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METADHERIN FUNCTIONS AS A LAMININ RECEPTOR THAT IS ESSENTIAL FOR METASTASIS AND IS ASSOCIATED WITH POOR SURVIVAL IN OSTEOSARCOMA

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A

DISSERTATION

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In Partial Fulfillment
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DOCTOR of PHILOSOPHY

By

Limin Zhu

Houston, Texas
April, 2014

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DEDICATION

To my devoted parents, thank you for your unconditional love. You are always there for me to support me in every possible way.

To my husband, thank you for loving me. You have brought countless happiness to my life.

To Dr. Wanda Schroeder, thank you for your guidance and support throughout my undergraduate education in Wesleyan College. You inspired my passion for biomedical sciences.

To all the osteosarcoma patients, you are the ultimate motivation of my research. Keep fighting and never lose hope!

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METADHERIN FUNCTIONS AS A LAMININ RECEPTOR THAT IS ESSENTIAL FOR METASTASIS AND IS ASSOCIATED WITH POOR SURVIVAL IN OSTEOSARCOMA

Limin Zhu, B.A.

Supervisory Professor: Dennis P. M. Hughes, M.D. Ph.D.

Osteosarcoma is a highly invasive bone malignancy in which metastasis accounts for the vast majority of death and morbidity in patients. Understanding the mechanisms controlling metastasis is essential for improving patient survival in this disease. In order to improve the clinical outcomes for patients with poor prognosis, it is urgent to find new therapeutic targets to block metastasis in this disease. Recent studies have shown that Metadherin (MTDH) plays an essential role in mediating tumorigenesis and metastasis in a variety of human cancers. Our study assessed the role of MTDH in osteosarcoma metastasis and elucidated the mechanisms underlying its metastasis-promoting activity.

To evaluate the expression of MTDH in primary and metastatic lesions of osteosarcoma, two tissue microarrays containing patient-derived primary and metastatic tumor specimens were examined by immunohistochemical staining with anti-MTDH antibody. We also examined MTDH in a cDNA array expression database made from pretreatment diagnostic biopsies of high-grade osteosarcoma patients to further assess the correlation between MTDH expression and clinical outcome. We used western blot, qPCR, and flow cytometry to measure the expression of MTDH in a panel of osteosarcoma cell lines. In parallel experiments we used MTDH-specific shRNA to reduce endogenous MTDH expression, and blocked cell surface MTDH by anti-MTDH antibodies. The impact of MTDH inhibition was assessed in vitro using transwell migration assays and matrigel

invasion assays. In addition, we developed an orthotopic xenograft mouse model to study the relationship between MTDH expression and osteosarcoma pulmonary metastasis. To investigate the role of MTDH in cell-extracellular matrix (ECM) interaction and to identify the extracellular binding partner for cell surface MTDH, a series of adhesion assays were performed, followed by bidirectional co-immunoprecipitation.

We have demonstrated that MTDH is up-regulated in human osteosarcoma cell lines and patient-derived specimens compared with normal human osteoblasts. Overexpression of MTDH is more profound in metastatic lesions compared to primary tumors and is correlated with poor clinical outcomes in osteosarcoma patients. MTDH knockdown and blockade of cell surface MTDH significantly reduced migration and invasion in osteosarcoma cells. In the *in vivo* experiments, down-regulation of MTDH in osteosarcoma cells delayed primary tumor growth and prohibited pulmonary metastasis. Both in vitro and in vivo studies confirmed the critical role of MTDH in the invasive and metastatic capacity of osteosarcoma cells. More importantly, we have identified the significance of cell surface localization of MTDH in mediating osteosarcoma motility and invasiveness. We showed that MTDH exists as a type II membrane protein in osteosarcoma cells and its expression on cell surface is facilitating cell invasion by means of modulating cell adhesion to the ECM through interaction with Laminin. In total, these observations establish MTDH as a promising target for therapeutic interventions in metastatic osteosarcoma. The novel connection between MTDH and extracellular laminin also establishes a new paradigm for the function of MTDH in mediating tumor cell metastasis.

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Chapter 1. Introduction

Biology of Osteosarcoma

Osteosarcoma is the most common type of primary bone cancer in pediatric patients (1). It derives from primitive mesenchymal stem cells or osteoblasts and is characterized by the production of neoplastic osteoid or immature bone by tumor cells (2). Osteosarcoma occurs primarily in growing adolescents and young adults, with a peak incidence in the second decade of life. Consistent with its high incidence during puberty when bones grow rapidly, osteosarcoma usually arises from the metaphyseal regions of the long bones such as the distal femur and proximal tibia (3). As an exceedingly aggressive tumor, osteosarcoma has a high tendency to spread to distant organs in the body. The lung is the most frequent site for metastasis, followed by the bones (4). About 15-20% of patients present with overt lung metastases and nearly all of the remainder have micrometastatic disease at diagnosis (5, 6). The standard therapy consists of surgical removal of any resectable primary tumor and metastases, combined with 6-9 months of neoadjuvant and adjuvant chemotherapy. Current chemotherapy regimens include four agents: doxorubicin (adriamycin), cisplatin, high-dose methotrexate with leukovorin rescue, and ifosfamide (2). The addition of liposomal muramyl tripeptide phosphatidylethanolamine (mifamurtide) to therapeutic regimens demonstrated clinical benefit and has been approved by the European Union to treat high-grade, resectable, non-metastatic osteosarcoma (7).

Although the modern multimodal therapy yields a survival of approximately 70% for patients without overt disease at diagnosis, the clinical outcome for metastatic osteosarcoma remains poor: fewer than 30% of patients presenting with metastases survive 5 years after initial diagnosis (8). The prognosis for patients with refractory or recurrent disease is even worse. Since current treatment options for osteosarcoma patients have very limited efficacy

against recurrent metastatic disease, most studies in this field have been focused on identifying key regulatory pathways and molecular events that mediate critical steps of metastasis.

Essential pathways in osteosarcoma metastasis

Migration and Invasion

The process of tumor metastasis involves a complex cascade of events. The first step is to interact with and migrate through the extracellular matrix (ECM) and basement membrane that comprise barriers against invading cells. The pivotal role of matrix metalloproteinases (MMPs) including MMP-2 and MMP-9, as well as other proteases such as m-calpain, has been repetitively implicated in osteosarcoma metastasis (9-11). Previous studies also suggested that the Wnt/β-catenin pathway and Src signaling promote metastasis in osteosarcoma through modulation of tumor cell migratory ability (12). The Notch pathway, including its major components Notch receptor 1, 2, and the downstream target gene Hes1, has been recently identified to be important regulator of osteosarcoma invasion (13, 14).

Survival in the bloodstream

After tumor cell invade into the circulatory system, it is essential for disseminated tumor cells to acquire anoikis resistance in order to survive in the absence of intercellular adhesions and cell-ECM interactions. Several molecular mechanisms contribute to the evasion of anoikis in osteosarcoma, including overexpression of specific integrins, Focal adhesion kinase (FAK)-independent activation of PI3K/Akt signaling, activation of the Src, NFκB and Wnt/β-catenin pathways, and upregulation of anti-apoptotic genes of the BcL family (15).

Extravasation and adherence

The next step of metastasis is to exit the circulatory vessels, invade into the distant organ, and adapt to the local microenvironment. The process of extravasation and metastatic colonization are primarily facilitated by proteinases and chemokines (16, 17). Commonly expressed chemokines in osteosarcoma include CXCR-3 and CXCR-4. Binding of these chemokines to their ligands, CXCL-9, -10, -11, and -12, which are abundantly expressed in the lung, not only mediate adherence of circulating tumor cells, but trigger other essential survival pathways as well (16, 18-24). Ezrin, a membrane-cytoskeleton linker protein overexpressed in variety of cancers, is associated with a higher risk of metastasis and poor survival in both animal models and pediatric patients with osteosarcoma (25-27). Previous studies suggested that ezrin facilitates adherence of metastatic osteosarcoma cells to lung tissues by mediating membrane organization and interactions between tumors cells and the lung microenvironment (28). Ezrin also promotes survival and proliferation of the newly arrived osteosarcoma cells in the lung through β4-integrin mediated activation of the PI3K/Akt and MAPK survival pathways (17, 22, 25, 26, 29).

Dormancy

It is frequently observed that osteosarcoma patients who present without radiographic evidence of metastasis at diagnosis develop metastatic relapse within 2-3 years after initial resection of the primary tumor (30, 31). This phenomenon is likely explained by the prolonged survival of single cells or micrometastases in the lung environment, which is defined as dormancy. When being triggered to start proliferating again, theses small lesions can quickly develop into gross metastases. Despite the clinical significance of tumor dormancy, the biological processes regulating dormancy and tumor outgrowth from dormant

state in osteosarcoma are still poorly understood. Previous studies indicated that tumor dormancy is controlled by overexpression of the anti-apoptotic protein Bcl-xL, α5β1-integrin mediated activation of NF-κB signaling, and the ratio between ERK and p38-MAPK proteins (17, 22, 32, 33). Since vascularization is an essential prerequisite for tumor expansion, metastatic cells usually increase the expression of anti-angiogenic proteins to suppress tumor outgrowth and maintain dormancy (34, 35). Recent research suggests that ECM, which is the source of various growth and survival signals, serves as an important mediator of tumor dormancy for metastatic cells (32, 35). Micrometastases usually remain in the dormant state in the absence of connection to the ECM in the lung environment, while proper anchorage to the ECM could activate dormant cells and stimulate them to proliferate via β1-integrin signaling. Additionally, dormancy has been shown to be related to a subpopulation of cancer stem-like cells (CSCs) which have the ability to self-renew and populate a growing tumor (36-40). With increased capacity for DNA repair and upregulation of anti-apoptotic proteins, CSCs could survive under metabolic and environmental stresses for a long time (41-46).

Neovascularization

Tumor growth and progression are often dependent on a sufficiency of nutrients and growth factors supplied by the blood vessels. Therefore, neovascularization and aberrant proliferation are prerequisites for the sustained expansion of metastatic lesions in the lung. Simultaneous upregulation of a number of pro-angiogenic factors like VEGF, FGF, HGF, PDGF, TGF-α and Ang-1 and downregulation of anti-angiogenic factors such as thrombospondin-1, PEDF, and troponin I induce rapid neo-angiogenesis (47-51). Elevated expression of growth factor receptors and proteolytic enzymes, including EGFR, IGF-1R,

PDGFR, and MMPs, also contributes to survival and proliferation in metastatic osteosarcoma cells in the lung (52).

Evasion of immune system

Another important feature of the metastatic osteosarcoma cells that survive either in the circulatory system or at the sites of metastasis is the ability to evade the host immune surveillance. Downregulation of the cell surface receptor HLA class 1 prevents the tumor cells from being recognized by the host cytotoxic T-lymphocytes (52, 53). Metastatic osteosarcoma cells can also modulate the activity of the host immune system by inducing the expression of a group of immunosuppressive molecules such as IL-10 (22, 54). The Fas/FasL signaling pathway has also been implicated in immune evasion of osteosarcoma cells. Impaired downstream signaling of Fas/FasL pathway or downregulation of Fas expression on the surface of osteosarcoma cells inhibits Fas-induced cell death and prevents the activation of cytotoxic natural killer cells, resulting in an increase in metastatic potential. In support of this notion, patient-derived tumor specimens from osteosarcoma pulmonary metastases have been shown to be Fas-negative, and low Fas expression is associated with worse prognosis (55-59).

In spite of tremendous past and ongoing efforts, our knowledge of the mechanisms underlying cell invasion and metastasis in osteosarcoma is still limited. Previous attempts to target pathways mentioned above have not demonstrated much clinical efficacy and there has been little improvement in the treatment of metastatic osteosarcoma over the last decade. To improve the clinical outcomes for patients with poor prognosis, it is urgent to find new therapeutic targets to block metastasis in this disease. We became interested in metadherin

(MTDH) because it has emerged in recent years as an oncogene that is critically involved in tumor pathogenesis and progression.

Molecular Cloning of MTDH

Metadherin (MTDH), also known as astrocyte elevated gene-1 (AEG1) and Lyric, was first identified in 2002 as a novel late response gene induced by HIV infection or treatment with TNF-α or viral glycoprotein gp120 in primary human fetal astrocytes (60-62). In 2004, Brown and Ruoslahti named this protein MTDH as they identified its metastasis-promoting function in mouse breast cancer cell through an in vivo phage screening (63). The mouse-rat ortholog of MTDH was cloned in the same year as a tight junction protein that co-localizes with ZO-1 in rat prostate epithelial cells and encodes the lysine-rich CEACAM-1 co-isolated protein (Lyric) (64). Human MTDH gene contains 12 exons and 11 introns and is located at chromosome 8q22, a region that is frequently amplified in many cancers (60). The full-length MTDH cDNA contains 3611 bps (excluding the poly-A tail) and the human mRNA encodes a single-pass transmembrane protein consisting of 582 amino acid residues with a predicted molecular mass of 64 kDa (60, 65). Alternative splicing and posttranslational modifications of MTDH may lead to different molecular weights detected by western blotting, ranging from 20kDa to 80kDa (64, 66).

MTDH structure and localization

According to BLAST analysis of MTDH, the structure of this gene has no similarity to any currently known genes (64). Initial protein motif analyses failed to identify any known functional domains or motifs in MTDH except a putative transmembrane domain (TMD) and three nuclear localization signals (NLS) (60, 63, 64, 66). The highly hydrophobic TMD, which is further confirmed by multiple independent protein structure prediction approaches,

is localized very close to the N-terminal, between 51-72 amino acid residues (67). The function of the three NLSs, at the locations between 79-91, 432-451, and 561-580 amino acid residues, was further characterized by Thirkettle and colleagues (68). They found that the extended NLS-1 (78-130 a.a.) and NLS-3 (546-582 a.a.) are responsible for nucleolar and nuclear localization of MTDH respectively, and the NLS-2 (415-486 a.a.) ubiquitination directs the cytoplasmic distribution of MTDH.

The subcellular localization and transmembrane orientation of this molecule have been a subject of great debate. Initial characterization of MTDH suggested that it localizes predominantly to the cytoplasm, endoplasmic reticulum, perinuclear region, nucleus, and inside the nucleolus in various cell types (60, 66, 69). However, immunofluorescence detection and FACS analysis of non-permeabilized mouse mammary tumor cells revealed cell surface localization of MTDH (63). The orientation of cell surface MTDH remains controversial: while most investigators believe that MTDH has a Type Ib topology based on functional analyses and the C-terminal localization of the experimentally verified NLSs, Brown and Ruoslahti have demonstrated a type II orientation for MTDH in breast cancer cells and proposed a putative lung-homing domain (LHD) which they suggested mediates lung metastasis through interactions with lung endothelial cells (63, 66, 69).

Figure 1

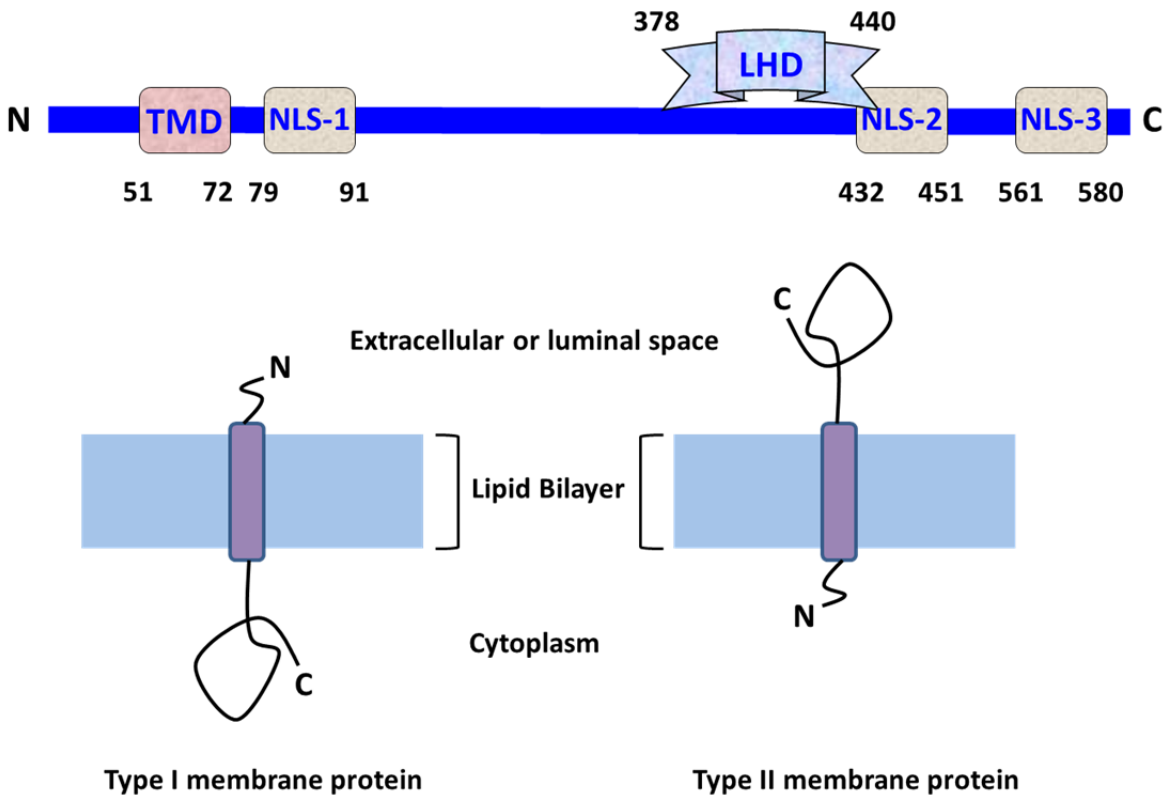


Figure 1. Schematic diagram of the full-length MTDH protein structure and topology on membrane. The numbers denote amino acid positions. TMD: transmembrane domain; NLS: nuclear localization signal; and LHD: lung homing domain.

MTDH in cancer

MTDH is ubiquitously expressed in all human normal tissues at varying levels (60). However, numerous studies over the past decade have demonstrated that MTDH expression is significantly upregulated in many types of solid tumors including glioma, oligodendroglioma, neuroblastoma, melanoma, brain, head and neck, breast, prostate, esophageal, lung, gastric, renal, liver, and colorectal cancer when compared with normal tissues (60, 63, 65, 70-84). Consistent with the high incidence of MTDH overexpression in

cancer, recent clinical studies have provided convincing evidence of an association between high MTDH expression level and advanced tumor stage as well as poor patient prognosis (68, 70, 78, 80-83). These observations strongly suggest that MTDH could be employed as a powerful diagnostic or prognostic marker in a variety of cancer types.

In parallel with evaluation of MTDH as a biomarker for cancer, mounting evidence from functional studies indicates a pivotal role of MTDH in diverse aspects of tumor malignancy including aberrant proliferation, angiogenesis, invasion, metastasis, and chemoresistance (69, 77, 85). In addition to its unique ability to modulate gene expression changes that are common in cancer, MTDH promotes tumor progression through activation and integration of multiple pro-tumorigenic signal transduction pathways (65, 67). Ras signaling increases MTDH expression (86), which subsequently promotes cancer cell growth, survival, and invasion through the activation of PI3K/Akt (69), NF-κB (87, 88), and Wnt/β-catenin pathways (77). MTDH-induced activation of PI3K/Akt signaling protects cells from apoptosis and facilitates angiogenesis (85, 89). By activating NFκB and its downstream targets, MTDH increases proliferation, angiogenesis, inflammation, and invasion in tumor cells (16, 88). MTDH could also alter expression of a group of genes involved in invasion, chemoresistance, senescence, and angiogenesis through Wnt/β-catenin pathway (78)

Figure 2

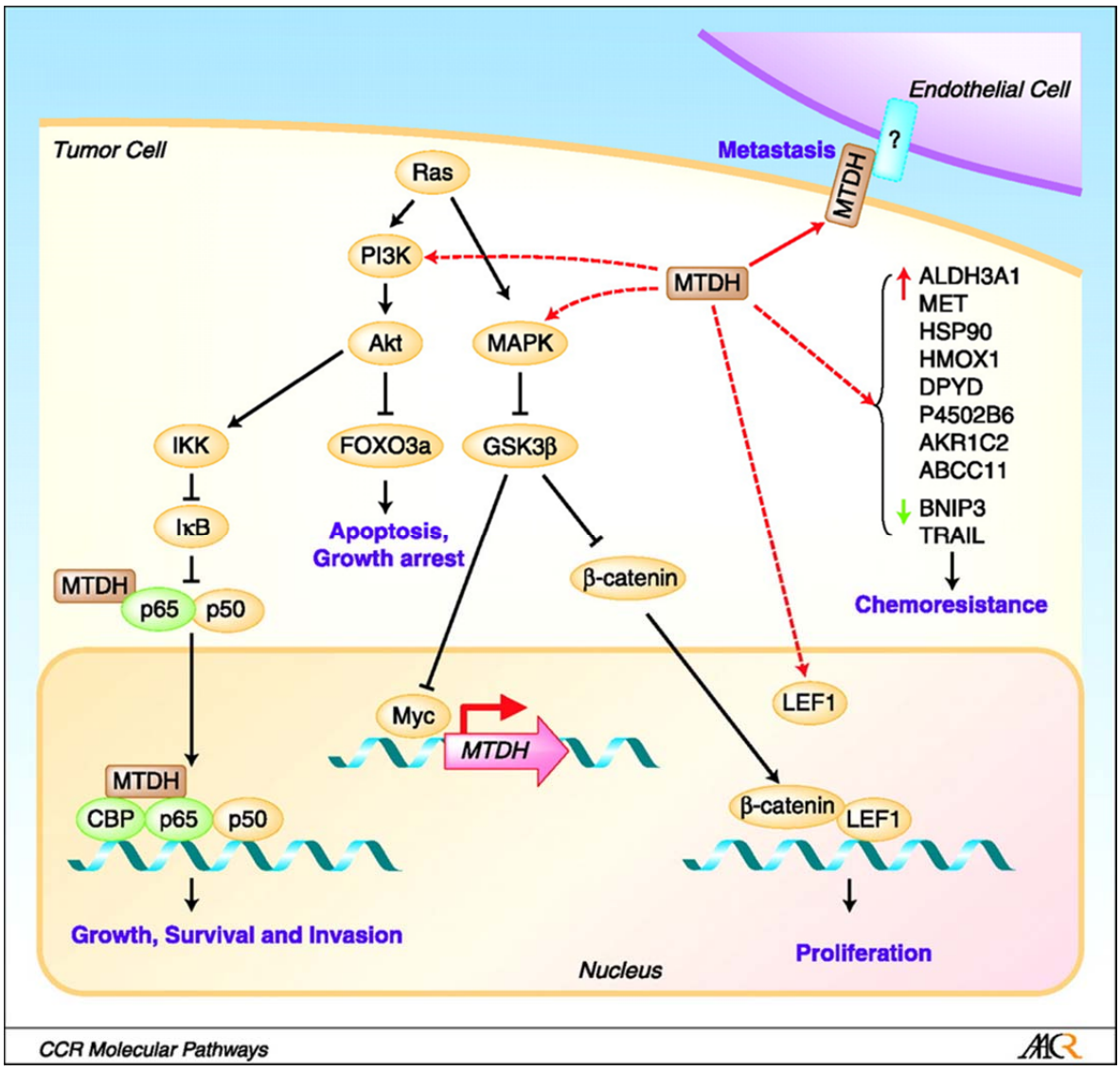


Figure 2. MTDH promotes tumor progression through the integration of multiple signaling pathways. Oncogenic Ha-Ras increases MTDH expression through the activation of the PI3K/Akt pathway, which phosphorylates and inactivates GSK3β, and subsequently enhances the stabilization and binding of c-Myc to the MTDH promoter. Activation of NFκB signaling is partially mediated by the direct interaction of MTDH with p65 and CBP. MTDH activates the Wnt/β-catenin pathway through increasing the activity of MAPK kinases ERK and p38, which phosphorylates GSK3β and stabilized β-catenin. Furthermore, MTDH increases the expression of LEF-1, a transcriptional cofactor for β-catenin. The important role of MTDH in broad spectrum chemoresistance is mediated by many downstream genes that promote the resistance to multiple chemotherapeutic agents. *Reprinted from Hu G et al. Clin Cancer Res 2009;15:(5615-5620) with permission from American Association for Cancer Research.*

MTDH and metastasis

As an important mediator of tumor development and progression, the invasion-promoting function of MTDH has been confirmed by multiple studies in various types of aggressive cancer including glioma, neuroblastoma, prostate cancer, breast cancer, hepatocellular carcinoma (HCC), head and neck squamous cell carcinoma, and colorectal carcinoma (63, 70, 73, 74, 78, 84, 90). Upregulation of MTDH leads to elevated expression of adhesion molecules, which facilitate both the extravasation and intravasation processes of metastasis. A recent study comparing metastatic potential of HCC cell lines with different MTDH expression displayed that cells with higher endogenous MTDH levels have better adhesive ability to microvascular endothelial cells than those with relatively lower MTDH expression (91). Additionally, MTDH-induced activation and secretion of MMPs, especially MMP1, MMP2 and MMP9, have been shown to promote metastasis through remodeling and degradation of ECM (70, 71, 84). In cancers of epithelial origin such as breast cancer and HCC, MTDH could enhance metastatic spread of tumor cells by inducing the epithelial-mesenchymal transition (EMT) process (92, 93).

Another important finding concerning the function of MTDH in metastasis is the identification of the putative extracellular lung-homing domain through a phage display experiment conducted in the breast cancer mouse model. It has been shown that overexpression of MTDH in human embryonic kidney cells enhances their localization to lung vasculatures. Neutralizing antibodies targeting this specific domain of MTDH displayed comparable inhibition of experimental pulmonary metastasis as siRNA-mediated MTDH knockdown in breast cancer cells. Based on these observations, it was postulated that MTDH favorably promotes pulmonary metastasis as it detects and binds to an unknown marker that

is primarily expressed on the surface of lung endothelial cells. However, the hypothesis that MTDH has a binding preference for the lung vascular bed remains questionable. Recent studies have demonstrated that MTDH not only promotes lung metastasis, but also enhances metastasis to other organs, such as the bone and brain in breast cancer, and liver in colorectal cancer (65, 94). To date, the molecular mechanisms of MTDH binding to endothelium remain elusive.

The role of MTDH in osteosarcoma

The first study to characterize the expression status of MTDH in osteosarcoma was conducted by Wang and colleagues in 2011(95). They performed immunohistochemical staining to examine the MTDH expression level in 82 paraffin-embedded surgical specimens, including 62 osteosarcoma samples and 20 normal bone tissues from patients who had received neoadjuvant chemotherapy. They found that MTDH was overexpressed in the majority of osteosarcoma samples assessed while MTDH expression was barely detectable in normal bone tissues. Spearman correlation analysis based on IHC staining score of theses specimens indicated that MTDH overexpression is strongly associated with clinical stages, classification, metastasis, and differentiation. Moreover, the average survival time in the low MTDH expression group was remarkably longer than that in high MTDH expression group. The only other report about the function of MTDH in osteosarcoma was focused on how MTDH mediates chemoresistance (96). It has been shown that MTDH confers chemoresistance in osteosarcoma cells by regulating ET-1/ETAR signaling pathway in a PI3K-dependent manner. Due to the lack of knowledge about the role of MTDH in osteosarcoma metastasis, we decided to focus our efforts on exploring this research topic.

Goal of dissertation

Osteosarcoma is the most common bone malignancy, causing significant morbidity and mortality in teenagers and young adults. With current treatment regimens combining chemotherapy and surgery, osteosarcoma patients with localized disease have a survival rate that approaches 70%. However, the clinical outcome for metastatic osteosarcoma remains poor. For patients who suffer from this fatal disease, a better understanding of how their cancer metastasizes will lead to novel therapeutic approaches that will significantly prolong their survival and improve the quality of their lives. To address this challenge, it is critical to identify and characterize promising proteins and key pathways responsible for osteosarcoma progression and metastasis and to develop their specific inhibitors.

Over the past 10 years, numerous studies have consistently demonstrated that high expression of MTDH is associated with increased tumor aggressiveness, metastasis, and decreased patient survival in a variety of human solid tumors. These observations establish MTDH as a promising target for therapeutic interventions. It will be important to know which tumors might benefit from such treatments. To date, however, very few studies have examined the functional role of MTDH in osteosarcoma and our knowledge about how MTDH expression affects osteosarcoma metastasis is very limited. The first goal of our research was to confirm the expression status of MTDH in osteosarcoma and to assess its impact on patient survival. Next, we wanted determine the sub-cellular localization and transmembrane orientation of MTDH in osteosarcoma. We planned to use both in vitro and in vivo approaches to explore the biologic function(s) of MTDH in osteosarcoma progression and metastasis. Further, we wished to elucidate the mechanism(s) underlying the metastasis-promoting activity of MTDH, which are very likely to have impacts beyond osteosarcoma.

Prior work on MTDH mechanisms have largely focused on its direct involvement in classical oncogenic pathways so that protein-level interactions have been scarcely studied. Currently, the means by which MTDH enhances cancer metastasis remain unclear. As mentioned earlier, Brown and Ruoslahti proposed that the cell surface MTDH contains an extracellular lung-homing domain which facilitates lung metastasis by binding to an unknown ligand on pulmonary endothelial cells (63). However, our preliminary data in a colon cancer cell line indicated that MTDH might regulate metastasis by modulating cell attachment to ECM components, which is completely independent of endothelial cell binding. One major goal of this research is to identify the potential MTDH-interacting protein(s) in ECM. Characterization of the association between MTDH and ECM component will help us gain better understanding of the metastasis-promoting function of MTDH and at the same time provide novel target(s) for developing anti-metastasis therapy. More importantly, the new findings will be applicable to a broad panel of invasive solid tumors with MTDH overexpression and therefore would benefit more patients.

Combining our preliminary results and current knowledge about the functions of MTDH in cancer, we hypothesize that MTDH overexpression promotes osteosarcoma cell migration and invasion in response to recognition of non-cellular protein component of ECM (Figure 3).

Figure 3

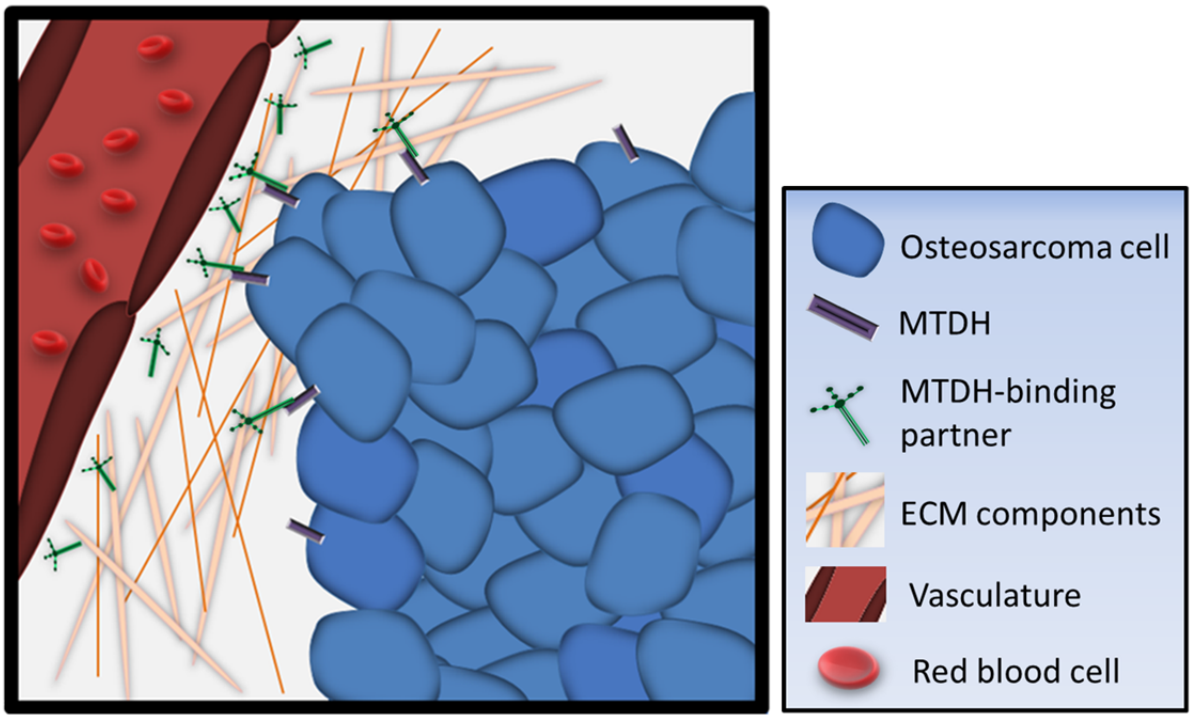


Figure 3. Hypothesis: MTDH interacts with specific extracellular ECM component to facilitate tumor cell invasion and migration in osteosarcoma.

Chapter 2. Materials and Methods

Cell lines and reagents

Human osteosarcoma cell lines HOS, CCHD, SAOS2, LM7, SJSA, MG63 were maintained in High Glucose Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with L-glutamine and 10% Fetal Bovine Serum (FBS) (Hyclone) in a humidified atmosphere at 37°C with 5% CO_2. The established osteosarcoma cell lines HOS, SAOS2, SJSA, and MG63 are available from ATCC. LM7, a subline of SAOS2 with high metastatic potential, was developed by repeated passaging of SAOS2 cells through pulmonary metastases in nude mice (97). CCHD is a stable OS cell line derived from pre-treatment biopsy of a proximal femur lesion in an 18-year-old male patient who presented with pulmonary metastases at M. D. Anderson Children's Cancer Hospital. The human fetal osteoblastic cell line hFOB (ATCC) was cultured at 34°C with 5% CO_2 in a 1:1 mixture of phenol-free Dulbecco's modified Eagle's medium/Ham's F-12 medium with 2.5 mM L-glutamine (DMEM-F12) (Invitrogen), supplemented with 10% FBS and 0.3 mg/ml G418.

Patient Samples

Formalin-fixed, paraffin-embedded tissue blocks (FFPE) of osteosarcoma cases were obtained from the files of the Department of Pathology, University of Michigan Medical Center, Ann Arbor, MI. The University of Michigan Institutional Review Board provided a waiver of informed consent to obtain these samples. After pathological review, a tissue microarray was constructed from the most representative area using the methodology of Nocito et al. (98). Each case was represented by two 1 mm or three 0.6 mm diameter cores, obtained from the most representative, non-necrotic area of the tumor. The osteosarcoma TMA containing primary tumor specimens from 49 patients and metastatic tumor specimens from 24 patients was used to evaluate the expression level of MTDH in osteosarcoma with

immunohistochemistry (IHC). All specimens were reviewed by Dr. Wei-Lien Wang, an experienced sarcoma pathologist at M.D. Anderson Cancer Center. Scoring of the tumor samples was based on IHC staining intensity (as shown in Fig.7), and the intensity of the signal was classified as 0 (no expression), 1 (weak expression), 2 (moderate expression) or 3 (strong expression).

Western blotting

Whole cell lysates from a panel of human osteosarcoma cell lines and normal human osteoblast cells were prepared as follows: Cells were detached from the culture plates with the aid of cell scrapers and washed with cold PBS. After 10-minute centrifugation at 13,000 rpm, cell pellets were resuspended and incubated in cold lysis buffer (25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40 and 5% glycerol) with protease inhibitor cocktail tablets (Roche Diagnostics) and phosphatase inhibitor cocktail 2 (Sigma) at 4°C for 20 minutes. Lysates were collected after being centrifuged at 13,000 rpm for 10 minutes. The protein concentrations were determined using the bicinchoninic acid (BCA) assay (Thermo Scientific). Whole-cell lysates were separated by SDS-PAGE on a 10% polyacrylamide gel and transferred to nitrocellulose membrane following standard procedures. To detect MTDH protein level, membrane was probed with rabbit-anti-MTDH antibody (1:1,000; Sigma-Aldrich), followed by horseradish peroxidase-conjugated anti-rabbit IgG (1:2,000; GE Healthcare). Beta-actin (Sigma-Aldrich) was probed as a loading control. Chemiluminescent signal was detected using Pierce ECL western blotting substrate (Thermo Scientific).

Real-time quantitative PCR

Total RNA was extracted from osteosarcoma cells with the RNeasy Mini Kit (Qiagen). cDNA was made using the Omniscript Reverse Transcriptase Kit (Qiagen) with oligo-dTs

(Invitrogen) according to the manufacturer's instruction. Real-time PCR analysis was performed by the iCycler iQ quantitative PCR system (Bio-Rad) using SYBR Green PCR Master Mix (Bio-Rad) following the protocol of the manufacturer. The primers used were as follows: MTDH (Forward) 5'-CACTGTCAATGGAGGAGGCT-3'; MTDH (Reverse) 5'-TGAACGGTCACTCCAACTCC-3'; GAPDH (Forward) 5'-GCATCCTGGGCTACACTGAG-3'; GAPDH (Reverse) 5'-CCACCACCCTGTTGCTGTAG-3'. Expression data were normalized to GAPDH expression in each sample and were analyzed according to the $2^{-\Delta\Delta Ct}$ method. The fold changes in gene expression were calculated relative to the mRNA level of the control cell line hFOB.

Flow cytometry

To measure the cell surface expression of MDTH, a panel of osteosarcoma cells were detached from culture plates by enzyme-free cell dissociation buffer (Invitrogen) and washed with cold PBS/1% bovine serum albumin (Fisher Scientific). The cells were incubated with normal rabbit IgG (1:100; Millipore) or rabbit anti-MTDH antibody (1:100; Sigma-Aldrich) on ice for 1 hour. The cells were then washed with PBS/1% BSA three times and blocked with PBS/1% BSA containing 10% goat serum. Cells were then incubated with PE-conjugated anti-rabbit IgG (1:50; Molecular Probes) on ice for 30 minutes and washed as above. Expression was then assessed using a FACSCaliber flow cytometer (BD Biosciences) and analyzed with Flowjo software (Tree Star Inc).

Retroviral transduction

To silence human MTDH gene, four pGFP-V-RS shRNA retroviral constructs (Origene) were used to generate 29-mer short hairpin RNAs (shRNA) targeting MTDH. The shRNA sequences for MTDH are:

SH-3: 5'-GAAATCAAAGTCAGATGCTAAAGCAGTGC-3'

SH-4: 5'-TGCTGAGCCAGTTTCTCAGTCTACCACTT-3'

SH-5: 5'-CATCACAGTTACCACCGAGCAACTTACAA-3'

SH-6: 5'-GGTGATTCTCATCTAAATGTTCAAGTTAG-3'

To generate retrovirus, Phoenix-Ampho cells (ATCC) were seeded in 6-well plates at a density of 1 million cells per well without antibiotics. After 12 hours, FuGENE 6 transfection reagent (Roche) was diluted in serum-free DMEM to generate 100 µl solution (90 µL DMEM and 10 µL FuGENE 6). After a 5-minute incubation, 3 µg of pGFP-V-RS vectors containing MTDH shRNA or scrambled control sequence were added to the diluted FuGENE 6 transfection reagents and co-incubated at room temperature for 30 minutes. Next, the transfection components were added to the cells and the culture plates were returned to the incubator. The transfection complex was removed and replaced with fresh DMEM at 12 hours after initial transfection. After 24 hours, medium containing virus was collected and centrifuged at 1500 rpm for 5 minutes. 2 ml of viral supernatant and 8 µg/ml Polybrene (Sigma) were combined and added to HOS and CCHD cells. The plates were centrifuged at 2500 rpm for 50 minutes and then incubated at 34°C overnight. Viral medium was replaced by fresh medium and cells were returned to standard culture conditions. After being selected for puromycin resistance for 7 days, the stably transduced cells were analyzed by western blot for MTDH protein expression, and knockdown was confirmed by densitometric analysis

(Image J Processing Software, NIH). Cells transduced with SH-4 and SH-6 displayed the highest MTDH knock-down efficiency, and therefore were used in parallel with cells transduced with scrambled control plasmid in subsequent assays as indicated in individual experiments.

Cell proliferation assay

Cells were seeded in triplicate into 6-well plates at the density of $2x10^4$ and $5x10^4$ cells/well respectively for HOS and CCHD cell lines. The number of viable cells was counted after 2, 4, and 6 days of culture by using an automated Vi-Cell Analyzer (Beckman Coulter). Cells were prepared as follows: medium was removed from the culture plates and the cells were rinsed with PBS to remove the dead cells and debris. Cells were co-incubated with 0.5 ml of 0.01 M HEPES/0.015 M $MgCl_2$ buffer at room temperature for 5 minutes. Next, 50 µl of 0.132 M Bretol (Ethylhexadecyldimethylammonium bromide) with 0.525 M glacial acetic acid was added to the cells and the culture plates were agitated for 10 minutes at room temperature. Cells were then fixed by the fixative solution (0.9% NaCl and 0.5% formalin). 2 ml of the solution containing cell nuclei was transferred into an autosampler cup for further processing by the automated Vi-Cell Analyzer.

Matrigel invasion assay

The invasive ability of osteosarcoma cells was evaluated by using 24-well BD BioCoat Matrigel invasion chambers with 8-µm pore size (BD Biosciences). Briefly, $2x10^4$ or $5x10^4$ cells suspended in 300 µl of serum-free DMEM medium were seeded in triplicate into the upper chamber of the system. The lower chamber contained 750 µl DMEM medium with 10% FBS as a chemoattractant. In the antibody-blocking experiments, rabbit-anti- MTDH antibody (Simga-Aldrich) or normal rabbit IgG (Millipore) were used at a final concentration

of 7.5 μg/ml to treat HOS and CCHD cells during the incubation period. After 48h incubation at 37°C, the migrated cells were fixed, stained with Hema-3 (Fisher Diagnostics), and counted under a microscope at 100-fold magnification.

Cell migration assay

In vitro cell migration was performed in the 24-well Corning Transwell polycarbonate membrane cell culture inserts with 8-μm pore size (Corning). The $3x10^4$ cells suspended in 100 μl serum-free DMEM were seeded into the upper part of each chamber, whereas the lower compartments were filled with 600 μl of DMEM with 10% FBS. Normal rabbit IgG (Millipore) and rabbit-anti-MTDH antibody (Sigma-Aldrich) at a final concentration of 7.5 μg/ml were used in the antibody-blocking experiments. After incubation for 24 h at 37°C, the migrated cells were fixed, stained with Hema-3, and counted under a microscope at 100-fold magnification.

Animal Model

To be sure that in vivo experiments were not compromised by growth of contaminating non-transduced cells, clones were made from the MTDH shRNA #4 and scrambled control CCHD cells described above by limiting dilution and visual confirmation of clonality. Several were tested, and a clone whose growth characteristics matched the bulk population most closely was chosen for in vivo experiments. These cells were then retrovirally labeled with firefly luciferase as described (99). Luciferase-labeled CCHD cells (1×10^6 cells suspended in 15μl of sterile PBS) were injected into the right tibia of 6-week-old NOD/SCID/IL2Rγ-deficient mice (Jackson Laboratories). Primary tumor growth and development of metastasis were monitored weekly through noninvasive bioluminescent imaging using an IVIS 100 animal imaging system (Xenogen). Mice were sacrificed at 6

weeks after inoculation, the lungs inflated with 10% formaldehyde via transtracheal injection, and the primary tumors and lungs fixed in formalin and embedded in paraffin. Five micron sections were mounted on glass slides for analysis, and H&E staining was performed by our core laboratory. Metastatic nodules in lungs were quantified by direct microscopic visualization and counting of a single lung section and confirmed by an experienced sarcoma pathologist. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Immunohistochemistry

Immunohistochemistry was performed using formalin-fixed, paraffin-embedded tissue sections. Heat Induced Epitope Retrieval (HIER) was performed on paraffin-embedded tissues with 0.1M Sodium Citrate buffer, pH 6.0. After being blocked with 4% fish gelatin in PBS for 20min, sections were incubated with rabbit-anti-MTDH antibody (1:300; Sigma-Aldrich) in a humidified chamber at 4°C overnight. Slides were incubated with rabbit-on-rodent HRP-polymer (Biocare) or Mach 4 HRP polymer (Biocare) for 30min at room temperature and staining was developed using DAB followed by Hematoxylin counterstaining. Images were analyzed using a Leica light microscope.

Immunofluorescence

Osteosarcoma cells were seeded in 4-well Lab-Tek chamber slides (Nunc) at a density of $1x10^5$ cells per well in standard tissue culture conditions. Medium was removed after 12 hours and cells were fixed in -20°C acetone and 1:1 acetone and chloroform mixture for 5 minutes each. Cells were blocked with 4% fish gelatin in PBS and incubated with rabbit-anti-MTDH antibody (1:300; Sigma-Aldrich) in a humidified chamber at 4°C overnight. Slides were incubated with Alexa 488-labeled anti-rabbit secondary antibody (1:1,000,

Molecular Probes) for 1h and nuclei were counterstained with Hoechst (1:10,000) for 5 minutes. Slides were mounted with anti-fade mounting media (glycerol/PBS containing N-propyl gallate) and a coverslip. Images were taken on a Nikon fluorescence microscope.

Cell adhesion assay

Cells were harvested, suspended in serum-free DMEM medium, and seeded in triplicates ($2x10^4$ cells/well) onto 96-well plates pre-coated with Matrigel, Fibronectin, Laminin, or Collagen-type IV (BD Biosciences). In the antibody-blocking experiments, rabbit-anti- MTDH antibody (Sigma-Aldrich) or normal rabbit IgG (Millipore) were used at a final concentration of 7.5 μg/ml to treat HOS and CCHD cells during the period of co-incubation. After 1h incubation at 37°C in a humidified incubator containing 5% CO_2, unattached cells were removed by rinsing three times with PBS, Meanwhile, the input control groups were left unwashed and the plates were centrifuged at 1300 rpm for 3min to force the cells onto the bottom of the well. The remaining attached cells were fixed and stained with Hema-3 (Fisher Diagnostics). The number of cells from three different fields of each well was counted at 10X magnification. The percentage of adhesion was calculated by dividing the average cell number of PBS-washed group with that of the respective input control group.

Co-Immunoprecipitation

To investigate whether MTDH interacts with extracellular Laminin through formation of a protein complex, HOS cells were cultured under standard conditions until they reached 80-90% confluence and then treated with matrigel (1:100 dilutions) for 1h at 37°C. The cells were then thoroughly washed with PBS to remove any unbound ECM components. Whole-cell lysates were made and subjected to co-immunoprecipitation with anti-MTDH antibody (Sigma-Aldrich), anti-Laminin antibody (Abcam), or normal IgG (Millipore) following

manufacturer's instructions. The precipitated proteins were then transferred to nitrocellulose membrane and probed with anti-MTDH, anti-Laminin, and anti-Fibronectin (Abcam) antibodies.

Statistical analyses

Triplicate samples were analyzed in each assay, and all experiments were conducted at least three times. Data are represented as mean ± standard error of mean (SEM). Statistical analysis was performed using two-tailed Student's t test (GraphPad Software Inc). Log-rank test was used for assessment of survival curves. A p-value of <0.05 was considered statistically significant in all cases.

Chapter 3: Elevated MTDH expression is associated with metastasis and poor outcome in several cancers including osteosarcoma

Rationale

As mentioned earlier, MTDH has been found to be widely upregulated in many types of aggressive solid tumors and is often associated with poor survival (68, 70, 78, 80-83). Accumulating evidence from functional studies of MTDH confirmed the importance of this protein in many aspects of tumor growth and progression including metastasis (69, 77, 85). To determine whether MTDH is involved in promoting metastasis in aggressive human tumors, we started with analyzing the expression patterns of MTDH in microarray databases of various cancer patients. We hypothesized that MTDH expression in distant metastases is greater than that in primary tumors, and MTDH overexpression correlates with higher risk for metastasis and worse survival in patients.

To obtain the relevant clinical information that contains survival and metastasis data, we used publicly available cancer microarray databases (Oncomine, and R2: microarray analysis and visualization platform) and osteosarcoma tissue microarray (a kind gift from Dr. Dafydd G. Thomas, Department of pathology, the University of Michigan Hospitals and Clinics, Ann Arbor, Michigan) to assess the correlation between MTDH expression and clinical outcome in patients with breast cancer, prostate cancer, melanoma, and osteosarcoma. Since MTDH has been rarely studied in osteosarcoma and the role of MTDH in osteosarcoma metastasis remains unclear, we have concentrated our efforts in studying this disease.

Results

High MTDH expression is associated with metastasis and poor survival in multiple aggressive human cancers

Analysis of MTDH mRNA levels in primary and metastatic lesions in two melanoma datasets and one prostate cancer dataset revealed that metastatic lesions exhibited significantly higher level of MTDH than primary tumors (1.8 fold in Haqq Melanoma, p=0.005; 1.7 fold in Xu Melanoma, p=2.19E-6; and 3.9 fold in Chandran Prostate, p=1.75E-10) (Figure 4). To determine whether MTDH expression is predictive of distant metastatic relapse, we compared the MTDH mRNA level in primary tumor samples obtained from patients who developed metastasis during the 3-year follow-up period versus those who did not. Significantly higher level of MTDH (4.1 fold difference, p=1.25E-5) was observed in patients who progressed to metastatic relapse, indicating that MTDH overexpression is closely related to high metastatic potential of melanoma (Figure 5). In three independent datasets, we further explored the relationship between MTDH expression and clinical outcome in patients with melanoma and breast cancer (Figure 6). Analysis of stage III melanoma patient data indicated that MTDH overexpression is correlated with shorter survival from metastasis. Significantly shortened overall survival and metastasis-free survival were observed in the high MTDH-expressing group as compared to the low MTDH-expressing group in breast cancer patients. These results imply that MTDH is overexpressed in metastasis and may serve as an important prognostic marker of metastasis risk and survival for cancer patients.

Figure 4

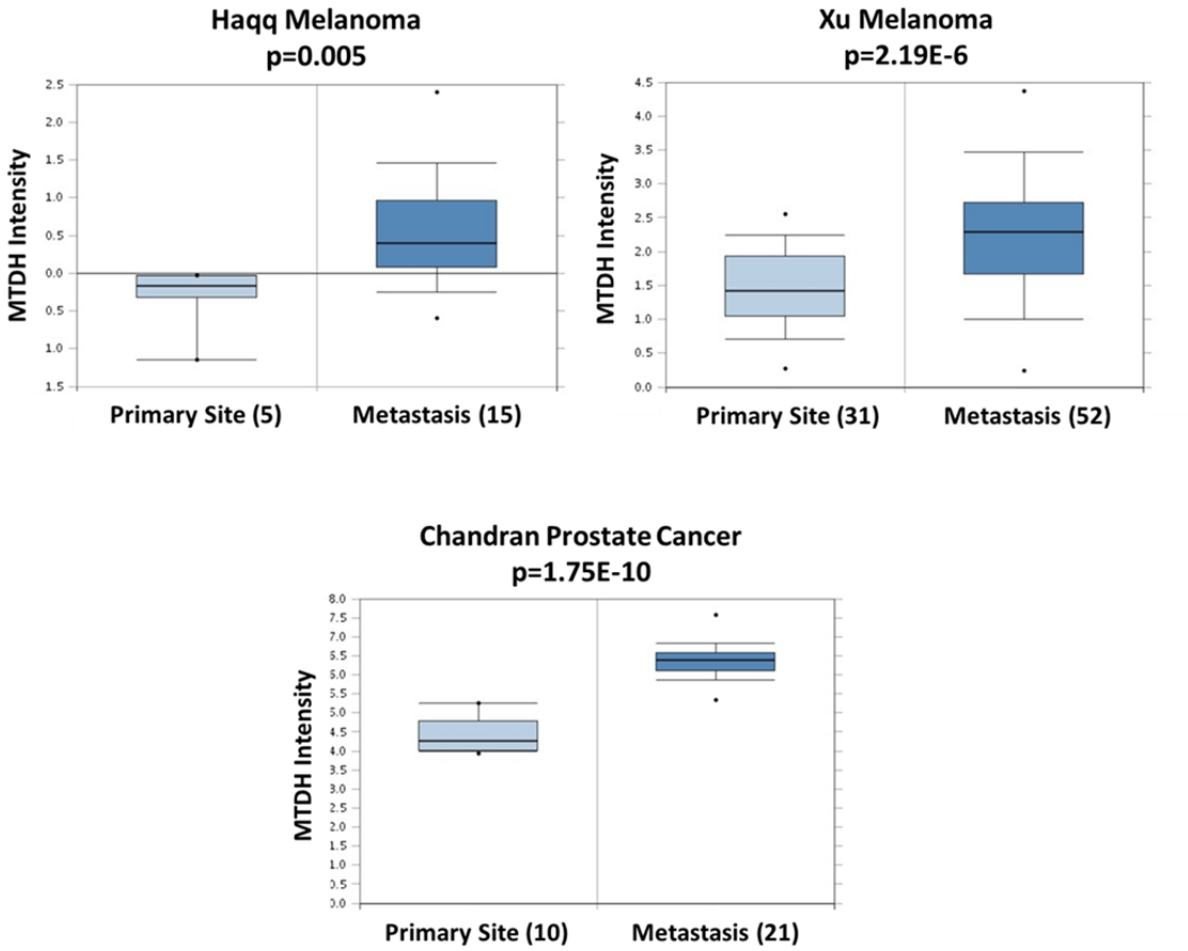


Figure 4. MTDH expression is elevated in metastatic lesions. Comparison of MTDH mRNA levels between unpaired primary tumors and metastatic lesions was performed in two independent melanoma datasets (5 primary samples and 15 metastatic samples in Haqq Melanoma dataset; 31 primary samples and 52 metastatic samples in Xu Melanoma dataset) and one prostate cancer dataset (10 primary samples and 21 metastatic samples). All datasets were accessed through the Oncomine Research Edition (https://www.oncomine.org).

Figure 5

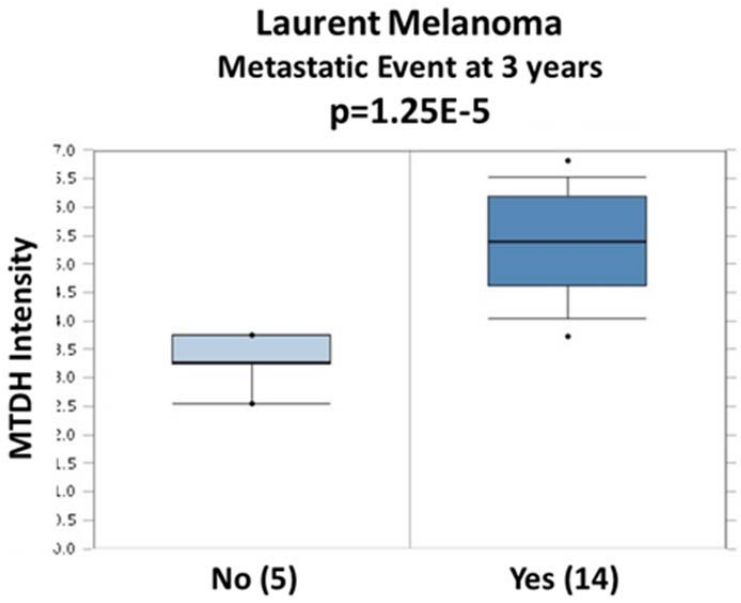


Figure 5. MTDH overexpression is associated with high incidence of metastatic relapse. Comparison of MTDH mRNA expression levels between the patients with and without newly developed metastatic lesions during follow-up period was performed in Laurent melanoma dataset (19 samples total). Melanoma dataset was accessed through the Oncomine Research Edition (https://www.oncomine.org).

Figure 6

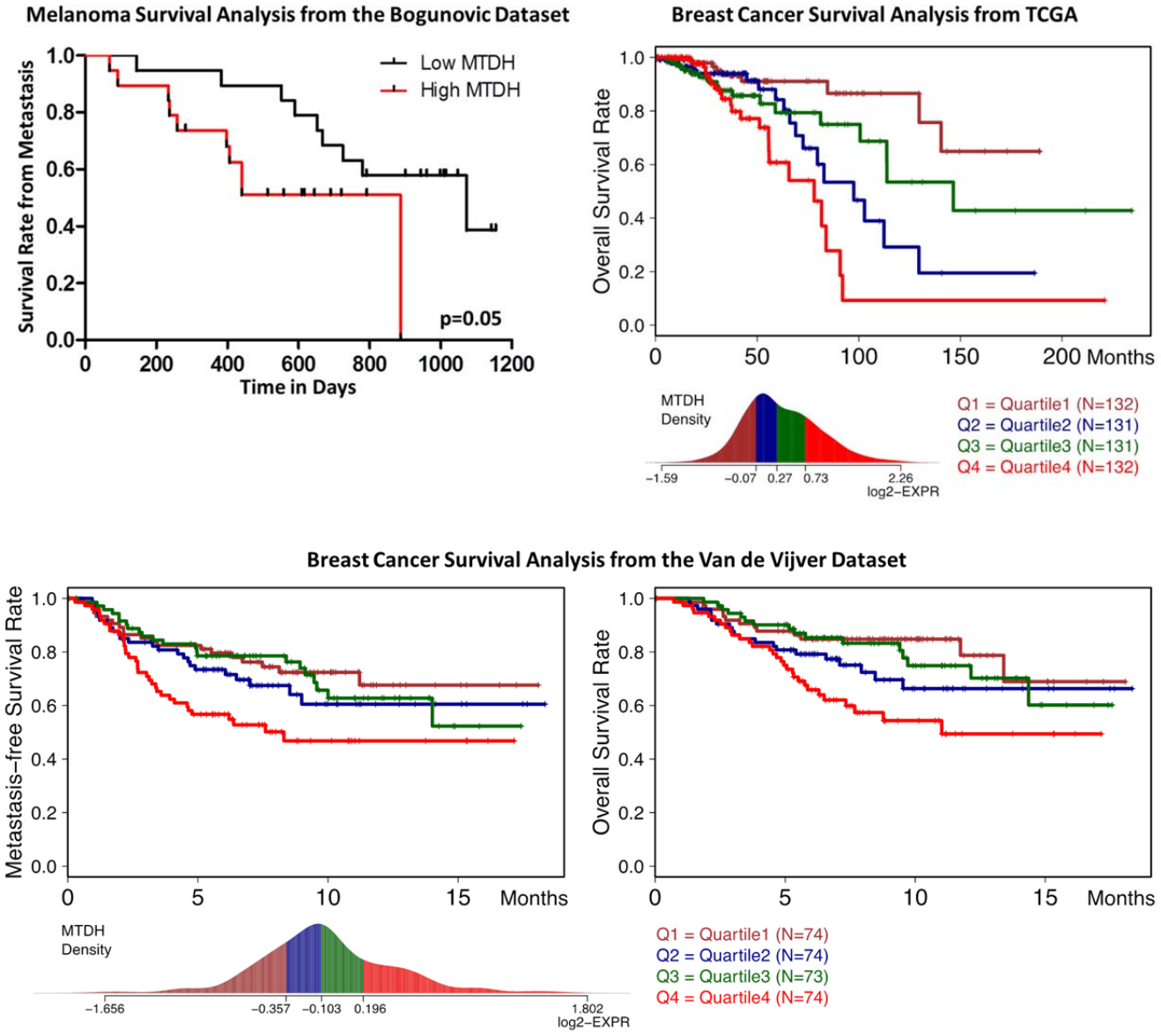


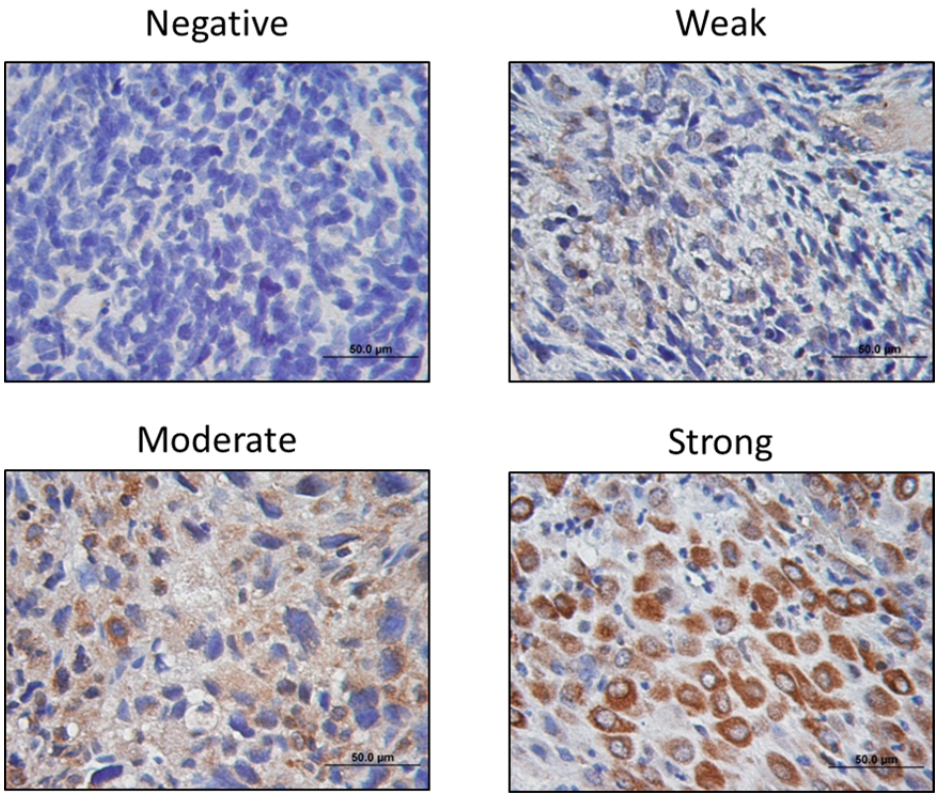
Figure 6. High MTDH expression is associated with poor clinical outcome in melanoma and breast cancer patients. Kaplan-Meier survival analysis was used to assess the prognostic significance of MTDH in overall survival and metastasis-free survival of patients with melanoma and breast cancer. Melanoma and breast cancer datasets were accessed through the Oncomine Research Edition (https://www.oncomine.org). The log-rank test was used to calculate the p value.

MTDH expression in patient-derived osteosarcoma samples

To evaluate the expression of MTDH in primary and metastatic lesions of osteosarcoma, a tissue microarray containing 49 primary tumor samples and 24 metastatic tumor samples were examined by IHC staining with anti-MTDH antibody. MTDH expression level was reported to be very low in normal bone tissues (95), but we observed substantial MTDH staining in patient-derived osteosarcoma samples (Figure 7). Among the 49 primary tumor samples, 18/49 (37%) had no detectable MTDH, 17/49 (35%) displayed weakly positive staining, 10/49 (20%) were moderately positive, and the remaining 4 samples (8%) had strong MTDH expression. In comparison, the staining intensity for MTDH was more abundant in metastatic tumor samples: only 3/24 (12%) scored negative, 9/24 (37%) were weakly positive, 10/24 (42%) were moderately positive and 2/24 (9%) displayed strong staining of MTDH (Table 1).

IHC analysis of the MTDH expression levels in 9 pairs of matched primary osteosarcoma and metastases revealed that metastatic lesions had a significant 2.5 fold increase in average MTDH staining intensity in comparison with their primary tumor counterparts ($p<0.05$, Figure 8). Of the 9 patients, 7 patients had increased MTDH expression from primary to metastatic tumor, 1 patient had equal MTDH expression in primary and metastatic tumor, 1 patient displayed decreased MTDH in metastatic lesions. Taken together, these observations suggested that MTDH is overexpressed in the majority of osteosarcoma samples. In addition, increased MTDH expression could be detected in metastatic lesions compared with primary tumors.

Figure 7


Negative
Weak
Moderate
Strong
50.0 µm

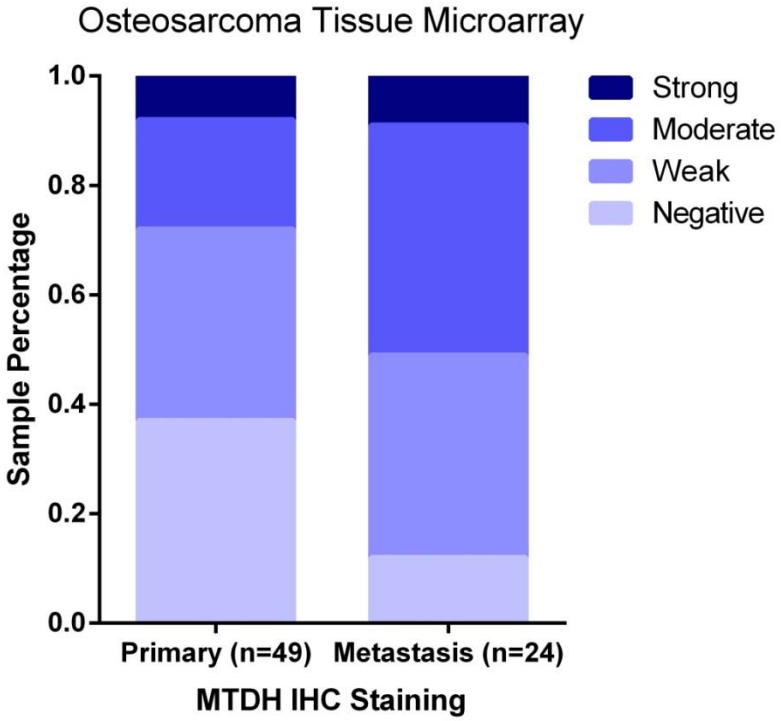

Osteosarcoma Tissue Microarray
Sample Percentage
1.0
0.8
0.6
0.4
0.2
0.0
Strong
Moderate
Weak
Negative
Primary (n=49)
Metastasis (n=24)
MTDH IHC Staining

Figure 7. MTDH is highly expressed in osteosarcoma patient samples. Representative MTDH immunohistochemical staining images of human osteosarcoma tissue microarray were taken using a Leica light microscope. Stacked histogram displayed here represents the proportion of samples in which MTDH expression was negative, weak, moderate or strong, per the scale shown in the top panel. All specimens were reviewed by an experienced sarcoma pathologist at M.D. Anderson Cancer Center.

Table 1

MTDH Expression		
Expression Score	**n (%)**	
	Primary Tumor	Metastatic Lesion
0	18 (37%)	3 (12%)
1	17 (35%)	9 (37%)
2	10 (20%)	10 (42%)
3	4 (8%)	2 (9%)
Total	**49 (100%)**	**24 (100%)**

Table 1. Summary of osteosarcoma tissue microarray immunostaining data. Expression score: 0-negative expression, 1-weakly positive expression, 2-moderately positive expression, 3-strongly positive expression.

Figure 8

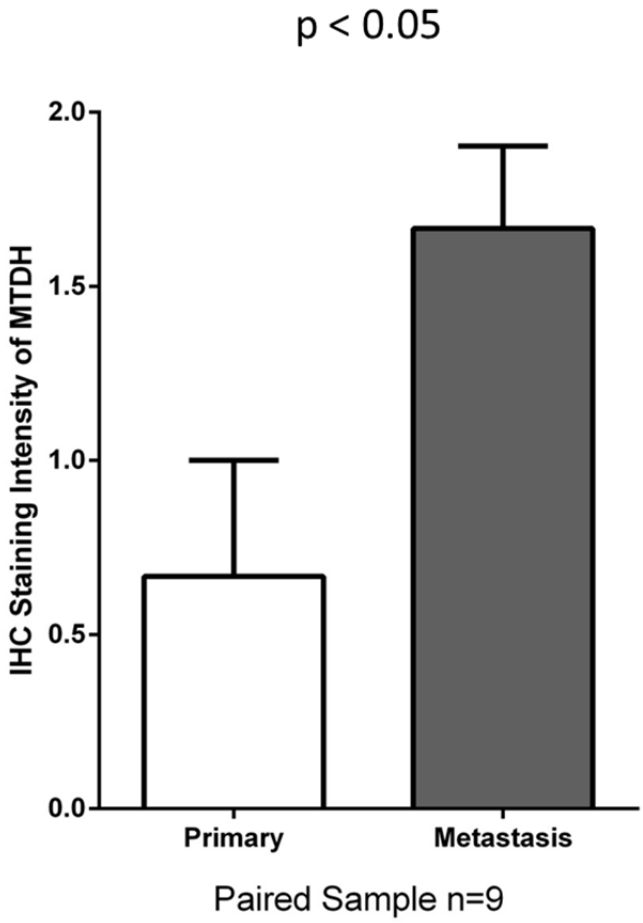


Figure 8. MTDH expression increases from primary to metastatic tumors in osteosarcoma. Comparison of MTDH expression between primary tumors and distant metastatic lesions was conducted on paired samples obtained from 9 osteosarcoma patients. Scoring of the tumor samples was based on IHC staining intensity. The expression score was classified as 0 (negative expression), 1 (weak expression), 2 (moderate expression) or 3 (strong expression).

Table 2

MTDH Expression		
Patient ID	Expression Score	
	Primary Tumor	Metastatic Lesion
1	0	**1**
2	0	**3**
3	1	**2**
4	1	**1**
5	0	**1**
6	3	**2**
7	1	**2**
8	0	**2**
9	0	**1**
Mean Score	0.67	1.67

Table 2. Comparison of MTDH expression scores in paired samples from the osteosarcoma tissue microarray. Expression score: 0-negative expression, 1-weakly positive expression, 2-moderately positive expression, 3-strongly positive expression. Red number represents increased MTDH expression score in metastatic lesion; black number represents equal MTDH expression score in metastatic lesion; blue number represents decreased MTDH expression score in metastatic lesion.

MTDH overexpression correlates with metastasis and poor survival in osteosarcoma patients

To further assess the correlation between MTDH expression and clinical outcomes of osteosarcoma patients, Kaplan-Meier survival analyses were performed on pretreatment diagnostic biopsies of 88 high-grade osteosarcoma patients. 19 samples from patients who presented with metastasis at diagnosis were excluded from the analysis. The other 71 samples were categorized into high (n=42) and low (n=29) MTDH-expressing groups according to MTDH gene expression levels. Our results revealed that patients with high MTDH expression had significantly poorer metastasis-free survival ($p < 0.05$) and relatively decreased overall survival ($p = 0.10$) rate compared with the low MTDH-expressing group (Figure 9). In this respect, MTDH may serve as an important prognostic biomarker of metastasis risk and survival for patients with osteosarcoma. With the available online resources, we also compared MTDH expression in tumor samples from patients who developed metastatic disease within 5 years versus those who did not within the same time frame. MTDH level was found to be significantly higher in the patients who developed metastatic relapse within 5 years after initial diagnosis of osteosarcoma (Figure 10).

Figure 9

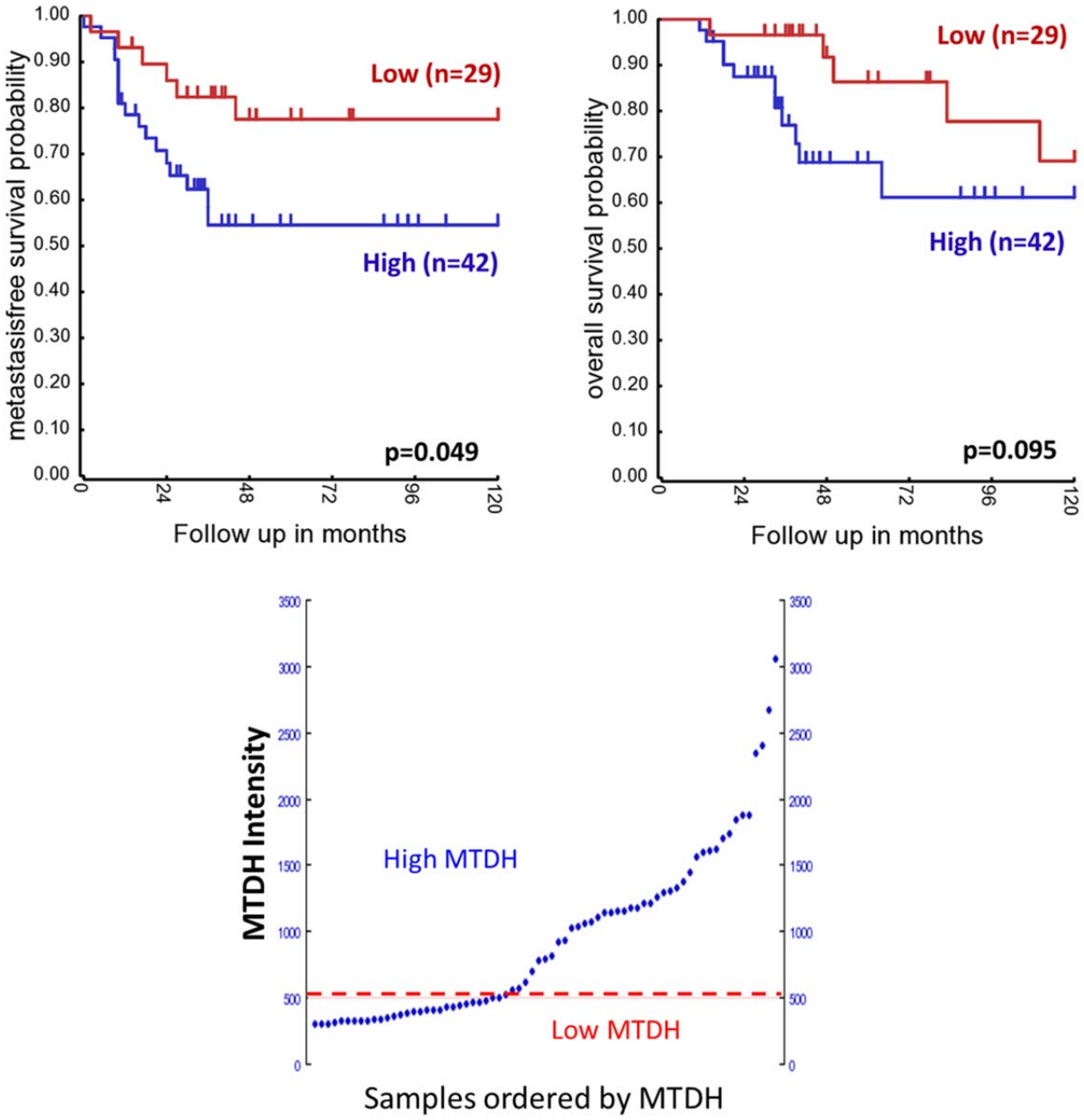


Figure 9. High MTDH expression correlates with worse clinical outcome in genomic screen of high-grade osteosarcoma samples. Kaplan-Meier survival estimates of metastasis-free and overall survival were generated from the Kuijjer Mixed Osteosarcoma Database - Cleton-Jansen Lab. The dataset was accessed through the R2: microarray analysis and visualization platform (http://r2.amc.nl). The log-rank test was used to calculate the p value.

Figure 10

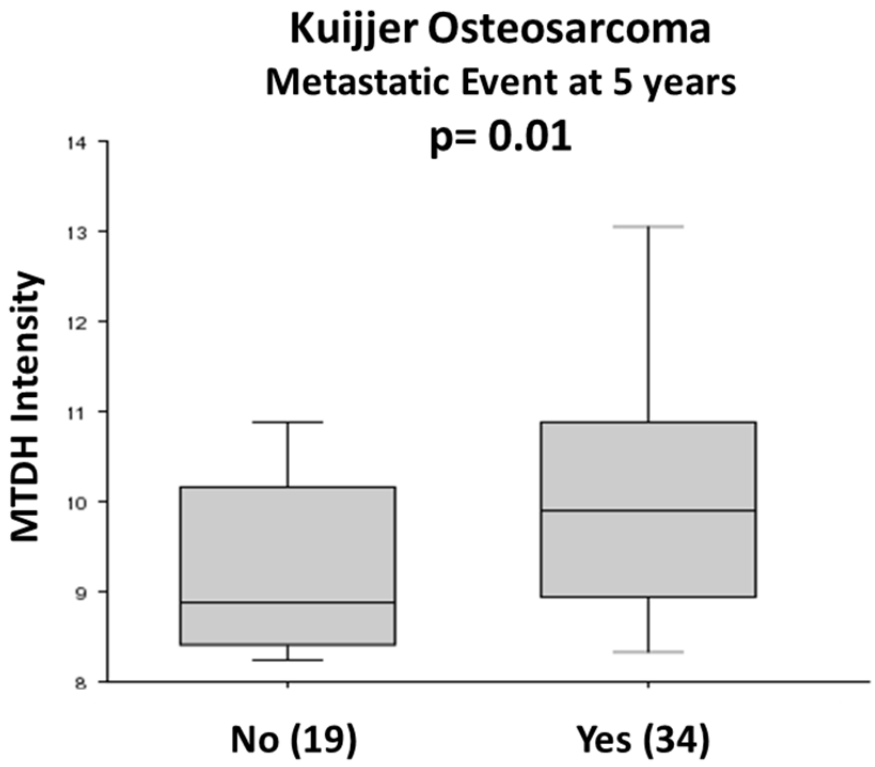


Figure 10. MTDH overexpression predicts high risk of metastasis in osteosarcoma. Comparison of the MTDH expression in primary tumors between patients with and without newly developed metastatic disease within 5 years after diagnosis was performed in the Kuijjer osteosarcoma dataset (53 samples total). The dataset was accessed through the R2: microarray analysis and visualization platform (http://r2.amc.nl).

Summary

By comparing MTDH expression between primary and metastatic tumor samples in melanoma, prostate cancer, and osteosarcoma, we confirmed that MTDH is upregulated during the metastatic process. As shown in Figure 5 and 10, MTDH overexpression has been associated with higher risk of metastatic relapse in melanoma and osteosarcoma. Our survival analysis in Figure 6 and 9 further revealed that high MTDH expression accelerates tumor progression and predicts shorter overall and metastasis-free survival in several aggressive cancers including osteosarcoma. These observations are consistent with previous findings that MTDH is a powerful marker for tumor aggressiveness and a predictor for poor patient prognosis (68, 70, 78, 80-83). As depicted in Figure 8, the significant increase in MTDH expression from primary to metastatic tumors in the same patient spurred our interest in understanding the mechanisms by which MTDH causes more aggressive disease. All the clinical evidence described in this chapter supported the significance of MTDH overexpression in osteosarcoma metastasis and warranted further exploration in this disease model.

Chapter 4. MTDH is overexpressed on osteosarcoma cell surface as a type II transmembrane protein

Rationale

A strong correlation between high MTDH expression and distant metastasis has been implicated in two independent osteosarcoma clinical datasets presented in Chapter 3. This finding triggered our interest to further explore the function(s) of MTDH in osteosarcoma, and particularly in osteosarcoma metastasis. To continue the functional studies of MTDH in osteosarcoma cell models, we had to examine the expression status of MTDH in each of the osteosarcoma cell lines included in this study through western blotting and real-time quantitative PCR.

Another important goal of this chapter is to investigate the distribution of MTDH protein in osteosarcoma cells through immunofluorescence microscopy because the subcellular localization of a protein is tightly linked to its function. To date, there is still great uncertainty and controversy over the potential localization of MTDH in cancer cells as some studies showed cytoplasmic and perinuclear localizations of MTDH while others suggested that MTDH primarily expressed inside the nucleus (60, 66, 69). In 2004, Brown and Ruoslahti provided the first and only evidence that supports the cell-surface localization of MTDH in a study about the role of MTDH in breast cancer metastasis (63). Considering the significance of cell surface proteins in cancer, we proposed that MTDH is abundantly expressed on the surface of osteosarcoma cells. Moreover, we wanted to use flow cytometry to determine the transmembrane topology of MTDH in osteosarcoma since the localization of the long C-terminal domain of MTDH would provide clues to better understand its functions and the underlying mechanisms.

Results

MTDH expression profile in various osteosarcoma cell lines

After MTDH overexpression was confirmed in osteosarcoma samples in chapter 3, we wanted to explore the expression of MTDH in established osteosarcoma cell lines. We began by assessing MTDH expression at the protein level. According to western blotting analysis, MTDH protein was overexpressed in all 6 osteosarcoma cell lines in comparison with the normal control hFOB cells: 16 fold increase in CCHD, 10 fold increase in LM7, 10 fold increase in SAOS2, 15 fold increase in SJSA, 9 fold increase in HOS, and 11 fold increase MG63 (Figure 11). In the meantime, real-time PCR analyses demonstrated that osteosarcoma cell lines had upregulated MTDH at the level of transcription as well (Figure 12). Compared to the normal control cell line hFOB, the mRNA expression of MTDH was increased by 10.6 fold, 1.8 fold, 2.5 fold, 3.5 fold, 2.1 fold, and 2.3 fold in CCHD, LM7, SAo2, SJSA, HOS, and MG63 cell lines respectively.

Figure 11

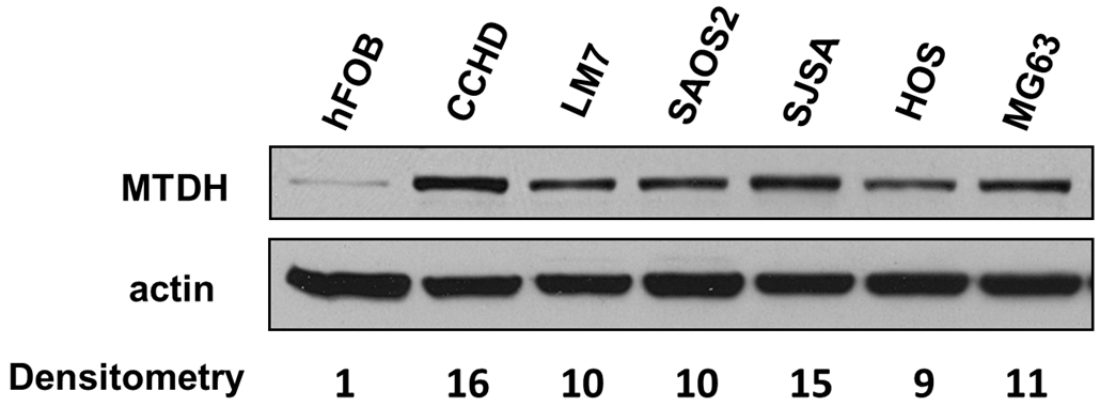


Figure 11. MTDH expression is upregulated in osteosarcoma cells. The protein expression of MTDH in normal human fetal osteoblastic cells (hFOB) and a panel of human osteosarcoma cell lines was analyzed by Western blot. Beta-actin was used as a loading control.

Figure 12

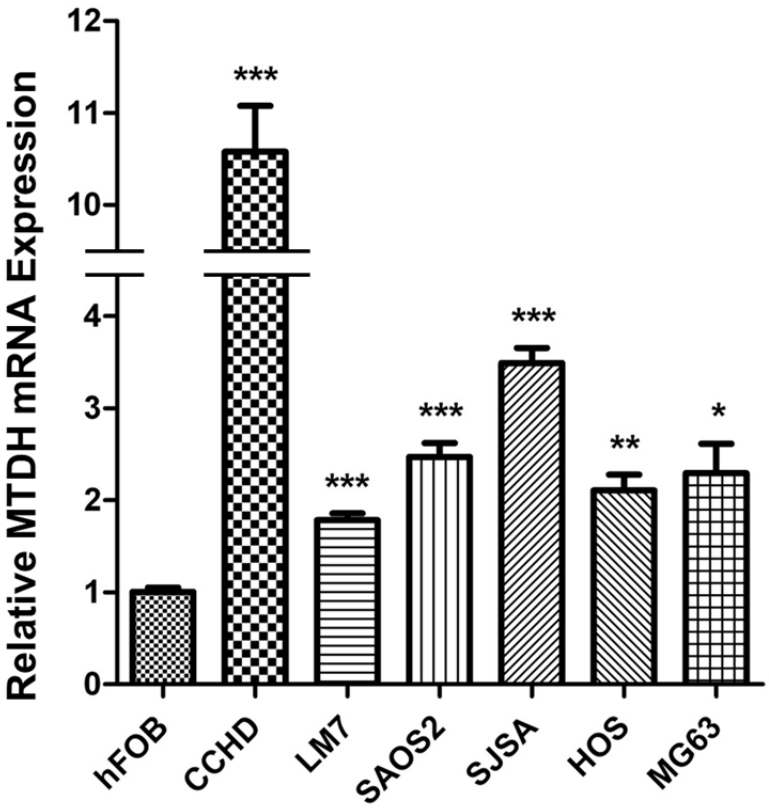


Figure 12. MTDH is upregulated in osteosarcoma cells at the mRNA level. Relative mRNA expression of MTDH in hFOB and indicated osteosarcoma cell lines was determined by real-time PCR. Expression data were normalized with GAPDH. Fold change of gene expression in each osteosarcoma cell line was relative to hFOB. *P≤0.05, **P≤0.01, ***P≤0.001 vs. hFOB cells. Bar graphs represent mean ± SEM.

Localization of MTDH in osteosarcoma cells

To determine the localization of MTDH in osteosarcoma cells, HOS and CCHD cells were examined by immunofluorescence (IF) microscopy using anti-MTDH antibody and Alexa 488-labeled secondary antibody (Figure 13). IF analysis of fixed and permeabilized HOS and CCHD cells demonstrated diffuse cytoplasmic staining of MTDH. Concentrated immunoreactivity for MTDH was also detected at the cell edges in both CCHD and HOS

cells. No significant nuclear distribution could be found. These observations implied that MTDH protein is primarily localized to the cytoplasm and cell membrane in osteosarcoma cells.

Figure 13

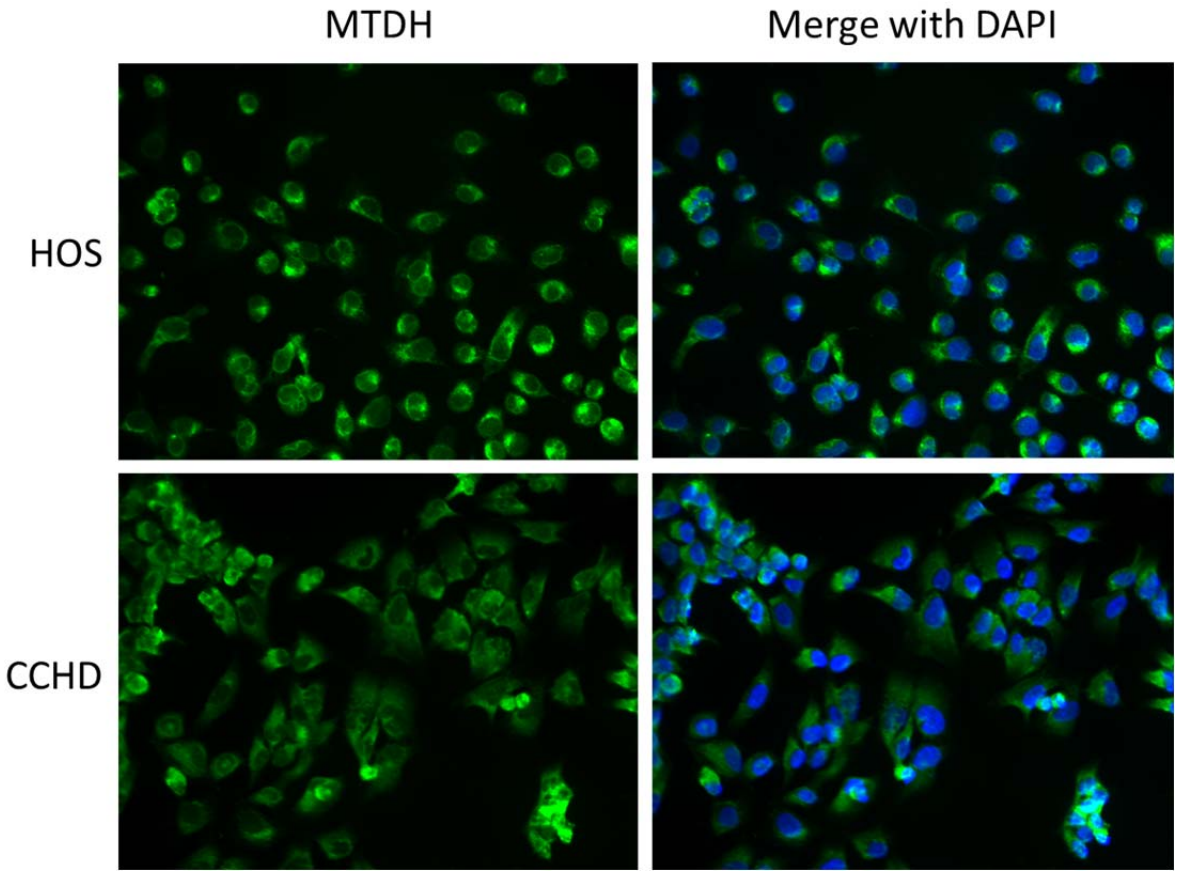


Figure 13. Expression and localization of MTDH in osteosarcoma cells. Acetone-fixed HOS and CCHD cells were stained with rabbit-anti-MTDH primary antibody followed by Alexa 488-labeled anti-rabbit secondary antibody. Cell nuclei were counterstained with Hoechst. Green fluorescence represents the immunoreactivity for MTDH. Blue fluorescence represents staining of nuclei. All images were taken on a Nikon fluorescence microscope under 100X magnification.

Cell surface expression and membrane topology of MTDH in osteosarcoma cells

To confirm the cell surface localization of MTDH, we used flow cytometry to evaluate the expression and topology of MTDH protein on the cell membrane. Antibodies specific for amino acid residues 315-461 of MTDH protein were allowed to bind only to the outside of non-permeabilized osteosarcoma cells, ensuring that any signal detected was from the outer cell membrane. While normal hFOB cells had virtually no detectable cell surface MTDH expression, all osteosarcoma cell lines assessed expressed abundant level of cell surface MTDH protein (Figure 14). The results indicated that, in osteosarcoma at least, MTDH is localized at the cell surface as a type II transmembrane protein with a short cytoplasmic N-terminal domain and a long extracellular C-terminal domain which could be involved in potential interactions with extracellular components.

The type II topology of MTDH in osteosarcoma cells was further confirmed by western blot analyzing phosphorylation of serine residues located on the C-terminal segment (Figure 15). While there were multiple proteomic studies that identify serine-phosphorylated peptide fragments apparently derived from MTDH, we could not detect any phosphoserine signal in our immunoprecipitated MTDH samples. This observation, contrary to previous findings from multiple phosphorylation studies, suggested that the C-terminal domain of MTDH which is rich in phosphorylation sites localizes to the extracellular space, indicating a type II topology of MTDH protein in osteosarcoma cells.

Figure 14

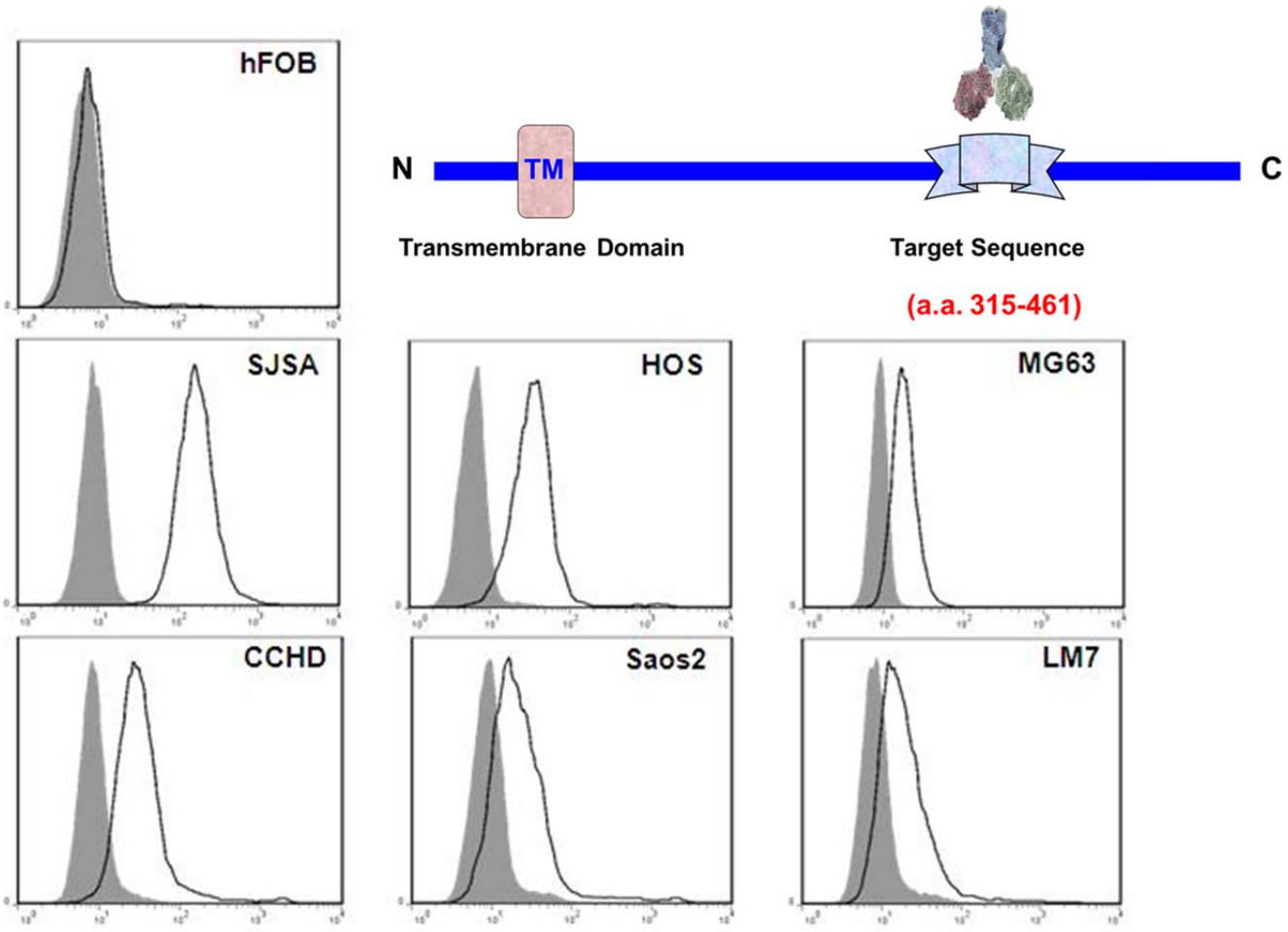


Figure 14. Cell surface expression and membrane topology of MTDH in osteosarcoma cells. Cell surface expression of MTDH was assessed in non-permeabilized hFOB and six osteosarcoma cell lines by flow cytometry using rabbit-anti-MTDH (315-461 a.a.) antibody in combination with a secondary PE-conjugated antibody. Representative histograms are shown.

Figure 15.

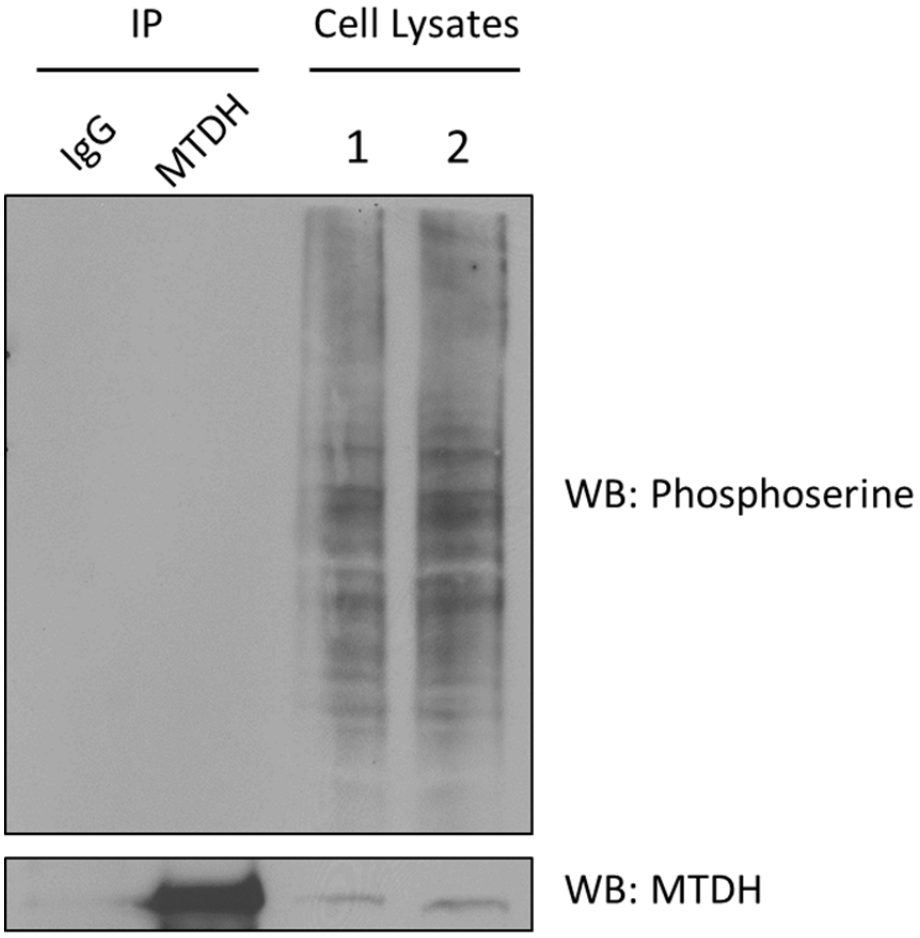


Figure 15. Analysis of phosphorylation status of MTDH in osteosarcoma cells. Whole-cell lysates of HOS cells were subjected to co-immunoprecipitation with anti-MTDH antibody or normal IgG. The precipitated proteins (lane 1 and lane 2) and the whole cell lysates (lane 3 and lane 4) were transferred to nitrocellulose membrane and probed with anti-phosphoserine and anti-MTDH antibodies.

Summary

In this chapter we explored the expression, localization, and topology of MTDH in established osteosarcoma cell lines through multiple experimental approaches. We have demonstrated through western blotting and real-time quantitative PCR that compared to normal human osteoblasts, osteosarcoma cell lines ubiquitously expressed high level of MTDH at both protein and mRNA levels. Immunofluorescence analysis of the distribution of MTDH protein in HOS and CCHD cells displayed uniform cytoplasmic and cell membrane localization. By performing flow cytometry analysis on non-permeabilized osteosarcoma and control cells with an anti-MTDH antibody that targets the C-terminal domain of MTDH, we confirmed the overexpression of MTDH on cell surface and demonstrated that MTDH is a type II transmembrane protein in osteosarcoma cells. This topology was supported by further evidence obtained from the analysis of MTDH phosphorylation status in HOS cells.

Chapter 5. Inhibition of MTDH suppresses invasive properties of osteosarcoma cells

Rationale:

Previous studies of clinical samples have established a positive correlation between high MTDH expression level and poor patient prognosis (68, 70, 78, 80-83). The data collected in Chapter 3 revealed a significant increase in MTDH expression in metastatic lesions compared to primary tumors. In the meantime, high expression of MTDH has been associated with increased risk of metastasis in osteosarcoma. Given the results presented in Chapter 4 that MTDH is overexpressed in osteosarcoma cell lines as compared with normal human osteoblasts, we hypothesized that MTDH expression promotes metastasis-associated behaviors in osteosarcoma cells.

In this chapter, we wanted to investigate the function(s) of MTDH, particularly those expressed on the cell surface, in osteosarcoma metastasis by assessing the impact of MTDH inhibition on osteosarcoma cell proliferation, migration, and invasion in vitro. We planned to use two different approaches to inhibit MTDH: silencing endogenous MTDH gene expression by retrovirus-mediated shRNA, and blocking surface-bound MTDH with anti-MTDH antibody. For the experiments performed in this chapter, we have chosen HOS and CCHD cell lines, both of which have abundant expression of MTDH and are highly invasive according to literature and previous studies conducted in our lab. We anticipated that MTDH inhibition leads to decreased cell invasion and migration ability.

Results

Knockdown of endogenous MTDH reduces proliferation in osteosarcoma cells

To examine the effects of MTDH downregulation on osteosarcoma cells, we used retrovirus-mediated shRNA targeting MTDH to stably knock down MTDH expression in osteosarcoma cell lines HOS and CCHD. After a week of selection by puromycin, the knockdown efficiency of MTDH in each cell line was validated by western blotting analysis. A significant reduction in MTDH protein level (80% and 90% in SH-4 and SH-6 respectively) was observed in cells transduced with MTDH-specific shRNA constructs when compared with the control cells transduced with a scrambled sequence (Figure 16).

Figure 16.

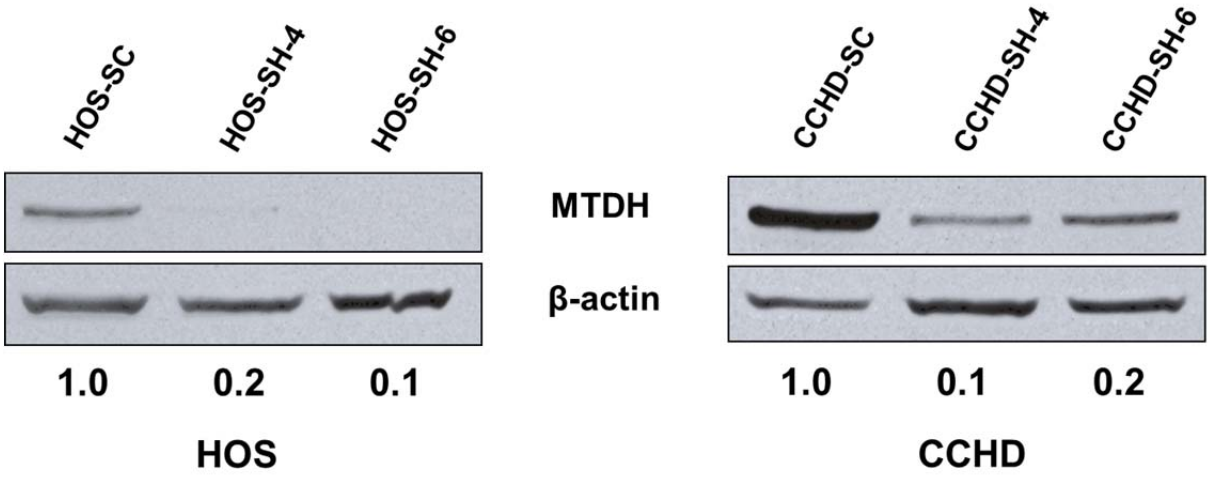


Figure 16. Retrovirus-mediated shRNA targeting MTDH effectively reduces MTDH expression in HOS and CCHD cells. MTDH knockdown in HOS and CCHD was confirmed by western blotting analysis of whole cell lysates extracted from control and MTDH-knockdown osteosarcoma cells. (SC: scramble control; SH-4: MTDH-specific shRNA construct #4; SH-6: MTDH-specific shRNA construct #6). Beta-actin was used as a loading control. Densitometric analysis (Image J Processing Software, NIH) was applied to calculate the knockdown efficiency.

Next, we used an automated Coulter counter (Vi-Cell) to evaluate the effect of MTDH silencing on the proliferation rate of HOS and CCHD cells for a period of 6 days (Figure 17). MTDH depletion caused a modest reduction in cell yield of HOS cells on day 6 ($p<0.01$). We also observed a significant decrease in proliferation of MTDH-knockdown CCHD cells compared with control cells on day 4 and day 6 ($p<0.001$).

Figure 17

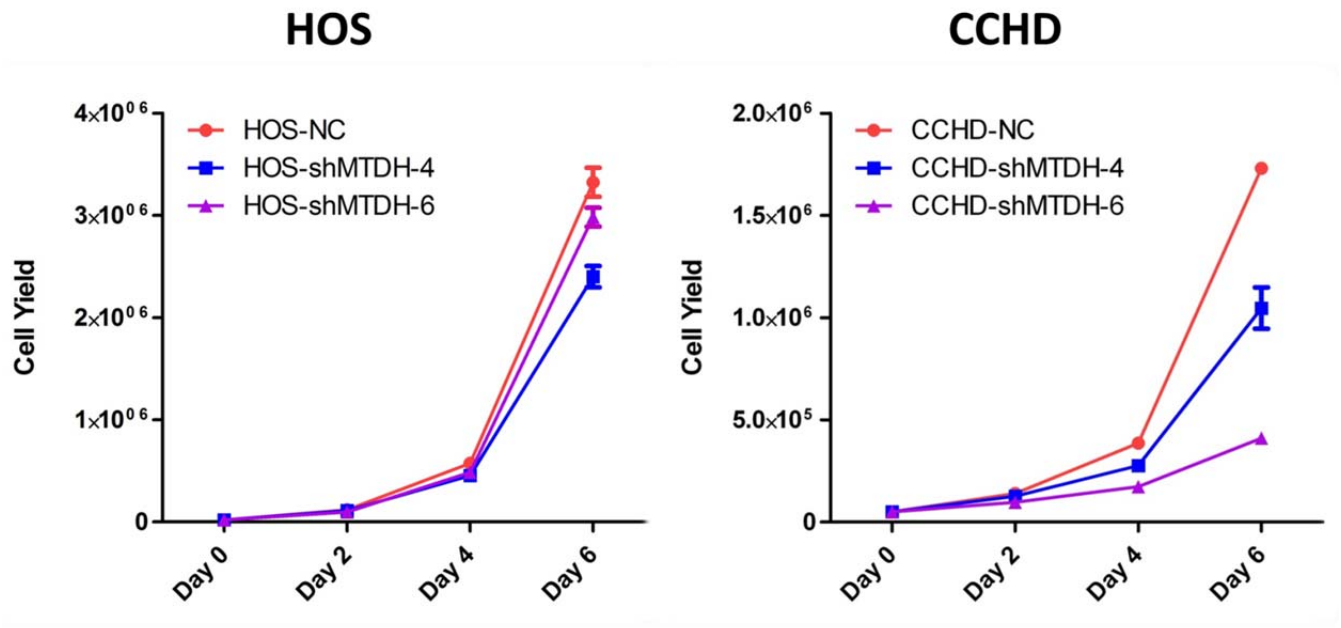


Figure 17. Downregulation of MTDH reduces cell proliferation in osteosarcoma cells. Cell proliferation was determined by automated cell counting after 0, 2, 4, and 6 days of incubation of control cells (NC) and MTDH-knockdown cells (shMTDH-4 and shMTDH-6). Results are shown as mean ± SEM of three replicates and they are representative of three independent experiments.

Downregulation of MTDH has marginal effect on clonogenic ability of osteosarcoma cells

To determine whether MTDH knockdown alters clonal growth of osteosarcoma cells, we performed colony formation assay on control and MTDH-knockdown HOS and CCHD cells. When compared to the control HOS cells (137.3±4.3 colonies), a minor decrease in the number of colony formed was observed in HOS-SH-4 cells (114.0±7.1 colonies, $p<0.05$) while no significant reduction was found in HOS-SH-6 cells (130.3±7.9 colonies, ns). Similar results were shown in CCHD cells. CCHD-SH-4 cells (288.7±9.6 colonies, $p<0.05$) and CCHD-SH-6 cells (240.0±5.3 colonies, $p<0.001$) formed slightly fewer colonies than the control CCHD-NC cells (332.7±6.6 colonies). These results indicated that MTDH expression has very limited effect on clonogenic ability of osteosarcoma cells.

Figure 18.

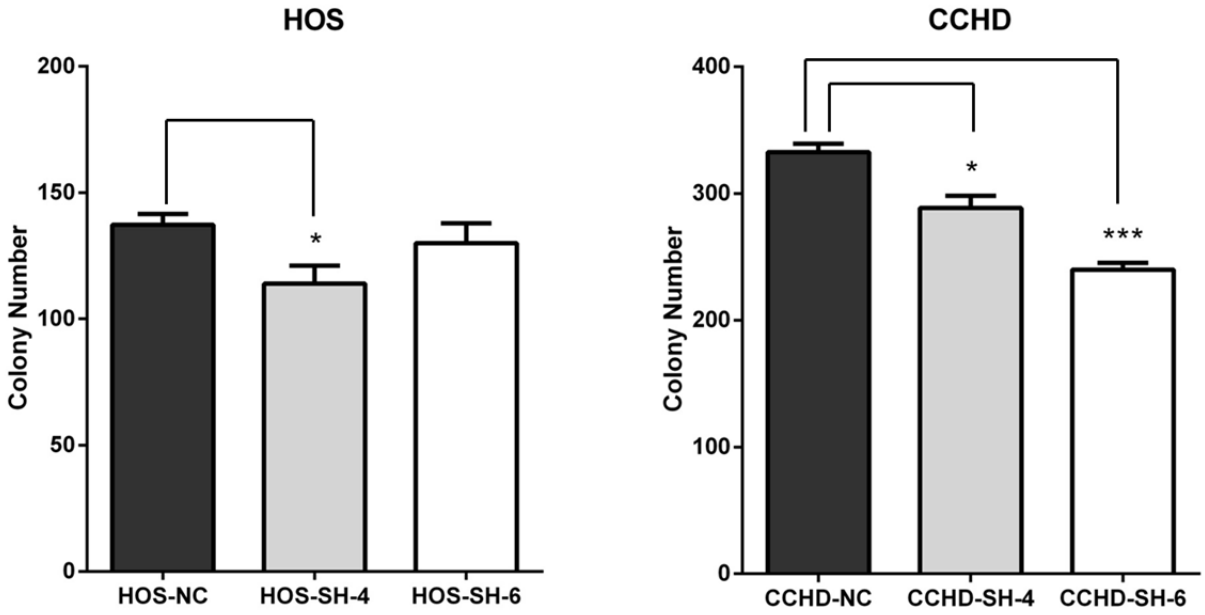


Figure 18. MTDH knockdown slightly decreases the colony formation ability of osteosarcoma cells. Control and MTDH-knockdown HOS and CCHD cells were seeded in 6-well plates at very low density in triplicates and cultured under standard conditions. After 2 weeks of incubation, cells were fixed and stained with 0.05% crystal violet PBS solution. Colonies with 50 or more cells were counted. Results are shown as mean ± SEM of three replicates. *P≤0.05, **P≤0.01, ***P≤0.001 vs. control cells.

MTDH knockdown inhibits osteosarcoma cell migration and invasion

To explore the functional role of MTDH in osteosarcoma metastasis, we used transwell migration assay and matrigel invasion assay to examine the effects of MTDH knockdown on cell aggressiveness. It has been shown that downregulation of MTDH by shRNA triggered a significant reduction in the number of migrated cells of HOS and CCHD after 24 hours when compared to control cells (HOS: 429.3±7.0 cells/field in SH-4 and 310.0±80.5 cells/field in SH-6 vs. 1201.0±98.4 cells/field in NC; CCHD: 128.7±39.7 cells/field in SH-4 and 107.7 ±22.1 cells/field in SH-6 vs. 402.3±73.7 cells/field in NC) (Figure 19). We have also demonstrated in both HOS and CCHD cells that efficient knockdown of MTDH, compared to scrambled control shRNA, resulted in profound reduction in invasive ability of osteosarcoma cells in vitro (HOS: 135.0±32.8 cells/field in SH-4 and 163.7±10.4 cells/field in SH-6 vs. 565.0±43.7 cells/field in NC; CCHD: 18.7±0.7 cells/field in SH-4 and 16.7±4.1 cells/field in SH-6 vs. 216.0±31.9 cells/field in NC) (Figure 20). Taken together, these data strongly suggested that MTDH promotes osteosarcoma aggressiveness by mediating cell invasion and migration.

Figure 19

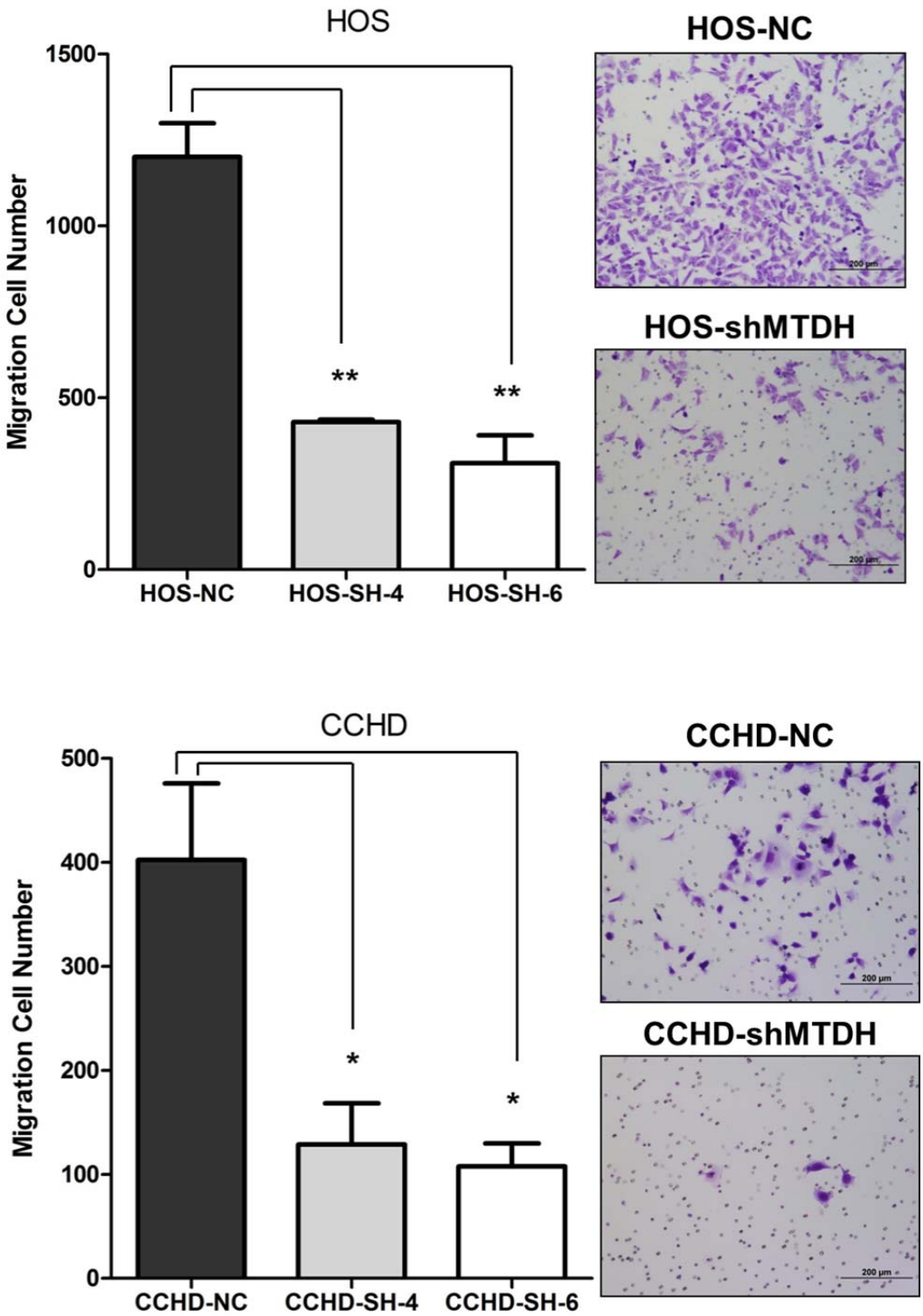

HOS
Migration Cell Number
1500
1000
500
0
**
**
HOS-NC
HOS-SH-4
HOS-SH-6
HOS-NC
HOS-shMTDH
200 μm
200 μm
CCHD
Migration Cell Number
500
400
300
200
100
0
*
*
CCHD-NC
CCHD-SH-4
CCHD-SH-6
CCHD-NC
CCHD-shMTDH
200 μm
200 μm

Figure 19. Silencing endogenous MTDH inhibits migration of HOS and CCHD cells. In vitro cell migration was performed in the 24-well Corning Transwell polycarbonate membrane cell culture inserts with 8-μm pore size (Corning). After incubation for 24 hours, the migrated cells were fixed, stained with Hema-3, and counted under a microscope. Representative pictures (right) and quantification of migrated cells (left) from transwell migration assays are displayed. Results are shown as mean ± SEM (cells/field) of three replicates and are representative of three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001 vs. control cells.

Figure 20

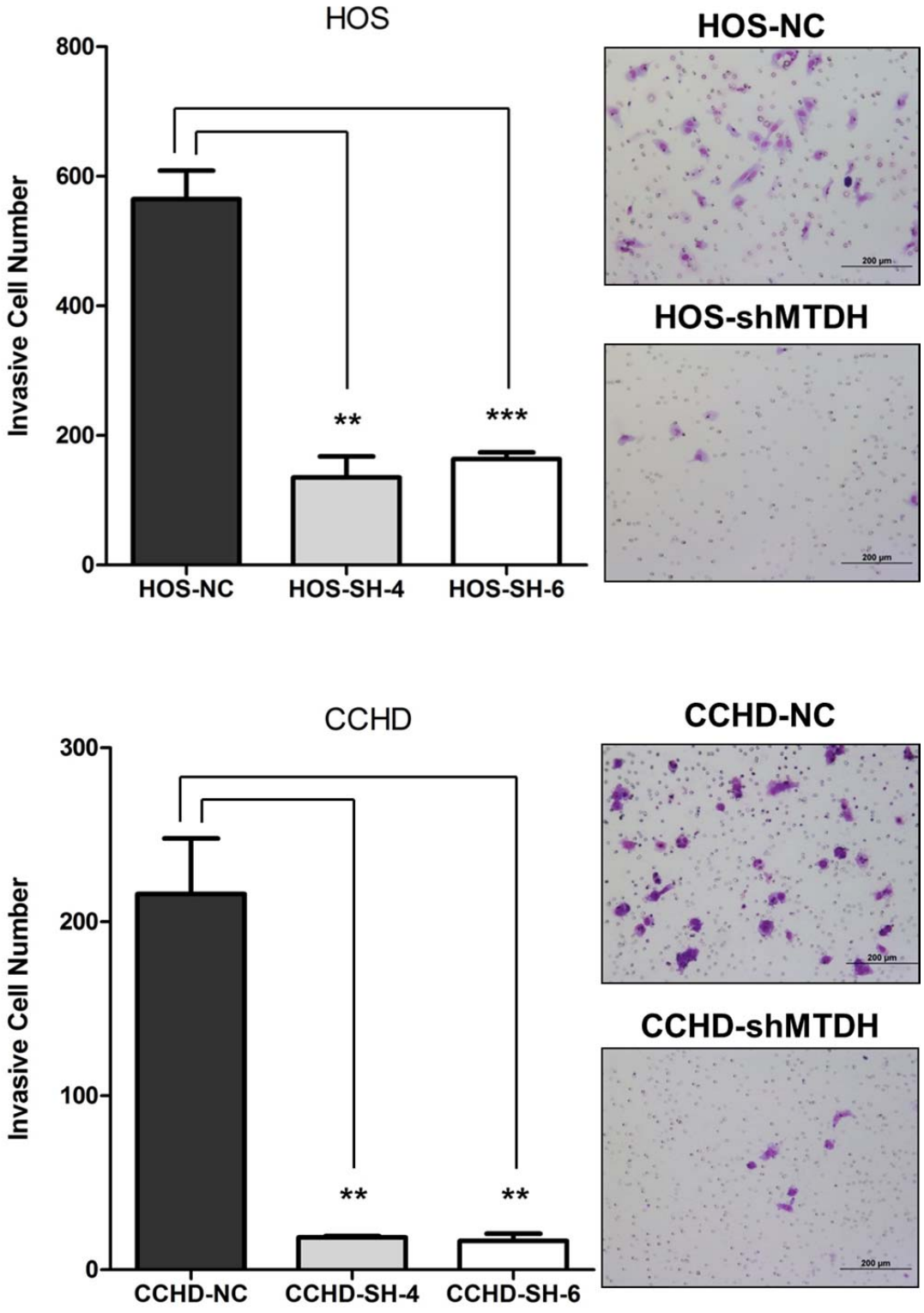

HOS
Invasive Cell Number
800
600
400
200
0
**
***
HOS-NC
HOS-SH-4
HOS-SH-6
HOS-NC
HOS-shMTDH
200 μm
200 μm
CCHD
Invasive Cell Number
300
200
100
0
**
**
CCHD-NC
CCHD-SH-4
CCHD-SH-6
CCHD-NC
CCHD-shMTDH
200 μm
200 μm

Figure 20. Downregulation of MTDH leads to decreased invasive ability of osteosarcoma cells. The invasive ability of osteosarcoma cells was evaluated by using 24-well BD BioCoat Matrigel invasion chambers with 8-μm pore size. After 48h incubation, the penetrated cells were fixed, stained with Hema-3, and counted under a microscope Representative pictures (right) and quantification of penetrated cells (left) from matrigel invasion assays are shown. Results are shown as mean ± SEM (cells/field) of three replicates and are representative of three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001 vs. control cells.

Blockade of cell surface MTDH with antibody inhibits migration and invasion

Since we have defined the cell-surface localization for MTDH in chapter 4, we wished to know if steric interference with MTDH's extracellular functions might cast a similar effect. Transwell migration and matrigel invasion assays were performed on HOS and CCHD cells in the presence of an anti-MTDH antibody specific for an epitope within the extracellular C-terminal domain compared to an isotype control antibody. A significant reduction in migration was demonstrated in both HOS and CCHD cells incubated with the MTDH-blocking antibody when compared with cells treated with the control IgG (HOS: 197.0±20.0 cells/field in anti-MTDH group vs. 994.0±92.0 cells/field in normal IgG group; CCHD: 304.0±7.0 cells/field in anti-MTDH group vs. 688.0±33.9 cells/field in normal IgG group) (Figure 21). Results from the invasion assay revealed that blocking cell surface MTDH with anti-MTDH antibody effectively decreased in vitro invasiveness of HOS and CCHD cells (HOS: 95.3±25.2 cells/field in anti-MTDH group vs. 299.0±49.2 cells/field in normal IgG group; CCHD: 138.7±17.3 cells/field in anti-MTDH group vs. 345.0±51.0 cells/field in normal IgG group) (Figure 22).

To determine whether the effects on cell migration and invasion from anti-MTDH antibodies resulted from antibody-induced cellular cytotoxicity, cell viability was evaluated under the exact same conditions. Anti-MTDH antibody did not affect cell viability at the indicated concentration within the same period of time (Figure 23). Taken together, these results suggested that MTDH located on the cell surface is essential for mediating cell motility and invasiveness in osteosarcoma.

Figure 21

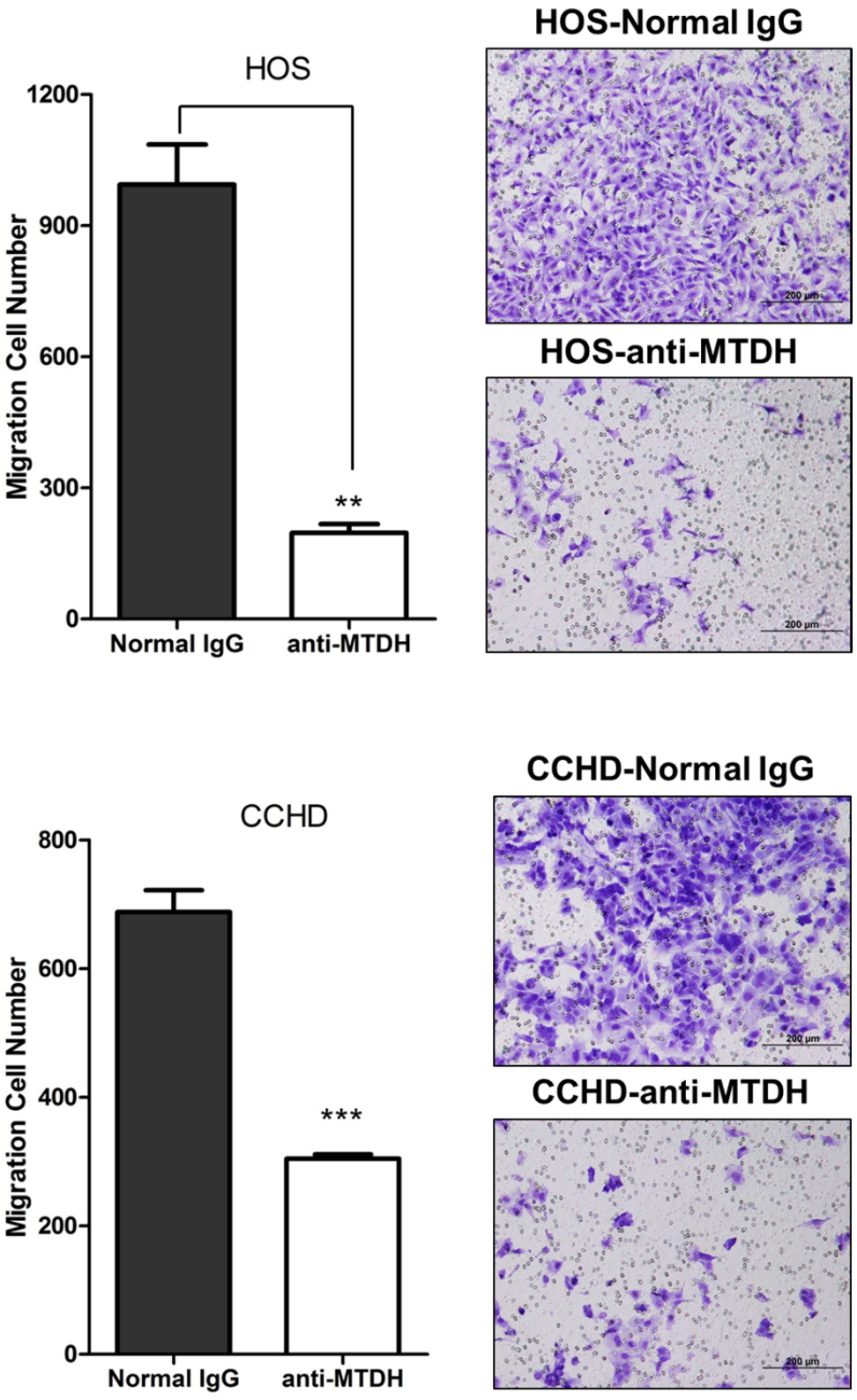

HOS
Migration Cell Number
1200
900
600
300
0
**
Normal IgG
anti-MTDH
HOS-Normal IgG
HOS-anti-MTDH
200 μm
CCHD
Migration Cell Number
800
600
400
200
0
***
Normal IgG
anti-MTDH
CCHD-Normal IgG
CCHD-anti-MTDH
200 μm

Figure 21. Inhibition of cell surface MTDH leads to significantly decreases motility of osteosarcoma cells. HOS and CCHD cells were co-incubated with rabbit IgG or rabbit-anti-MTDH antibody at a final concentration of 7.5 μg/ml. After 24-hour of incubation in the transwell Boyden chamber, the migrated cells were fixed, stained with Hema-3, and counted under a microscope. Representative pictures (right) and quantification of migrated cells (left) from migration assay are displayed. Results are shown as mean ± SEM (cells/field) of three replicates and are representative of three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001 vs. control cells.

Figure 22

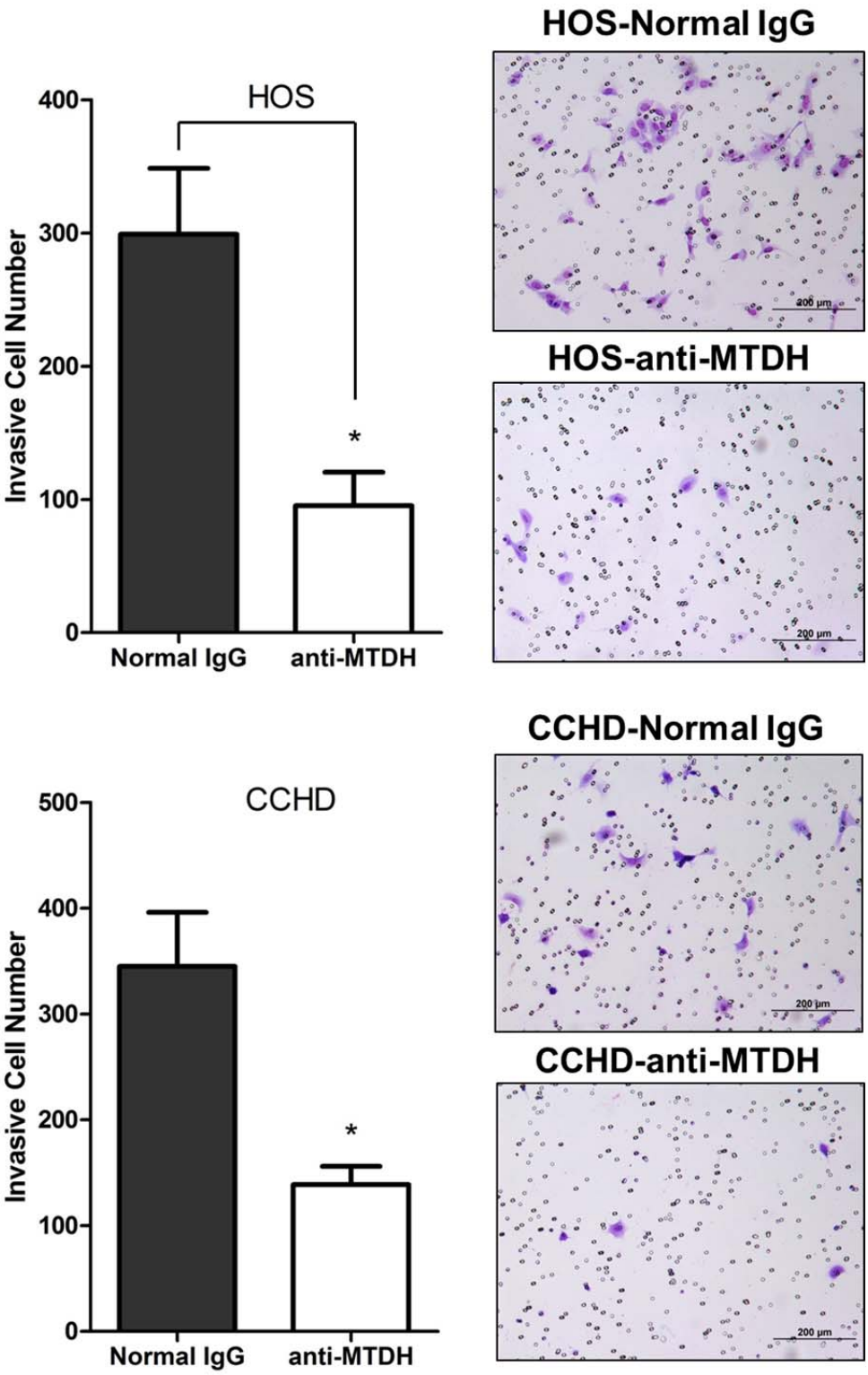

HOS
Invasive Cell Number
400
300
200
100
0
*
Normal IgG
anti-MTDH
HOS-Normal IgG
200 μm
HOS-anti-MTDH
200 μm
CCHD
Invasive Cell Number
500
400
300
200
100
0
*
Normal IgG
anti-MTDH
CCHD-Normal IgG
200 μm
CCHD-anti-MTDH
200 μm

Figure 22. Anti-MTDH antibody inhibits osteosarcoma cell invasiveness. HOS and CCHD cells were co-incubated with rabbit IgG or rabbit-anti-MTDH antibody at a final concentration of 7.5 μg/ml. After 48-hour of incubation in the invasion chamber, the penetrated cells were fixed, stained with Hema-3, and counted under a microscope. Representative pictures (right) and quantification of penetrated cells (left) from invasion assay are displayed. Results are shown as mean ± SEM (cells/field) of three replicates and are representative of three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001 vs. control cells.

Figure 23

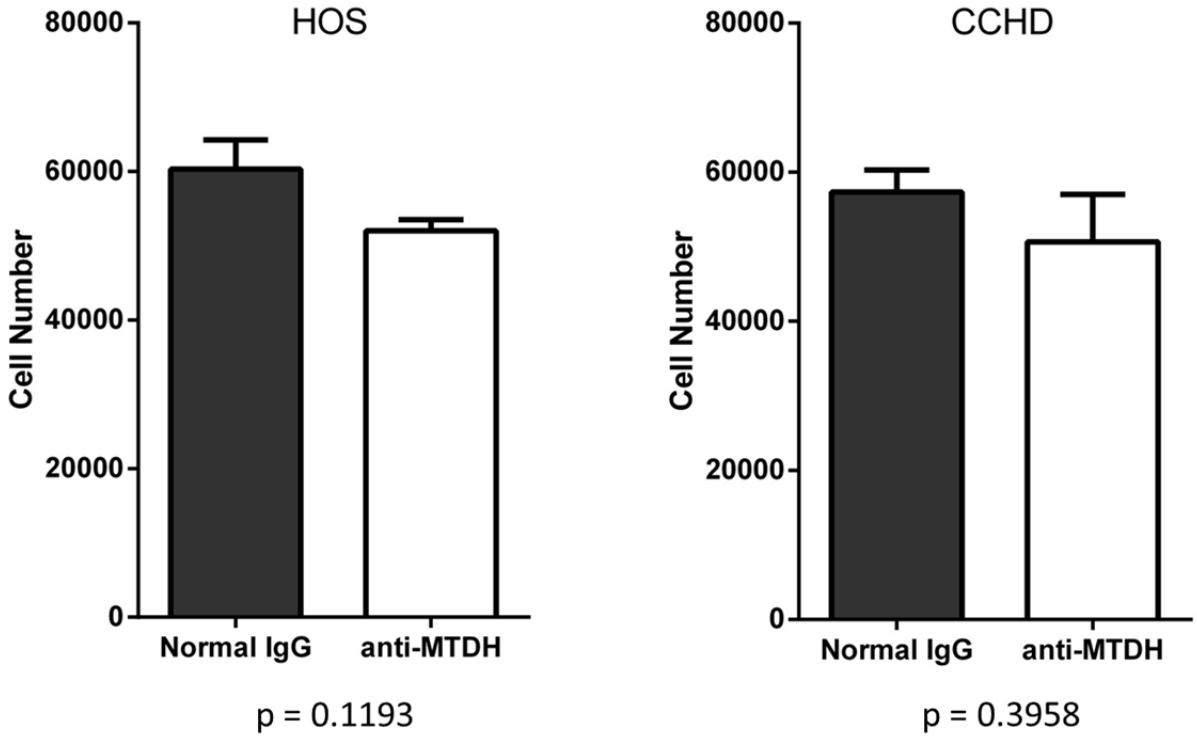


Figure 23. Anti-MTDH antibody has no significant impact on osteosarcoma cell proliferation at indicated concentration. HOS and CCHD cells were co-incubated with rabbit IgG or rabbit-anti-MTDH antibody at a final concentration of 7.5 μg/ml. After 48-hour of incubation under standard culture conditions in a 6-well plate, cell yield was determined by automated cell counting using Vi-Cell. Results are shown as mean ± SEM of three replicates and are representative of three independent experiments.

Summary

In this chapter we investigated the biological significance of MTDH in the aggressive behavior of osteosarcoma. The impacts of MTDH knockdown on osteosarcoma cell proliferation, colony formation, migratory and invasive abilities were tested by shRNA-mediated gene silencing in HOS and CCHD cells. In the MTDH knockdown studies, we have shown that downregulation of MTDH had limited effect on the proliferative and clonogenic ability of osteosarcoma cells. However, we observed a more significant reduction in transwell migration and matrigel invasion of MTDH-knockdown cells compared with the control cells. To further understand the function(s) of MTDH located on the cell surface, we used an anti-MTDH antibody which targets the extracellular C-terminal domain to interfere with MTDH's extracellular functions and assessed its impacts on osteosarcoma cell migration and invasion. We have shown that blocking MTDH expressed on the cell membrane significantly decreased osteosarcoma cell motility and effectively inhibited cell invasion through matrigel. Altogether, these results indicate that MTDH plays a pivotal role in mediating pro-metastatic activities in osteosarcoma cell models.

Chapter 6. Knockdown of MTDH inhibits pulmonary metastasis in vivo

Rationale:

Given the results presented in Chapter 5 that MTDH knockdown decreases the invasive properties of osteosarcoma cells in vitro, we thus decided to evaluate the role of MTDH in osteosarcoma metastasis in an animal model. Since high MTDH expression promotes osteosarcoma cell migration and invasion, and is associated with increased risk of metastasis in clinical datasets, we anticipated that MTDH knockdown would decrease the metastatic potential of osteosarcoma cells and therefore inhibit pulmonary metastasis in vivo.

To better mimic the whole processes of tumor progression and metastasis, we developed a novel bioluminescent orthotopic osteosarcoma xenograft murine model with CCHD cells. The stable MTDH-knockdown and scrambled control CCHD cells were labeled with luciferase and injected into the tibia of 6-week-old NOD/SCID/IL2Rγ-deficient mice. Primary tumors were allowed to develop, followed by spontaneous pulmonary metastases. Both the primary tumor and the lung metastases were monitored weekly by IVIS spectrum imaging system and the luciferase signals were quantified. After the 6-week incubation period, all mice were sacrificed and lungs were resected for further analysis.

Results

MTDH knockdown impedes primary tumor growth and inhibits pulmonary metastasis

While in vitro analyses are helpful in determining a protein's role in specific behaviors like proliferation, migration and invasion, assessment of metastasis requires in vivo analysis. To determine whether MTDH expression is essential for metastasis, we utilized luciferase-labeled CCHOD cells, transduced with either the same MTDH-specific shRNA used in Chapter 5 or scrambled control shRNA. To ensure that our results were not compromised by contamination with untransduced cells, which might have an advantage in both proliferation and metastatic potential, clonal populations were derived from both the MTDH-knockdown and scrambled control cells. One clonal line from each group was used for all in vivo studies.

One million tumor cells were injected into the tibia of each NOD/SCID/IL2Rγ-/- xenograft host mouse (n=7 of MTDH-knockdown and control groups) and tumor growth was measured weekly by bioluminescence imaging. Quantification of the bioluminescent signals indicated that MTDH knockdown impeded primary tumor growth (Figure 24B) and inhibited development of spontaneous lung metastases (Figure 24C). All mice in the control group developed massive lung metastases 6 weeks after cancer cell inoculation, and some mice demonstrated multiple metastatic sites including bones, livers, and kidneys (Figure 24A). By contrast, mice in the MTDH-knockdown group had smaller primary tumors and significantly fewer lung metastases. Three mice showed no evidence of metastatic disease and the other four mice developed 5 or fewer metastatic lesions. Both primary and metastatic tumor burden of the control group was remarkably higher than that of the MTDH-knockdown group.

Figure 24

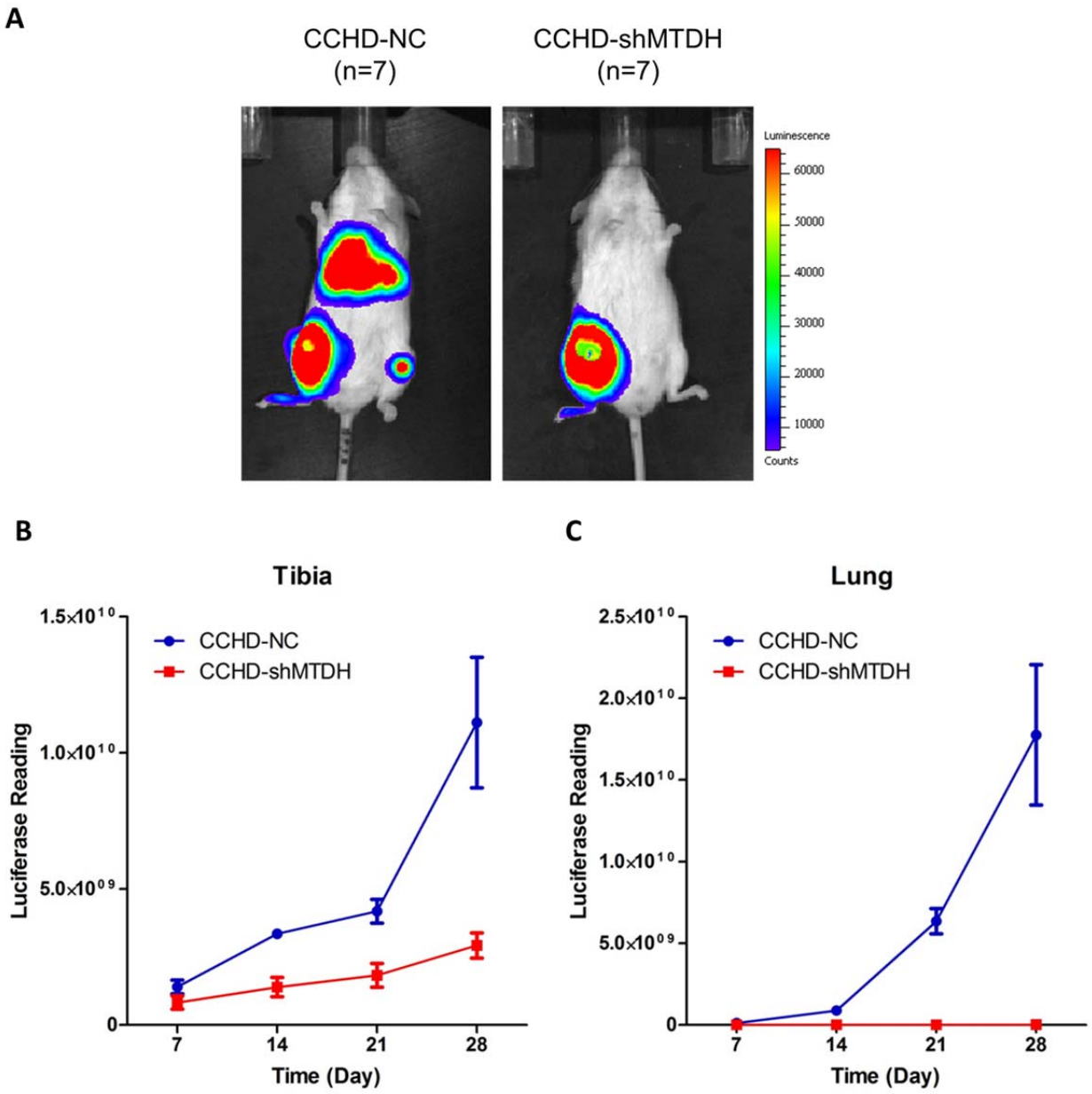


Figure 24. Knockdown of MTDH reduces primary tumor growth and inhibits pulmonary metastasis. A) Displayed are images of representative mice at the fourth week after injection. Primary tumor and lung metastasis burden of NOD/SCID/IL2Rγ-deficient mice inoculated orthotopically with luciferase-labeled CCHD cells with or without MTDH knockdown were monitored weekly by bioluminescent imaging. Quantification of the bioluminescent signals emitted from primary tumor (A) and lung metastases (B) are shown. Each data point represents mean ± SEM of 7 mice.

Tumor-bearing mice from both groups were sacrificed at 6 weeks after inoculation and the mouse lungs were harvested, photographed, and weighed. Microscopic examination of the H&E stained lung sections revealed that downregulation of MTDH significantly decreased the number of metastatic nodules developed in lung (Figure 25).

Figure 25

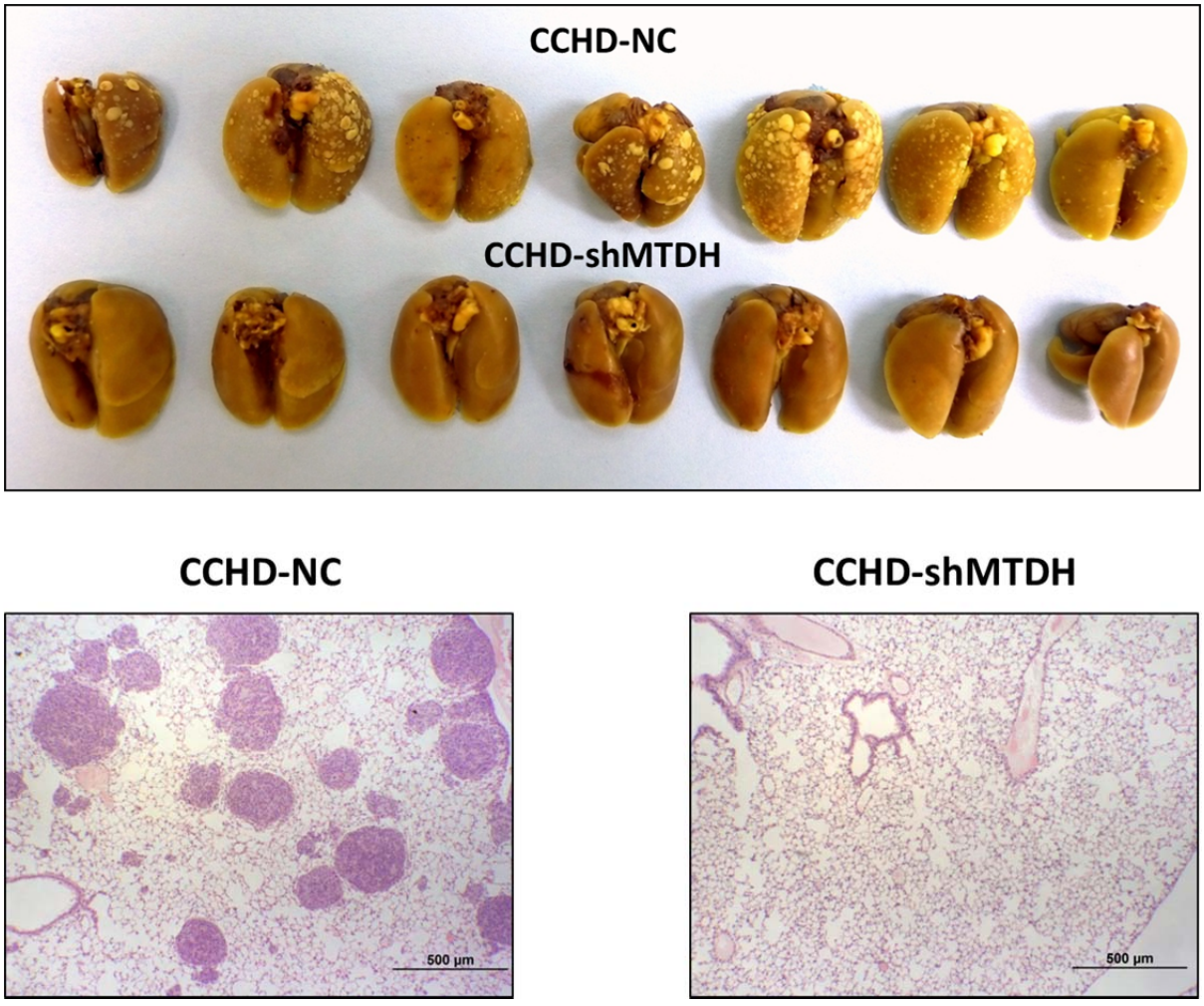


Figure 25. Downregulation of MTDH inhibits lung metastasis in vivo. Image of whole lungs isolated from mice sacrificed at six weeks after tumor cell inoculation (top). Representative H&E stained lung sections of mice at the sixth week after intratibial injection of one million CCHD-NC or CCHD-shMTDH cells (bottom).

Further analysis of the resected mouse lungs revealed that the mean lung weight ($p<0.05$, Figure 26A) and the mean number of metastatic nodules ($p<0.0001$, Figure 26B) were remarkably reduced in the MTDH-knockdown group. The mean lung weight of mice inoculated with MTDH-knockdown cells was 23% less than that of the control group. In the meantime, an average of 11.29±1.52 metastatic tumor nodules were detected per field in lungs isolated from the control group, while mice in the MTDH-knockdown group developed an average of 0.51±0.26 metastatic nodules per field.

Figure 26

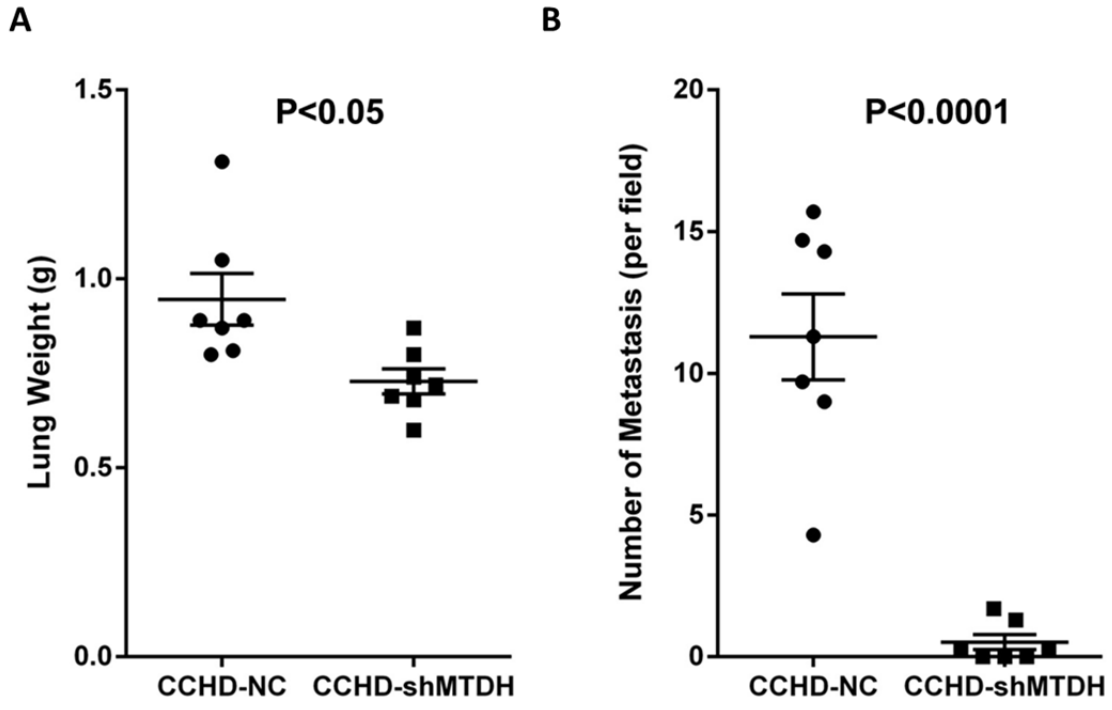


Figure 26. MTDH knockdown decreases lung tumor burden and reduces the number of metastatic nodules. A) Whole lungs isolated from mice sacrificed at the sixth week post injection were weighed. Data are displayed as a scatter dot blot. B) Quantified lung metastases are depicted as the number of metastatic nodules per field in a scatter dot blot. Lines and whiskers represent mean ± SEM of 7 mice.

To be certain that the reduction in metastasis was not solely due to the reduced proliferation of primary tumors, a parallel group of mice (n=3) inoculated with MTDH-knockdown cells were allowed to live to 12 weeks after initial inoculation, during which time the primary tumors reached a size comparable to that of the control group near the termination of the experiment while no sign of lung metastasis was detected through bioluminescent imaging (Figure 27). When their lungs were analyzed, no metastatic nodule was identified. Hence, the low rate of metastasis observed in the MTDH-knockdown group was due to the substantially reduced metastatic capacity of these cells.

Figure 27

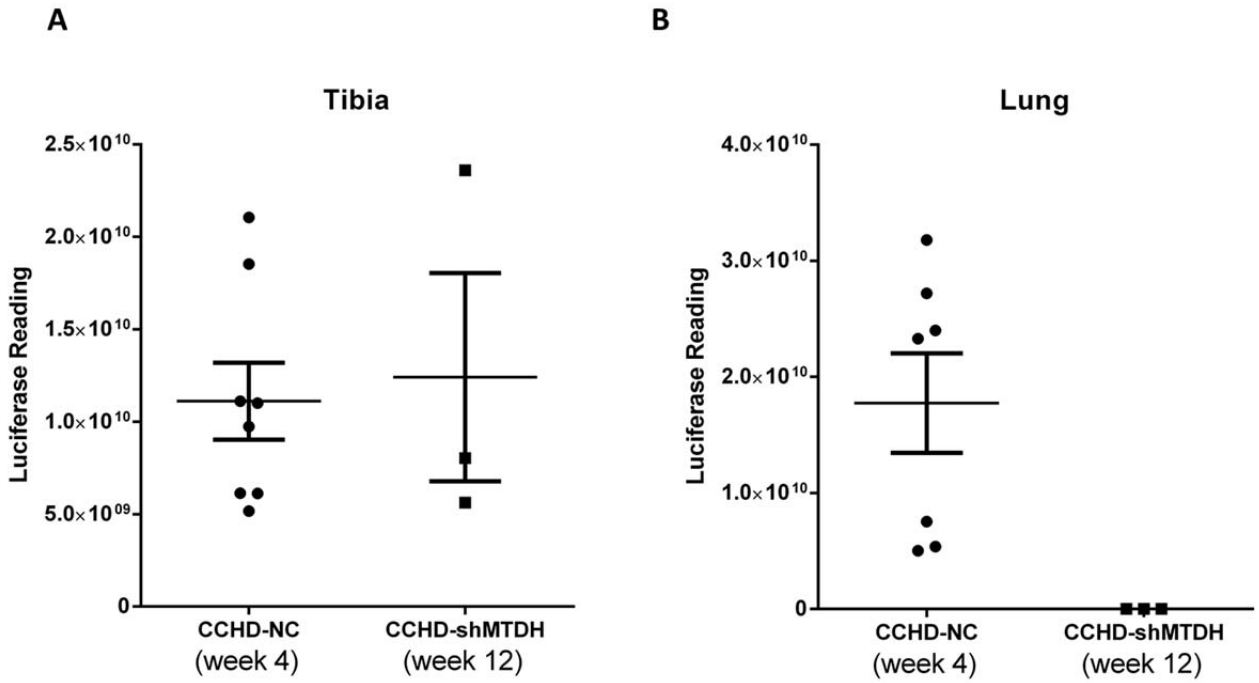


Figure 27. Inhibition of metastasis observed in MTDH-knockdown group is due to decreased metastatic ability of osteosarcoma cells. Quantification of the bioluminescent signals emitted from primary tumor (A) and lung metastases (B) are shown here. Each data point represents mean ± SEM of 7 mice in control group and 3 mice in CCHD-shMTDH group.

Metastasized osteosarcoma cells have upregulated MTDH expression

Immunohistochemical analysis of primary and metastatic tumors harvested from mice of control and MTDH-knockdown groups demonstrated that MTDH expression was dramatically enhanced in pulmonary metastatic nodules in comparison to that in primary tumors (Figure 28). When we examined the tissue samples from the four mice that developed lung metastasis in MTDH-knockdown group, we found that the rare metastatic nodules demonstrated re-expression of MTDH, while MTDH expression was still suppressed in primary tumors.

Figure 28

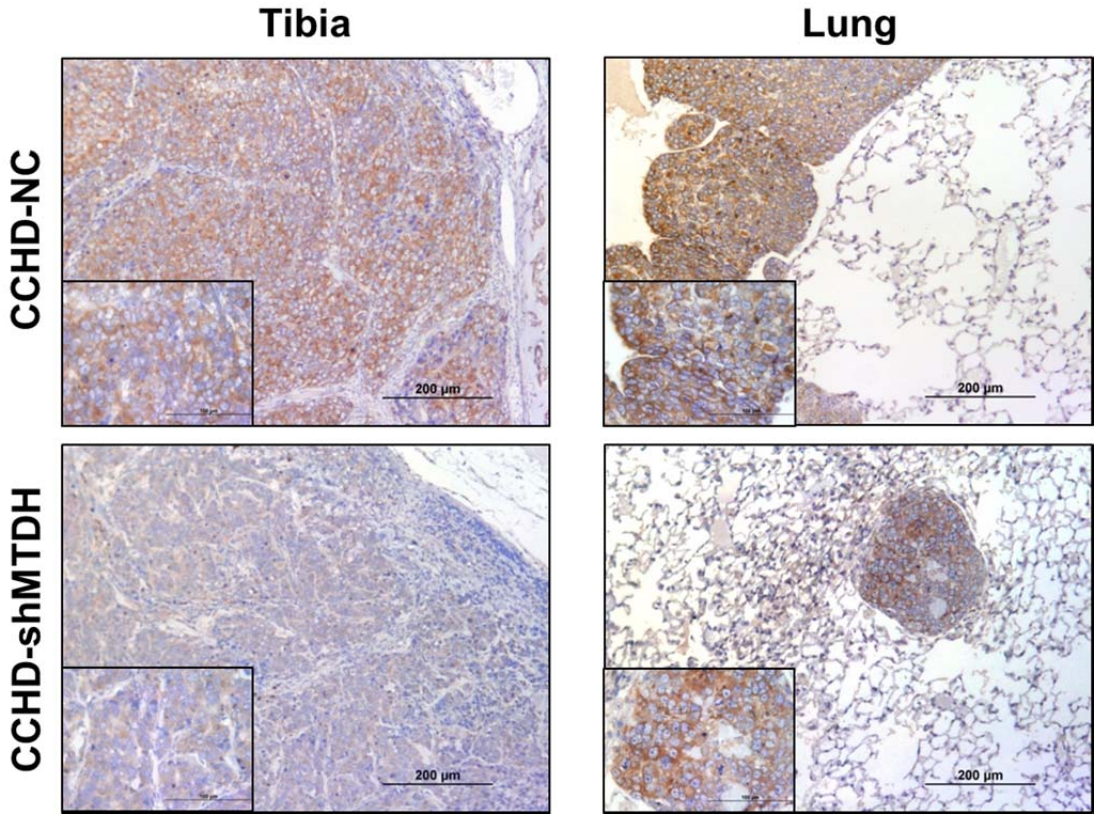


Figure 28. Metastasized osteosarcoma cells display upregulated MTDH expression. Representative examples of MTDH-immunostaining intensity in primary tumors and lung metastases are shown here. Images of higher magnification are displayed in the bottom left corner.

Summary

Based on conclusion from Chapter 5 that MTDH promotes osteosarcoma cell migration and invasiveness in vitro, we hypothesized that knockdown of MTDH would inhibit pulmonary metastasis of osteosarcoma in vivo. To test this hypothesis, we used non-invasive bioluminescence imaging to investigate the role of MTDH in tumor progression and metastasis in an orthotopic osteosarcoma xenograft mouse model.

By comparing the intensity of bioluminescent signal emitted from tibia and lung between control mice and the mice inoculated with stable MTDH-knockdown cells, we demonstrated that downregulation of MTDH in osteosarcoma cells delayed primary tumor growth and inhibited pulmonary metastasis in vivo. Further analysis of the resected lungs revealed that MTDH knockdown decreased metastatic tumor burden and significantly reduced the number of metastatic nodules developed in lung.

Additionally, immunohistochemical analysis of primary tumor and lung sections from experimental animals revealed that osteosarcoma cells that have successfully metastasized to the lung demonstrated upregulated MTDH level. This observation is consistent with the patient data (Figure 8) obtained from osteosarcoma tissue microarray analysis presented in Chapter 3. It is worth mentioning that rare pulmonary metastases of mice bearing MTDH-knockdown tumors had re-expressed MTDH despite arising from a clonal population expressing shRNA specific for MTDH. Although the mechanism by which aggressive osteosarcoma cells overcome shRNA-mediated gene silencing is unknown, this finding suggested a pivotal role of MTDH in promoting osteosarcoma metastasis.

Chapter 7. MTDH modulates cell adhesion to ECM through interaction with Laminin

Rationale

Through functional, in vivo analyses, we have clearly demonstrated in previous chapters that knocking down endogenous MTDH by shRNA inhibits osteosarcoma invasion and metastasis. More importantly, MTDH expressed on the cell membrane has been found to play an essential role in mediating osteosarcoma cell invasiveness as our results showed that steric interference of the extracellular domain of MTDH by anti-MTDH antibody significantly decreased cell motility and inhibited cell invasion through matrigel. Since invading through the extracellular matrix is a prerequisite for tumor cell metastasis, these observations led us to reason that surface-bound MTDH mediates interaction and signal transduction between tumor cells and non-cellular protein components of the extracellular matrix (ECM), which facilitates cell motility and invasive properties.

To search for extracellular binding partners for MTDH, a series of adhesion assays were performed to evaluate the adhesion ability of osteosarcoma cells with or without MTDH inhibition to major components of ECM, including Fibronectin, Collagen, and Laminin. After we identified the candidate MTDH-interacting protein, we performed bi-directional co-immunoprecipitation to confirm the interaction. Whole cell lysates of HOS cells cultured in standard conditions or briefly treated with diluted matrigel were subjected to anti-MTDH immunoprecipitation, followed by SDS-PAGE separation and western blotting. By identifying this novel connection between MTDH and ECM, we wished to establish a new paradigm for the function of MTDH in mediating tumor cell metastasis.

Results

MTDH mediates osteosarcoma cell adhesion to ECM protein Laminin

To determine whether MTDH is involved in modulation of cell adhesion to ECM, we performed a set of adhesion assays on 96-well plates pre-coated with matrigel and different ECM proteins, comparing control and MTDH-knockdown cells (Figure 29). HOS and CCHD cells with downregulated MTDH displayed a significantly decreased adhesion to matrigel and Laminin compared with their control counterparts. However, there were no statistically significant differences in adhesion to Fibronectin and Collagen IV in both HOS and CCHD cell lines.

Since we showed that cell-surface MTDH plays a pivotal role in promoting cell invasion, we next investigated whether blocking the cell surface-bound MTDH with antibody affected the ability of osteosarcoma cells to adhere to the ECM proteins (Figure 30). MTDH-specific antibody decreased adhesion of HOS cells to matrigel, Laminin, and Fibronectin by approximately 70%, 80%, and 20% respectively as compared with that observed in the control cells treated with normal IgG (Figure 30A). Similar results were seen with CCHD cells, with 70%, 90% and 50% reduction in cell attachment to matrigel, Laminin, and Fibronectin in the presence of MTDH-blocking antibody (Figure 30B). Meanwhile, adhesion of the two cell lines to Collagen IV was not significantly altered by anti-MTDH antibody. These results suggested that the surface-bound MTDH plays a key role in mediating adhesion of cancer cells to the ECM. This effect seemed likely to be achieved through interaction with Laminin, which is a major component of the basement membrane (100).

Figure 29

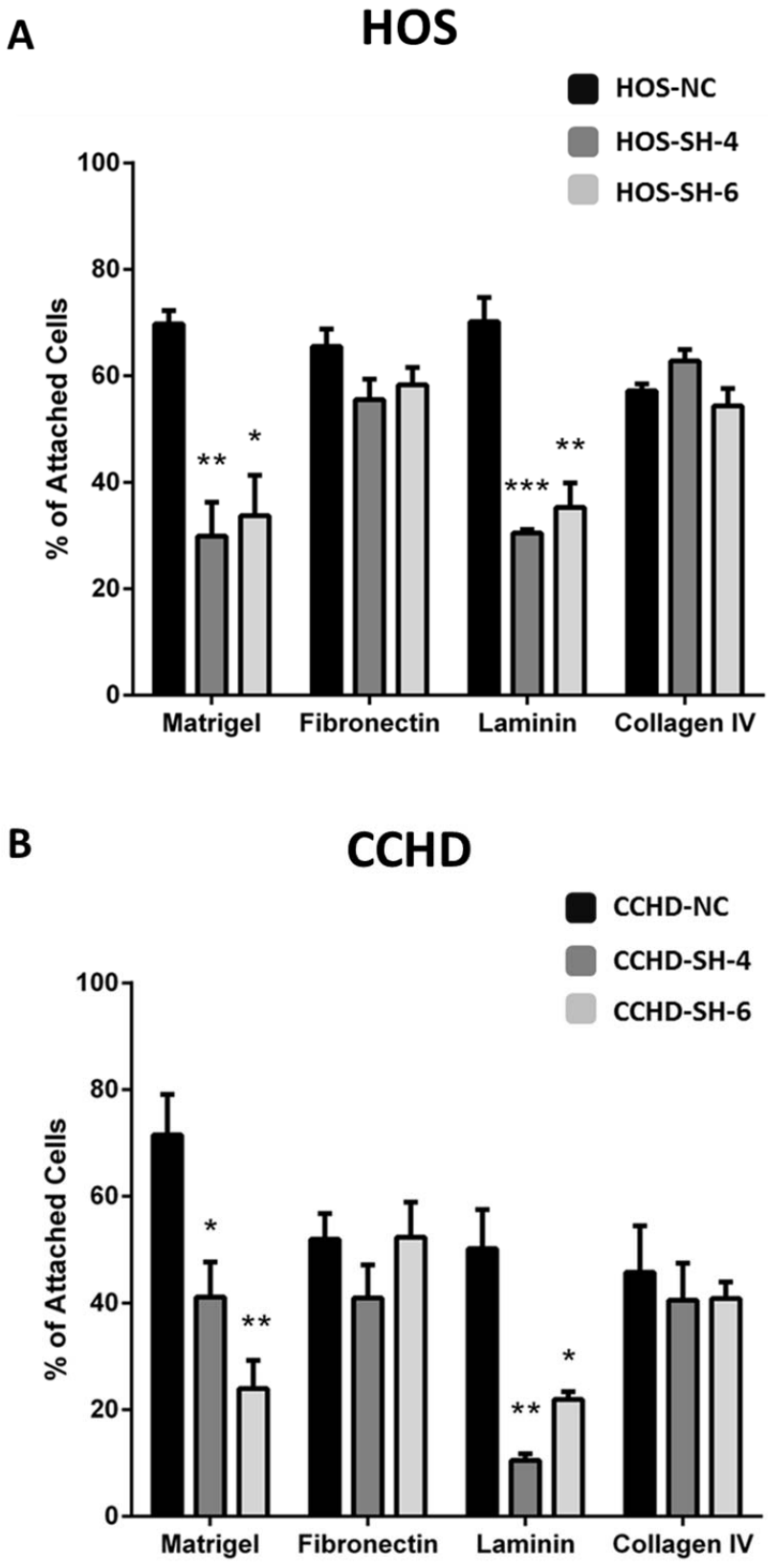

A
HOS
HOS-NC
HOS-SH-4
HOS-SH-6
% of Attached Cells
100
80
60
40
20
0
**
*
***
**
Matrigel
Fibronectin
Laminin
Collagen IV
B
CCHD
CCHD-NC
CCHD-SH-4
CCHD-SH-6
% of Attached Cells
100
80
60
40
20
0
*
**
**
*
Matrigel
Fibronectin
Laminin
Collagen IV

Figure 29. Downregulation of MTDH decreases osteosarcoma cell adhesion to matrigel and Laminin. HOS (A) and CCHD (B) cells were harvested, suspended in serum-free DMEM medium, and seeded in triplicate onto 96-well plates pre-coated with Matrigel, Fibronectin, Laminin, or Collagen IV. After 1-2h incubation, unattached cells were removed by rinsing three times with PBS while the input control groups were left unwashed. Histograms represent the percentage of cells remaining bound to each substrate compared to unwashed controls. Data represent mean ± SEM of three replicates from independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001 vs. control cells.

Figure 30.

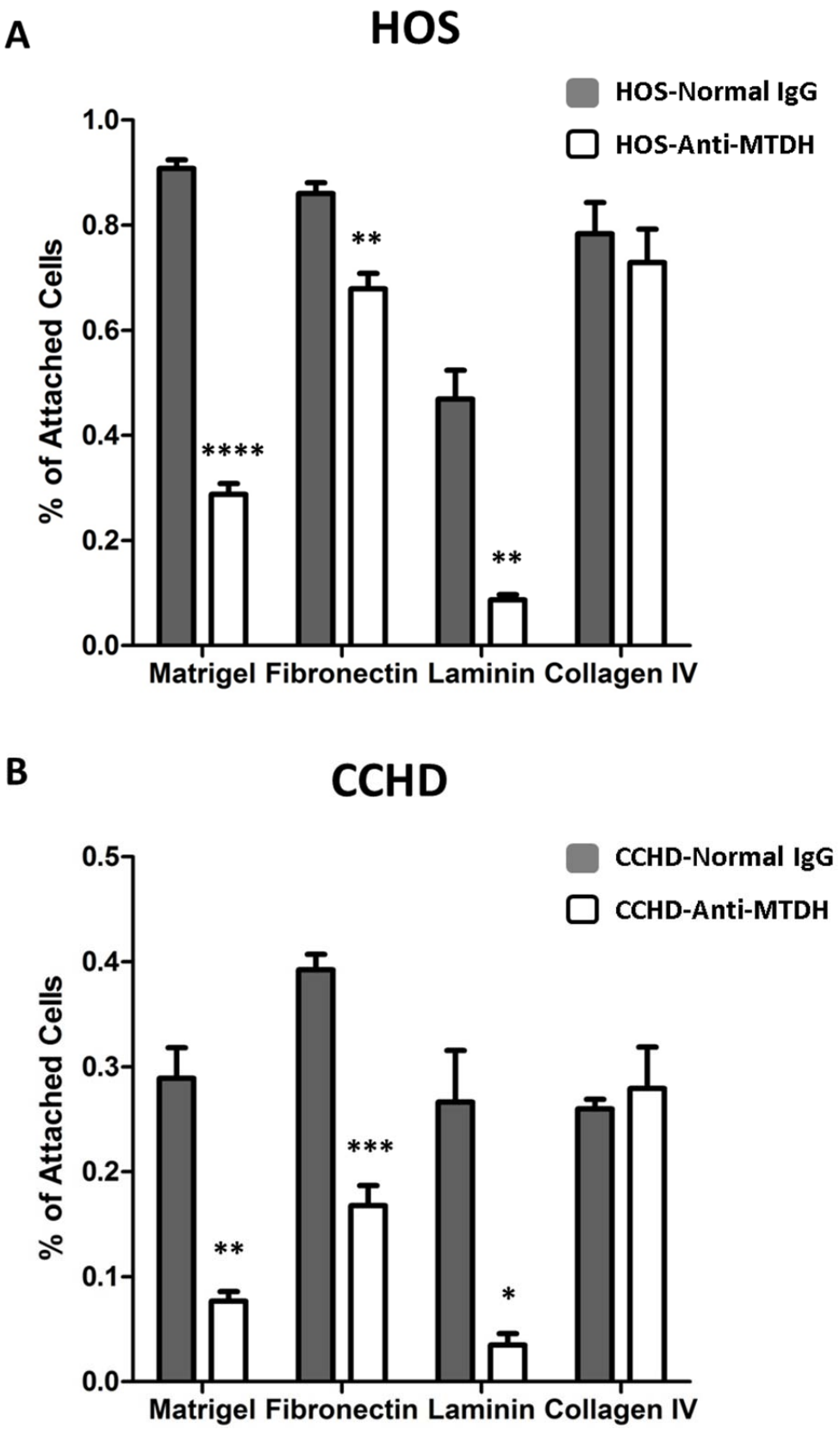

A
HOS
HOS-Normal IgG
HOS-Anti-MTDH
% of Attached Cells
1.0
0.8
0.6
0.4
0.2
0.0
****
**
**
Matrigel Fibronectin Laminin Collagen IV
B
CCHD
CCHD-Normal IgG
CCHD-Anti-MTDH
% of Attached Cells
0.5
0.4
0.3
0.2
0.1
0.0
**
***
*
Matrigel Fibronectin Laminin Collagen IV

Figure 30. Blocking cell surface MTDH inhibits osteosarcoma cell adhesion to matrigel and Laminin. HOS (A) and CCHD (B) cells were treated with rabbit anti-MTDH antibody or normal rabbit IgG at a final concentration of 7.5μg/ml. After 1-2h incubation in 96-well plates pre-coated with Matrigel, Fibronectin, Laminin, or Collagen IV, unattached cells were removed by rinsing three times with PBS while the input control groups were left unwashed. Histograms represent the percentage of cells remaining bound to each substrate compared to unwashed controls. Data represent mean ± SEM of three replicates from independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001 vs. control cells.

Identification of Laminin as an MTDH-interacting protein

To validate the interaction between MTDH and extracellular Laminin, we performed bi-directional co-immunoprecipitation (co-IP) experiments with antibodies targeting these two proteins. Whole cell lysates extracted from HOS cells cultured under standard conditions or exposed briefly to diluted matrigel were subjected to immunoprecipitation with anti-MTDH antibody. Western blot analysis with anti-Laminin antibodies demonstrated that Laminin could only be detected in the cells pre-treated with diluted matrigel (Figure 31). Reciprocal co-IP was performed with anti-Laminin antibody. Western blotting of the immunoprecipitated protein complex with anti-MTDH antibody indicated that MTDH was pulled down along with Laminin from the cells co-incubated with diluted matrigel (Figure 32). These results suggest that MTDH and Laminin sustain a strong protein-protein interaction in osteosarcoma cell model.

To determine the specificity of the interaction between MTDH and Laminin, we investigated the potential connection between MTDH and Fibronectin which is another major protein component of ECM. Whole cell lysates from control cells or cells co-incubated with diluted matrigel were subjected to anti-MTDH co-IP and probed with anti-Fibronectin antibody in western blot. We could not detect Fibronectin being co-immunoprecipitated with MTDH in this experiment (Figure 33). These data indicate that MTDH located on the cell surface regulates cell-matrix adhesion by interacting with extracellular Laminin, thus having a key role in promoting invasiveness and metastasis.

Figure 31

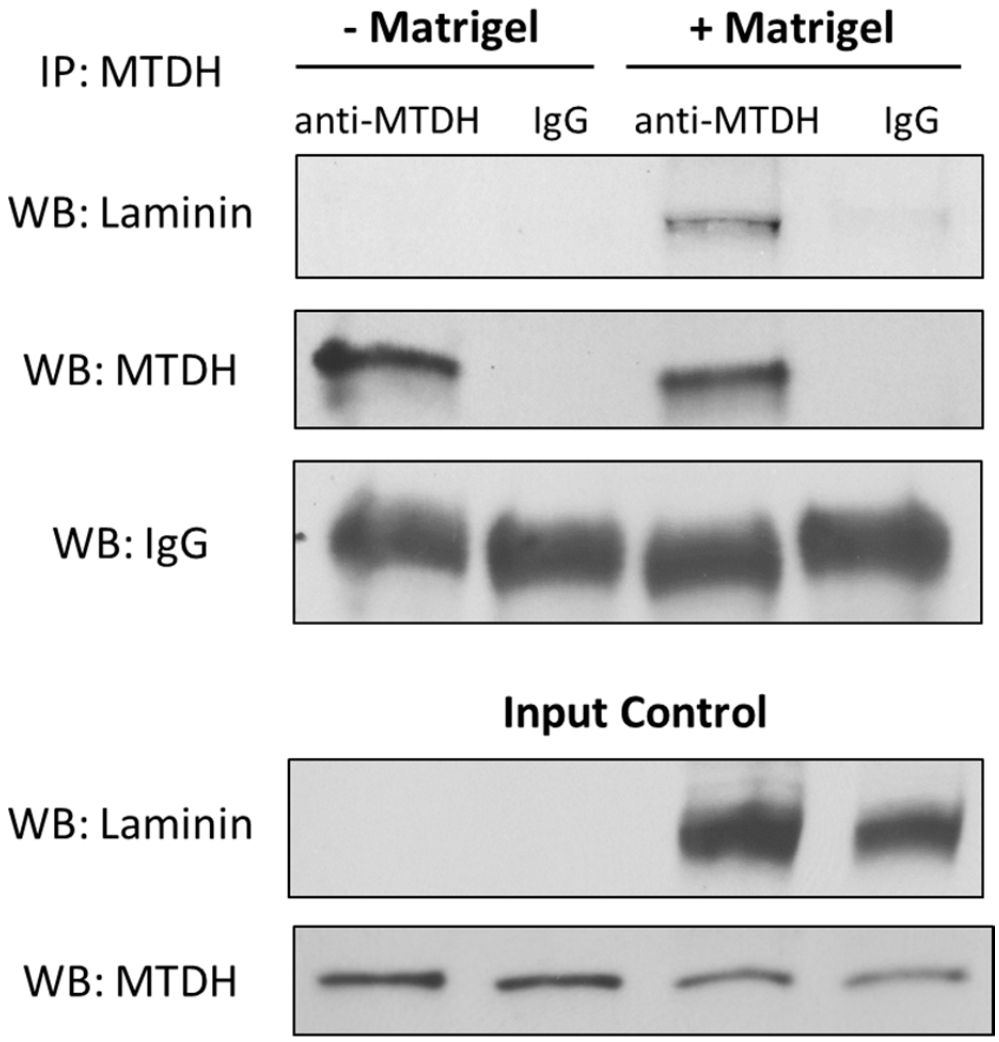


Figure 31. Identification of Laminin as MTDH-interacting partner. Lysates from HOS cells without exposure to matrigel (lane 1 and lane 2) or HOS cells briefly exposed to extracellular matrix proteins contained in matrigel (lane 3 and lane 4) were immunoprecipitated with anti-MTDH antibody or normal rabbit IgG, and immunoblotted with anti-Laminin antibody. Input control displayed at the bottom represents the protein level of MTDH and Laminin in each sample before co-IP. IP: immunoprecipitation; WB: western blot.

Figure 32

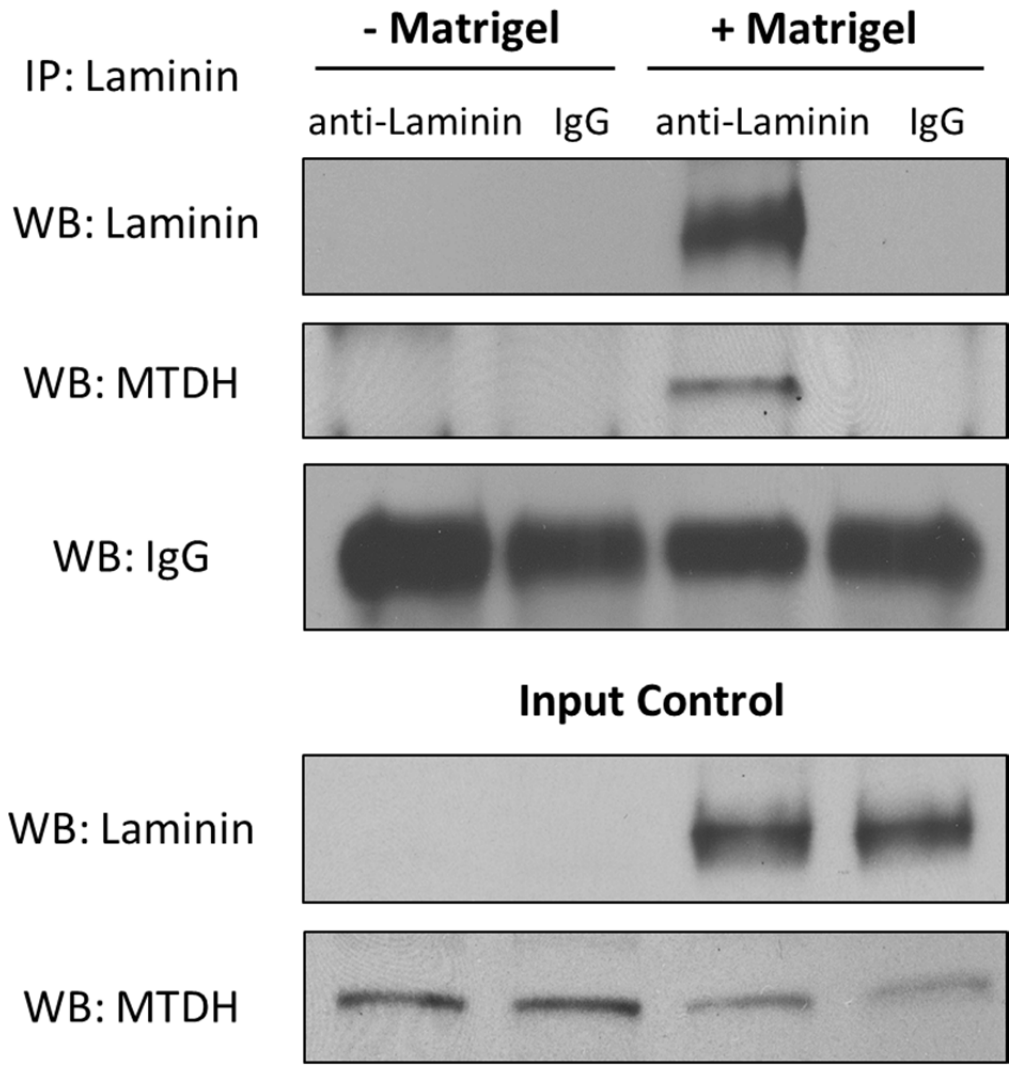


Figure 32. MTDH is pulled down with Laminin in the same protein complex in a co-IP experiment. Lysates from HOS cells without exposure to matrigel (lane 1 and lane 2) or HOS cells briefly exposed to extracellular matrix proteins contained in matrigel (lane 3 and lane 4) were immunoprecipitated with anti-Laminin antibody or normal rabbit IgG, and immunoblotted with anti-MTDH antibody. Input control displayed at the bottom represents the protein level of MTDH and Laminin in each sample before co-IP. IP: immunoprecipitation; WB: western blot.

Figure 33

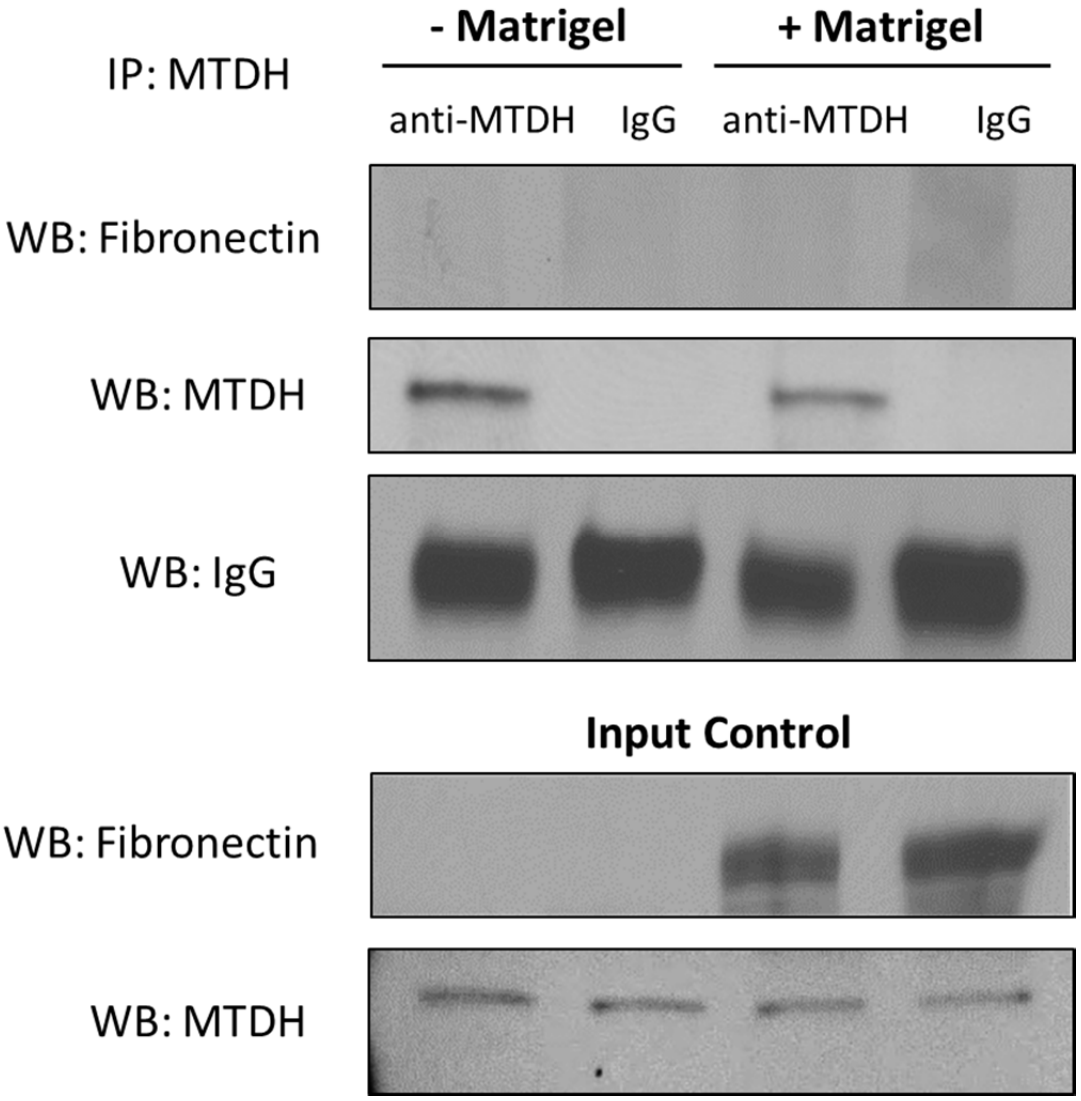


Figure 33. MTDH has no evident interaction with Fibronectin in ECM. Lysates from HOS cells without exposure to matrigel (lane 1 and lane 2) or HOS cells briefly exposed to extracellular matrix proteins contained in matrigel (lane 3 and lane 4) were immunoprecipitated with anti-MTDH antibody or normal rabbit IgG, and immunoblotted with anti-Fibronectin antibody. Input control displayed at the bottom represents the protein level of MTDH and Fibronectin in each sample before co-IP. IP: immunoprecipitation; WB: western blot.

Summary

In this chapter we hypothesized that MTDH expressed on the cell surface modulates cell-ECM interactions by binding to non-cellular ECM proteins. We propose that the protein-protein interaction between surface-bound MTDH and extracellular ECM proteins promotes tumor cell adhesion to ECM, therefore facilitating cell migration and invasion through ECM and basement membrane barriers. To test this hypothesis and identify the candidate interacting-partner of MTDH, we evaluated cell adhesive ability to matrigel and various ECM protein components in HOS and CCHD cells with constitutive MTDH knockdown or blockade of MTDH at the cell surface, followed by a bi-directional co-IP.

Through a series of cell adhesions assays, we observed significantly decreased cell adhesion to matrigel and Laminin in MTDH-knockdown osteosarcoma cell lines as compared to control osteosarcoma cells after 1-2 hours of incubation time. Interestingly, MTDH-knockdown cells showed very little difference in cellular adhesive ability to Fibronectin and Collagen IV when compared to the control cells. Similarly, we saw a remarkable reduction in cellular adhesion to marigel and Laminin for HOS and CCHD cells treated with antibodies targeting the extracellular domain of MTDH in relation to the control cells co-incubated with equal concentrations of normal IgG. A modest decrease in adhesion to Fibronectin was observed in the antibody-blocking experiments while no difference was detected in cell adhesion to Collagen IV. These results suggest that Laminin could be the candidate protein involved in the interaction between cell surface MTDH and the ECM. Bi-directional co-IP with anti-MTDH antibody and anti-Laminin antibody confirmed that these two proteins exist in the same protein complex and sustain a strong protein-protein interaction. The fact that Fibronectin, another major component of ECM, could not be co-

immunoprecipiated with MTDH supported the specificity of the interaction between MTDH and Laminin. By establishing this novel connection between MTDH and extracellular Laminin, our research not only confirms the importance of the cell surface localization of MTDH in osteosarcoma, but also provides a mechanism that is involved in the metastasis-promoting actions of MTDH.

Chapter 8. Discussion: Implications of Results and Future Directions

Implications of Results

Accumulating evidence suggests that the aberrant MTDH expression observed in many malignant tumors is often correlated with poor clinical outcomes (68, 70, 78, 80-83). The critical role of MTDH in tumor cell proliferation, chemoresistance, angiogenesis, invasion and metastasis has been verified by a number of functional studies performed in various cancer models (63, 69, 70, 73, 74, 77, 78, 84, 85, 90). However, most of the recent studies about MTDH focus exclusively on common adult cancers such as breast cancer and liver cancer. There was no report on expression status of MTDH in osteosarcoma when we started this project. Since the most common cause of mortality associated with osteosarcoma is pulmonary metastasis, identification and characterization of new molecular targets for the development of anti-metastasis therapeutic strategies will be valuable for treating this disease. Moreover, the mechanism of how MTDH mediates tumor cell aggressiveness and metastasis remained largely unknown. To extend our understanding of the biological functions of MTDH and to identify novel therapeutic targets for osteosarcoma, we established three objectives for our research: 1) investigating the expression pattern of MTDH in osteosarcoma patient samples and its relationship to metastatic risk and patients' outcome in clinic; 2) identifying the function(s) of MTDH in tumor progression and metastasis of osteosarcoma; 3) elucidating the mechanism(s) by which MTDH mediates tumor aggressiveness. To achieve these three goals, we conducted a series of studies using clinical datasets and specimens, osteosarcoma cell models, and an orthotopic osteosarcoma xenograft mouse model. In the present study we have shown that MTDH, a cell surface protein correlated with worse outcome in osteosarcoma and many other cancers, plays a decisive role in multiple steps of tumor metastasis.

MTDH is widely expressed in osteosarcoma and correlates with metastasis

We have demonstrated that MTDH is highly expressed in both osteosarcoma cell lines and patient tumor specimens. Western blot and Q-PCR analyses of a panel of six osteosarcoma cell lines and the normal control cell line derived from human fetal osteoblasts revealed that osteosarcoma cells had upregulated MTDH expression both at the protein and mRNA levels (Figure 11 and 12). Evaluation of MTDH expression in a tissue microarray containing 73 osteosarcoma samples showed that MTDH could be detected in 63% of primary osteosarcoma tumors and 88% of metastatic tumors specimens (Figure 7 and Table 1). More importantly, after examining IHC staining intensities of MTDH in 9 matched pairs of primary and metastatic osteosarcoma specimens, we found that MTDH expression was remarkably increased from primary to metastatic tumors in the same patient (Figure 8 and Table 2). Similarly, we observed in three other clinical datasets of melanoma and prostate cancer that metastatic tumor lesions had significantly higher level of MTDH expression than primary tumor specimens (Figure 4)

In support of this notion, analyses of survival data and related clinical outcomes in melanoma and breast cancer datasets revealed that patients with high MTDH expression developed metastatic disease earlier and had a shorter overall survival than the low-MTDH group (Figure 5 and 6). Comparative analysis of metastasis-free survival in a cohort of high-grade osteosarcoma patients has also demonstrated that MTDH overexpression correlated with a higher risk of metastatic relapse and is associated with worse clinical outcome (Figure 9 and 10). These results strongly indicated that MTDH may act as a metastasis-promoting gene in various cancers including osteosarcoma, and could be an ideal prognostic biomarker for predicting osteosarcoma metastasis. The fact that the trend toward reduced overall

survival in the MTDH-high cohort did not reach statistical significance probably reflects the small sample size (n=71 total). The analysis should be repeated with a larger number of clinical samples when they become available for osteosarcoma.

MTDH promotes osteosarcoma metastasis by modulating cell motility and invasiveness

The biological process of invading and filtrating into the surrounding ECM and the basement membrane is the first step of cancer metastasis. Therefore, the metastatic potential of a tumor cell is usually dependent on its motility and invasive ability. Previous studies in glioma and head and neck squamous cell carcinoma have demonstrated that MTDH promotes proteolytic digestion of the connections between tumor cells and ECM through activation of the MMPs including MMP1, MMP2 and MMP9 (70, 71, 84). Our data are consistent with recent findings in other tumor models showing that MTDH promotes metastatic dissemination of tumor cells through modulating cell migration and invasion.

Both in vitro and in vivo studies clearly demonstrated that blocking MTDH is an effective way to inhibit the metastatic process, at least in these osteosarcoma models. Silencing endogenous MTDH by shRNA abrogated in vitro migration and invasion of human osteosarcoma cells without severely affecting their proliferation and clonogenic ability (Figure 17-20). In vivo experiments performed in an osteosarcoma mouse model of spontaneous pulmonary metastasis demonstrated that constitutive knockdown of MTDH delayed primary tumor growth and inhibited pulmonary metastasis (Figure 24-26). In addition, we saw a remarkable increase in MTDH expression in metastatic lesions in the lung as compared to the primary tumors resected from the mouse tibia (Figure 28), which is similar to what has been observed in clinical osteosarcoma samples (Figure 8). Overall our

data indicated a key role of MTDH in inducing the invasive properties in osteosarcoma cells. These findings have important clinical implications because MTDH, as a pivotal metastasis-associated gene overexpressed in osteosarcoma, could be an ideal molecular target for preventing and treating metastatic disease.

Functional significance of the cell surface localization of MTDH

Regarding the mechanism by which MTDH mediates metastasis, Brown and Ruoslahti had proposed that cell-surface MTDH contains an extracellular lung-homing domain which mediates pulmonary metastasis by binding to an unknown ligand specifically expressed on lung endothelial cells (63). This report was the first to indicate the importance and potential functions of MTDH expressed on the surface of tumor cells. By using flow cytometry with an antibody reactive to amino acid residues 315-461 on the C-terminal segment of MTDH, we confirmed that MTDH localizes to the cell surface and has type II membrane topology in osteosarcoma (Figure 14 and 15). Our data demonstrated that osteosarcoma cells displayed abundant level of MTDH on cell surface as compared to the control osteoblasts (Figure 14).

It is known that cell-surface proteins are often involved in mediating interactions between cells and its microenvironments. The cell-surface localization of MTDH in osteosarcoma provided revealing clues about its function and narrowed down the list of potential proteins that it may interact with. Through a series of in vitro functional studies, we have demonstrated that the cell surface distribution of MTDH is critical for its metastasis-promoting function in osteosarcoma. Steric interference of the biological functions of the cell surface MTDH with antibodies targeting the extracellular C-terminal segment could efficiently inhibit cell migration, invasion, and adhesion to the ECM. Altogether, our

preliminary data in osteosarcoma cell models indicated that the invasion-promoting functions of surface-bound MTDH are independent of endothelial cell binding, which is inconsistent with the model proposed in Brown and Ruoslahti's paper that MTDH contains an extracellular lung-homing domain.

MTDH functions as a cell surface receptor for Laminin

The processes of invasion and metastasis involve a complex cascade of events. One important property of metastatic tumor cells is the ability to attach to and migrate through basement membranes which comprise barriers against invading cells. Tremendous effort has been made in the past to define the role of proteases in degrading tumor matrix and surrounding tissue planes, but the mechanisms underlying tumor cell recognition, attachment to, and manipulation of these non-cellular stromal components remain incompletely characterized.

Through a set of cell adhesion assays using osteosarcoma cells transduced with MTDH-specific shRNA or scrambled control sequence, we found that inhibition of endogenous MTDH resulted in significantly decreased adhesion to marigel and Laminin in vitro, while cell attachment to Fibronectin and Collagen IV was not affected (Figure 29). Similar effects were observed when we blocked the surface-bound MTDH with the anti-MTDH antibodies (Figure 30). Considering the fact that matrigel is a gelatinous mixture of ECM proteins that resembles the complex extracellular microenvironment, MTDH may serve as an important mediator of cell-ECM interactions. The impaired cell adhesion to Laminin observed when MTDH was inhibited further suggested that Laminin could be the extracellular binding partner of MTDH. This hypothesis was validated by the bi-directional

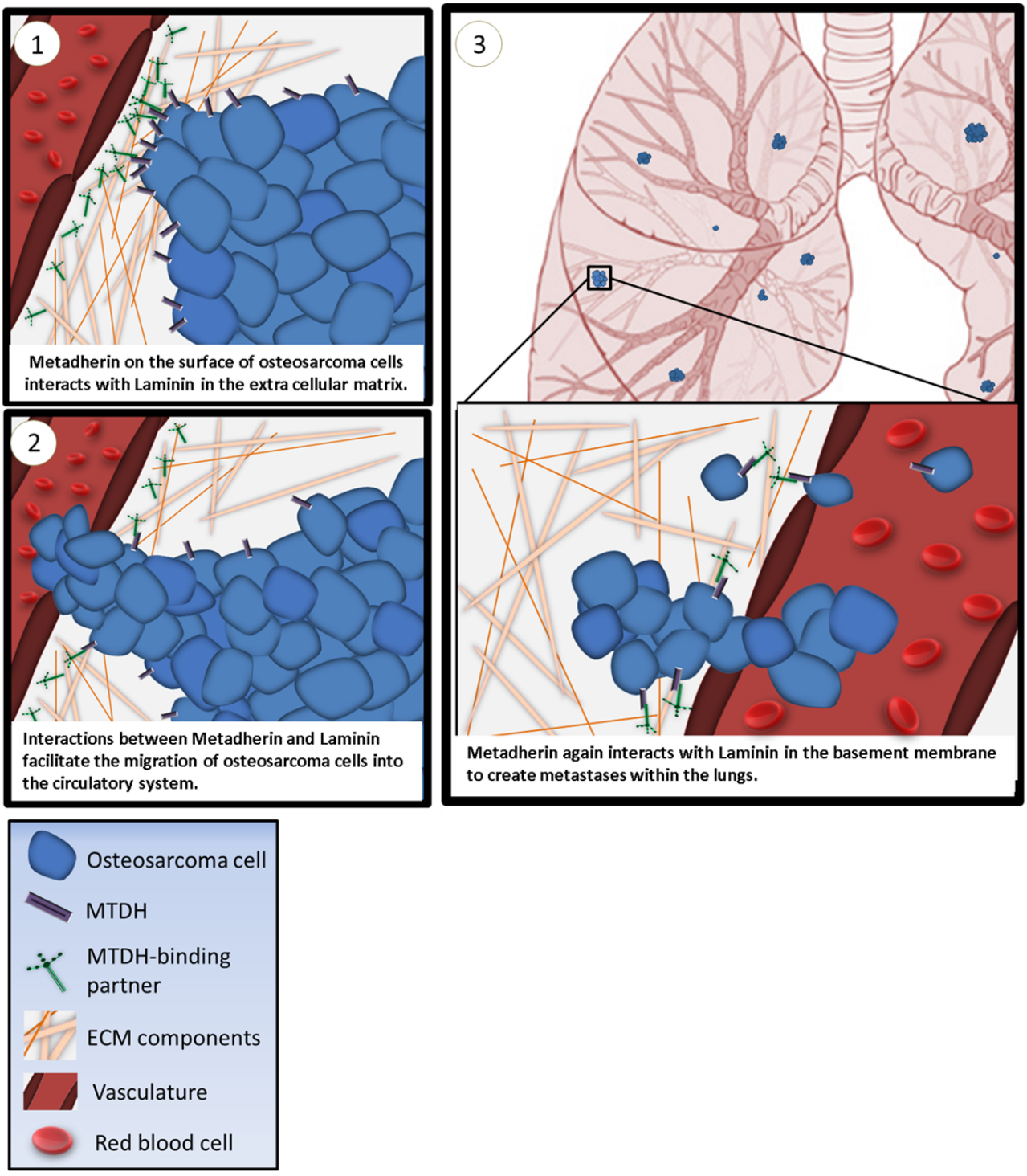
co-immunoprecipitation performed with anti-MTDH and anti-Laminin antibodies, which demonstrated that MTDH and Laminin exist in the same protein complex when tumor cells interact with ECM (Figure 31 and 32). Our current study provides the first evidence that MTDH is a key regulator of cell-matrix adhesion in osteosarcoma, through which cell motility and invasiveness could be directly affected. Moreover, we have identified MTDH as a novel cell-surface interacting partner for extracellular Laminins. It will be of future interest to determine the region of MTDH that mediates binding with Laminin, which will enable better characterization of this interaction.

Laminins, a family of secreted glycoproteins, are major components of extracellular matrix and basement membranes (100). They are composed of three different subunits, the α-chain, β-chain, and γ-chain arranged in a cruciform shape (101). Laminins could form independent protein networks through connections with other ECM proteins such as Collagen IV, Enactin, Fibronectin, and Perlecan (102). They could also interact with cells through binding with the cell surface proteins including integrin receptors and various glycoproteins (102). Through these interactions, Laminins have diverse biological functions including promoting cell adhesion and migration, initiating cell signaling pathways, and mediating cell differentiation and polarity (103). The significance of Laminin in tumor metastasis is well characterized (104). There is further evidence in osteosarcoma supporting a positive relationship between cell invasiveness and its ability to attach to Laminin (105, 106). By discovering the new connection between MTDH and extracellular Laminin in osteosarcoma, we improve our understanding of the mechanisms by which MTDH promotes metastasis. More importantly, these findings will establish a new paradigm for the function of MTDH in mediating tumor cell invasiveness. It is known that cancer cells need to invade

through basement membranes multiple times during their metastatic spread: starting with initial escape from the primary tumor, followed by intravasation and extravasation at secondary sites (107). For osteosarcoma, like many solid tumors, the spreading process also involves invasion into adjacent soft tissues and muscles. By incorporating this information into our present findings, here we propose a new model for the function of MTDH in metastasis (Figure 34).

Figure 34. Schematic model of MTDH function. In the upper left panel, a mass of tumor cells (blue) is seen extending via mass migration through extracellular matrix proteins and stromal elements toward a nearby blood vessel. Laminin, normally present predominantly in the basement membrane, is depicted just outside endothelial cells. MTDH mediates attachment to the basement membrane and orients the migration of cells toward the vessel. In the lower left panel, tumor cells have breached the endovascular space and access the bloodstream. On the right, the upper panel depicts lungs with metastatic tumors, and the lower panel depicts how MTDH-Laminin interactions might help mediate exit from the endovascular space once the pulmonary endothelial lining is disrupted.

Figure 34


1
Metadherin on the surface of osteosarcoma cells interacts with Laminin in the extra cellular matrix.
2
Interactions between Metadherin and Laminin facilitate the migration of osteosarcoma cells into the circulatory system.
3
Metadherin again interacts with Laminin in the basement membrane to create metastases within the lungs.
Osteosarcoma cell
MTDH
MTDH-binding partner
ECM components
Vasculature
Red blood cell

Future Directions

Mechanistic Studies

Since we have identified the connection between MTDH and Laminin when tumor cells interact with the extracellular microenvironments, alternative strategies for blocking the interactions between MTDH and Laminin may provide additional approaches for preventing metastatic progression in addition to targeting MTDH itself. Further mechanistic studies to define the protein domain structures, interactions and functions of MTDH are warranted. Our next step will be to determine if this interaction is governed by a direct physical association or mediated by another protein located on the cell surface or inside the ECM. To address this question, fluorescence resonance energy transfer (FRET) microscopy or bimolecular fluorescence complementation (BiFC) could be employed to display whether a close physical association between MTDH and Laminin exists in osteosarcoma.

Future experiments will include mapping the specific protein domain on MTDH that is responsible for binding with extracellular Laminins through a series of protein truncation and site mutagenesis approaches. We are currently collaborating with the Center for Biomolecular Structure and Function of MD Anderson Cancer Center to develop purified protein fragments of MTDH. We propose to test each of these protein fragments for their ability to block the binding of osteosarcoma cells to Laminin. We will then use smaller fragments of the portion that blocks the binding to map out the region on MTDH that mediates the binding of the whole molecule to Laminin. These results will provide grounds for the development of therapeutic strategies that interferes with the interaction between MTDH and ECM. In the meantime, we plan to perform a yeast two-hybrid screening with

full-length MTDH cDNA and cDNA library from human osteosarcoma cell to identify potential intracellular MTDH-interacting proteins.

We recognize that the interaction between MTDH and Laminin might be mediated by other cell-surface proteins or ECM constituents. Previous studies have shown that integrins, a family of integral membrane glycoproteins that mediate cell-cell and cell-ECM interactions, are the most common receptors for Laminins in various cell systems (108). The α, β, and γ-chains of Laminin have all been shown to possess integrin-binding sites (108). Clustering of integrins on cell surface has been shown to promote adhesion, migration, invasion, and survival of tumor cells both in vitro and in vivo in a variety of human cancers (109-111). Knowing the essential function of integrins in tumor malignancy and their close association with Laminins, we want to investigate whether integrins are involved in the interaction between MTDH and Laminin. Since the expression pattern of integrins in osteosarcoma remains unclear, we will determine the expression level of various integrins in osteosarcoma cells by q-PCR and western blot, followed by co-immunoprecipitation experiments to study the association among MTDH, Laminins, and integrins.

Translational Implications

Our results collectively suggest that overexpression of cell-surface protein MTDH promotes metastasis in osteosarcoma. The therapeutic potential of manipulating MTDH is highlighted by the fact that the overall expression of this protein is dramatically upregulated, not only in osteosarcoma as shown here, but also in many other solid tumors (60, 63, 65, 70-77). More importantly, by identifying its cell surface localization we have provided

promising evidence that this protein will be easily accessible to therapeutic agents, which strongly supports the development of targeted therapies against MTDH.

During the past 25 years, the therapeutic antibody technologies have undergone considerable development (112). Chimeric, humanized, and completely human monoclonal antibodies are gradually becoming important treatment options for cancer patients (113). These monoclonal antibody products, either in unconjugated forms or linked to cytotoxic molecules such as conventional chemotherapeutic agents, prodrug-converting enzymes, radioisotopes, and natural toxins, could recognize cell surface antigens that are specifically expressed on cancer cells and facilitate localized delivery and release of the toxic agents to the tumor sites (114-116). With the aid from the activated host immune system, therapeutic antibodies are able to induce a series of cytotoxic events such as cell cycle arrest and apoptosis to achieve clinical responses (115). Due to the high specificity and efficacy of antibody-based therapeutics, six types of these drugs have been approved by the FDA for the treatment of cancer (112). Knowing that MTDH is localized to the cell membrane as a type II transmembrane protein in osteosarcoma, its extracellular C-terminal segment which contains 510 amino acid residues could be an ideal target for antibody-based immunotherapy. We expect the development of humanized monoclonal anti-MTDH antibody to improve the current therapeutic regimens for osteosarcoma patients and extend their survival.

Another approach to target MTDH in clinic is to develop chimeric antigen receptor (CAR)-based therapies. This novel type of immunotherapy which incorporates the exquisite specificity of monoclonal antibodies with the long-term persistence of cytotoxic T cells has shown clinically-significant anti-tumor activities in a number of adult and pediatric malignancies (117, 118). In addition, current clinical trials in adults and pediatric patients

have demonstrated that the CAR therapy has high specificity and limited off-tumor toxicity for both short-term and long-term treatments (119-121). Since the efficacy of CAR therapy depends on the affinity of T cells to tumor cell-surface antigens, an ideal targeted protein should have abundant expression on the surface of tumor cells while having limited expression on normal tissue. Considering the discrete overexpression of MTDH in numerous cancer types, potential MTDH-targeted CAR therapy will have a wide range of applications in treating solid tumors in which effective control of metastasis is pivotal in improving clinical outcomes.

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VITA

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