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PTEN-Dependent Stabilization of MTSS1 Inhibits Metastatic Phenotype in Pancreatic Ductal Adenocarcinoma

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) presents at metastatic stage in over 50% of patients. With a survival rate of just 2.7% for patients presenting with distant disease, it is imperative to uncover novel mechanisms capable of suppressing metastasis in PDAC. Previously, we reported that the loss of metastasis suppressor protein 1 (MTSS1) in PDAC cells results in significant increase in cellular migration and invasion. Conversely, we also found that overexpressing MTSS1 in metastatic PDAC cell lines corresponds with not only decreased metastatic phenotype, but also greater overall survival. While it is known that MTSS1 is downregulated in late-stage PDAC, the mechanism behind that loss has not yet been elucidated. Here, we build off our previous findings to present a novel regulatory mechanism for the stabilization of MTSS1 via the tumor suppressor protein phosphatase and tensin homolog (PTEN). We show that PTEN loss in PDAC cells results in a decrease in MTSS1 expression and increased metastatic potential. Additionally, we demonstrate that PTEN forms a complex with MTSS1 in order to stabilize and protect it from proteasomal degradation. Finally, we show that the inflammatory tumor microenvironment, which makes up over 90% of PDAC tumor bulk, is capable of downregulating PTEN expression through secretion of miRNA-23b, potentially uncovering a novel extrinsic mechanism of MTSS1 regulation. Collectively, these data offer new insight into the role and regulation of MTSS1in suppressing tumor cell invasion and migration and help shed light as to what molecular mechanisms could be leading to early cell dissemination in PDAC.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer-related deaths in the United States with a 5-year survival rate of 9% [1,2]. With research currently struggling to identify new biomarkers and methods for earlier detection, most patients present with advanced disease. Whereas 39% of PDAC patients present with regional or distant disease, an astounding 53% of patients afflicted with PDAC are diagnosed at metastatic stage [3,4]. This accounts for 92% of all patients diagnosed with PDAC being diagnosed after the primary tumor has already spread. This phenomenon is due in part to the asymptomatic nature of the disease and a lack of reliable early detection methods [5,6].

Abbreviations: 3T3, NIH3T3 mouse fibroblast cells; CAF, cancer-associated fibroblast; CAFM, CAF-conditioned media; CHX, cyclohexamide; DMSO, dimethyl sulfoxide; EpM, epithelial-conditioned media; IgG, immunoglobulin G; IP, immunoprecipitate; MOE, MTSS1 overexpression; MTSS1, metastasis suppressor protein 1; PDAC, pancreatic ductal adenocarcinoma; PTEN, phosphatase and tensin homolog; TrCP, transducin repeat containing protein; VEC, vector.

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Because of this late disease presentation, less than 20% of patients will be viable candidates for surgical resection, the treatment that still has the best outcome for pancreatic cancer patients [7,8]. These challenges combine to make PDAC an incredibly difficult disease to diagnose at localized stage, thereby highlighting the importance of designing and implementing innovative approaches to harness the disease before it is able to spread. Specifically, elucidation of the molecular mechanisms responsible for the early tumor cell dissemination observed in PDAC that can lead to metastasis is an area that still requires much focus [9].

One of the molecular mechanisms that has recently been shown to be critical for preventing tumor cell dissemination is the regulation of metastasis suppressor protein 1 (MTSS1) [10]. Also known as Missing in Metastasis (MIM), MTSS1 is believed to prevent metastasis by increasing Rac-GTP levels in order to inhibit cell-cell junction disassembly, thereby elevating actin assembly at the cell-cell contacts and preventing cellular dissemination [11]. While very little is known about the mechanism of action and molecular relationships of MTSS1, it has been found to play a role in combatting cellular migration and invasion in PDAC [10]. However, while the functional role of MTSS1 in PDAC has recently been uncovered, the regulation of MTSS1 in PDAC is still an enigma.

Multiple microRNAs have been shown to be capable of regulating MTSS1 expression [12–15], whereas ubiquitination-driven destruction and DNA methylation have also been found to play a role in MTSS1 downregulation as well [16–18]. Additionally, MTSS1 has also been shown to have affinity for protein phosphatases, such as PTPδ and PTPN11, in a range of different cancer subtypes [19,20]. The center of the MTSS1 protein is rich in proline, serine, and threonine residues [21]. These residues, all containing hydroxyl groups, interact and bind with these phosphatases, which then may provide a functional link between the MTSS1 protein and signal transduction pathways that prevent an invasive state from arising. This interaction is intriguing because one of the most commonly implicated tumor suppressors involved in cancer progression is a phosphatase known as phosphatase and tensin homolog (PTEN).

Here, we present evidence of a novel regulatory mechanism for MTSS1 stabilization via the canonical tumor suppressor protein PTEN. PTEN expression has been found to be crucial in minimizing the lethality of PDAC through regulation of the PI3K/AKT pathway [22]; however, its role in PDAC metastasis has only been briefly studied [23–25]. In this manuscript, we demonstrate that loss of PTEN in PDAC cells not only leads to a more invasive and migratory phenotype, but also results in decreased in MTSS1 expression. We find that PTEN positively regulates the stability and protein level of MTSS1. Furthermore, we demonstrate that PTEN is a protein phosphatase for MTSS1 and inhibits the interaction between MTSS1 and its E3 ligase $SCF^{\beta\text{-}TRCP}$ to block the proteasome degradation of MTSS1. Finally, we introduce a new, cell-extrinsic mechanism for the downregulation of PTEN via miRNA-23b from the PDAC tumor microenvironment, suggesting a novel role for both the tumor microenvironment and PTEN in PDAC metastatic regulation.

Materials and Methods

Cell Culture and Transfection

Cancer-associated fibroblast (CAF) cell line UH1301-63 was obtained from Melissa L. Fishel, Ph.D. (Department of Pediatrics, IU School of Medicine), and prepared for study as previously described [26]. Fibroblast nomenclature was reduced for purposes of simplicity with "S63" referring to UH1301-63 CAFs. Other commercial cell lines were purchased from ATCC. S63, NIH3T3, PANC-1, MIA PaCa-2, and 293T cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin with 5% CO_2 at 37°C. Cells were trypsinized and passaged at approximately 90% confluence. Cell transfection was performed using Lipofectamine 2000 (Invitrogen), unless otherwise noted. Cells were harvested at 24 hours post-transfection for protein analysis, unless otherwise noted.

Cell Authentication

Fibroblast and epithelial cell lines were authenticated by Genetica DNA Laboratories. All cell lines were a 97% to 100% match to the correct cell line in both the ATCC and DSMZ database. The CAFs were previously determined not to have a match in either database, were non-tumorigenic in mice, and did not have a mutation for K-RAS [26].

Virus Production and Transduction

For overexpression of PTEN or MTSS1, 293T cells at 60% confluence were transfected with retroviral plasmid (5 μg) containing vector control, PTEN or MTSS1, VSVG (3 μg), and GAG (2 μg). For knocking down PTEN or MTSS1, 293T cells at 60% confluence were transfected with lentiviral plasmid (5 μg) containing Scramble, shPTEN or shMTSS1, VSVG (3 μg), and GAG (2 μg). Virus was collected 48 hours after transfection. Virus containing media was then added to 70% confluent S63 or NIH3T3 cells supplemented with polybrene (8 μg/ml). Stable cell pools were selected with puromycin for 5 days. Verification was accomplished via Western blot analysis. For shMTSS1, the following target sequence was used: GCTGATG CATTGTGCATAT. The sequence was proven effective for both human and mouse MTSS1 gene as previously described [27]. For shPTEN, the following target sequence was used: CCACAAAT GAAGGGATATAAA. The sequence was proven effective for both human and mouse PTEN gene as previously described [28–31].

For shPTEN + MOE rescue experiments, 1.5×10^5 PANC-1 Scramble and shPTEN cells were plated in a 6-well setting. Twenty-four hours post-plating, shPTEN cells were transfected with MTSS1 Myc WT plasmid (2 μg) using the Lipofectamine 3000 Transfection Reagent (Thermo Fisher, L3000015) according to manufacturer's protocol.

Western Blotting Analysis

Protein lysates were prepared from S63, NIH3T3, PANC-1, MIA PaCa-2, 293T, and stable cell pools in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, and1 mM EDTA supplemented with a protease inhibitor cocktail tablet (Roche 11836153001). Cell lysate (40 μg) was resolved by gel electrophoresis (BioRad Mini Protean TGX Gel 400091313) and transferred to a nitrocellulose membrane. The membranes were blocked in 5% dry milk in TBS-T. Primary antibodies diluted in 5% dry milk and TBS-T include MTSS1 (Cell Signaling 4386S), PTEN (Cell Signaling 9559S), phospho-Akt (Ser473) (Cell Signaling 4060S), and β-actin (Cell Signaling 4970L) at 1:1000. Secondary antibodies used include anti-rabbit (Cell Signaling 7074S). Protein levels were detected by enhanced chemiluminescence (Thermo Scientific 32106) and quantitated with Image Lab software (Bio-Rad). Densitometry analysis was completed using ImageJ64 software.

For immunoprecipitation experiments, 500 μg of cell lysate was incubated with IgG and Protein A–Agarose (SIGMA P2545) for 3 hours at 4°C. Beads were washed three times with lysis buffer and centrifuged at 5000*g* for 5 minutes between each wash. Protein was eluted from beads with 50 μl of Laemmli sample buffer (Bio-Rad).