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Wnt Interaction and extracellular release of prominin-1/CD133 in human malignant melanoma cells

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Abstract

Prominin-1 (CD133) is the first identified gene of a novel class of pentaspan membrane glycoproteins. It is expressed by various epithelial and non-epithelial cells, and notably by stem and cancer stem cells. In non-cancerous cells such as neuro-epithelial and hematopoietic stem cells, prominin-1 is selectively concentrated in plasma membrane protrusions, and released into the extracellular milieu in association with small vesicles. Previously, we demonstrated that prominin-1 contributes to melanoma cells pro-metastatic properties and suggested that it may constitute a molecular target to prevent prominin-1-expressing melanomas from colonizing and growing in lymph nodes and distant organs. Here, we report that three distinct pools of prominin-1 co-exist in cultures of human FEMX-I metastatic melanoma. Morphologically, in addition to the plasma membrane localization, prominin-1 is found within the intracellular compartments, (e.g., Golgi apparatus) and in association with extracellular membrane vesicles. The latter prominin-1-positive structures appeared in three sizes (small, ~40 nm; intermediates ~40–80 nm, and large, >80 nm). Functionally, the down-regulation of prominin-1 in FEMX-I cells resulted in a significant reduction of number of lipid droplets as observed by coherent anti-Stokes Raman scattering image analysis and Oil red O staining, and surprisingly in a decrease in the nuclear

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Conflict of Interest

The authors declare to have no conflict of interests.

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localization of beta-catenin, a surrogate marker of Wnt activation. Moreover, the T-cell factor/lymphoid enhancer factor (TCF/LEF) promoter activity was 2 to 4 times higher in parental than in prominin-1-knockdown cells. Collectively, our results point to Wnt signaling and/or release of prominin-1-containing membrane vesicles as mediators of the pro-metastatic activity of prominin-1 in FEMX-I melanoma.

Keywords

prominin-1; melanoma; Wnt; catenin; vesicles

Introduction

The incidence of melanoma is steadily increasing in Caucasian populations worldwide, with approximately 70,000 new cases per year in the United States alone, while its associated mortality has not changed considerably over the last decades [1]. Although many studies have recently focused on the identification and characterization of cancer stem cells, not enough attention has been devoted to the cell sub-populations responsible for metastatic dissemination. This is particularly striking for metastatic melanoma (MM), cause of six out of every seven deaths associated with all types of skin cancer [1]. The phase of “escaping” from the primary tumor occurs very early in melanoma progression, such that 15–30% of the clinical stage I or II melanoma patients have histopathologically identifiable metastases in their sentinel lymph nodes [2]. Attempts to reduce the primary tumor mass are therefore destined to fail, because of the early presence of potentially metastatic cells in the general circulation, lymph nodes or as micrometastatic foci. Potentially metastatic cells may represent a minority of the bulk population of melanoma cells that must be specifically targeted to prevent metastatic dissemination. Biological and pharmacological differences between primary and metastatic melanoma are well established [3]. However, at this moment, there is a paucity of molecular targets that can be utilized to treat MM.

In a study on fresh human MM specimens, a prominin-1 (CD133) positive sub-population with tumor-initiating property accounting for 0.2–0.8% of the total tumor cell population was identified [4]. Several studies have reported the expression of prominin-1 in a sub-population of melanoma cells in most, if not all patients investigated [4–7]. We have also investigated the role of this cell surface antigen in human FEMX-I MM cells, which are originally derived from a lymph node metastasis of a melanoma patient [8]. FEMX-I cells express on their surface about 400,000 prominin-1 molecules/cell in 100% of them [9]. Moreover, we have reported that down-regulation of prominin-1 by short hairpin RNA (shRNA) reduced cell growth, motility, capacity to invade matrigel, a substance mimicking the basement membrane, metastatic capacity, and decreased the formation of spheroids under stem cell culture conditions [9]. Independently, we have also described the release of small prominin-1-containing membrane vesicles from neuro-epithelial and hematopoietic stem and progenitor cells, and hypothesized a certain role of this molecule as intercellular messenger [10–12]. A link between prominin-1 and the Wnt pathway was suggested by the observation that down-regulation of prominin-1 increased the gene expression of several Wnt inhibitors [9]. Reportedly [13], melanoma cells with metastatic potential have constitutive activation of Wnt signaling pathway with nuclear and cytoplasmic accumulation of β -catenin, and TCF/LEF family is involved in human melanoma from different stages of tumor progression, with LEF-1 mRNA expression being predominant in highly migrating cells from MM [14]. In the present study, based on the comparison of parental and prominin-1-knockdown FEMX-I melanoma cells, we report that prominin-1 activates the Wnt pathway, as shown by nuclear accumulation of β -catenin and active transcription from a reporter plasmid of the TCF/LEF binding site. Also, we report for the first time the

extracellular release of prominin-1-containing membrane microvesicles from melanoma cells.

Material and Methods

Cell Culture and cytotoxicity assay

The FEMX-I cell line was originally derived from a lymph node metastasis of a patient with MM [8]. FEMX-I cells were cultured in RPMI (Mediatech Inc., Manassas, VA, <http://www.cellgro.com>) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, <http://www.atlantabio.com>) at 37°C in a 5% CO₂ humidified incubator. Cells were used between passages 3 and 15 and tested routinely for mycoplasma contamination by the Venor GeM mycoplasma detection kit (Sigma-Aldrich, St. Louis, MO). For preparation of microvesicles, cells were enzymatically detached and plated in serum-free medium, consisting of Dulbecco's modified Eagle's medium/Ham's F-12 low osmolality medium in the presence of B27 supplement (both from Gibco, Grand Island, NY, <http://www.invitrogen.com>) and growth factors (1,000 IU/ml leukemia inhibitory factor plus 10 ng/ml basic fibroblast growth factor, and 20 ng/ml epidermal growth factor), as previously described [9].

Down-regulation of prominin-1

The vector pSUPER.retro.neo + GFP (pSUPER) from OligoEngine (Seattle, WA, <http://www.oligoengine.com>) was used to generate retroviral plasmids that express shRNAs based on the prominin-1cDNA, corresponding to nucleotides 773–792 (5'-GACCCAACATCATCCCTGT-3'; GenBank accession no. NM006017) and EGFP, as previously described [9]. As control vectors, we used the same retroviral plasmid carrying shRNA sequences nonspecific to any human gene and EGFP, to produce the control cell line FEMX-I/GFP. Generation of retroviral producers, transduction and selection of prominin-1 knockdown clones were previously described [9].

Lentiviral vector transduction

Cells were transduced with VSV-pseudotyped lentiviral particles expressing firefly luciferase under the control of the TCF/LEF promoter, and the puromycin N-acetyltransferase gene under the control of the hPGK promoter (pCignal/luc) (SABiosciences, Frederick, MD), using the Retronectin method as follows: retroviral supernatants were preloaded onto recombinant fibronectin (Retronectin, Takara Shuzo, Japan)-coated plates and centrifuged at 950xg for 30 minutes at 4°C. The operation was repeated a second time with fresh supernatant. The supernatant was then removed and the plates washed with PBS before addition of cells. Transduced cells were isolated via selection with 2 µg/ml puromycin.

Prominin-1-EGFP fusion plasmid and transfection

The eukaryotic expression vector plasmid enhanced GFP (pEGFP)-N1-prominin-1, containing the entire coding sequence of human prominin-1 fused in-frame to the N-terminus of GFP, was described [15]. In this plasmid, prominin-1-EGFP fusion protein is under the control of cytomegalovirus (CMV) promoter. Adherent cells in log phase of growth were enzymatically detached, resuspended at a density of 7.5×10^6 cells/mL in 200 µL of serum-free RPMI medium, and electroporated with 10 µg plasmid in a 0.4 cm-gap cuvette by a Gene Pulser Xcell instrument employing the following parameters: Voltage (150), pulse length (10 ms), field strength (0.6 kV/cm), n. of pulses (1).

Golgi staining

Golgi was stained by incubating cells for 16 hours with a baculovirus coding for a human Golgi-resident enzyme (N-acetylgalactosaminyltransferase 2) targeting sequence fused to emerald GFP (BacMam 2.0, Invitrogen, Carlsbad, CA; <http://www.lifetechnologies.com>),

NanoPro100 in-capillary nanofluidic immuno-detection system

Cells were first lysed with M-Per non-denaturing detergent formulation (Thermo Scientific, Rockford, IL, cat N. 78501) in the presence of protease and phosphatase inhibitors. Cell lysates (62.5 ng/ μ L) were prepared in Premix G2 pH 3–10 separation gradient containing pI standards and protease inhibitors. Separation time was 50 minutes at 15 MilliWatts. Rabbit 6B3 monoclonal anti-human β -catenin antibody (Cell Signaling Technology, Danvers, MA) was used as the primary antibody and anti-rabbit-HRP (Cell Signaling Technology, Danvers, MA) was used as the secondary antibody. The incubations were 110 and 55 minutes, respectively. The predicted theoretical pI (5.53) was different from the observed pI (approximately 7.3), presumably for post-translational modifications of β -catenin. In particular, acetylation of β -catenin has been described in several experimental systems [16–19].

Immunocytochemistry

Cells were seeded on poly-L-lysine coated chamber slides and grown overnight. Following aspiration of media, cells were washed rapidly with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and cell surface stained for 30 minutes with primary fluorochrome-labeled monoclonal antibodies (mAb). Incubation was carried out on ice, to prevent antigen capping. In a separate set of experiments, cells were fixed in 4% paraformaldehyde (PFA), washed with PBS, permeabilized in 0.5% Tween 20 and blocked with goat serum. After washing with PBS, cells were incubated overnight at 4°C with primary antibodies in 1% BSA-PBS, followed by a 45-minutes incubation at room temperature with fluorochrome-labeled secondary antibody in 1% BSA in PBS. Chambers were then removed and slides were mounted using Vectashield mounting media (Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>) and stored at 4°C until viewing in a CKX41 fluorescence inverted microscope (Olympus, Center Valley, PA). Two-photon images were taken using a homebuilt multi-photon microscope, as previously described [20]. The excitation wavelength was at 924 nm. The epi-reflected emission signals arising from EGFP and DsRed were collected simultaneously with 500/40 nm and 600/40 nm bandpass filters, respectively.

Migration assays

In vitro migration assays were performed using 8 μ m-pore Multiwell HTS systems and BioCoat invasion chambers with Matrigel-coated 8- μ m pore poly(ethylene terephthalate) membrane inserts (both from BD Biosciences, San Jose, CA, <http://www.bdbiosciences.com>), according to the manufacturer's directions. The Matrigel layers of the invasion chambers were rehydrated with serum-free bicarbonate-containing medium, as suggested by the manufacturer. The lower chambers were filled with RPMI 1640 medium with 10% FBS. Following 24 hours of incubation, the cells on the upper side of the membrane were gently removed with wet cotton swabs. The invasive cells on the lower surface of the membrane were fixed in 4% PFA for 10 minutes, and then stained with 0.2% crystal violet in 30% ethanol for 1 hour before washing. The number of cells in 8–12 randomly selected microscopic fields (10X) was counted under an Olympus CKX31 microscope (Olympus America Corp., Center Valley, PA, <http://www.olympus-global.com>).

Flow cytometric analysis

The level of the cell surface expression of prominin-1 was measured using the Quantum Simply Cellular System (QSCS; Bangs Laboratories Inc., Fishers, IN, <http://www.bangslabs.com>). Five $\times 10^5$ cells/sample were incubated with saturating concentrations (10 $\mu\text{g/ml}$) of phycoerythrin (PE)-conjugated AC133 mAb for 30 minutes at 4°C. Standard curves of beads with fixed antibody-binding capacity and samples were analyzed on a FACSVantage flow cytometer (BD Biosciences). The antibody-binding capacity for each cell population was calculated using QuickCal v.2.3. Software (Bangs Laboratories Inc.), employing median histogram values and linear regression analyses.

Immunoblotting

W6B3C1 anti-prominin-1 (Millipore, Billerica, MA, <http://www.millipore.com>), was employed for immunoblotting according to the manufacturers' recommended conditions. Gel band densitometric quantification was performed employing the ImageJ64 software (rsbweb.nih.gov/ij).

Measurement of intracellular lipid droplets

Intracellular lipid accumulation was evaluated with Oil Red O (ORO) staining and coherent anti-Stokes Raman scattering (CARS) imaging at 2845 cm^{-1} , as described previously [21, 22].

Luciferase Assay

The preparation of cell extracts and measurement of luciferase activity were carried out using the Steady-Glo Luciferase Reporter Assay System according to recommendations by the manufacturer (Promega, Madison, WI). The assays for luciferase activity were done employing a 20/20 luminometer (Turner Biosystems, Sunnyvale, CA).

Immunoelectron microscopy

The 200,000 $\times g$ pellets obtained after differential centrifugation (see above) of 1 ml 6-day-conditioned media from either parental FEMX-1 melanoma cells or clone FEMX-I/prominin-1^{-7/5} were resuspended in 40 μl of 4% PFA in phosphate buffer. The suspensions were applied onto 400-mesh grids with formvar- and carbon-coating. The samples were blocked for 5 minutes with 0.1% glycine in PBS and twice for 10 minutes with 0.2% gelatine, 0.5% BSA in PBS (PBG). The grids were incubated for 1 hour at RT with either mAb AC141 (0.46 mg/ml, Miltenyi Biotec, Bergisch Gladbach, Germany) directed against human prominin-1 [23] or mAb PeliCluster CD63 (1:15, Sanquin, Amsterdam, The Netherlands) and after washing with PBG, samples were incubated for 1 hour with goat anti-mouse IgG coupled to 10-nm gold (British Biocell, Cardiff, UK). After subsequent washing steps with PBG and PBS, the grids were post-fixed for 5 minutes in 2% glutaraldehyde in PBS. The samples were negatively contrasted with 1.0% uranylacetate in water and viewed in a Morgagni electron microscope (FEI Company). Micrographs were taken with a Morada camera and AnalySIS software (Soft Imaging Systems). The images shown were prepared using Adobe Photoshop software.

Results

Intracellular distribution of prominin-1 in human FEMX-I melanoma cells and establishment of prominin-1-knockdown clones

We previously reported that the down-regulation of prominin-1 in FEMX-I melanoma resulted in decreased cell growth, motility, formation of spheroids under stem cell culture conditions and metastatic capacity [9]. To investigate the relationship between prominin-1

and metastatic potential of melanoma cells, we isolated by limiting dilution from the FEMX-I/SUPER⁷⁷³ prominin-1-knockdown cell line [9], two clones referred to as FEMX-I/prominin-1⁻/5 and FEMX-I/prominin-1⁻/6, which have a minimal residual expression of prominin-1 (Fig. 1).

The decrease in prominin-1 expression was confirmed by cell surface immunofluorescence employing either AC133 or AC141 mAb, which recognize distinct, glycosylation-dependent epitopes (Fig. 1A). The QSCS and flow cytometry analyses revealed that the number of cell surface prominin-1 molecules per cell is $3,800 \pm 1,100$ and $14,700 \pm 2,600$ for FEMX-I/prominin-1⁻/5 and FEMX-I/prominin-1⁻/6 clones, respectively, by comparison to $420,000 \pm 40,000$ for parental FEMX-I/GFP cells. Down-regulation of prominin-1 was also observed by immunoblotting of total cell lysates, using the glycosylation-independent W6B3C1 mAb (Fig. 1B). Under these conditions, 95.1 and 78% decrease in total prominin-1 expression was observed for FEMX-I/prominin-1⁻/5 and FEMX-I/prominin-1⁻/6, respectively (Fig. 1C). Interestingly, the immuno-labeling performed upon fixation and permeabilization of cells revealed an additional, intracellular pool of prominin-1 (Fig. 2A). Employing a baculovirus-based system encoding the emerald GFP containing the targeting sequence of a human Golgi-resident enzyme (N-acetylgalactosaminyltransferase 2), we found a significantly overlapping signal of prominin-1 with the Golgi apparatus (Fig. 2B). The two pools (surface + intracellular) of prominin-1 were also observed after transfection of FEMX-I cells with prominin-1-EGFP fusion protein indicating that this phenomenon is independent of an antibody-induced endocytosis process (Suppl. Fig. 1). However, it is interesting to note that such internalization and retrograde transport of prominin-1 to, or near, the Golgi complex might occur upon the binding of AC133 mAb to the cells at 37°C (Fig. 2C).

Down-regulation of CD133 impairs substrate and inter-cellular adhesion, cell motility and invasion

The knockdown of prominin-1 as observed in both clones, FEMX-I/prominin-1⁻/5 and FEMX-I/prominin-1⁻/6, resulted in a general change of cellular patterns such as the cell alignment and disturbances of intercellular orientation, suggestive of modifications in cell-matrix and/or cell-cell adhesion characteristics (Fig. 3A). Interestingly, the adhesion to tissue culture plastic was significantly delayed in cells lacking prominin-1 by comparison to the parental ones (Fig. 3B). We also found using a 24h-HTS transwell assay that FEMX-I/prominin-1⁻/5 and FEMX-I/prominin-1⁻/6 had a decrease in their migration potential of 93% ($p=0.001$) and 89% ($p=0.003$), respectively. Finally, although FEMX-I cells were able to invade through the Matrigel layer of BioCoat chambers, a significant impairment of invasion was observed for FEMX-I/prominin-1⁻/5 cells (Fig. 3C), in agreement with our previous observation [9].

Release of prominin-1-containing membrane vesicles

To determine whether FEMX-I release prominin-1 in association with membrane vesicles, as previously reported for neural progenitors and hematopoietic stem cells [12, 24], the 6-day-old conditioned culture FEMX-I medium was collected and subjected to differential centrifugation followed by immunoblotting of the recovered pellets for prominin-1. A strong prominin-1 immunoreactivity was observed in the $200,000 \times g$ pellet like in a whole cell lysate (Fig. 4A). Only a minor proportion of prominin-1 immunoreactivity was detected in other fractions, i.e. the $500 \times g$, and $10,000 \times g$ pellet (Fig. 4A). The association of prominin-1 with the $200,000 \times g$ pellet was specific, because other abundant proteins, including AHNK, myosin and β -catenin were not detected therein (data not shown). To assess the nature of the prominin-1⁺ material associated with the $200,000 \times g$ pellet, an electron microscopy (EM) analysis was undertaken. This revealed the presence of three different classes of membrane vesicles based on their size: small (< 40 nm), intermediate

(~40–80 nm) and large (> 80 nm); intermediate vesicles were prevalent and the larger ones rare (Fig. 4B). The immunogold labeling of prominin-1 revealed that both the intermediate and the larger membrane structures were positive (Fig. 4B, sub-panels a-a4, white arrowheads, arrows, respectively). The small membrane vesicles were rarely labeled for prominin-1 (Fig. 4B, sub-panels a-a4, black arrowheads) in contrast to CD63 (Fig. 4B, sub-panels b, c, black arrowheads), a tetraspan membrane protein highly enriched in exosomes from virtually any cell type [25], suggesting the presence of such vesicles in the 200,000 × g fraction.

Effects of prominin-1 on intracellular lipids

Interestingly, we found a significant intracellular pool of lipid in prominin-1⁺ cells using CARS microscopy, which is a reliable measure of lipids [26] (Fig. 5). Indeed, such intracellular lipid accumulation is observed in cells with an elongated morphology, where lipids tended to concentrate at two poles (Fig. 5A, B), whereas, in cells with spherical morphology, they concentrated toward one pole and appeared to be either on the membrane or close to it (Fig. 5C). Surprisingly, no intracellular lipid accumulation was detected in prominin-1⁻ cells (Fig. 5B, right panel). The correlation between prominin-1 expression and intracellular lipid accumulation was further confirmed with ORO staining. The number of lipid droplets per cells decreased from 23.2 (± 12.1 SD/cell) in FEMX-I cells to 1.3 (± 0.6) and 1.9 (± 1.2)/cell in FEMX-I/prominin-1⁻/5 and FEMX-I/prominin-1⁻/6 clones, respectively.

Prominin-1 mediates Wnt activation in parental FEMX-I cells

We previously reported that down-regulation of prominin-1 increased the gene expression of several Wnt inhibitors [9]. To further investigate the effect of prominin-1 on the Wnt pathway, we studied the intracellular distribution of β -catenin. While wild-type FEMX-I cells displayed nuclear localization of β -catenin, FEMX-I/prominin-1⁻/5 and FEMX-I/prominin-1⁻/6 cells had a predominantly plasma membrane staining, as detected by both two-photon microscopy and by conventional immunofluorescence (Fig. 6A). The β -catenin protein expression was quantified in wild type and prominin-1 knockdown FEMX-I cells separating proteins by isoelectric focusing-capillary electrophoresis, followed by in-capillary immuno-chemoluminescence detection (Fig. 6B). β -catenin expression was reduced by 2.8- and 1.7-fold in FEMX-I/prominin-1⁻/5 and FEMX-I/prominin-1⁻/6, respectively. No additional peaks were observed (Fig. 6B), thus excluding the presence of phosphorylated isoforms. Then, we transduced parental FEMX-I and FEMX-I/prominin-1⁻/5 with a lentiviral reporter construct, expressing firefly luciferase downstream of the TCF/LEF promoter (*TCF/LEF-luc*). In eight independent experiments, baseline Wnt activity was 85 ± 2% (mean ± S.D.) higher in parental FEMX-I than in FEMX-I/prominin-1⁻/5 cells. In both parental and prominin-1-knockdown cells, Wnt activity could be increased in a concentration-dependent fashion by the addition of Wnt3a, although at greater levels in parental cells (Fig. 6C). Interestingly, the addition of Wnt-3a ligand, a physiological activator of the canonical Wnt pathway, to FEMX-I/prominin-1⁻/5 cells for 5 hours resulted in a translocation of β -catenin into the nucleus (Fig. 6D), similar to the constitutive localization of β -catenin in untreated parental FEMX-I cells (Fig. 6A). The effect of prominin-1 knockdown on the Wnt pathway was confirmed in a different prominin-1-expressing cell model, the human Caco-2 colon carcinoma cell line. In this cell line, Wnt reporter activity was 47 ± 7% (mean ± S.D.) higher than in the prominin-1-knockdown counterpart. Transduction of FEMX-I and FEMX-I/prominin-1⁻/5 cells with a MAPK (Erk) reporter gave similar results, with 80 ± 25% (mean ± S.D.) higher reporter activity in parental cells. In contrast, transduction of 8 additional reporters constructs, for SMAD, Jun, NF κ B, Notch, p53, c-myc, HIF- α and E2F resulted in equal levels of luciferase activity in parental and prominin-1-knockdown cells (Suppl. Fig. 2).

To further establish the effect of prominin-1 expression on the Wnt pathway, we transduced FEMX-I cells in reverse order: first with TCF/LEF-luc, and then with prominin-1/shRNA. After selection of prominin-1 knockdown cells with G-418 for seven days, the Wnt reporter activity was decreased by 4.1 ± 0.5 -fold compared with parental cells, whereas the negative control had no effect on reporter activity. The effect was specific for the Wnt pathway, because prominin-1 shRNA transduction had no effect on the reporter activity of FEMX-I–c-myc-luc, stably transduced with a construct of c-myc-binding site upstream of the firefly luciferase (not shown).

Discussion

We previously reported that down-regulation of prominin-1 by short hairpin RNA reduced the metastatic capacity of FEMX-I melanoma and increased the expression of Wnt inhibitors [9]. To elucidate the mechanism of the pro-metastatic activity of prominin-1, and of its association with the Wnt pathway, we first decided to characterize the intracellular distribution of prominin-1. We observed in FEMX-I probed with AC133 mAb, a prominin-1 pool associated with plasma membrane and one associated with the Golgi. This finding was confirmed in cells transfected with prominin-1-GFP fusion plasmid, thus excluding experimental bias related to antibody penetration and accessibility. Interestingly, we also observed extracellular release of prominin-1-containing membrane microvesicles. In a previous study, Huttner et al. [27] detected prominin-1-containing microvesicles in the cerebrospinal fluid of glioblastoma patients. However, to the best of our knowledge, this is the first direct report of release of prominin-1-containing microvesicles from cancer cells. The origin of prominin-1⁺ membrane vesicles might be i) plasma membrane for the large ones (>80 nm); ii) microvillus, primary cilium or multivesicular bodies for the intermediate ones and iii) multivesicular bodies for the small ones, which are often labeled for CD63. The apparent co-localization of intracellular prominin-1 and Golgi presumably reflects the presence of prominin-1 in multivesicular bodies located in the proximity of Golgi. A role for microvesicles as mediators of extracellular communication has been established, with abundant experimental evidence to suggest their role in cancer progression (for a Review, see [28]). Since the function of microvesicles is dependent on the molecular cargo that they carry, the observation that microvesicles released by MM cells contain prominin-1 suggests that prominin-1 may have an important role in extracellular communication. Degradation of the basement membrane is a critical step in promoting tumor metastasis. We have found that prominin-1 knockdown results in reduced motility and misalignment, presumably because since the cell migration is impaired, the cells can not align properly. We have also observed loss of the capacity to invade through Matrigel, a basement membrane-mimicking substance. Interestingly, it has been previously shown that tumor-derived microvesicles often carry protein-degrading enzymes, including matrix metalloproteinases. By releasing these proteases, tumor cells can degrade the extracellular matrix and invade surrounding tissues.

Our finding of reduced intracellular lipid droplets, identified by both CARS and Nile Red staining, in prominin-1-knockdown cells supports the hypothesis that the well-known interaction of prominin-1 with cholesterol within membrane micro-domains might be important in maintaining an appropriate lipid composition within the plasma membrane [29]. Protein-lipid assemblies might be the essential structural determinant in the release process of prominin-1, thus supporting the concept that prominin-1-containing lipid rafts may host key determinants for the metastatic potential of the cancer cells. Also, frequent budding of vesicles in prominin-1⁺ cells might trigger membrane damage repair mechanism to replenish membrane components. Since lipid droplets serve as reservoirs for phospholipids, sterols, and membrane-bound proteins [30], it is plausible that increased intracellular lipid droplet accumulation constitutes an emergency response to membrane repair [31]. In addition, membrane fluidity and polarity play crucial roles in endothelial and

cancer cell motility [32–34]. Intracellular lipid accumulation has been observed together with changes in membrane polarity in migrating and circulating tumor cells [20]. While the relationship between prominin-1 expression and intracellular lipid accumulation is unclear, intracellular lipid level could potentially serve as a marker of prominin-1 expression level, changes of membrane fluidity, and metastatic potential of MM cells.

Based on our previous findings of a link between prominin-1 and Wnt in FEMX-I cells [9], we have now demonstrated that suppression of prominin-1 expression leads to loss of the nuclear localization of β -catenin and a reduction in canonical Wnt/ β -catenin signaling through TCF/LEF1 transcription factors. Interestingly, addition of Wnt3a to prominin-1-knockdown cells restored the nuclear localization of β -catenin, showing that in the absence of prominin-1 the Wnt pathway can still be activated by physiological ligands. β -catenin is a dual purpose protein: in normal cells, it is prevalently localized at the plasma membrane, where it provides a mechanical linkage between cell-to-cell junctional proteins (e.g., E-cadherin) and cytoskeletal proteins (e.g., β -catenin and actinin-4) (34, 35). By contrast, in tumor cells, β -catenin is often found in the cytoplasm and nucleus, where it associates with TCF family members to form a complex, which activates transcription of many genes, including pro-metastatic genes. A constitutive complex of β -catenin and LEF-1 has been detected in melanoma cell lines expressing either mutant β -catenin or mutant APC [35]; however, β -catenin mutations are rare in primary malignant melanoma, while its nuclear localization, a potential indicator of Wnt/ β -catenin pathway activation, is frequently observed in melanoma [36]. The relevance of Wnt signaling to the metastatic process has been previously unveiled by the observation that Wnt5a has an important role in human melanoma progression [37] and by the report that β -catenin is a central mediator of melanoma metastasis to the lymph nodes and lungs [38].

Interestingly, a clear association between prominin-1 and nuclear β -catenin exists for colon carcinoma [39]. Boivin et al. [40] suggested that tyrosine phosphorylation of prominin-1 by Src and Fyn tyrosine kinases in response to a, presently unknown, extracellular ligand, might activate the Wnt pathway, that would influence cell growth, motility and metastatic potential. Also, the presence of conserved tandem T-cell factor/lymphoid enhancer factor binding sites in the prominin-1 gene [16] suggests a feedback regulation of prominin-1 expression by the Wnt pathway. Consistent with our findings, Bak *et al.* [41] recently reported that the deacetylase HDAC6 physically associates with prominin-1 to negatively regulate its trafficking down the endosomal-lysosomal pathway for degradation and that prominin-1, HDAC6, and β -catenin physically associate as a ternary complex, resulting in activation of β -catenin signaling. We propose that in certain cancer progenitor cells prominin-1-mediated Wnt activation, probably linked to the dynamic organization of cholesterol-rich membrane microdomains, is responsible for the precise coordination and integration of the expression of multiple genes resulting in dynamic, pro-metastatic changes in cell adhesion and motility. This situation is potentially amenable to specific targeting by inhibition of prominin-1 and/or of the Wnt pathway.

Conclusions

Herein, we have first reported release of prominin-1-containing microvesicles from cancer cells. Our results point to Wnt signaling and/or release of prominin-1-containing microvesicles as mediators of the pro-metastatic activity of prominin-1 in FEMX-I melanoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- First report of release of prominin-1-containing microvesicles from cancer cells.
- Pro-metastatic role of prominin-1–containing microvesicles in FEMX-I melanoma.
- Down-regulation of prominin-1 results in decreased nuclear localization of b-catenin.
- Wnt signaling as mediator of the pro-metastatic activity of prominin-1

Figure 1. Prominin-1 knock-down in FEMX-I cells

A. Fluorescent images of parental FEMX-I/GFP and prominin-1-knockdown clones (^{-1/5} and ^{-1/6}) in adherent cultures upon cell surface labeling in the cold with PE-conjugated AC133 and AC141 mAbs, directed against different epitopes of prominin-1. B. Immunoblot analysis for prominin-1 (CD133). 20 μ g of total lysate from each cell line were loaded onto an SDS-PAGE and membranes were probed with W6B3C1 mAb. β -actin expression was used as loading control. C. Quantification of the expression level of prominin-1 in prominin-1-knockdown clones (^{-1/5} and ^{-1/6}) by flow cytometry (FC) (plasma membrane staining by AC133) and by immunoblotting (IB) (mAb W6B3C1). Bars, SD.

Figure 2. Intracellular localization of prominin-1

A. FEMX-I cells were fixed, permeabilized and probed with AC133 mAb, followed by fluorescein-labeled secondary antibody. B. Co-localization of prominin-1 (red) with the Golgi complex, which was visualized by 2-photon fluorescence microscopy upon infection with baculovirus encoding the emerald GFP carrying the human Golgi-resident enzyme (N-acetylgalactosaminyltransferase 2) targeting sequence. C. Retrograde Golgi transport of prominin-1 upon AC133 mAb binding and internalization. Accumulation of AC133-PE antibody (red) in or near the Golgi complex (blue) 1 h after treatment of FEMX-I cells with the antibody at 37°C. Golgi was visualized as described above. Dotted and dashed lines highlight the cell and nuclear (N) contours, respectively. Scale bars, 12 μ m.

Figure 3. Disorganized pattern of growth, delayed adhesion and loss of invasiveness of prominin-1-knockdown FEMX-I cells

A. Morphological appearance of FEMX-I/GFP, FEMX-I/prominin-1⁻⁵ and FEMX-I/prominin-1⁻⁶ cells growing in adherent cultures. Scale bars, 12 μm. B. The adhesion of FEMX-I/prominin-1⁻⁵ cells to tissue culture plastic in serum-supplemented medium is delayed by comparison to parental FEMX-I/GFP. Data represent the mean of three experiments; Bars, SD; Asterisks, $p < 0.01$ (unpaired Student's *t* test). C. Loss of invasiveness of FEMX-I/prominin-1⁻⁵ cells, as measured by the number of cells migrating through Matrigel layers of BioCoat chambers in 16 hours, compared to parental FEMX-I cells.

Figure 4. Prominin-1 is associated with extracellular membrane vesicles

A. Six-days old conditioned, serum-free medium from FEMX-I cells was subjected to differential centrifugation for 5 minutes at $300 \times g$, 5 minutes at $500 \times g$, 30 min at $10,000 \times g$ and 60 minutes at $200,000 \times g$. The resulting pellets and total cell lysate were analyzed by immunoblotting using W6B3C1 mAb against prominin-1. Beta-actin was used as control for equal loading. B. Six-days old conditioned serum-free medium from either FEMX-1 (a, b) or FEMX-I/prominin-1^{-/-} (c) cells were subjected to differential centrifugation, and the resulting $200,000 \times g$ pellets were processed for negative staining and prominin-1– (a-a4, c) or CD63– (b, c) immunogold labelling EM. Three major sizes of membrane vesicles (see cartoon) were immunolabeled for prominin-1: small (~ 40 nm, black arrowhead), intermediate (~ 40 – 80 nm, white arrowhead) and large (~ 80 nm, arrow) membrane vesicles. Note that small ones are infrequently labelled for prominin-1 in contrast to CD63, and large membrane vesicles are rare. Bars, 100 nm.

Figure 5. Lipid distribution in parental and prominin-1-knockdown FEMX-I cells by coherent anti-Stokes Raman scattering (CARS)

The contrast mechanism for CARS derives from the CH_2 stretch vibrational frequency at 2845 cm^{-1} . A. Three representative single frames of parental FEMX-I cells taken along the vertical axis at 1-micron interval, and the merge image of approximately 20 single frames. CARS, red; prominin-1, green. B. Representative images of either FEMX-I cell (left panel) or FEMX-I/prominin-1^{-/-}/5 cell (right panel) with elongated morphology where the intracellular lipid (CARS, red) is observed in parental cell but absent in prominin-1 (green) knockdown cell. D. Representative image of FEMX-I cell with spherical morphology where a polar distribution of lipid is visualized with CARS microscopy.

Figure 6. Changes in localization and intracellular levels of β -catenin and Wnt activation in prominin-1-knockdown FEMX-I clones

A. Parental FEMX-I and prominin-1-knockdown clones $^{-/5}$ and $^{-/6}$ were stained with anti- β -catenin antibody and visualized with PE-labeled secondary antibodies by 2-photon (top panel) and wide-field (bottom panel) fluorescence. B. β -catenin expression was assessed using the NanoPro100 system, based on separating proteins by isoelectric focusing using capillary electrophoresis, followed by in-capillary immuno-chemoluminescence detection, showing pI migration. Note the expression of β -catenin was reduced in the prominin-1-knockdown clones by approximately 1.7- and 2.8-fold. Scale bars, 25 μ m. C. Parental FEMX-I and FEMX-I/prominin-1 $^{-/5}$ were transduced with a lentiviral reporter construct, expressing firefly luciferase downstream of the TCF/LEF promoter (*TCF/LEF-luc*). Wnt activation was measured by chemiluminescence detection of luciferase activity before and 5 hours after exposure to different concentrations of Wnt3a, a physiological activator of the canonical Wnt pathway. D. The addition of Wnt-3a to FEMX-I/prominin-1 $^{-/5}$ cells for 5 hours resulted in a translocation of β -catenin into the nucleus.