Received: 21 December 2017 | Accepted: 17 August 2018

DOI: 10.1002/jcp.27371

RAPID COMMUNICATION

WILEY Journal of Cellular Physiology

TRPM4 channel is involved in regulating epithelial to mesenchymal transition, migration, and invasion of prostate cancer cell lines

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Funding information
Fondo Nacional de Desarrollo Científico y Tecnológico, Grant/Award Numbers: 1151435, 1120286, 1151446, 11170840 and 1161288; UNAB, Grant/Award Number: DI-741-15/N; FONDAP, Grant/Award Number: 15010006; Iniciativa Científica Milenio, Grant/Award Number: P09-016-F; Comisión Nacional de Investigación Científica y Tecnológica, Grant/Award Number: Anillo ACT172101

Abstract

Transient Receptor Potential Melastatin 4 (TRPM4) is a Ca^{2+}-activated and voltage-dependent monovalent cation channel, which depolarizes the plasma cell membrane, thereby modulating Ca^{2+} influx across Ca^{2+}-permeable pathways. TRPM4 is involved in different physiological processes such as T cell activation and the migration of endothelial and certain immune cells. Overexpression of this channel has been reported in various types of tumors including prostate cancer. In this study, a significant overexpression of TRPM4 was found only in samples from cancer with a Gleason score higher than 7, which are more likely to spread. To evaluate whether TRPM4 overexpression was related to the spreading capability of tumors, TRPM4 was knockdown by using shRNAs in PC3 prostate cancer cells and the effect on cellular migration and invasion was analyzed. PC3 cells with reduced levels of TRPM4 (shTRPM4) display a decrease of the migration/invasion capability. A reduction in the expression of Snail1, a canonical epithelial to mesenchymal transition (EMT) transcription factor, was also observed. Consistently, these cells showed a significant change in the expression of key EMT markers such as MMP9, E-cadherin/N-cadherin, and vimentin, indicating a partial reversion of the EMT process. Whereas, the overexpression of TRPM4 in LnCaP cells resulted in increased levels of Snail1, reduction in the expression of E-cadherin and increase in their migration potential. This study suggests a new and indirect mechanism of regulation of migration/invasion process by TRPM4 in prostate cancer cells, by inducing the expression of *Snail1* gene and consequently, increasing the EMT.

KEYWORDS
EMT, invasion, prostate cancer, Snail1, TRPM4

1 | INTRODUCTION

Prostate cancer is one of the most diagnosed cancers in the world and the fifth cause of cancer-related death in men (Torre et al., 2015). This type of cancer is characterized by its progression to androgen-independent growth after the conventional treatment, and the subsequent development of resistant metastatic lesions, leading to a deterioration in the patient's health (Bellmunt & Oh, 2010; Ferraldeschi, Welti, Luo, Attard, & de Bono, 2015). Throughout this disease's natural history, many genetic and molecular alterations are described, including the loss of tumor suppressor genes such as *PTEN*, *TP53*, and *NKX3.1*; genetic fusions such as TMPRSS2-ERG and the oncogenic mutation of PIK3CA, and the Androgen Receptor (AR; Barbieri et al., 2013; Grasso et al., 2012; Khemlina, Ikeda, & Kurzrock, 2015). Also, in recent years different research groups have noted the relevance of the aberrant expression and function of several ion channels belonging to the Transient Receptor Potential (TRP) family such as TRPM8, TRPC6, and TRPV6 in the development and progression of prostate cancer (Gkika & Prevarskaya, 2011; Prevarskaya, Flourakis, Bidaux, Thebault, & Skryma, 2007; Valero, Morenilla-Palao, Belmonte, & Viana, 2010; Van Haute, De Ridder, & Nilius, 2010). Interestingly, several research groups have describes the overexpression of the Transient Receptor Potential Melastatin 4 (TRPM4) in prostate cancer cells lines and prostate tumor samples (Holzmann et al., 2015; Sagredo et al., 2017). TRPM4 is a monovalent nonselective cation channel, activated by an increase in the concentration of intracellular Ca^{2+}. Its activity translates a local increase of calcium in membrane depolarization, affecting the driving force for external Ca^{2+} entry and modulating several intracellular Ca^{2+} dependent signaling pathways (Fliegert et al., 2007; Guinamard, Demion, & Launay, 2010; Launay et al., 2002). Under physiological conditions, TRPM4 is involved in different cellular functions (Guinamard et al., 2010). For example, TRPM4 participates in insulin secretion (Cheng et al., 2007), T-cell proliferation (Launay et al., 2004), mast cells activation (Shimizu et al., 2009; Vennekens et al., 2007), and endothelial (Sarmiento et al., 2014) and dendritic cell migration (Barbet et al., 2008). Also, TRPM4 has been described as being localized in the focal adhesion (FA) complexes, playing a pivotal role in FA turnover and lamellipodial actin cytoskeleton dynamics (Cáceres et al., 2015). Under pathological conditions, such as prostate cancer, TRPM4 mRNA is upregulated in the transition from Prostatic Intraepithelial Neoplasia (PIN) to prostate carcinoma (Ashida et al., 2004). TRPM4 protein overexpression is also associated with an increased risk of biochemical recurrence after the radical prostatectomy (Berg et al., 2015) and has been identified as an important gene involved in androgen-independent prostate cancer progression (Schinke et al., 2014), suggesting its participation in cancer progression. Furthermore, the knockdown of TRPM4 in prostate cancer cell lines DU145 and PC3 revealed a decrease of cellular migration capacity, suggesting an important role of TRPM4 expression in the invasive behavior of this tumor (Holzmann et al., 2015). Nevertheless, the possible molecular mechanism exerted by this channel in the progression and invasiveness of prostate cancer cells remains unknown. Recently, we described TRPM4 as a positive regulator of prostate cancer cell proliferation through the inhibition of GSK-3β and the stabilization of β-catenin, promoting its transcriptional functions and the expression of genes related to cell-cycle progression (Armisén et al., 2011; Sagredo et al., 2017). Interestingly, GSK-3β is a key component of many intracellular signaling pathways and one of the principal negative regulators of the transcription factor (TF) Snail1, a key TF involved in activation and maintenance of EMT (Wang, Shi, Chai, Ying, & Zhou, 2013; Zheng et al., 2013; Zhou et al., 2004). This transcriptional and phenotypical program is characterized by changes in cellular polarity, the loss of epithelial markers (Thiery, Acloque, Huang, & Nieto, 2009), together with the acquisition of mesenchymal characteristics and motile/invasive properties, allowing tumor cells to migrate out of the site of origin, reach distant tissue, and establish new lesions as metastatic colonization (Heerboth et al., 2015; Karlsson, Gonzalez, Welin, & Fuxe, 2017). Furthermore, knockdown of TRPM4 in HeLa cells produces an increase in the expression of E-cadherin (Armisén et al., 2011), a well-known epithelial marker (Kalluri & Weinberg, 2009), suggesting a relation between the expression of TRPM4 and the activation maintenance of the EMT program in cancer cells. Moreover, TRPM4 knockdown in endothelial cells leads to a decrease in endothelial protein markers and an increased expression of fibrotic and extracellular matrix markers, indicating a cellular specific and context-dependent effect of TRPM4 on the EMT process (Echeverría et al., 2015). These scenarios suggest a plausible relation between TRPM4 expression and the EMT program regulation in prostate cancer cells. This study presents further evidence showing that TRPM4 expression could alter the EMT process in prostate cancer cells and it has an effect on cellular migration and invasion phenotypes. Silencing of TRPM4 in high invasive and androgen insensitive PC3 cells correlates with a reduction of cell migration and invasion, a decrease in metalloproteinase activity, and a shift in E-cadherin/N-cadherin expression, a well described marker of the EMT process. Also, PC3 shTRPM4 cells show a reduced expression of the transcription factor Snail1, one of the EMT master regulators and other mesenchymal markers. These changes are correlated with a reduction of the inhibitory phosphorylation on GSK-3β (phospho-S9), enzyme that regulates the stability of Snail1, suggesting a possible molecular mechanism. Moreover, the overexpression of TRPM4 in androgen-sensitive LnCaP cells, whose TRPM4 expression is lowest than PC3 (Sagredo et al., 2017), leads to an increase of their migration capacity and a decrease in E-cadherin protein expression. These results reveal a new and indirect mechanism for migration/invasion regulation by TRPM4 expression in prostate cancer cell lines through the control of key

components of the EMT program, and indicate new roles for this channel in tumor progression.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Prostate cancer cells, PC3 and LnCaP, were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (Corning Inc., Corning, NY) supplemented with 10% v/v fetal bovine serum (FBS; Corning Inc.) and penicillin/streptomycin (Hyclone Laboratories, Logan, UT).

2.2 | cDNAs from patient samples

Prostate Cancer cDNA Array II was acquired from Origene (Origene, Rockville, MO). This cancer panel contemplates complementary DNAs (cDNAs) extracted from 48 samples, covering 8-normal tissues and 22-Stage IIA, 11-III, 2-IV, and 5-N/R tumoral tissues. All samples have its pathology verification and clinical report (Supporting information, Patient's clinical information).

2.3 | Antibodies

Mouse anti-TRPM4 (Origene, TA500381), mouse anti-GSK-3β (BD Biosciences, San Jose, CA, 610201), rabbit antiphospho-S9 GSK-3β (Origene, TA303847), mouse anti-E-Cadherin (Cell Signaling Technology, Danvers, MA, 5296), rabbit Anti-N-Cadherin (Cell Signaling Technology, 4061), rabbit anti-Snail1 (Santa Cruz Biotechnology, Santa Cruz, CA, sc-10432), mouse anti-VIM (Santa Cruz Biotechnology, sc-373717), rabbit anti-FAK (Cell Signaling Technology, 3285), anti-p-FAK(Tyr397; Cell Signaling Technology, 3283) as loading controls, mouse anti-α-tubulin (Sigma Aldrich, St. Louis, MO, T5168), or mouse anti-HSP70 (Origene, TA309356) were used.

2.4 | Drugs

For the inhibition of GSK-3β kinase activity, SB 216763 (Tocris Bioscience, Bristol, UK, 1616) was added to the growth media (10 μM for 8 hr). For the proteasome inhibition, MG-132 (Tocris Bioscience, 1748) was added to the growth media (20 μM for 8 hr).

2.5 | Transfection and transductions

PC3 cells were transduced with a commercial pre-packaged lentiviral vector (SBI, Palo Alto, CA) coding an shRNA against TRPM4 mRNA (ShTRPM4) or a scrambled control ShRNA (ShControl; Sagredo et al., 2017). Cells were grown in culture media with 0.8 μg/ml Puromycin as selection media (Corning Inc.). LnCaP cells were transfected with pcDNA4TO/TRPM4b (human) plasmid or an empty vector (mock), using Lipofectamine LTX with Plus Reagent (Invitrogen, Carlsbad, CA) and growth media was supplemented with 50 μg/ml Zeocin (Corning Inc.) for selection.

2.6 | Immunoblotting

Cells lysates were prepared in a RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% v/v TritonX-100, 1% w/v sodium deoxycholate, and 0.1% w/v sodium dodecyl sulfate (SDS)) and supplemented with a protease (Calbiochem, San Diego, CA) and phosphatase (Roche Life Sciences, Mannheim, Germany) inhibitor cocktail as described previously (Sagredo et al., 2017). Protein lysates (30 μg per lane) were resolved on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred onto a nitrocellulose membrane. Membranes were blocked in 5% w/v bovine serum albumin (Winkler, Santiago, Chile), and then incubated with primary antibodies at 4°C overnight. All primary antibodies were detected using appropriate Horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence reagent (SuperSignal WestPico Chemiluminescent Substrate, Thermo Scientific, Waltham, MA) and images were obtained using the ChemiScope3500 Mini chemiluminescence imaging system (Clinx Science Instruments, Shanghai, China).

2.7 | RT-qPCR

We used the Prostate Cancer cDNA Array II (Origene) as a cDNA sample or cellular RNA was extracted using TRIzol (Invitrogen), followed by DNAse treatment (TURBO DNase, Ambion, Austin, TX). Total of 1 μg of RNA was reverse-transcribed using an AffinityScript quantitative real-time polymerase chain reaction (qRT-PCR) cDNA Synthesis Kit (Agilent Technologies, Inc., Santa Clara, CA) and diluted five times. Quantitative expression analysis was performed using specific oligonucleotide primers and a Brilliant II SYBR Green qRT-PCR Master Mix (Agilent Technologies, Inc.). The reactions were made in the Eco Real-Time PCR (Illumina, San Diego, CA) using the following program: 95°C for 15 s, 58°C for 15 s, and 72°C for 15 s at 40 cycles. Expression values were calculated using the ΔΔCt method and expressed as the fold change relative to control samples. *GAPDH* was used as a housekeeping gene. The primer sequences (5′→3′) used:

GAPDH (Fw GTTGCTGTAGCCAAATTCGTTGT,
Rv GGTGGTCTCCTCTGACTTCAACA),
SNAIL1 (Fw AGGCTCGAAAGGCCTTCAACT,
Rv TGTGGCTTCGGATGTGCATC)
TRPM4 (Fw TCGGCAAAGTACAGGGCAAC,
Rv AGGCGCAAGTGGGAGATGAC)
E-CADHERIN (Fw GCACCGGTCGACAAAGGACA,
Rv AGTCCCAGGCGTAGACCAAGA)

2.8 | Wound healing assay

Cells were seeded in 6-well plates at confluence. Before the assay, the cell culture medium was changed to serum-free RPMI 1640 (Corning Inc.), the confluent cell monolayers were wounded by manually drawing a gap with a plastic pipette tip (P200). The ability of cells to migrate into the cleared section was monitored by Cytation 3 Multi-Mode

Reader microscopy (Biotek Instrument, Winooski, VT) at specific time points (0, 3, 6, 9, and 12 hr). The images acquired were analyzed using ImageJ (NIH) software to measure the closure of the wounded area. The percentage of the nonhealed scratched area (*S*) for each replicate at specific time points was calculated as follows: % of the nonhealed scratched area = [*S* (at specific time)/*S* (at starting time)] x 100%. All the experiments were done in triplicate.

2.9 | Migration assay

Cell migration was assayed using the transwell chamber (Corning Inc.) assay. Briefly, cells (2.5×10^4 cells/well) in 500 µl of serum-free RPMI 1640 (Corning Inc.) medium were seeded into the top of the upper chamber (8 µm-pore Boyden chambers). Wells were filled with 1% FBS RPMI 1640 (Corning Inc.) as a chemoattractant (conditioned medium). Cells were allowed to migrate to a conditioned medium for 12 hr under the standard cell culture conditions. Finally, cells adhering to the upper surface of the filter were removed using a cotton tip applicator. Cells that migrated, on the opposite side of the filter, were stained with 0.1% crystal violet solution in 20% methanol, photographed using the Cytation 3 Multi-Mode Reader (Biotek Instrument, Winooski, VT) and counted. The data represent three interdependent experiments, each in triplicate.

2.10 | Invasion assay

The cell invasion assay was carried out using the transwell chamber invasion assay. Briefly, cells (2.5×10^4 cells/well) in 500 µl of serum-free RPMI 1640 medium were seeded into the top of the upper chamber (8 µm-pore Boyden chambers coated with Matrigel, Becton Dickinson-BioCoat). Wells were filled with 1% FBS RPMI 1640 as a chemoattractant (conditioned medium). Cells were allowed to invade a conditioned medium for 22 hr under standard cell culture conditions. Finally, cells adhering to the upper surface of the filter were removed using a cotton applicator. The invasive cells, on the opposite side of the filter, were stained with 0.1% crystal violet solution in 20% methanol, photographed using the Cytation 3 Multi-Mode Reader (Biotek Instrument, Winooski, VT) and counted. The data represent three interdependent experiments, each in triplicate.

2.11 | Zymography assay

Cells were cultured for 48 hr up to 90% confluence. The growth medium was changed to serum-free RPMI 1640 (Corning Inc.), and cells were cultured for a further 30–36 hr. The medium was collected and centrifuged at 1,500 g for 10 min at 4°C. Supernatants were collected as conditioned medium for further study. Samples were mixed with loading buffer, under nonreducing conditions (10% v/v glycerol, 2% SDS, 62.5 mM Tris, pH 6.8, 0.02% bromophenol blue), and applied to a 10% Tris/HCl acrylamide gel containing 0.1% Gelatin, for the determination of proteolytic activity. Electrophoresis was performed at 120 V at 4°C for approximately 2–2.5 hr. Resolved proteins were renatured by incubating the gels in 2.5% Triton X-100 solution for 30 min at room temperature. Thereafter, gels were quickly washed three

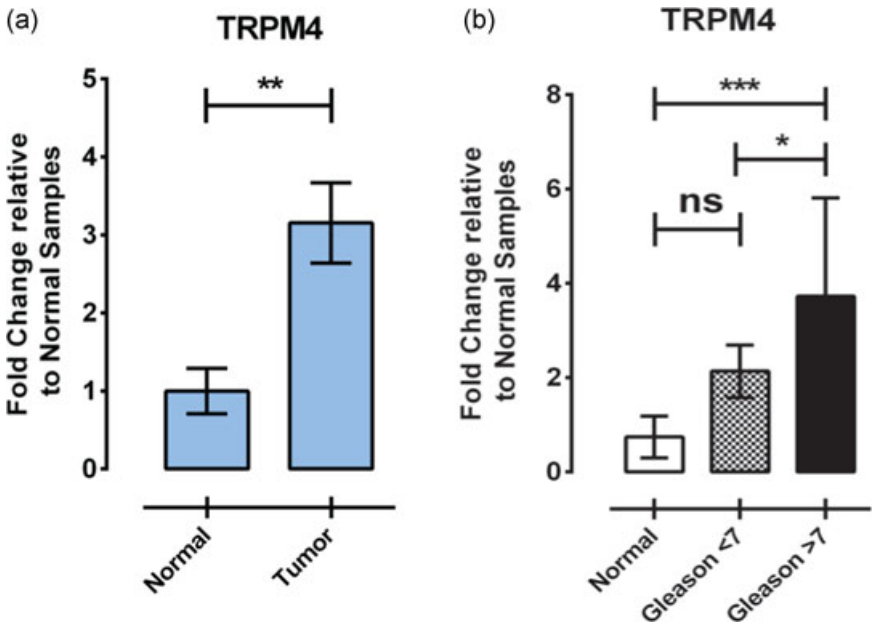

FIGURE 1 TRPM4 mRNA expression in human prostate cancer samples and grouped by Gleason score. (a) qPCR was performed to compare the increased expression of TRPM4 mRNA in 39 tumor samples compared with eight normal controls. cDNA was obtained using the commercial Origene Prostate cancer Panel II. Mean ± *SEM* *t* test of one tail. $^{**}p \leq 0.01$. (b) Tumor samples were pooled by Gleason score (> 7 and < 7). *TRPM4* gene expression increases significantly in tumors with Gleason score > 7. Mean ± *SEM* are shown, one-way ANOVA multicomparisons. NS: $p > 0.05$, $^{*}p \leq 0.05$, and $^{***}p \leq 0.001$. cDNA, complementary DNA; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction; SEM, standard error of mean [Color figure can be viewed at wileyonlinelibrary.com]

times in water, and then incubated in zymography buffer (50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 1.25 mM $CaCl_2$) overnight at 37°C. Proteolytic activity in the gels was visualized as a negative staining line with Coomassie brilliant blue stain. The images acquired were analyzed using ImageJ (NIH) software, (Rasband, 2015).

2.12 | Statistical analysis

qRT-PCR, western blot, and functional assays were examined by Student's *t* test with Welch correction or by the analysis of variance (ANOVA) test, whichever was applicable. The $p < 0.05$ was consid-

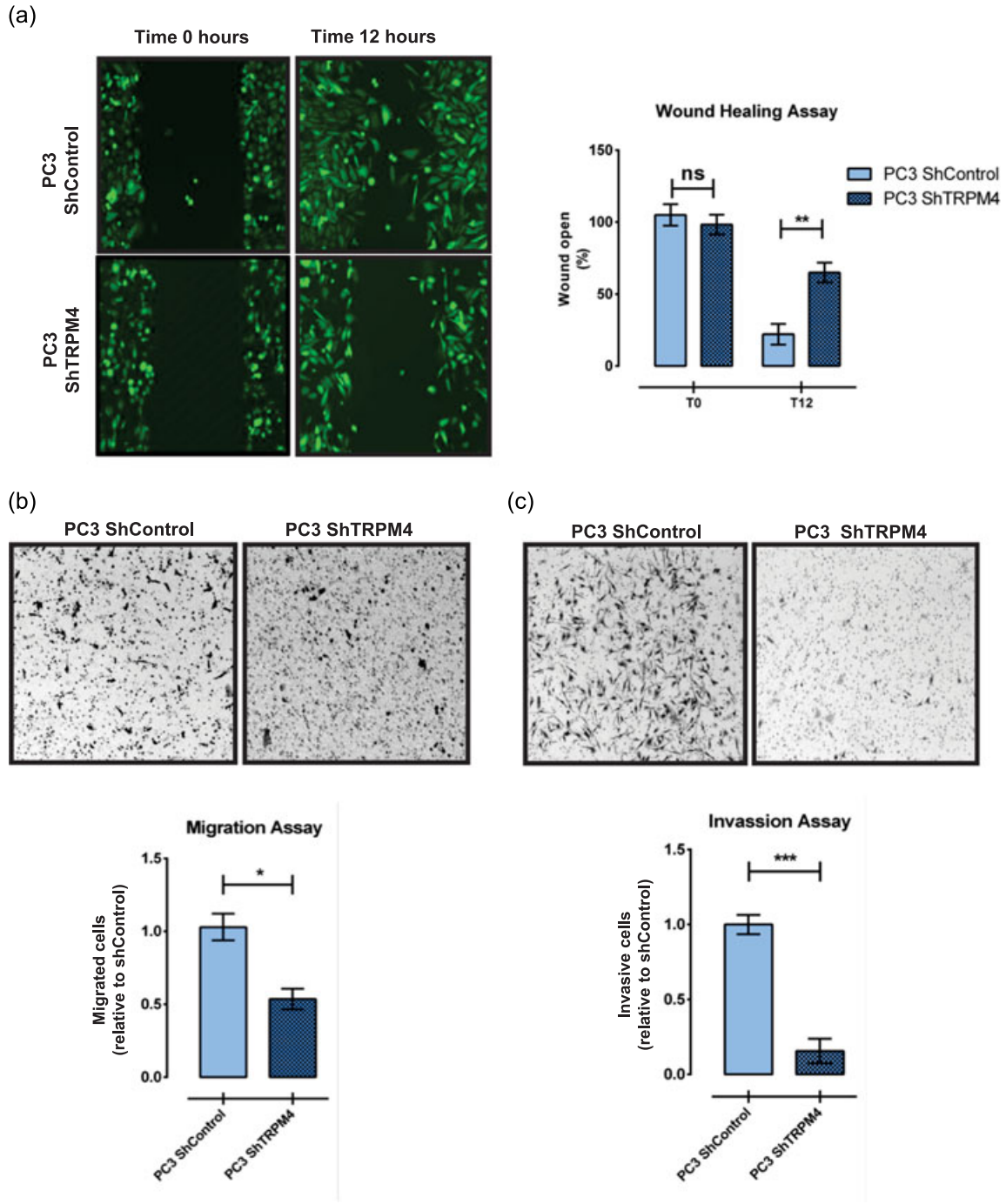

FIGURE 2 TRPM4 expression affects the migration and invasion processes in PC3 prostate cancer cell lines. (a) and (b) Knockdown of TRPM4 in PC3 cells decreases cellular migration. (a) Representative images of wound healing assay in PC3 ShControl and ShTRPM4 cells. Graph shows the percentage of the nonhealed scratched area after 12 hr of incubation. The area was quantified in at least three independent experiments. (b) Knockdown of TRPM4 in PC3 cells decreases the transwell migration mediated by chemoattractant (1% FBS). Graph shows the total cell count normalized to PC3 control in at last three independent experiments. (c) Knockdown of TRPM4 in PC3 cells decreases the Matrigel invasion. Graph shows the total cell count normalized to PC3 control in at last three independent experiments. In (a–c), Means ± *SEM* are shown, *t* test with Welch correction, $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$. SEM, standard error of mean [Color figure can be viewed at wileyonlinelibrary.com]

ered statistically significant. At least three independent experiments were performed for each analysis. Statistical analysis was performed using Prism 5.0 (GraphPad Software, San Diego, CA).

3 | RESULTS

3.1 | TRPM4 mRNA is overexpressed in high grade Gleason score prostate cancer samples

Overexpression of TRPM4 has been reported in various types of tumors including prostate cancer (Berg et al., 2015; Holzmann et al., 2015; Loo et al., 2017; Suguro et al., 2006). Given this scenario, we focused on finding a correlation between the TRPM4 mRNA expression and the Gleason score, a widely used grading system for estimating tumor aggressiveness in prostate cancer patient samples (Gordetsky & Epstein, 2016; Stark et al., 2009). As expected, we found that TRPM4 mRNA expression was significantly higher in tumor samples compared with normal samples (Figure 1a). However, this overexpression was only significant in prostate tumors with a high Gleason scores (> 7) and not in the lower and intermediate grades (Gleason Scores < 7; Figure 1a,b). Tumors with a Gleason score > 7 are more aggressive, less differentiated, and with a high probability of invasion to other tissues (Donohue et al., 2006; Rusthoven et al., 2014). These results are consistent with a previous work (Holzmann et al., 2015) were the immunohistochemistry analysis of TRPM4 in samples with Prostatic Intraepithelial Neoplasia (PIN) or areas with an increased Gleason score showed a medium or strong signal of TRPM4 expression.

3.2 | Migration and invasion of prostate cancer cell lines are related to TRPM4 expression

Since TRPM4 mRNA expression is higher in tumors with a high Gleason score, which are prone to invade and metastasize to distant organs (Donohue et al., 2006), we assessed the role of TRPM4 expression in prostate cancer cell's migration and invasion. We used two different cell models: PC3 cells, which are highly invasive and express high levels of TRPM4. In these cells, the expression of this channel was knock-down by using lentiviral particles coding a shRNA against TRPM4 mRNA. On the other hand, TRPM4 was over-expressed by transient transfection in LnCaP cells, which are noninvasive and express lower levels of the channel (Liu et al., 2011; Sagredo et al., 2017; Supporting Information Supplementary Figure 1). These cell lines resemble two stages of prostate cancer progression. The migration capabilities of PC3 ShControl and PC3 ShTRPM4 cells were measured by wound-healing and Transwell assays (Figure 2). PC3 ShTRPM4 cells showed a significant diminution of their migration capability compared with control cells (Figure 2a). After 12 hr of incubation, PC3 ShTRPM4 cells were not able to close the artificial wound, whereas PC3 ShControl closed over 60% of the artificial wound (Figure 2a). Using the Transwell assay, PC3 ShTRPM4 cells show a significant reduction of their migration capabilities mediated by a chemoattractant (1% FBS) compared with PC3 ShControl cells (Figure 2b), indicating the importance of TRPM4 expression in the migration process as reported in previous research (Cáceres et al., 2015; Holzmann et al., 2015). Moreover, the invasion of PC3 cells into the Matrigel support was significantly reduced after TRPM4 knockdown (Figure 2c), suggesting a role of TRPM4 expression in the invasion properties of PC3 cells, as well. Interestingly, overexpression of TRPM4 in LnCaP cells increased cellular migration, but had no evident effect on the invasiveness of these cells (Supporting Information Supplementary Figure 2a–c). These results suggest that the overexpression of TRPM4 alone is not enough to promote invasiveness properties in LnCaP cells and that other molecular and genetic changes are needed in these cells in order for them to acquire invasive capacities.

3.3 | Knockdown of TRPM4 results in a reduction of the Snail1 transcription factor and increased expression of epithelial markers in prostate cancer cell lines

We recently showed that TRPM4 expression regulates the inhibitory phosphorylation of GSK-3β on serine 9, without significant changes in the total amount of this enzyme (Sagredo et al., 2017). This intracellular kinase is a central regulator of several signaling proteins such as β-catenin (Liu et al., 2002), NFAT (Beals, 1997), TSC2 (Buller et al., 2008), and the transcription factor (TF) Snail1 (McCubrey et al., 2014). This TF is one of the main core proteins of the EMT program (De Craene et al., 2005; Puisieux, Brabletz, & Caramel, 2014; Wang et al., 2013). Snail1 promotes a mesenchymal phenotype through the transcriptional repression of E-cadherin, Occludin, and Cytokeratin, among other cell adhesion proteins (Ohkubo & Ozawa, 2004; Xu, Lamouille, & Derynck, 2009). It also induces a reduction in cell to cell adhesion and promotes the expression of different mesenchymal markers, such as N-cadherin (Kim, Yi, Kim, & Choi, 2014), vimentin (Kim et al., 2014; Myong, 2012), and fibronectin (Porta-de-la-Riva et al., 2011). Furthermore, Snail1 regulates other important TFs to maintain mesenchymal phenotypes, such as Zeb1, Twist, and Slug (Lamouille, Xu, & Derynck, 2014; Zeisberg & Neilson, 2009). Snail1 stimulates the expression of matrix metalloproteinases (MMP) that cleave and remodel the extracellular matrix (Yokoyama et al., 2003), thereby modifying cell-matrix adhesions, and facilitating cellular migration, and invasion (Deryugina & Quigley, 2006). Knockdown of TRPM4 expression in PC3 cells results in a significant reduction of total Snail1 protein levels (Figure 3a), which correlates with an increase in the GSK-3β activity of these cells (Sagredo et al., 2017 and Supporting Information Supplementary Figure 3). Accordingly, PC3 ShTRPM4 cells show a significant increase of E-cadherin and a decrease in N-cadherin protein levels compared with control cells (Figure 3b,c). Furthermore, mRNA expression of vimentin was reduced and the tight junction protein claudin1 was increased after TRPM4 knockdown (Supporting Information Supplementary Figure 4a,b), reinforcing the relationship between Snail1 reduction and the partial loss of mesenchymal markers. Interestingly, an

immunofluorescence assay for detection of Vimentin in PC3 ShTRPM4, and PC3 ShControl, shows a different pattern of distribution and reveals the differences in cellular morphology between both models, with the shTRPM4 cells being related to a more rounded phenotype (Supporting Information Supplementary Figure 5). The mRNA levels of canonical EMT TFs, such as Twist1, Slug, and Zeb1 were also significantly decreased after TRPM4 knockdown (Supporting Information Supplementary Figure 4c). Conversely, the overexpression of TRPM4 in LnCaP cells resulted in a significant decrease of E-cadherin protein and mRNA levels (Figure 3d,e), indicating a transcriptional effect on E-cadherin following TRPM4 overexpression. Altogether, this data suggests that reduction of Snail1 protein levels in PC3 shTRPM4 cells also affect its transcriptional capacity. Accordingly, the activity of metalloproteinase 2 and 9, known to be upregulated by the transcriptional activity of Snail1 (Haraguchi, 2009; Lamouille et al., 2014), was assessed by zymography. Our results

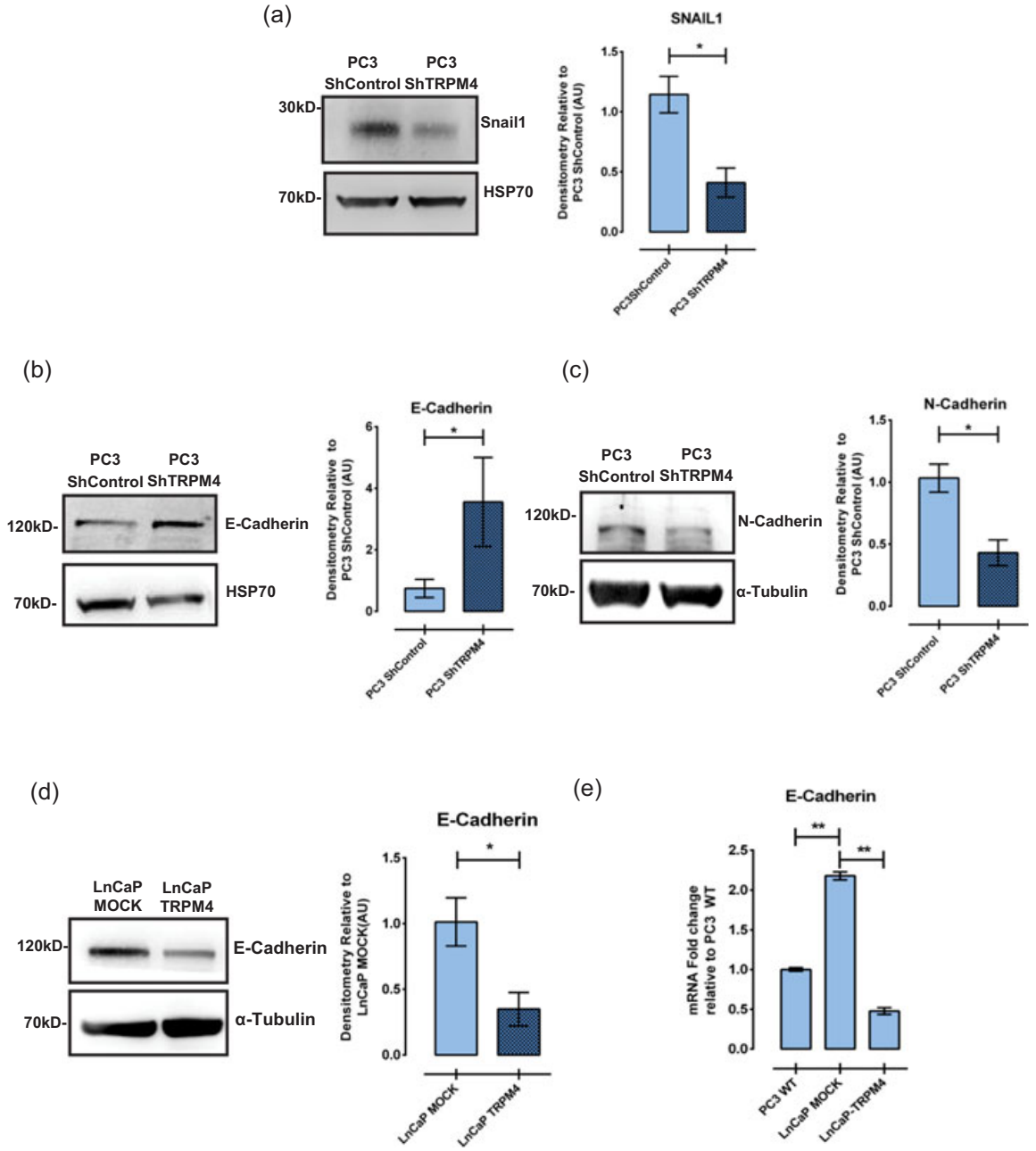

FIGURE 3 TRPM4 knockdown reduces Snail1 protein levels and increases E-Cadherin. (a) Knockdown of TRPM4 in PC3 cells is related to a reduction of Snail1 protein levels. Representative western blots and densitometries of three independent experiments (mean ± *SEM*) are shown. *t* test with Welch correction, $^{*}p \leq 0.05$. (b) and (c) PC3 ShTRPM4 evidence an increase of the epithelial marker E-cadherin and a reduction of the mesenchymal markers N-cadherin, respectively. Representative western blots and densitometries of three independent experiments (mean ± *SEM*) are shown. *t* test with Welch correction, $^{*}p \leq 0.05$. (d) and (e) Overexpression of TRPM4 in LnCaP cells correlated with a decrease of E-cadherin protein and mRNA levels compared to mock cells. Representative western blots and densitometries of three independent experiments (mean ± *SEM*) are shown. *t* test with Welch correction, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$. mRNA, messenger RNA; SEM, standard error of mean [Color figure can be viewed at wileyonlinelibrary.com]

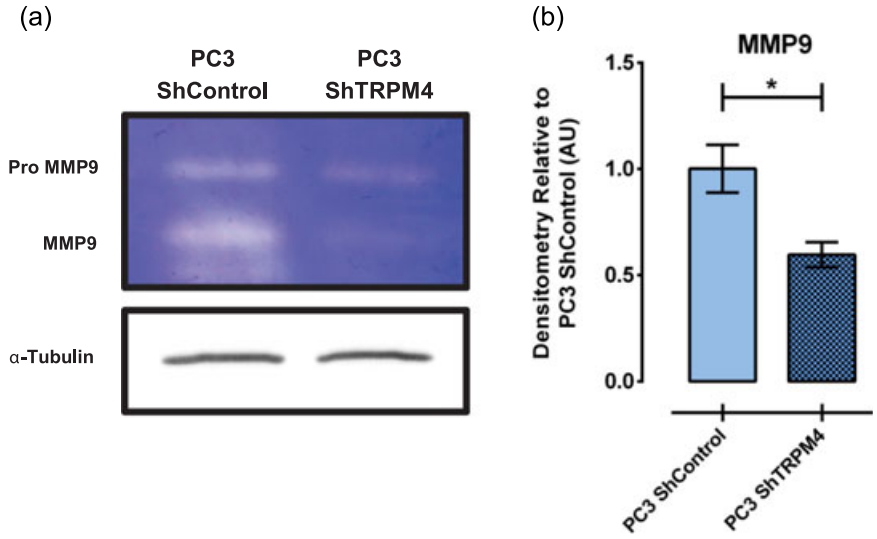

FIGURE 4 Effect of TRPM4 knockdown in PC3 cells on MMP9 activity. (a) Zymography assay for gelatinase activity dependent of MMP9. PC3 ShTRPM4 cells show a lower gelatinase activity for MMP9 compared with PC3 ShControl cells. Representative images and densitometries of at least three independent experiments (mean ± *SEM*) are shown. $p \leq 0.05$; *t* test with Welch correction. SEM, standard error of mean [Color figure can be viewed at wileyonlinelibrary.com]

show a decrease of MMP9 metalloproteinase activity in PC3 ShTRPM4 cells compared with control cells (Figure 4a) but no detectable changes in MMP2 activity (Supporting Information Supplementary Figure 6). These results suggest an alteration in the transcriptional function of Snail1 after TRPM4 knockdown, indicating that this channel could be a regulator of the EMT process.

Phosphorylation of Snail1 by GSK-3β is required for proteasomal-mediated Snail1 degradation (Zhou et al., 2004). As shown previously, PC3 ShTRPM4 cells have decreased levels of pSer9 GSK-3β. As this is an inhibitory phosphorylation, this data suggest an increased activity of GSK-3β in these cells, which in turn could explain the decrease of Snail1 protein upon TRPM4 knockdown. Thus, we evaluated the steady-state levels of Snail1 protein after the incubation with GSK-3β and proteasome inhibitors SB216763 and MG-132, respectively in PC3 shControl and shTRPM4 cells (Figure 5a). Snail1 protein levels accumulated upon GSK-3β and proteosome inhibition in PC3 ShControl as well as in PC3 ShTRPM4 cells, compared with DMSO control conditions. However, the protein levels of Snail1 in PC3 ShTRPM4 were not able to equate the protein levels of PC3 ShControl under both inhibitory conditions, indicating that by knocking down TRPM4, another mechanism controlling the steady-state levels of Snail1 was affected. Interestingly, a reduction of Snail1 mRNA expression after TRPM4 knockdown in PC3 cells was observed (Figure 5b). Consistently, an increase of Snail1 mRNA was detected after TRPM4 overexpression in LnCaP cells (Figure 5c). Together, these results suggest that TRPM4 associates to the regulation of the levels of Snail1 protein by affecting Snail1 gene expression and not through regulation of the GSK-3β/proteosome pathway.

4 | DISCUSSION

Metastasis is responsible for the death in most patients with cancer (Mehlen & Puisieux, 2006). The metastatic signaling is a complex process divided into several steps, including detachment of cancer cells from the primary tumor, invasion, intravasation, circulation survival, extravasation, and distant organ colonization (Martin, Ye, Sanders, Lane, & Jiang, 2013; Van Zijl, Krupitza, & Mikulits, 2011). EMT is involved in the metastatic cascade of many solid tumors and is characteristic of this event (Wang & Zhou, 2013). The EMT program involves transcriptional and phenotypic changes, characteristic of embryonic development in vertebrates, and includes the loss of cellular polarization and epithelial markers such as E-cadherin, together with the acquisition of invasive features and mesenchymal markers such as N-cadherin (Kalluri & Weinberg, 2009; Wang & Zhou, 2013). Deregulation of the EMT process has been extensively studied during tumor evolution, and is associated with an invasive behavior in prostate cancer (Grant & Kyprianou, 2013; Montanari et al., 2017).

Previous data shows that knocking down TRPM4 in HeLa cells results in higher levels of the E-cadherin compared with control cells, suggesting an alteration in its mesenchymal characteristics (Armisén et al., 2011). Similarly, in this study, we observed that PC3 ShTRPM4 cells express higher levels of E-cadherin compared with PC3 ShControl cells. In addition, PC3 ShTRPM4 cells showed a decreased expression of N-cadherin and vimentin, suggesting a reversal of the mesenchymal phenotype to a more epithelial one. Also, the morphology of the PC3 ShTRPM4 changes and appear more rounded than the controls cells that exhibit a more elongated morphology (Supporting Information Supplementary Figure 5), reinforcing the results that indicate an alteration of the mesenchymal phenotype. Since the EMT process is intimately related to the acquisition of motile and invasive characteristics (Guarino, 2007), we determined the relevance of knocking down TRPM4 in PC3 cells. In line with previous observations (Holzmann et al., 2015), migration of PC3 ShTRM4 cells was impaired compared with control cells. Also, and complementing those experiments, we studied the effect of TRPM4 knockdown on the invasion activity, a complex process that involves the protrusive formation of an invadopodia, and the degradation and remodeling of the extracellular matrix by proteolytic enzymes (Mareel & Leroy, 2003). Our experiments, showed a decrease in

the invasion capacity of PC3 ShTRPM4 compared with control cells, indicating the importance of TRPM4 expression in the invasiveness properties of PC3 cells. Notably, the knockdown of TRPM4 its related to a diminution of activating phosphorylation (Tyr397) on Focal Adhesion Kinase (FAK) (Cáceres et al., 2015) a key regulators of FA turnover. In accordance with these results, PC3 ShTRPM4 exhibits a diminution of FAK phosphorylation (Supporting Information Supplementary Figure 7), suggesting a direct role of TRPM4 in cellular migration of prostate cancer cells. Effect that could be independent of its activity as regulator of the stability of transcription factors as β-Catenin and Snail1. However, further works would be necessary to address these questions. Interestingly, the overexpression of TRPM4 in LnCaP cells, whose TRPM4 expression is significant lower than PC3 and quite similar than nontransformed RWPE-1 cells (Sagredo et al., 2017) is associated with a decrease of E-cadherin and an increase of cellular migration, without any evident effect on invasion. These results indicate that overexpression of TRPM4 alone in the noninvasive LnCaP cells (Yang, Loda, & Sytkowski, 1998) is not enough to generate an invasive phenotype, and other genetic and molecular changes are needed.

Unexpectedly, the downregulation of TRPM4 in LnCaP cells, using a lentiviral TRPM4 knockdown approach produces a significant reduction of several EMT markers by qPCR (Supporting Information Supplementary Figure 8), suggesting that in cells with low abundance of TRPM4, the inhibition of this channel is related with the loss of mesenchymal markers. Nowadays, the transcription factors, which initiate and maintain the EMT process, have been extensively studied and are well defined (Goossens, Vandamme, Van Vlierberghe, & Berx, 2017; Sánchez-Tilló et al., 2012). The analysis of Snail1, a key EMT transcription factor, in PC3 ShTRPM4 cells showed a significant reduction of their protein levels, suggesting a change compatible with a mesenchymal-phenotype reversion to one that is more epithelial. Snail1 has the ability to induce EMT, by the direct repression of E-cadherin gene expression, an increase of N-cadherin, vimentin, MMP9 mRNA synthesis (Figures 3 and 4), and other important EMT TFs, such as Twist1, Slug, and Zeb1 (Supporting Information

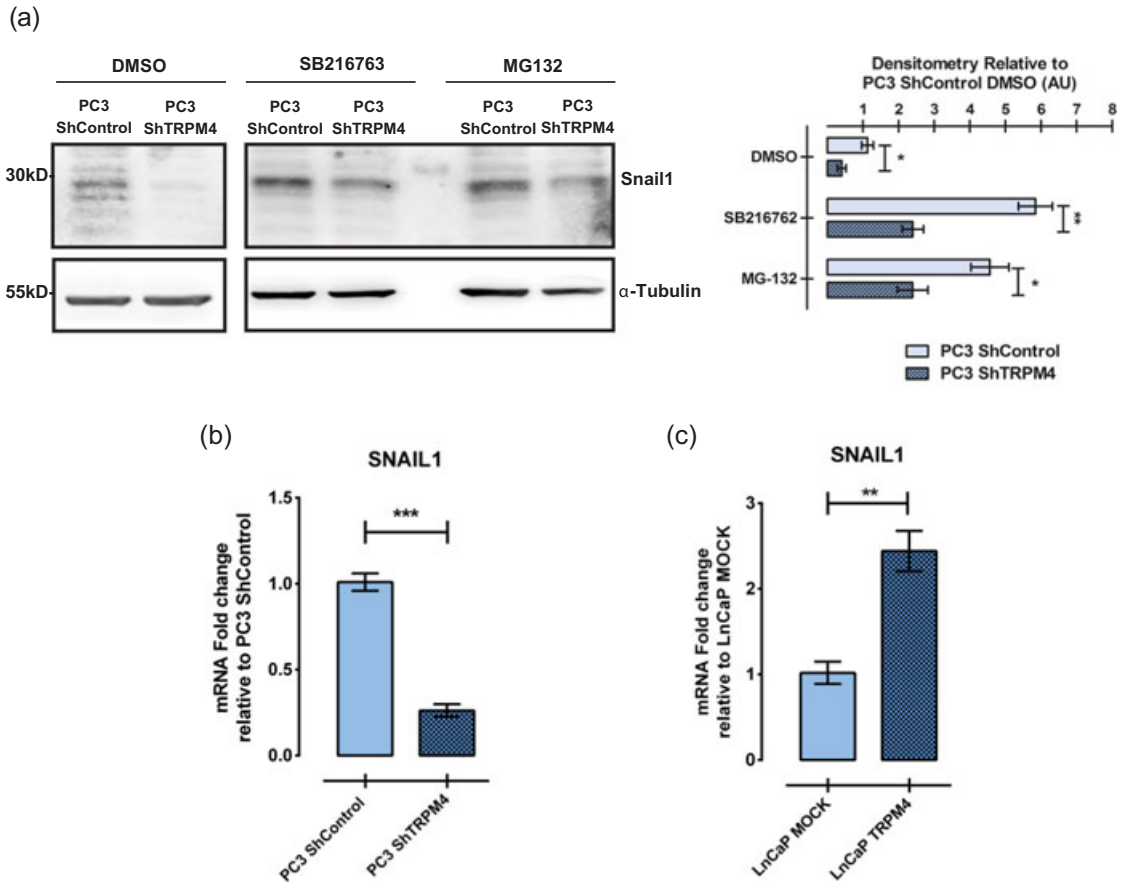

FIGURE 5 TRPM4 expression in PC3 cells affect Snail1 protein levels and mRNA expression. (a) PC3 ShControl and PC3 ShTRPM4 cells were incubated with the GSK-3β inhibitor SB216763 (SB), the proteasome inhibitor MG-132, or DMSO as a control vehicle for 8 hr before the protein extractions. An increase in the total amount of Snail1 was observed with SB 216763 and MG-132 incubation in both cellular models compared with the vehicle controls (DMSO). Representative western blots and densitometries of three independent experiments. Mean ± *SEM* are shown *i* test $^{*}p \le 0.05$, $^{**}p \le 0.01$. (b) TRPM4 Knockdown in PC3 cells is related to a decrease of SNAIL1 mRNA. Relative mRNA expression of Snail1 in at least three independent experiments are shown (mean ± *SEM*) $^{***}p \le 0.001$; *t* test with Welch correction. (c) Overexpression of TRPM4 by transient transfection in LnCaP cells correlated with an increase of SNAIL1 mRNA expression in at least three independent experiments compared with MOCK control cells (mean ± *SEM*) $^{**}p \le 0.01$. *t* test with Welch correction. mRNA, messenger RNA; SEM, standard error of mean [Color figure can be viewed at wileyonlinelibrary.com]

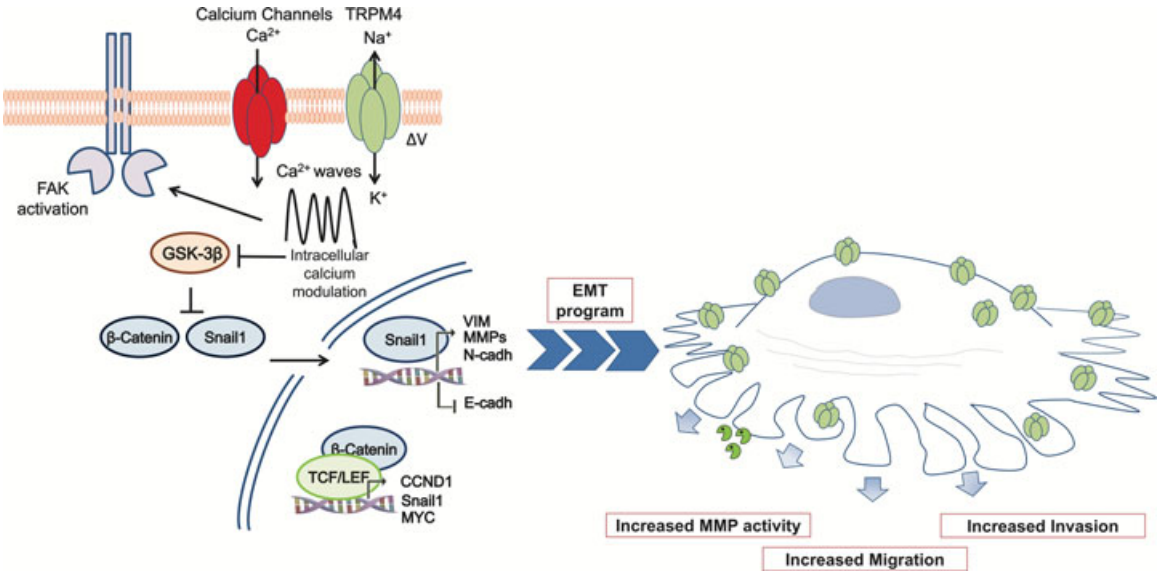

FIGURE 6 Molecular model of TRPM4 and its relation with the epithelial to mesenchymal transition. TRPM4 expression and function modulate the inhibitory phosphorylation of GSK-3β promoting the β-Catenin stability and its transcriptional activity on several genes involved in proliferation as Myc and CCND1 (Sagredo et al., 2017) and EMT markers such as Zeb, Vimentin, and Snail1. Also, this effect could regulate the protein levels of Snail1, promoting the invasive phenotype thought the regulation of the EMT markers as E-Cadherin, N-Cadherin, MMPs, and others. Interestingly, TRPM4 it is involved directly in the focal adhesion turnover promoting the cellular migration (Cáceres et al., 2015). These finding highlight this ion channel as a new potential target for future cancer therapies [Color figure can be viewed at wileyonlinelibrary.com]

Supplementary Figure 4) among others (Barrallo-Gimeno & Nieto, 2005; Cano et al., 2000; Peinado, Ballestar, Esteller, & Cano, 2004). In addition, the degradation of Snail1 depends on GSK-3β activity (Figure 3a), similar to β-catenin, another important EMT inductor described in cancer cells (Kim et al., 2012; Liu et al., 2002). Interestingly, under normal cell culture conditions, PC3 ShTRPM4 cells show decreased levels of Snail1 protein compared with control cells. Using the drug SB216763, a GSK-3β inhibitor, or MG132 a proteasome inhibitor, we observed an increase in the total amount of Snail1 in both cellular models. However, the accumulation of Snail1 in PC3 ShTRPM4 cells does not reach the levels detected in PC3 ShControl, suggesting that the lower levels of Snail1 in PC3 ShTRPM4 are due to a lower mRNA transcription of the *SNAIL1* gene (Figure 5b). This observation may be explained because of a decrease in total β-catenin and its cotranscriptional activity (Sagredo et al., 2017). In this line, it has been reported that TGF-β3 induced the expression of LEF-1, which interact with β-catenin to form a transcriptional complex that induce *SNAIL1* gene transcription (Medici, Hay, & Olsen, 2008), indicating a direct effect of β-catenin on the Snail1 expression. Interestingly, the Snail1 promoter has several functional response elements as AP1, AP4, and E-boxes sites sensitive to different signaling pathways as SMAD, LEF1, NF-κB, ERK1/2, and among others (Barberà et al., 2004; Taylor, Parvani, & Schiemann, 2010), suggesting, that not only a direct effect on the Snail1 protein levels could be mediated by TRPM4 expression and its effect on GSK-3β activity, but also, the downregulation of TRPM4 could affect the Snail transcriptional regulation by several calcium sensitive signaling pathways (De Herreros, Peiró, Nassour, & Savagner, 2010; Li et al., 2011; Medici, Hay, & Goodenough, 2006; Peinado, Quintanilla, & Cano, 2003).

The results presented in this study highlight the relevance of TRPM4 expression in the EMT regulation and its consequences on invasion properties of PC3 cells. Also, supports previous works showing altered expression of several ion channels in cancer cells and its effect on the EMT induction or reversion (Fortunato, 2017; Lai et al., 2013; Rapetti-Mauss et al., 2017; Restrepo-Angulo, Sánchez-Torres, & Camacho, 2011). Interestingly, the Ca^{2+} permeable TRPM7 channel was described as a partial regulator of the EMT process through the EGF-induced expression of the mesenchymal marker vimentin in the breast cancer cell MDA-MB-468 (Davis et al., 2013). These research works revealed a mechanism whereby the EMT induction is abrogated by intracellular calcium chelation, indicating the importance of ion channel functions in the remodeling of intracellular calcium dynamics and their consequences in STAT3 signaling pathway. A downregulation of TRPM4 in PC3 cells has been shown to correlate with a significant decrease in intracellular calcium influx after endoplasmic reticulum Ca^{2+} depletion compared with control cells (Sagredo et al., 2017). Thus, it is plausible that calcium sensitive intracellular signaling pathways could be affected by the downregulation of TRPM4 and its function as calcium regulator. For example, the expression of Twist transcription factor and others EMT markers as vimentin, N-Cadherin, and CD44 were significantly impaired after the intracellular calcium chelation (Davis et al., 2013) and several calcium signaling pathways are deregulated in different stages of cancer progression (Stewart, Yapa, & Monteith, 2015).

Until our knowledge, this is the first work that describes the effect of TRPM4 expression ion channel under EMT markers. Our results indicate a new relation of migration/invasion regulation by TRPM4 expression in prostate cancer cell through the expression control of key components of the EMT program. TRPM4 expression could regulate the levels of Snail1 protein and the expression of its mRNA promoting molecular and phenotype changes as E-Cadherin repression or metalloproteases expressions. All these changes were summarized in a schematic molecular model (Figure 6). Also, TRPM4 mRNA overexpression correlated with the aggressiveness of prostate cancer (Gleason score > 7). Tumors with a Gleason score > 7 are more aggressive, less differentiated, and with a high probability of invasion (Donohue et al., 2006; Rusthoven et al., 2014). For these reasons we decide to segregates the patients using the Gleason score > 7 as cut off, and then analyzed the TRPM4 mRNA, suggesting a correlation of TRPM4 expression with the aggressiveness of this disease, indicating new roles for this channel in tumor progression. Whether or not the ion channel activity of TRPM4 on intracellular calcium dynamics regulation is important in the EMT induction and maintenance is still unknown and further works could address this question.

ACKNOWLEDGMENTS

This study was supported by FONDECYT grants 1120286 (R.A.), 1151446 (R.A.), 1151435 (K.M.), 11170840 (C.E) and 1161288 (F.S), UNAB DI-741-15/N (F.S.), Millennium Institute on Immunology and Immunotherapy P09–016-F (F.S.), and CONICYT Doctoral fellowships 21100538 and 21130361 (A.I.S and E.A.S). This study received institutional support from Fondecyt-FONDAP grant 15010006 (A.S.) and Anillo en Ciencia y Tecnología ACT172101 (K.M.).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interests.

AUTHOR CONTRIBUTIONS

K.M and R.A conceived and designed the project. A.I.S, E.A.S, RO.A, V.P, and C.E performed the experiments. K.M, A.I.S, E.A.S, RO.A, C.E, V.P, A.S, L.M and F.S analyzed and interpreted the data. A.I.S, K.M and R.A wrote the manuscript.

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SUPPORTING INFORMATION

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How to cite this article: Sagredo AI, Sagredo EA, Pola V, et al. TRPM4 channel is involved in regulating epithelial to mesenchymal transition, migration, and invasion of prostate cancer cell lines. *J Cell Physiol*. 2019;234:2037–2050. https://doi.org/10.1002/jcp.27371