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Midkine promotes tetraspanin–integrin interaction and induces FAK-Stat1α pathway contributing to migration/invasiveness of human head and neck squamous cell carcinoma cells

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ABSTRACT

The heparin-binding growth factor, MK, promoting tumorigenesis and survival was found to associate with α6β1 integrins. We showed for the first time that MK interacted with TSPAN1 and facilitated the association between TSPAN1 and integrin α6β1 proteins in head and neck squamous cell carcinoma (HNSCC) cells. We found that MK mediated an integrin-dependent tyrosine phosphorylation of FAK and activation of paxillin and Stat1α pathways. As result, downstream target genes implicated in cell migration and invasiveness (e.g. MMP-2 and MMP-26) were overexpressed. We observed that RNAi silencing of the critical signaling intermediates led to decrease of MK-induced migration/invasiveness of HNSCC cells. The major finding of this study is a novel MK-triggered signaling mechanism implicated in migration and invasiveness of HNSCC cells.

Cell surface transmembrane proteins (e.g. receptors and integrins) transduce the extracellular signals triggering intracellular pathways leading to gene transcription controlling cell cycle, apoptosis and cell migration/invasiveness of tumor cells [3,9,26]. Epithelial cells migrate by assembling/disassembling cell–cell and cell–matrix adhesions and by forming connections between the adhesion molecules (e.g. integrins) and their cytoskeleton, thus generating contractile forces required for motion [3,14,20,35,36]. Integrins are shown to mediate adhesion to the extracellular cell matrix (ECM) and regulate the tumor cell invasiveness [5,14,23–26,32].

Tetraspanins were found to cooperate with integrins in regulation of cell motility in normal and tumor microenvironment [3,9]. Tetraspanins enhance assembling of multimolecular complexes between cell surface proteins (e.g. extracellular integrin-α chain domains) and linking them to downstream signaling intermediates [4,9,12,32].

The heparin-binding growth factor midkine (MK) that promotes the growth, tumorigenesis, survival, and migration was recently found to associate with α4β1- and α6β1-integrins [2,15,16,19,21]. We suggested that exposure of epithelial cells (e.g. HNSCC) to MK might trigger the complex formation of integrins with tetraspanins necessary to regulate cellular functions associated with tumorigenesis (e.g. cell migration/invasiveness).

Materials and methods

Antibodies and cells. Human full-length MK, N-truncated MK (MKN) and C-terminal truncated MK (MKC) were purchased from Bachem AG/Bachem America, Inc. We used a mouse monoclonal antibody against human MK (PA1-25539), a mouse polyclonal antibody against human TSPAN1 (PA1-57842), a rabbit polyclonal antibody against Stat1α (OPA1-03001) and a mouse monoclonal antibody against human matrix metalloproteinase (MMP)-2 (MA1-26370) obtained from ABR-Affinity BioReagents Inc. A mouse monoclonal antibody against phosphorylated Stat1α (p-Stat1α Y701, #612132), a rabbit polyclonal antibody against human FAK (#556368) and a mouse monoclonal antibody against human phosphorylated FAK (p-FAK-Y397, clone 14, #611723) were obtained from BD/Bioscience-Pharmingen. A rabbit polyclonal antibody against phosphorylated paxillin (p-paxillin-Y-31, sc-14035-R), rabbit (sc-10730) and goat (sc-6597) polyclonal antibodies against human integrin-α6 (sc-10730) were obtained from Santa Cruz Biotechnology. We used a mouse monoclonal antibody against human Matrilysin-2 (MMP-26, #CL2MMP26, Cedarlane Lab. Ltd), a rabbit polyclonal antibody against human integrin-β1 (#2387.00.02, Strategic Diagnostics, Inc.) and a rabbit polyclonal antibody against human paxillin (#RB-10643-P0, Lab Vision). The horseradish peroxidase-conjugated and agarose-bound secondary antibodies (anti-mouse, anti-rabbit or anti-goat immunoglobulins (IgG) were obtained from GE Healthcare Bio-Sciences Corp.

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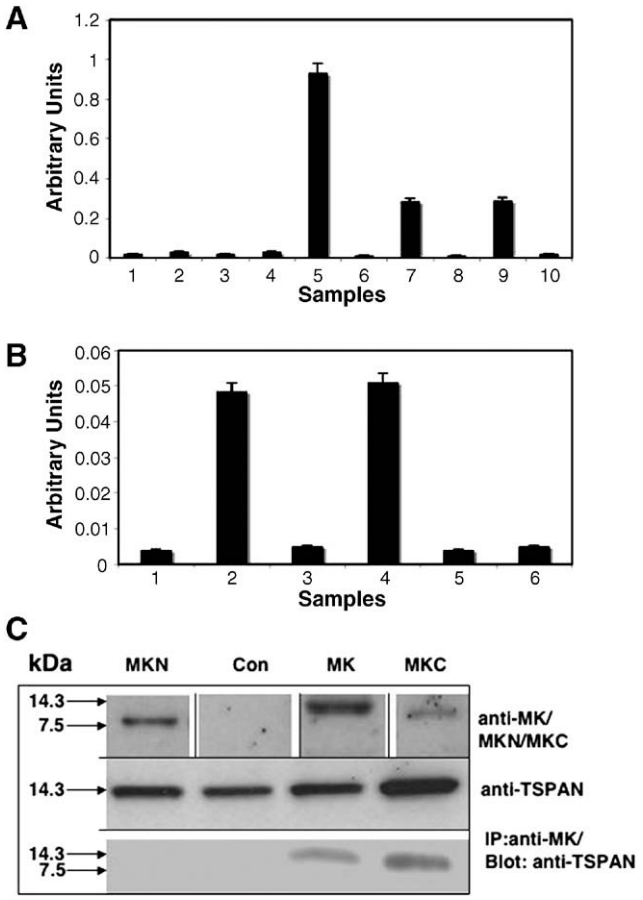

Fig. 1. MK associated with the TSPAN1 in vitro. (A) 2-Hybrid assay. Positive clones were analyzed by a quantitative α-galactosidase assay measured at A_{410}/A_{600}. Samples: 1, pGal4-AD-SV40 + pGal4-BD-lamin C (sample 1, negative control); 2, pGal4-BD + pGal4-AD; 3, pGal4-BD-MKN + pGal4-AD-TSPAN1; 4, pGal4-AD + pGal4-BD-MKN; 5, pGal4-BD-p53 + pGal4-AD-SV40 (positive control); 6, pGal4-AD + pGal4-BD-MK; 7, pGal4-BD-MK + pGal4-AD-TSPAN1; 8, pGal4-AD + pGal4-BD-MKC; 9, pGal4-BD-MKC + pGal4-AD-TSPAN1; 10, pGal4-BD + pGal4-AD-TSPAN1. (B) ELISA assay. AH109 yeast cells were transfected with the following prey plasmids: Samples: 1, 3 and 5 - pGal4-AD; 2, 4, and 6- pGal4-AD-TSPAN1. 100 μg of yeast extracts were panned against immobilized MK (1 ng, samples 1 and 2) or MKC (1 ng, samples 3 and 4, or MKN (1 ng, samples 5 and 6) in 96-wells plates. IgG-conjugated activity was measured with the alkaline phosphatase substrate at A_{410}. (C) MK associated with TSPAN1 in 029 cells. 029 cells were incubated with control medium, MK, MKN or MKC (3 nM each) for 30 min. Total lysates were precipitated with antibody against MK (1:100) and blotted with indicated antibodies (dilution 1:500 each).

029 HNSCC cells (expressing wild type p53 and p63) were isolated at the Johns Hopkins University School of Medicine [8].

Yeast 2-Hybrid Screening. A yeast 2-hybrid screen was performed using the Matchmaker Gal4 system 3 with four human 2-hybrid cDNA libraries (Startagene), including normal keratinocytes, HeLa cells, kidney, and brain embryonic cells [10]. We generated pGal4-BD-MK (containing complete MK, residues 1–143), pGal4-BD-MKC (containing C-terminal domain of MK, residues 82–143), or pGal4-BD-MKN (containing N-terminal domain of MK, residues 1–81) and introduced them into *Saccharomyces cerevisiae* strain AH109 as described [10]. Positive clones were grown in liquid SD/ Leu^-Trp^- medium for 3 days and then analyzed by a quantitative α-galactosidase assay [10].

ELISA binding analysis. The wells of microtiter plates were coated with purified MK, MKC or MKN (1 μg/ml) in bicarbonate buffer (pH 9.6) for 2 h at room temperature or overnight at 4 °C [10]. Yellow substrate (*p*-nitrophenyl-phosphate) for alkaline phosphatase (Sigma) was added and the phosphatase activity was estimated from the absorbance at A410 nm measured by a microtiter plate reader (Biotrack, Pharmacia) [10].

RT-PCR assay. One microgram of total RNA [10] was used to generate a cDNA from each sample with one-step RT-PCR kit (Qiagen) and custom primers for MMP-26; sense, 5′-AACAATTCC ATCGGAATGGG-3′, antisense, 5′-CTGGGAATCAGAGCTCCATA-3′, for MMP-2, sense, CCCAAGGAGAGCTGCAACCT-3′ and antisense, ACCAT GGGCGGCAACGCTGA-3′. As an internal control, the GAPDH region was amplified with the following primers; sense, 5′-GAGAAGG CTGGGGCTCATTT-3′ and antisense, 5′-CAGTGGGGACACGGAAGG-3′. PCR products were visualized with ethidium bromide after 1% agarose electrophoresis and quantified [10].

Immunoprecipitation. Cells were lysed in buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Brij-96, 1 mM PMSF, 0.5 mM NaF, 0.1 mM Na_3VO_4, 2× complete protease inhibitor cocktail), sonicated five times for 10 s time intervals, and clarified for 15 min at 15,000*g*. Supernatants (e.g. total lysates, 500 μl) were clarified by adding 5 mg of normal rabbit/mouse/goat IgG for 1 h at room temperature followed by the second antibodies against rabbit/mouse/goat IgG bound to agarose for 1 h at room temperature, and then spun down at 15,000*g* for 15 min. Resulting supernatants were precipitated with indicated primary antibodies overnight at cold room and then with secondary antibodies bound to agarose beads for 1 h at room temperature. The beads boiled in SDS-sample buffer were loaded on 4–12% SDS–PAGE and immunoblotted [10].

Expression knockdown by RNA interference. Scrambled pSM2 c-shRNAmir (negative control), and pSM2 c-U6-shRNAmir constructs for TSPAN1 (#RHS1764-9499461) and FAK (PTK2, RHS1764-97188355) were purchased from Open Biosystems and used accordingly [10]. We used the ratio of 10^8 pfu over 10^6 cells between pSM2 c-U6-shRNAmir retroviral particles and 029 cells [10].

We also used Integrin-β1 (H00003688-R07), FAK/PTK2 (H00005747-R04), Stat1α (H00006772-R01, paxillin (H00005829-R01), and scrambled control small interfering RNA/DNA chimeras (siRNA, Novus Biologicals, CO/Abnova) [31]. SiRNA chimeras (∼1–2 nM) were introduced into 029 cells (2×10^4–10^5, ∼30–40% confluence) seeded in 6- or 24-well plates using 2–4 μl of Lipofectamine RNAiMAX (#13778-150, Invitrogen) in RPMI medium w/o antibiotics for 24 h per manufacturer's recommendations [31,33].

Results and discussion

MK/TSPAN1 interactions in vitro

The heparin-binding mitogen MK is overexpressed in human cancers and might contribute to cell migration/invasiveness [37]. To define the molecular partners of MK that could potentially regulate cell migration/invasiveness, we employed a yeast 2-hybrid analysis. We generated bait plasmid, pGal4-BD-MK (residues 1–143) and then screened four human 2-hybrid cDNA libraries [10]. Screening of a total 3.5×10^8 yeast transformants yielded 121 positive clones, including the extracellular domain of TSPAN1 (residues 110–211) [10]. To examine the interaction of MK with TSPAN1, we generated additional baits harboring the C-terminus domain of MK (MKC; residues 82–143) or the N-terminus domain of MK (MKN, residues 1–81) designated as pGal4-BD-MKC or pGal4-BD-MKN, respectively. Pairs of bait (pGal4-BD-MK, pGal4-BD-MKC or pGal4-BD-MKN) and prey plasmids were introduced into AH109 yeast cells [10] and the protein interactions were monitored by a liquid α-galactosidase assay, or by an ELISA assay (Fig. 1A and B). In both assays, we observed that MK and MKC formed protein complexes with TSPAN1 in yeast, while MKN failed to do so (Fig. 1A and B).

We further analyzed the endogenous MK/TSPAN1 interactions by incubating HNSCC 029 cells with 3 nM MK, 3 nM MKN or 3 nM MKC for 30 min. Total lysates were precipitated with an antibody against MK followed by a subsequent immunoblotting with antibodies against TSPAN1 that was directed against the full-length TSPAN1 expressed in *Escherichia coli* (from ABR-Affinity