixed in EAF (ethanol–acetic acid–formol saline fixative, 40:5:10:45% v/v) and processed for histology. Paraffin sections were stained with hematoxylin and eosin. All microscopic images were acquired using IP-Lab software (Scanalytics Inc.) in combination with a monochrome CCD camera (Retiga SRV, 1,392 × 1,040 pixels) and a RGB filter (Slikder Module; QImaging) attached to a motorized microscope (Leica DM6000). Quantification of tumor size and number was carried out by ImageJ image analysis software.

Cell migration, elongation, and scatter experiments

Following overnight serum starvation, cells were harvested and resuspended in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 0.1% FBS. Subsequently, 50,000 cells were applied to a transwell insert with 8-μm pore size (Corning Life Sciences), which was incubated in DMEM supplemented with 10% FBS. Cells were allowed to migrate for 8 hours at 37°C. Migrated cells were fixed (75% methanol and 25% acetic acid) and stained (0.25% Coomasie blue, 45% methanol, and 10% acetic acid in H_2O). Single cell migration on vitronectin-coated (500 ng/mL) culture dishes was followed for 24 hours by time-lapse microscopy and analyzed using ImageJ image analysis software. Cell elongation was determined based on length and width ratios, measured after 24 hours. A cell was considered elongated when the ratio length/width was larger than 2. MCF7 cell scattering on vitronectin-coated (500 ng/mL) culture dishes was visualized by time-lapse microscopy in DMEM supplemented with 0.1% FBS. Gap closure assays were carried
out according to manufacturer’s recommendations (Applied Biophysics). In short, 40,000 MCF7 cells were seeded per insert and cultured overnight. After removal of the insert, cells were allowed to migrate for 24 hours, and migration was followed by time-lapse microscopy. Where indicated, cells were incubated in the presence of different concentrations Y27632 (Sigma-Aldrich) and GSK429286 (Seleckbio) Rho-kinase inhibitors.

**Fluorescent staining of focal adhesions, E-cadherin, and F-actin**

Images were taken with a Leica TCS SP5 confocal (Leica Microsystems) equipped with a 63× water-immersion objective and LAS-AF acquisition software (Leica Microsystems). Cells were cultured overnight on vitronectin-coated (500 μg/mL) glass coverslips in DMEM containing 0.1% FBS. Where indicated, cells were next incubated for 2 hours in the presence of 5 μM Y27632 Rho-kinase inhibitor (Sigma-Aldrich). Cells were fixed in 3.7% formaldehyde, permeabilized in 0.1% Triton X-100, and stained for pTyr118 paxillin (1:100; Life Technologies), E-cadherin (1:200; BD Biosciences) and F-actin, using Alexa-568–conjugated Phalloidin (1:100; Life Technologies). The average number of focal adhesions per cell was quantified using an ImageJ analysis routine (macro). Series of paxillin images (pixel size, 0.11 μm2) were normalized with respect to intensity/contrast, background was subtracted and cell boundaries were detected by manually setting the appropriate threshold. The original image was subjected to a rolling ball filter of radius 10 pixels, which effectively suppresses staining irregularities while retaining contrast in the focal adhesions. Further thresholding and the “Analyze Particles” plugin (settings: particle size 30–500 pixels; circularity 0.1–1.0) were used to determine the number of focal adhesions. Photomicrographs of at least 60 cells were analyzed for each condition.

**Detection of pSer19 myosin light chain and focal adhesion–associated pTyr118 paxillin on Western blot**

For detection of pSer19 myosin light chain in control and TRPM7 shRNA–transduced MDA-MB-231 cells, cells were lysed in Laemmli buffer supplemented with 1 μM MgCl2 and 1:200 Benzoate Nuclease (Merck) and left on ice for 30 minutes. Focal adhesion–associated proteins were extracted from MDA-MB-231 cells, as described previously (20). Proteins were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. Blots were incubated with rabbit polyclonal anti-pTyr118 paxillin antibody (1:750; Life Technologies) or anti-pSer19 myosin light chain antibody (1:100; Life Technologies) and mouse monoclonal γ-tubulin (1:10,000; Sigma Aldrich) antibodies, followed by horseradish peroxidase–conjugated secondary antibodies (1:5,000; Dako). Proteins were detected using ECL Western blot reagent (GE Healthcare) and exposing the blots to film.

**Statistical analysis**

Statistical data are expressed as mean ± SD or ± SEM, as indicated in the text. Statistical differences were tested with 2-sided, unpaired Student t tests, and P less than 0.05 was considered statistically significant.

**Results and Discussion**

**TRPM7 mRNA expression levels in primary tumors are associated with breast cancer progression and metastasis formation, independent from standard clinical parameters**

Immunohistochemistry on primary breast cancer tissue samples showed that TRPM7 protein is expressed by epithelial cells that align mammary glands and by breast tumor cells. Perinuclear staining of breast carcinoma cells was observed with accentuation of the nuclear membrane, with or without diffuse staining of the cytoplasm (Fig. 1A). We explored the prognostic value of TRPM7 mRNA levels in breast cancer, using microarray-based gene expression data from breast cancer specimens, obtained by resection of the primary tumor at diagnosis (discovery cohort; n = 368) (Supplementary Table S1; ref. 17). After dichotomization based on the median TRPM7 expression level, the TRPM7–high group (n = 184) was found to exhibit a significantly shorter recurrence-free survival as compared with the TRPM7–low group (n = 184; HR, 1.42; 95% CI, 1.01–2.01; P = 0.042; Fig. 1B). Even stronger was the association of TRPM7 with distant metastasis-free survival interval (HR, 1.85; 95% CI, 1.22–2.81; P = 0.003; Fig. 1B).

In 3 additional breast cancer cohorts (n = 190, 244, and n = 216), we did not detect a significant association of TRPM7 with disease outcome. Discordances between microarray-based datasets remain a serious problem, often reflecting differences in patient populations, probe selection, and mRNA abundance. We therefore sought for independent validation by carrying out quantitative real-time PCR (qPCR) experiments in a highly similar, independent breast cancer patient cohort (validation cohort; n = 144; Supplementary Table S1). TRPM7 mRNA expression was associated with disease recurrence (HR, 1.88; 95% CI, 1.06–3.33; P = 0.029) and occurrence of distant metastases. Although the HR was similar as in the discovery cohort, the latter association did not reach statistical significance (HR, 1.84; 95% CI, 0.91–3.71; P = 0.085), possibly because of a lower number of events (Fig. 1C).

We next assessed the association between TRPM7 expression and standard clinical parameters using the combined discovery and validation cohorts (n = 512). In support of its association with disease progression, TRPM7 was found enriched in high-grade primary tumors (P = 0.02; Supplementary Table S2A). Other prognostic parameters, including tumor size and ER status, were not associated with TRPM7 expression levels and TRPM7 expression was similar between breast cancer subtypes (P = 0.28; Supplementary Table S2B and C). However, because the dominant prognostic feature in all microarray studies of ER-positive breast cancer is proliferation, we cannot exclude that TRPM7 is an indirect indicator of proliferation in the ER-positive subgroup of tumors.

Univariate Cox regression analysis indicated that histologic grade, tumor size, and TRPM7 expression levels are strong predictors of both disease recurrence and the occurrence of distant metastases (Table 1; P < 0.01). Importantly, multivariate analysis revealed that TRPM7 mRNA expression is an independent prognostic marker for both disease recurrence (P = 0.02) and occurrence of metastases at distant sites (P = 0.01; Table 1), after correction for standard clinical parameters.
parameters. Whereas future research must address to what extent TRPM7 levels indeed have prognostic value, our results indicated a strong and independent association between TRPM7 expression levels and breast cancer progression.

**TRPM7 knockdown interferes with the metastatic potential, but not proliferation, of invasive, triple-negative breast cancer cells in vivo**

To establish a causal relation between TRPM7 expression levels and metastasis formation, shRNA-mediated knockdown was carried out by lentiviral transduction of invasive, triple-negative MDA-MB-231 breast cancer cells. Knockdown efficiency was about 80%, as determined both by qPCR and by measuring TRPM7 autophosphorylation using an *in vitro* kinase assay (Supplementary Fig. S1A and B). A number of studies has shown that TRPM7 knockdown can affect cell viability and proliferation *in vitro* (21, 22). However, we observed that MDA-MB-231 TRPM7 shRNA cells proliferated normally and showed no loss in cell viability (Fig. 2A and Supplementary Fig. S2A). We next compared *in vivo* metastasis formation of MDA-MB-231 control and TRPM7 shRNA cells that were made to express a luciferase reporter gene. Following injection of cells in the tail vein of immunodeficient Rag2−/−/Il2rg−/− mice, luciferase-based noninvasive bioluminescence imaging was used to monitor dissemination and growth of tumor cells *in vivo*. Consistent with earlier reports describing
TRPM7 knockdown impairs migratory properties of invasive, triple-negative breast cancer cells in vitro

To examine how TRPM7 may affect the ability of tumor cells to spread to distant sites, we studied the consequences of TRPM7 knockdown on cytoskeletal organization and cell behavior. Whereas control MDA-MB-231 cells exhibited a characteristic spindle-shaped (mesenchymal) morphology with actin-rich protrusions at the leading edge, this elongated morphology was lost upon TRPM7 knockdown (percentage elongated control cells: 63.3% vs. TRPM7 shRNA: 2.8% vs. TRPM7 shRNA: 30.0%; n = 512). TRPM7-low, n = 256; TRPM7-high, n = 256. Bold numbers, \( P < 0.05 \), statistically significant.

### Abbreviation
CI, confidence interval.

### Table 1. TRPM7 is a predictor of breast cancer recurrence and metastasis, independent of standard clinical parameters

<table>
<thead>
<tr>
<th>Factors</th>
<th>Categories</th>
<th>HR (95% CI)</th>
<th>( P )</th>
<th>Univariate analysis</th>
<th>HR (95% CI)</th>
<th>( P )</th>
<th>Multivariate analysis</th>
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</thead>
<tbody>
<tr>
<td>A. Recurrence-free survival</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Age</td>
<td>&gt;50 vs. &lt;50 y</td>
<td>0.74 (0.53–1.03)</td>
<td>0.07</td>
<td></td>
<td>0.82 (0.54–1.24)</td>
<td>0.34</td>
<td></td>
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<tr>
<td>Histologic grade</td>
<td>2 &amp; 3 vs. 1</td>
<td>2.23 (1.37–3.76)</td>
<td>&lt;0.01</td>
<td></td>
<td>1.85 (1.10–3.11)</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Lymph node status</td>
<td>Positive vs. negative</td>
<td>1.24 (0.93–1.66)</td>
<td>0.15</td>
<td></td>
<td>1.24 (0.79–1.94)</td>
<td>0.34</td>
<td></td>
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<tr>
<td>Tumor size</td>
<td>&gt;2 cm vs. &lt;2 cm</td>
<td>1.93 (1.41–2.63)</td>
<td>&lt;0.01</td>
<td></td>
<td>1.71 (1.19–2.47)</td>
<td>&lt;0.01</td>
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</tr>
<tr>
<td>Syst. Adj. treatment</td>
<td>Yes vs. no</td>
<td>1.02 (0.75–1.37)</td>
<td>0.92</td>
<td></td>
<td>0.85 (0.52–1.41)</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td>Positive vs. negative</td>
<td>0.65 (0.45–0.93)</td>
<td>0.02</td>
<td></td>
<td>0.80 (0.51–1.26)</td>
<td>0.34</td>
<td></td>
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<tr>
<td>TRPM7</td>
<td>High vs. low</td>
<td>1.52 (1.13–2.03)</td>
<td>&lt;0.01</td>
<td></td>
<td>1.52 (1.08–2.12)</td>
<td>0.02</td>
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<tr>
<td>B. Distant metastasis free survival</td>
<td></td>
<td></td>
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<tr>
<td>Age</td>
<td>&gt;50 vs. &lt;50 y</td>
<td>1.28 (0.80–2.05)</td>
<td>0.30</td>
<td></td>
<td>1.19 (0.68–2.08)</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td>2 &amp; 3 vs. 1</td>
<td>2.50 (1.34–4.67)</td>
<td>&lt;0.01</td>
<td></td>
<td>2.11 (1.12–4.00)</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Lymph node status</td>
<td>Positive vs. negative</td>
<td>1.72 (1.21–2.43)</td>
<td>&lt;0.01</td>
<td></td>
<td>1.45 (0.86–2.43)</td>
<td>0.16</td>
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<tr>
<td>Tumor size</td>
<td>&gt;2 cm vs. &lt;2 cm</td>
<td>2.03 (1.40–2.96)</td>
<td>&lt;0.01</td>
<td></td>
<td>1.70 (1.10–2.64)</td>
<td>0.02</td>
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<tr>
<td>Syst. adj. treatment</td>
<td>Yes vs. no</td>
<td>1.39 (0.95–2.04)</td>
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<td></td>
<td>0.86 (0.47–1.57)</td>
<td>0.62</td>
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<tr>
<td>Estrogen receptor</td>
<td>Positive vs. negative</td>
<td>0.91 (0.57–1.47)</td>
<td>0.70</td>
<td></td>
<td>0.98 (0.54–1.77)</td>
<td>0.94</td>
<td></td>
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<tr>
<td>TRPM7</td>
<td>High vs. low</td>
<td>1.75 (1.19–2.58)</td>
<td>&lt;0.01</td>
<td></td>
<td>1.69 (1.13–2.54)</td>
<td>0.01</td>
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</table>

Note: Univariate and multivariate Cox proportional hazards modeling of factors associated with recurrence-free survival (A) and distant metastasis-free survival (B) in the combined discovery and validation cohorts (\( n = 512 \)). TRPM7-low, n = 256; TRPM7-high, n = 256. Bold numbers, \( P < 0.05 \), statistically significant.

TRPM7 knockdown expression effectively interferes with the metastatic potential of invasive human breast cancer cells in vivo.

TRPM7 knockdown cell line (Supplementary Fig. S1A and B, Supplementary Fig. S2D). We additionally reexpressed a mouse TRPM7 cDNA into the TRPM7 knockdown cells, which contained one mismatch with respect to the (human
specific) shRNA. We confirmed by qPCR analysis as well as by in vitro kinase assays that expression of TRPM7 was restored to about 70% of that in the control cells (Supplementary Fig. S1C and D). Reexpression of TRPM7 was sufficient to rescue the elongated morphology (percentage of elongated cells: 56.2% ± 4.7%, n = 400, 4 exp, P < 0.01) and

Figure 2. Reduced TRPM7 expression interferes with the metastatic potential of MDA-MB-231 human breast cancer cells in vivo. A, proliferation and overall viability of control and TRPM7 knockdown MDA-MB-231 cells, determined by MTS assays. Measurements were carried out at different time points, indicated on the x-axis. Metabolic activity is expressed as the amount of produced Formazan, determined by photospectrometry and normalized to initial metabolic activity. Data are presented as mean ± SEM of 2 independent experiments that were carried out in triplicate. B, representative bioluminescence images of mice 7 days after intravenous injections with MDA-MB-231 control or TRPM7 shRNA cells. C, quantification of bioluminescence in the lung region of mice for up to 30 days after injection. Data are presented as mean ± SEM of n = 5 mice in each group. D, bioluminescence images of mice taken 30 days after injection. Photon fluxes are to the same scale. Dagger in C and D indicates a mouse that had to be euthanized 22 days after injection with control MDA-MB-231 cells, because of a large tumor in the head region. Bioluminescence image in D was taken at day 21. Subsequent quantifications were carried out on the 9 remaining mice. E, quantification of mean lung tumor size in mice injected with MDA-MB-231 control or TRPM7 shRNA cells. Error bars represent SEM for n = 3 mice in each group. F, quantification of the number of lung tumors per mouse measured in resected lung tissue from mice injected with MDA-MB-231 control or TRPM7 shRNA cells. Error bars represent SEM for n = 3 in each group. ***P < 0.001. For size distribution, see Supplementary Fig. S2B. G, representative hematoxylin and eosin staining on lung tissue collected 30 days after injection with MDA-MB-231 control or TRPM7 shRNA cells. Prominent tumors in lung tissue from mice injected with TRPM7 shRNA cells (bottom) are indicated by arrows. Scale bar, 1 mm.
restore the migratory properties of MDA-MB-231 TRPM7 knockdown cells (transwell: 95.3% ± 1.2%, 2 exp, \( P < 0.05 \); single-cell migration: \( 25.3 \pm 1.6 \mu \text{m/h}, n > 200 \), 4 exp, \( P < 0.01 \); Fig. 3).

**Reduced TRPM7 expression levels confer a contractile phenotype onto triple-negative breast tumor cells and induce cell-substrate adhesion assembly**

In addition to the functional changes, immunofluorescent staining of MDA-MB-231 cells revealed the redistribution of filamentous actin to the cell cortex and a strong increase in the number of focal adhesions, especially in the periphery of TRPM7 knockdown MDA-MB-231 cells, relative to mock-transduced control cells (control: 13.8 ± 0.62 TRPM7 shRNA: 29.9 ± 0.85, \( n > 100 \), \( P < 0.001 \); Fig. 4A and B). Focal adhesions are mechanosensitive adhesion structures whose assembly and disassembly need to be tightly regulated by a combination of myosin II–based cellular tension and local \( \text{Ca}^{2+} \) signaling events to allow optimal cell migration (24–29). Increased focal adhesion assembly is generally associated with high cytoskeletal tension, accompanied by tyrosine phosphorylation of focal adhesion components such as paxillin as well as increased myosin light chain (MLC) phosphorylation (24). Indeed, the increase in focal adhesions observed in the TRPM7 knockdown cells was reflected by a rise in Tyr118-phosphorylated paxillin and Ser19-phosphorylated MLC on a Western blot (Fig. 4C and Supplementary Fig. S4A). Reexpression of TRPM7 reduced focal adhesion content (18.2 ± 0.89, \( n = 45 \), \( P < 0.001 \)) and reverted paxillin phosphorylation (Fig. 4).

Figure 3. TRPM7 contributes to the malignant phenotype of MDA-MB-231 breast cancer cells in vitro. A, representative phase-contrast images of control and TRPM7 shRNA MDA-MB-231 cells. Scale bar, 50 μm. B, quantification of elongated MDA-MB-231 cells, TRPM7 shRNA cells, and TRPM7 shRNA cells made to reexpress a mouse TRPM7 cDNA (rescued cells). Elongation is presented as percentage (± SEM; 4 independent experiments, \( n > 400 \) of cells that have a length of more than twice the width). ****, \( P < 0.01 \); ***, \( P < 0.001 \). C, quantification of serum-induced transwell migration by MDA-MB-231 cells, TRPM7 shRNA cells, and the rescued cell line. Data, normalized to the number of control MDA-MB-231 cells, are from 5 independent experiments carried out in duplicate in which the rescued cell line was included twice, and represent mean ± SEM. Migration was scored after 8 hours. ****, \( P < 0.01 \); *, \( P < 0.05 \). D, representative trajectories of migrating control (\( n = 10 \)) and TRPM7 shRNA (\( n = 10 \)) MDA-MB-231 cells followed for 24 hours. E, quantification of single cell migration speed. Shown is migration speed (μm/h, mean ± SEM) of 4 independent experiments, each carried out in duplicate (\( n > 200 \) per cell line), ***, \( P < 0.01 \).
ER-positive breast cancer cells exhibit reduced cell migration speed and enforced cell–cell adhesions upon TRPM7 knockdown

In addition to basal-like tumors, represented by the triple-negative MDA-MB-231 breast cancer model, a large part of the patient dataset consisted of ER-positive, luminal type breast cancer patients. To validate our observations in these tumors, we knocked down TRPM7 in noninvasive, ER-positive MCF7 human breast cancer cells (Supplementary Fig. S1E). This significantly reduced migration of MCF7 cells in gap-closure assays (control: 11.5 ± 0.5% vs. TRPM7 shRNA: 21.2 ± 1.7% gap remaining after 24 hours, 3 exp, P < 0.01; Fig. 5A and B). In these epithelial-like cells, TRPM7 knockdown predominantly affected cell–cell adhesion rather than cell-substrate adhesion. Unlike the control shRNA-transduced cells, the TRPM7 knockdown cells were able to maintain cell–cell contacts upon serum deprivation, a condition known to induce scattering of epithelial cells (ref. 30; Fig. 5C). Although increased MLC and paxillin phosphorylation were not observed in MCF7 TRPM7 shRNA cells (data not shown), confocal microscopy revealed profound effects on cytoskeletal organization and cell–cell contacts (Fig. 5D).
Similar to our observations in MDA-MB-231 cells, TRPM7 knockdown induced the redistribution of filamentous actin to the cell cortex. Moreover, cell–cell interactions seemed to be enforced in MCF7 TRPM7 shRNA cells, evident from increased cell–cell contact area and enrichment of E-cadherin at these interfaces. Cadherin-containing cell–cell adhesions, known as adherens junctions, show functional and structural similarities to focal adhesions (4). Like focal adhesions, adherens junctions are highly dynamic multi-protein complexes that act in close association with the actomyosin cytoskeleton to translate mechanical signals into cellular responses. Moreover, their formation and size are directly associated with myosin II–based cellular tension (5).

**Pharmacologic inhibition of cytoskeletal tension rescues the TRPM7 knockdown phenotype**

Our results indicated that TRPM7 knockdown confers a contractile phenotype onto breast cancer cells and consequently, impairs their migratory and metastatic properties. Consistent with this notion, inhibition of myosin II–based cytoskeletal tension using the Y27632 Rho-kinase inhibitor (31) was sufficient to revert the phenotype of TRPM7 knockdown in MDA-MB-231 cells to the characteristic elongated morphology of control cells (elongated cells: 54.5%/C6 3.0%, n > 100, 2 exp, P < 0.05; Fig. 6A), while reducing MLC- and paxillin phosphorylation, and the number of focal adhesions back to control levels (15.7%/C6 0.26, n > 100, P < 0.001; Fig. 6B and C, Supplementary Fig. S4A and B). A similar effect was
observed with the structurally unrelated Rho-kinase inhibitor GSK429286 (ref. 32; Supplementary Fig. S4A and B). Strikingly, Rho-kinase inhibition restored serum-induced transwell migration of TRPM7 knockdown cells [TRPM7 shRNA: 63.9% ± 7.0% vs. TRPM7 shRNA + Y27632 (5 μmol/L): 116.6% ± 17.9%, 3 exp, P < 0.05] without affecting MDA-MB-231 control cell migration (Fig. 6D and Supplementary Fig. 4C). Likewise, gap-closure speed of MCF7 TRPM7 shRNA cells was rescued by Rho-kinase inhibition (Fig. 6E and Supplementary Fig. S4D). In contrast to MDA-MB-231 cells, low concentrations of Rho-kinase inhibitors already significantly increased gap-closure speed of MCF7 control cells. However, much higher concentrations of these compounds were required to maximize gap-closure speed of MCF7 TRPM7 shRNA cells, supporting the notion that TRPM7 knockdown increases cellular tension.

Although our observations are not in agreement with the general notion that increases in Rho-ROCK signaling positively correlate with cell migration and metastasis formation (6, 33, 34), it is well known that actomyosin contractility,
adhesion dynamics, and the mechanical properties of the substrate have to be tightly balanced to maximize migration velocity (26, 27, 35, 36). Hence, overassembly of either focal adhesions or adherens junctions in the TRPM7 knockdown cells, both a consequence of increased cytoskeletal tension, likely interferes with optimal cell migration (Supplementary Fig. S4E). Altogether, our results indicated that TRPM7 is part of the mechanosensory machinery that regulates cellular tension and steers adhesion dynamics to allow cell migration and metastasis formation.

Conclusions

TRP channels play a prominent role in translating mechanical forces into biochemical signals, although in most cases it remains to be established whether they are directly activated by mechanical stimulation. Activation of these proteins not only leads to changes in local Ca\textsuperscript{2+} concentrations but also triggers other signaling mechanisms that influence cell behavior and differentiation (37). Mice deficient in TRPM7 show widespread defects in early embryonic development, pointing at a nonredundant role for this channel kinase in organ development (38). Defects in mechanotransduction, especially those that affect cellular tension, are known to contribute to disease progression (39, 40). Hence, we propose that TRPM7-guided cell adhesion and migration are normal attributes of epithelial and mesenchymal cells, required during organ development, but when spuriously activated in cancer cells contribute to metastasis formation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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