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FHOD1 formin is upregulated in melanomas and modifies proliferation and tumor growth

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ABSTRACT

The functional properties of actin-regulating formin proteins are diverse and in many cases cell-type specific. FHOD1, a formin expressed predominantly in cells of mesenchymal lineage, bundles actin filaments and participates in maintenance of cell shape, migration and cellular protrusions. FHOD1 participates in cancer-associated epithelial to mesenchymal transition (EMT) in oral squamous cell carcinoma and breast cancer. The role of FHOD1 in melanomas has not been characterized. Here, we show that FHOD1 expression is typically strong in cutaneous melanomas and cultured melanoma cells while the expression is low or absent in benign nevi. By using shRNA to knockdown FHOD1 in melanoma cells, we discovered that FHOD1 depleted cells are larger, rounder and have smaller focal adhesions and inferior migratory capacity as compared to control cells. Importantly, we found FHOD1 depleted cells to have reduced colony-forming capacity and attenuated tumor growth *in vivo*, a finding best explained by the reduced proliferation rate caused by cell cycle arrest. Unexpectedly, FHOD1 depletion did not prevent invasive growth at the tumor margins. These results suggest that FHOD1 participates in key cellular processes that are dysregulated in malignancy, but may not be essential for melanoma cell invasion.

1. Introduction

Cells undergo substantial functional alterations in order to obtain proliferative, invasive and metastatic properties which are associated with malignancy. Alterations that involve cell shape, signalling, adhesion, and migration depend on efficient remodelling of the actin cytoskeleton. Actin filaments not only determine shape but also form a scaffold for motor proteins that provide the contractile force for invasive movement and serve as regulators of intracellular signalling cascades. Dynamic actin filament remodelling is controlled by a multitude of actin-associated molecules [1]. Several classes of actin nucleating proteins assist the assembly of actin monomers into filamentous structures. Among them, the formin family is the largest.

Formins are large multi-domain proteins, which are defined by the presence of a highly conserved formin homology 2 (FH2) domain. This domain mediates nucleation or elongation of actin filaments. Other domains, present in most formins, regulate activation/inactivation and subcellular filament targeting. The biochemical properties of the individual formins are distinct and sometimes even counteract each other: once activated, they may nucleate, elongate, bundle, cap and even severe actin filaments. On the cellular level, individual formins have been shown to participate in the formation of diverse cellular protrusions, adhesions, and influence serum response factor-mediated transcription [2–4].

FHOD1 (formin homology 2 domain containing protein 1) is an efficient capping and bundling protein of actin filaments. In contrast to the majority of formin proteins, it does not elongate actin filaments *in vivo* [5]. The participation of FHOD1 in cytoskeletal remodelling and cell migration has been established in fibroblasts, melanoma cells and breast cancer cells [6,7]. FHOD1 is one of formins regulating transcription from the serum response element (SRE) [7,8]. This occurs through changes in the pool of monomeric actin. With increased actin filament polymerization, the pool of monomomeric actin is reduced, which allows the release of megakaryoblastic leukemia 1 (MKL-1, a.k.a. MAL or myocardin related transcription factor A, MRTF-A) which translocates/accumulates in nuclei [9]. MKL-1 binds to the SRE,

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inducing transcription from many cytoskeletal and cell cycle progression associated genes [10,11]. FHOD1 has further been found to be essential for nuclear movement and centrosome orientation in fibroblasts polarizing for migration [12,13].

There are very few studies on formin expression and significance in clinical cancer. FMNL1 (Formin-like protein 1) is over-expressed in non-Hodgkin lymphoma [14], and high FMNL2 (Formin-like protein 2) expression is associated with metastasis in colorectal cancer [15]. Recently, we found that high expression of FMNL2 in localized cutaneous melanoma is associated with increased risk of recurrence during follow-up. In cell lines, FMNL2 expression was dependent on ERK MAPK signalling, a pathway commonly over activated melanoma [16]. In another study, we found that FHOD1 expression increased with cancer-associated EMT, a process that mediates invasion [17]. Intriguingly, FHOD1 has been shown to directly interact with components of the ERK MAPK pathway [18], which suggests that FHOD1 could, if expressed in melanoma, be a link between altered signalling and cellular behaviour. With this background, we wanted to investigate FHOD1 expression in melanocytic neoplasms and explore its roles in melanoma cell lines.

In this study, we found that FHOD1 is expressed in most melanomas. In order to study the functional role of FHOD1 in melanoma, we stably silenced FHOD1 expression in the WM164 melanoma cell line using shRNA constructs. Studying the effects of FHOD1 depletion, we found changes in cell morphology, migration, colony formation, cell-spreading assays and cell cycle analysis *in vitro*. Importantly, we found that tumor growth is reduced after FHOD1 depletion in a mouse melanoma xenograft model. Taken together, all these results suggest a role for FHOD1 in melanoma tumorigenesis.

2. Material and methods

2.1. Tumor histology and immunohistochemistry

The mouse melanoma xenografts were paraffine embedded, sectioned and hematoxylin and eosin stained. For immunohistochemistry, slides were sectioned at 3 μm, stained for Ki-67 and cleaved caspase 3 using a Ventana Discovery XT autostainer device (Ventana Medical Systems, Tucson, AZ). After a standard pretreatment with Cell Conditioning Solution CC1 (Ventana), the slides were incubated with a Ki-67 polyclonal antibody (1:1000, Chemicon International, Billerica, MA) for 36 mins or Cleaved Caspase 3((D3E9) 1:100, Cell Signalling Technology, Danvers, MA) for 40 min, respectively. OminMap anti-Rb HRP using ChromoMap DAB, both from Ventana, were used for detection. FHOD1 immunohistochemistry was performed using a Labvision Autostainer device with a BrightVision Poly-HRP-anti-Rabbit IgG detection kit according to the manufacturer's protocol (Immunologic, Duiven, the Netherlands). The slides were pressure cooked for 2 min for antigen retrieval. The primary antibody against FHOD1 (1:150, Sigma-Aldrich, St Louis, MI) was incubated for 1 h, the secondary antibody for 30 min. This antibody has been extensively characterized previously [17]. Diaminobenzene was used as chromogene.

The tumor histology was evaluated by microscopy of HE-stainings. For analysis of cell diameter and of Ki-67 staining, four photomicrographs were taken at 400x magnification from different tumors in each treatment group. The diameter of at least 50 cells was measured from each micrograph.

For the study of FHOD1 expression in nevi and melanoma, 8 benign nevi and 10 melanoma +3 paired metastatic melanoma FFPE samples were sectioned and immunohistochemically stained as described above for mouse xenografts. The samples were collected from the tissue archive of the Department of Pathology at Turku University Hospital with the approval of the Joint Committee on Ethics of the University of Turku and Turku University Hospital.

2.2. Cell lines and culture conditions

Metastatic melanoma cell lines WM164 and WM239 (BRAF V600E and BRAF V600D, respectively) were cultured in RPMI 1640 medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Biowest), 5 mM Ultraglutamine I (Lonza, Basel, Switzerland), 1 x non-essential amino acids (NEAA) (Lonza), and 100 U/ml penicillin-streptomycin (Gibco). Cutaneous Bowes melanoma cell line (BRAF WT) and the metastatic SK-Mel-28 cells (BRAF V600E mutated) were cultured in EMEM (Gibco) and MEM (Gibco), respectively, supplemented with 10% FBS, 5 mM Ultraglutamine I, and 100 U/ml penicillin-streptomycin. Malme-3 M (BRAF V600E mutated) metastatic cells were maintained in DMEM (Lonza) containing all the mentioned supplements.

2.3. Western blotting

Cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with inhibitors (1xComplete Mini -protease inhibitor cocktail [Roche] and 1XPhosStop a phosphatase inhibitor cocktail [Roche]). Insoluble cell debris was removed by centrifugation (21,000g for 30 min at 4 °C). The protein concentration of lysates was determined by the Bradford method (BioRad) before adding 5x Laemmli to samples. Equal amounts of total protein were separated by SDS-PAGE and transferred to nitrocellulose membrane (Whatman PROTRAN, PerkinElmer). The membranes were blocked with 5% dry milk in TBST (TBS, 0.1% Tween) and immunoblotted with different antibodies diluted in 5% bovine serum albumin (BSA) in TBST.

The rabbit anti-human FHOD1 antibody (Sigma-Aldrich) was used at 1:1000. The mouse anti-α-tubulin antibody (Sigma-Aldrich) was used 1:10,000 as a control for protein loading. The secondary antibodies were HRP-conjugated swine anti-rabbit and HRP-conjugated rabbit anti-mouse immunoglobulins (1:2500, Dako) diluted in block solution. Membranes were washed three times with TBST between the different steps.

2.4. Generation of stable FHOD1 knockdown cell lines

For establishment of stable FHOD1 knockdown cell lines, 300×10^3 WM164 cells were transfected in a 24 well plate using 2 μl of Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) and 50 ng of pRFP-C-RS HuSH shRNA RFP vectors (Origene, Rockville, MD, USA) containing either the scrambled shRNA cassette TR30015 (scramble control), human FHOD1 targeting shRNAs FI351919 (CGCTGTGCCAAGGTGGACTTTGAACAGCT; shRNA 1) or FI351920 (CCGAGACAGAGAAGTTCTCAGGTGTGGCT; shRNA 2). The transfection medium was changed after 24 h and the cells were kept in selection medium containing 1.0 μg/ml puromycin (Sigma) for 2 weeks. Next, the cells were transferred to 10 cm dish and colonies expressing RFP (red fluorescence protein) were isolated, amplified and analysed for FHOD1 expression by western blotting.

The FHOD1 shRNA clones were stable in culture and maintained a low FHOD1 level for at least 15 passages in medium which contained 0.5 μg/ml puromycin. Similar passages of the 3 clones were used in all the following studies.

2.5. Cell Immunofluorescence stainings and microscopy

Cells were plated on gelatin (Sigma-Aldrich) precoated coverslips (13 mm) and grown in complete medium for 24 h. Next, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature. The coverslips were washed with PBS and blocked with 3% BSA, 5% dry milk, 0.5% triton X-100 in PBS for 45 min. The rabbit anti-FHOD1 (1:200, Millipore) antibody was incubated 1 h at room temperature. Next, the coverslips were incubated with Alexa Fluor 568 goat anti-