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The TMEFF2 tumor suppressor modulates integrin expression, RhoA activation and migration of prostate cancer cells☆

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ABSTRACT

Cell adhesion and migration play important roles in physiological and pathological states, including embryonic development and cancer invasion and metastasis. The type I transmembrane protein with epidermal growth factor and two follistatin motifs 2 (TMEFF2) is expressed mainly in brain and prostate and its expression is deregulated in prostate cancer. We have previously shown that TMEFF2 can function as a tumor suppressor by inhibiting cell migration and invasion of prostate cells. However, the molecular mechanisms involved in this inhibition are not clear. In this study we demonstrate that TMEFF2 affects cell adhesion and migration of prostate cancer cells and that this effect correlates with changes in integrin expression and RhoA activation. Deletion of a 13 basic-rich amino acid region in the cytoplasmic domain of TMEFF2 prevented these effects. Overexpression of TMEFF2 reduced cell attachment and migration on vitronectin and caused a concomitant decrease in RhoA activation, stress fiber formation and expression of αv, β1 and β3 integrin subunits. Conversely, TMEFF2 interference in 22Rv1 prostate cancer cells resulted in an increased integrin expression. Results obtained with a double TRAMP/TMEFF2 transgenic mouse also indicated that TMEFF2 expression reduced integrin expression in the mouse prostate. In summary, the data presented here indicate an important role of TMEFF2 in regulating cell adhesion and migration that involves integrin signaling and is mediated by its cytoplasmic domain.

1. Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer deaths in American men [1]. While organ-confined prostate cancer is successfully treated by surgical methods, no curative treatment is available for the metastatic form of the disease, which is responsible for the mortality associated with this disease. Prostate cancer cells are known to metastasize to numerous organs, with the bone, liver, and lymph nodes being the most common [2]; however, the molecular mechanisms that drive the metastatic cascade in prostate cancer are poorly understood. Understanding these mechanisms and the molecules involved in the metastatic cascade is critical to developing strategies for maximizing the efficacy of prostate cancer treatment.

Integrins are members of a family of transmembrane glycoprotein receptors that mediate cell–cell and the interactions with the extracellular matrix (ECM) [3]. By interacting with cytoskeletal-associated proteins, integrins provide a link between the extracellular environment and the cytoskeleton inside the cells. Integrins are heterodimers composed of non-covalently associated α and β subunits that can recognize and bind multiple ECM ligands, triggering a variety of signal transduction events that modulate diverse cellular processes including proliferation, survival, gene expression, adhesion and migration [3,4]. Evidence of altered integrin signaling has been demonstrated in several types of cancer, including prostate cancer. These changes correlate with tumor growth, invasion, and metastatic potential [5,6].

Several integrins, α2β1, α3β1, α5β1, α6β1, αvβ1, αIIbβ3, and αvβ3 are expressed in prostate cancer cells [6,7]. Of those, αvβ3 and β1 seem to play important roles in bone metastases, the main site of metastatic prostate cancer [7–9]. While not typically expressed in epithelial cells [8], integrin αvβ3 is expressed in prostate cancer. Its expression correlates with disease progression, metastatic potential [5–8], and with prostate cancer cell adhesion to vitronectin, a major extracellular component of mature bone [10]. Integrin β1 is upregulated in specimens from prostate cancer patients, and antibodies against β1 integrins inhibit binding of PC3 prostate cancer cells to human bone marrow endothelial cells [7,11], suggesting that β1 integrin mediates bone metastasis.

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Integrins signal bidirectionally [12–15]. Intracellular stimuli (inside-out signaling) can promote a conformational change in the integrin that lead to higher affinity for its ligand [12–14]. Ligand binding (outside-in signaling) ultimately leads to integrin activation and clustering into large mature focal adhesions (FAs) which generate downstream signaling events in a temporal fashion [12,15]. One of the changes that takes place early following integrin activation is the cytoskeletal rearrangements that regulate stress fiber formation and promote cell spreading and initiation of migration [16]. These events involve integrin-mediated modulation of specific cellular kinases and of RhoA activity [14,17]. RhoA is essential to remodeling actin fibers, regulation of actomyosin contractility, and rear cell detachment during motility [18]. RhoA is also activated via G-protein coupled receptors (GPCRs) that couple to the heterotrimeric G12/13 proteins, highlighting a cooperative relationship between integrin and GPCR signaling. Cross talk has also been described between integrins and growth factor receptor signaling to affect cell spreading, migration, growth and survival [19,20].

TMEFF2 is an evolutionarily conserved type I transmembrane protein expressed in the embryo, and selectively in the adult brain and prostate [21–23]. A role for TMEFF2 in prostate cancer was suggested by studies indicating that TMEFF2 expression is altered in a significant fraction of primary and metastatic prostate tumors [22,23]. We have described that TMEFF2 functions as a tumor suppressor, and that this role correlates, at least in part, with its ability to interact with SARDH to modulate cellular levels of sarcosine [24]. TMEFF2 overexpression blocked basal and sarcosine-induced cellular invasion of prostate epithelial RWPE cells, while TMEFF2 knockdown in 22Rv1 prostate cancer cells promoted increased cellular migration/invasion [25]. While these results highlight a role for TMEFF2 in the invasion of prostate cells, the molecular mechanism(s) involved in this process are not known. Here we report that TMEFF2 expression inhibits spreading and migration of RWPE2 prostate cancer cells on vitronectin. This inhibition correlates with a defect in FA and stress fiber formation and in RhoA activation and requires the presence of the cytoplasmic tail of TMEFF2. Importantly, TMEFF2 downregulates the expression of several integrins in RWPE2 cells indicating that the motility effects observed are integrin-mediated. The results presented point to an important role of TMEFF2 in modulating integrin signaling and prostate cell motility.

2. Materials and methods

2.1. Cell culture and plasmids

The 22Rv1, RWPE1 and RWPE2 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). The human prostate epithelial cell line RWPE1 and its Ki-ras transformed tumorigenic derivative, RWPE2, were cultured in KSF medium (Invitrogen, Carlsbad, CA). The human prostate carcinoma cell line 22Rv1 was maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA). TMEFF2 full length and ΔGA expression constructs were previously described [24,26]. The development of a system for inducible expression of FL_TMEFF2 and TMEFF2_ΔGA in RWPE2 cells was achieved using the Clontech's Tet-On Advanced system (Clontech, Mountain View, CA) essentially as described before for RWPE1 cells [24]. To inducibly express TMEFF2, cultures were grown in the presence of doxycycline (250 ng/ml; Sigma, St. Louis, MO). 22Rv1 cells transduced with pLKO.1 vectors containing shRNA to TMEFF2 or scramble control were described before [25].

2.2. Reagents

Antibodies recognizing TMEFF2 from cell lysates were purchased from Abcam (Cambridge, MA) and Sigma (St. Louis, MO). For TMEFF2 detection from mouse tissue lysates a SDIX, custom antibody was utilized (SDIX, Newark, DE). Other antibodies utilized in this study are: SV40 T-antigen (Abcam; Cambridge, MA), ITGAV, ITGB1 and ITGB3 (BD Biosciences, Franklin Lakes, NJ), ITGB3 (for mouse tissue lysates, Sigma, St. Louis, MO), ITGA5 (Millipore, Billerica, MA), ribosomal protein S6, β-actin, phospho-FAK, and FAK (Cell Signaling, Danvers, MA). Antibodies used for the immunofluorescence studies are listed in Section 2.5. The U0126 MAPK inhibitor or the inactive analog U0124 was purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA) was from Sigma (St. Louis, MO).

2.3. Mice

Animals were maintained in accordance with the Institutional Animal Care and Use Committee of East Carolina University. Transgenic TMEFF2 mice (129/Sv background, Lineberger Cancer Center Transgenic mouse facility) were crossed and maintained in a C57BL/6 background (backcrossed for over seven generations to C57BL/6). TRAMP mice (FVB background) were purchased from the Jackson laboratory (Bar Harbor, ME; stock number 008215) and crossed to C57Bl/6J (stock number 000664). The F1 generation derived from this cross, was then crossed to the transgenic TMEFF2 mouse and the TRAMP/TMEFF2 and the TRAMP progeny were selected after genotyping by PCR using tail genomic DNA. The TRAMP mice were genotyped as specified by the Jackson Laboratory (Bar Harbor, ME). The TMEFF2 mice were genotyped using primers 5′-GGAATTGCTCTGGTTATGATG-3′ and 5′-CAAATGTGGT ATGGCTGATTATG-3′.

2.4. Cell migration assays

Cell migration was measured using either a wound healing assay or Boyden chambers. Both assays were performed in the presence of 1 μg/ml aphidicolin (Sigma, St. Louis, MO) to prevent proliferation. For wound healing assays, 70 μl of cells (from a 3 to 7×10^5 cells/ml suspension) was loaded into each well of a culture plate insert (ibidi, Verona, WI). After 24 h incubation, the insert was removed to allow cell migration into the wound. Wound healing process was monitored by taking pictures at 0, 10, 24, or 48 h after removal of the insert, using an EVOS FL cell imaging system (Life Technologies, Carlsbad, CA). When specified, 2 μg/ml of CT04 (Cytoskeleton, Denver, CO) was added to the fresh medium after insert removal.

Cell migration was also assayed using Boyden chambers with non-coated 8 μM pore size membranes (BD Biosciences, Franklin Lakes, NJ). Cells (5×10^4) were suspended in 200 μl of serum-free medium and loaded into the upper chamber. The lower chamber was filled with 500 μl of medium supplemented with 20% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA) that was used as a chemoattractant. After 20 h of incubation, the cells that had migrated to the lower surface of the membrane were fixed with 70% ethanol for 10 min, followed by staining with 0.1% crystal violet (Sigma, St. Louis, MO) and photographed. To assay cell migration towards vitronectin or fibronectin the following modifications were implemented: i) the lower surface of the membrane in the Boyden chambers was treated with 10 μg/ml of the indicated ECM proteins or BSA as control (prepared in PBS) for 16 h at 4 °C; ii) cells ($0.5–1 \times 10^4$) were suspended in 200 μl of KSF base medium and loaded into the upper chamber; iii) the lower chamber was also filled with KSF medium; and iv) the culture was maintained for 1–2 days.

2.5. Cell spreading assay and immunofluorescence

Round cover glass slips (Fisher Scientific, Hampton, NH) were coated with collagen (40 μg/ml; BD Biosciences, Franklin Lakes, NJ), laminin (10 μg/ml; Sigma, St. Louis, MO), fibronectin (40 μg/ml; Millipore, Billerica, MA), or vitronectin (2 μg/ml; Promega, Fitchburg, WI) by incubation at 4 °C overnight and rinsed twice with PBS. Once coated, the cover glass slips were placed inside the wells of 12-well culture plates and 40,000 cells were loaded onto each well. Following 3 h incubation, pictures were taken for 10 random fields for each cover glass using an EVOS FL cell imaging system (Life Technologies, Carlsbad, CA) and