Olfactomedin 4 suppresses prostate cancer cell growth and metastasis via negative interaction with cathepsin D and SDF-1

Ling Chen1, Hongzhen Li1, Wenli Liu1, Jianqiong Zhu1, Xiongce Zhao2, Elizabeth Wright2, Liu Cao3, Ivan Ding3 and Griffin P. Rodgers1,2,∗

1Molecular and Clinical Hematology Branch, National Heart, Lung and Blood Institute and 2National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA. 3Key Laboratory of Cell Biology of Ministry of Public Health, China Medical University, Shenyang, China and 4Translational Research Program, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA.

The human olfactomedin gene (OLF4M) encodes an olfactomedin-related glycoprotein. OLFM4 is normally expressed in a limited number of tissues, including the prostate, but its biological functions in prostate are largely unknown. In this study, we found that OLFM4 messenger RNA was reduced or undetectable in prostate cancer tissues and prostate cancer cell lines. To study the effects of OLFM4 on prostate cancer progression, we transfected PC-3 prostate cancer cells with OLFM4 to establish OLFM4-expressing PC-3 cell clones. The OLFM4-expressing PC-3 cell clones were found to have decreased proliferation and invasiveness compared with vector-transfected control PC-3 cells in vitro. In addition, nude mice injected with OLFM4-expressing PC-3 cells demonstrated reduced tumor growth and bone invasion and metastasis compared with mice injected with vector-transfected control cells. Mechanistic studies revealed that OLFM4 may exhibit its anticancer effects through regulating cell autophagy by targeting cathepsin D, as OLFM4 reduced cathepsin D protein levels and enzymatic activity and attenuated cathepsin D-induced cancer cell proliferation. In addition, overexpression of OLFM4 abrogated stromal cell derived factor-1 (SDF-1)-induced PC-3 cell invasiveness in a Matrigel invasion assay partially through blocking SDF-1-mediated AKT phosphorylation. Coimmunoprecipitation and immunofluorescence staining studies in OLFM4-expressing PC-3 cells demonstrated a direct interaction between OLFM4 and cathepsin D or SDF-1. Taken together, these results suggest that OLFM4 negatively interacts with cathepsin D and SDF-1 and inhibits prostate cancer growth and bone metastasis.

Introduction

Prostate cancer is the most commonly diagnosed solid tumor and the second leading cause of cancer-related deaths in American men (1), largely due to invasion and metastasis. Proteinases and cytokines/chemokines are major factors participating in this organ-specific metastatic progression. Cathepsins are lysosomal proteinases that are involved in the proliferation, invasion and metastasis of cancers (2–5). Overexpression of cathepsin D, a tumor autocrine mitogen, has been reported in invasive prostate cancer and found to be associated with a poor prognosis (6–8). Several groups of chemokines have also emerged as important mediators of prostate cancer invasion and metastasis. Chemokines mediate tumor cell adhesion, migration and invasion during metastasis to specific organs as they do in inflammation (9). The chemokine stromal cell derived factor-1 (SDF-1) and its receptor, CXCR4, have been found to be broadly involved in the development and progression of a variety of cancers (10), including prostate cancer (11). SDF-1/CXCR4 axis-mediated angiogenesis (12,13) and bone metastasis have been reported in prostate cancer (14), as well as in other cancer types (15,16). Blocking of SDF-1/CXCR4 signaling by antagonists, such as specific antibodies targeting CXCR4, has shown promise in the prevention and treatment of prostate cancer metastasis (17).

OLF4M (human olfactomedin 4, also known as hGC-1, GW112 and hOlf D), was first cloned from myeloid precursor cells after granulocyte colony-stimulating factor treatment (18). OLFM4 messenger RNA (mRNA) is expressed mainly in the prostate, stomach, pancreas, small intestine, colon and bone marrow (18,19). We recently found that OLFM4 protein is a secreted glycoprotein that facilitates cell adhesion via interaction with lectins and cadherin on the cell surface (20). Increased expression of OLFM4 mRNA was detected in the crypt epithelium of inflamed colonic mucosa in inflammatory bowel diseases (21) and in gastric biopsies from patients and mice infected with Helicobacter pylori (22,23). High levels of OLFM4 mRNA have also been detected in lung (24), breast (24), gastric (25) and pancreatic cancer (26), as well as in colorectal adenomas and cancerous tissues (24,27,28). However, using a specific OLFM4 antibody, we found that OLFM4 protein is predominantly overexpressed in well-differentiated gastric and colon cancer, but not in poorly differentiated or metastatic lesions (29,30). Therefore, OLFM4 protein expression is probably cell or tissue dependent (31). Previous work has demonstrated that OLFM4 interacted with genes associated with retinoic-interferon-induced mortality (GRIM)-19 and attenuated retinoic acid-interferon β-induced apoptosis in HeLa cells and that ectopic expression of human OLFM4 in mouse Tramp-C1 cells promoted tumor growth in syngeneic C57/B16 mice (32). However, the biological functions of OLFM4 in human prostate cancer remain largely unknown.

In this study, we found that OLFM4 expression was significantly reduced in human prostate cancer tissues and prostate cancer cell lines. Ectopic OLFM4 expression inhibited human prostate cancer cell proliferation, growth and invasion and inhibited cancer cell bone metastasis in vivo. Lastly, a series of mechanistic studies revealed that the tumor-suppressive effects of OLFM4 are associated with cell autophagy and a reduction of cathepsin D and SDF-1 via a physical binding with OLFM4.

Herein, we report that both cathepsin D and SDF-1 could be targeted by the novel tumor suppressor OLFM4, resulting in inhibition of prostate cancer cell growth and bone metastasis in vitro and in vivo.

Materials and methods

Human prostate cell lines and tumor tissues

Human primary normal prostate epithelial (PRC, passage 2) and stromal (PRSC, passage 4) cells were purchased from Clonetics® and were cultured in the recommended media including basal medium and singleQuots. The human prostate cancer cell lines LNCaP, DU145, VCaP and PC-3, the breast cancer cell line MCF7 and promyelocytic leukemia cells were obtained from American Type Culture Collection, Manassas, VA and maintained in RPMI 1640 medium with 10% fetal bovine serum. Cell growth was determined either by cell number counting or by using the Cell titer 96 Aqueous Cell Proliferation Kit (Promega, Madison, WI). Frozen human benign prostatic hyperplasia or prostate cancer tissues and matched normal tissues were obtained from surgically resected materials from the University of Pennsylvania Medical.

Abbreviations: mRNA, messenger RNA; PCR, polymerase chain reaction; SDF-1, stromal cell derived factor-1; siRNA, small interfering RNA.

These authors contributed equally to this work.

Published by Oxford University Press 2011.
OLFM4 suppresses prostate cancer growth and metastasis

Center and Cooperative Human Tissue Network (Philadelphia, PA; IRB#94-H0010).

RNA preparation and reverse transcription–polymerase chain reaction
Total RNA was isolated from cell lines and frozen tissues using TRI Reagent® (Molecular research center, Cincinnati, OH). Reverse transcription and polymerase chain reaction (PCR) were performed as described previously (18). The semiquantitative reverse transcription–PCR for cathepsin D was done using Super Script III First Strand and Platinum PCR reagents (Invitrogen, Carlsbad, CA) and performed according to the manufacturer’s instructions. The cathepsin D-specific primers were 5’-GACACAGGACCTTCCCTCAT-3’ (forward) and 5’-CTCTGGGGCACGTTGTGAC-3’ (reverse). β-Actin was used as an internal control, with β-actin primers from Invitrogen. The PCR products were visualized on 2% agarose gels containing 0.5 μg/ml ethidium bromide.

Quantitative PCR on TissueScan Prostate Cancer Tissue 4PCR Panels (OriGene, Rockville, MD) was performed in an MX3000P detector (Agilent Technologies, Santa Clara, CA). The following parameters were used: 50°C (2 min), 95°C (10 min), followed by 45 cycles of 95°C (15 s), 60°C (1 min). The primers and probe to amplify OLFM4 and β-actin have been previously reported (33).

Plasmid construction and transfection
A 1536 bp fragment of the OLFM4 complementary DNA was subcloned into the pCDNA3.1/V5-His-TOPO vector (Invitrogen) using the BamHI and EcoRI sites. The complementary DNA insert was sequenced to verify its identity and the absence of mutation. Transfection of OLFM4 (or vector only) into PC-3 cells was performed using the ExGen reagent (Fermentas INC., Glen Burnie, MD). The PC-3 clones were maintained in RPMI 1640 growth medium. Cathepsin D plasmid (OriGene) and its small interfering RNA (siRNA) (Santa Cruz Biotechnology, Santa Cruz, CA) were transfected into DU145 cells using Lipofectamine 2000 (Invitrogen). After transfection for 2 days, the cells were cultured with selection medium containing 300 μg/ml G418.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, western blot and antibodies
Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blots were performed as described previously (34). The following antibodies were used for western blotting: anti-α-V5 (Invitrogen), anti-cathepsin D monoclonal antibody (BD Transduction Laboratories), β-actin, anti-Flag, anti-AKT pan and anti-pser473 (Cell Signaling Technology, Danvers, MA) and anti-NCIXR4 (Abcom).

Coimmunoprecipitation
For coimmunoprecipitation, the human complementary DNA clone expressing the SDF-1 gene with Flag tag was purchased from OriGene. Plasmids expressing the human OLFM4 gene with V5 tag or V5 tag only were constructed by our laboratory as described above. The two plasmids were cotransfected into 293T and PC-3 cells at 70% confluency. Immunoprecipitation was performed as described previously (35) with 5 μg/ml anti-Flag antibody (OriGene) or with 2 μg/ml anti-V5-tag antibody (Innogen) (20). Briefly, after 48 h of transfection, the cells were lysed with 1 ml immunoprecipitation buffer [10 mM Tris–HCl (pH 7.5), 5 mM ethylenediaminetetraacetic acid, 50 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN; ethylendiaminetetraacetic acid-free)] and the supernatant (400 l) was run in 20% Bis–Tris gel (Invitrogen) for 3 h at 4°C. Protein G-Sepharose (50 μl) was then added and gently mixed overnight at 4°C. The beads were washed three times with immunoprecipitation buffer and boiled in 50 μl of the sample-loading buffer (NuPAGE LDS sample buffer; Invitrogen) for 10 min. After a brief spin to remove the protein G-Sepharose beads, the supernatant (25 μl) was run in a 4–12% Bis–Tris gel (Invitrogen) for western blot analysis with anti-Flag or anti-V5 antibodies.

Colony formation in soft agar
A cell suspension (1 × 106 cells/ml) in 2 ml of 0.35% Noble agar with RPMI and 10% fetal bovine serum was overlaid into six-well plates containing a 0.6% agar base. Each experiment was performed in triplicate and colonies >0.2 mm in diameter were counted and recorded after culturing for three weeks (34).

Cell invasion assay
Cell invasion assays were performed by the method described by Lochter et al. (36) and modified at the counting step. To detect SDF-1-induced invasion, PC-3 cells were serum-starved for 4 h, then 1 × 105 cells in serum-free media were seeded onto inserts that were precoated with Matrigel in the upper chamber of a Transwell culture plate (BD). The lower chamber of the plate contained media with or without SDF-1 or with SDF-1 and anti-NCIXR4 antibody. Cell invasion was allowed to proceed for 22 h and invaded cells were stained and counted using a × 20 microscope objective (×200). The mean number of cells per high-powered field was calculated from triplicate wells.

Tumor growth and bone metastasis in nude mice
Animal experiments were performed following protocol K119MCHB06 (approved by the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health Animal Safety Committee). OLFM4-expressing PC-3 cell clones (1, 2 or 3) or vector-transfected control PC-3 cells (5 × 105) were injected subcutaneously into the right flank of 5- to 6-week-old male Balb/nude mice (The Jackson Laboratory, Bar Harbor, Maine). Tumor size was measured every week and volumes were calculated using the tumor volume = (W2 × L)/2 formula. All mice were euthanized 49 days after inoculation and all visible tumors removed and weighed. For determining bone invasion and metastasis, 5- to 6-week-old male Balb/nude mice were intravenously injected with 1 × 107 vector-transfected control PC-3 cells or OLFM4-expressing PC-3 clone 2 or clone 3 cells by tail vein. Bone- and skeletal-related invasion was tracked weekly and the sites and the numbers of osteolytic lesions were recorded. Histopathology with hematoxylin and eosin and X-rays were used for the final validation.

Cathepsin D activity assay
Purified human liver cathepsin D and the cathepsin D assay kit were purchased from Sigma, St. Louis, MO. Assays were performed according to the manufacturer’s instructions. Purified human OLFM4 protein was produced as described previously (20). Cathepsin D enzyme activities were measured with an automated assay performed at an excitation wavelength of 320 nm and emission wavelength of 460 nm at 25°C (FLUostar OPTIMA). Cathepsin D autocatalytic activities were performed with pure human cathepsin D (0.24 μM, C8696; Sigma) in an enzyme assay buffer (A3855; Sigma) with or without 0.27 or 0.54 μM pure human OLFM4 protein or Pepstatin A (PepA, 2 μM) at 37°C for 15 or 60 min. The reaction mixtures were then subjected to western blotting with OLFM4 antibody or cathepsin D antibody.

Flow cytometry analysis
PC-3 cell clones were grown in T75 flasks in RPMI 1640 plus 10% heat-inactivated fetal bovine serum (Invitrogen) to 80% confluency. The cells were trypsinized, removed from flasks and spun down at 1000 r.p.m. for 5 min at room temperature. The cells were resuspended with cold phosphate-buffered saline and the cell number adjusted to 1 × 107/ml. Cells were fixed with cold ethanol by adding ethanol drop-wise while vortexing until they were brought to 70% ethanol, then stored at −20°C. For staining, the cells were spun down, then washed twice with phosphate-buffered saline and stained using 1 μg/ml propidium iodide (Sigma) solution containing 5 μg/ml RNase for 30 min at 37°C. The cells were analyzed by flow cytometry (BD FACSCalibur; BD).

Autophagy assays
PC-3 cell clones were grown in T75 flasks in RPMI 1640 plus 10% heat-inactivated fetal bovine serum. For western blots, cell lysates were separated using 12% polyacrylamide gel electrophoresis and hybridized with a rabbit monoclonal antibody for LC3B (D11; Cell Signaling Technology) at 1:1000, then developed as described previously (34). For flow cytometry, cells were serum-starved for 3 days then exposed to 1 μg/ml acridine orange (Sigma) for 20 min, trypsinized and resuspended in phosphate-buffered saline. Approximately 10 000 cells were analyzed using the CD13-FE channel with a BD FACSCalibur flow cytometer. For fluorescence microscopy, cells were grown on tissue culture-treated chamber slides (BD Falcon). To detect autophagosomes, cells were exposed to 1 μg/ml acridine orange (Sigma) for 30 min and examined under a microscope after the slides were mounted with antifade reagent (Invitrogen). All images were acquired using an Olympus BX51 microscope and Qimaging Camera with Q Capture pro software (Qimaging, Surrey, Canada). Images were acquired using the ×60 Uplan Apo objective (1.42 oil), then imported into Adobe Photoshop for presentation.

Statistical analysis
Statistical analyses were performed using GraphPad Prism or SAS software. The data were analyzed by using the analysis of variance test, Tukey–Kramer honestly significant difference test or chi–square test, depending on the data to be analyzed. P < 0.05 was considered statistically significant.

Results
OLFM4 is significantly reduced or undetectable in human prostate cancer cell lines and cancer tissues
To determine OLFM4 mRNA expression in prostate tissue, two cultured normal prostate cell lines (epithelial and stromal) and four

987
prostate cancer cell lines were examined by reverse transcription–PCR. OLFM4 mRNA was detected in the normal epithelial cells, but not in the normal prostate fibroblasts or any of the four prostate cancer cell lines tested (Figure 1A). Similarly, OLFM4 mRNA was detected in 100% (8/8) of the normal prostate tissue samples tested, but only 24% (10/42) of the prostate tumor tissue samples tested ($P < 0.05$); however, OLFM4 mRNA was detected in 67% (4/6) of the benign prostatic hyperplasia tissue samples assayed (Figure 1B and C). OLFM4 mRNA was significantly reduced in prostate cancer tissue samples with high Gleason scores compared with normal prostate tissue (Figure 1D). These results indicated that OLFM4 mRNA expression was reduced with prostate cancer progression.

**OLFM4 expression inhibits prostate cancer cell growth in vitro and prostate tumor growth in vivo**

Because we observed a reduction or loss of OLFM4 mRNA in prostate cancer cells and prostate cancer tissues compared with the normal prostate, we speculated that OLFM4 might have a role during prostate cancer progression. Thus, we established a stable cell line overexpressing OLFM4 by transfecting OLFM4 plasmid into PC-3 prostate cancer cells and selecting with G418. We confirmed expression of OLFM4 by reverse transcription–PCR (data not shown) and western blotting (Figure 2A). Although OLFM4-expressing clones had varied growth rates in vitro, these cells grew more slowly than both PC-3 parental cells and vector-transfected control PC-3 cells (Figure 2B). In addition, the OLFM4-expressing PC-3 cells formed significantly fewer colonies in soft agar compared with vector-transfected control PC-3 cells (Figure 2C). In in vivo studies in which nude mice were subcutaneously injected with OLFM4-expressing clones of PC-3 cells and tumor volume measured over time, the tumors produced by OLFM4-expressing PC-3 cell clones (1, 2 and 3) had significantly reduced growth compared with tumors produced in mice injected with vector-transfected control PC-3 cells (Figure 2D, left and right panels).

Because of these observed inhibitory effects on cell growth in vitro and in vivo, we sought to determine whether OLFM4 was associated with enhanced apoptosis. We found that the cell cycle distribution and pre-G1 cell population of OLFM4-expressing PC-3 cell clones was similar to that of PC-3 parental cells and vector-transfected PC-3 control cells (supplementary Figure S1 is available at Carcinogenesis Online). Also, there was no difference in apoptosis in the vector-transfected control versus OLFM4-expressing PC-3 cells as measured by TUNEL assay or caspase 3 and 9 activity (data not shown). Collectively, these results suggested that OLFM4 inhibits proliferation of human prostate cancer cells by a mechanism not involving apoptosis.

**OLFM4 inhibits experimental bone invasion and metastasis of prostate cancer cells in vivo**

Previous studies have shown that OLFM4 is involved in cell adhesion (20), migration and cytoskeleton organization (30). Therefore, we examined the invasiveness and metastasis of OLFM4-expressing PC-3 cells. Expression of OLFM4 significantly reduced PC-3 cancer cell invasion by 60–70% in all three OLFM4-expressing PC-3 cell clones (Figure 3A). Moreover, in an in vivo model of invasion in which nude mice were intravenously injected with PC-3 cells transfected with either OLFM4 or vector control, increased expression of OLFM4 dramatically inhibited cancer cell skeletal invasion and bone metastasis (4.2 versus 55.9% for vector-transfected control PC-3 cells, $P < 0.05$; chi–square test). Nineteen of the 34 mice injected with vector-transfected control PC-3 cells developed bone metastases, whereas only one of eight mice injected with clone 2 (12.5%) and 0 of 16 mice injected with clone 3 (0%) exhibited metastatic tumor formation.
OLFM4 suppresses prostate cancer growth and metastasis

Fig. 2. Ectopic expression of the OLFM4 gene inhibits growth of prostate cancer cells in vitro and in vivo. (A) Stable expression of OLFM4 gene with V5 tag in PC-3 cell clones. Western blot with anti-V5 antibody (upper panel) and β-actin antibody as a loading control (lower panel) for PC-3 cell clones. 293T OLFM4-expressing clone 3 cells are shown as a positive control. (B) Cell proliferation of OLFM4-expressing PC-3 clones. The cell numbers were counted on the days indicated and presented as mean cell numbers (n = 3). *P < 0.05, **P < 0.01 (one-way analysis of variance test). (C) Anchorage-independent cell growth of OLFM4-expressing PC-3 clones in soft agar. The data represent the mean number of colonies from OLFM4-expressing PC-3 cell clones and the vector-transfected control PC-3 cells (n = 3). *P < 0.05; **P < 0.01 (Tukey–Kramer honestly significant difference test). (D) (left panel), Representative tumors 49 days after subcutaneous injection of nude mice with vector-transfected control PC-3 cells (upper panel) or OLFM4-expressing PC-3 clone 2 cells (lower panel). (D) (right panel), The mean tumor volume (n = 5–10) in nude mice was measured in vector-transfected control PC-3 cells or OLFM4-expressing PC-3 clone 1, 2 or 3 cells at the time points indicated. *P < 0.05 (analysis of variance test).

Fig. 3. OLFM4 inhibits invasion and bone metastasis of PC-3 cells. (A) Effects of OLFM4 expression on invasion of PC-3 cells. OLFM4-expressing PC-3 clone 1, 2 and 3 cells and vector-transfected control PC-3 cells were examined using a transwell invasion assay as described in the Materials and Methods. Data represent the mean number of cells counted per high-powered field (HPF) (n = 3). **P < 0.01 (Tukey–Kramer honestly significant difference test). (B) Nude mice (5–6 weeks old) were injected intravenously with vector-transfected control PC-3 cells or OLFM4-expressing PC-3 clone 2 or 3 cells. Tumor formation was evaluated weekly for 13 weeks after injection. Data represent the percentage of mice with bone metastatic tumor growth. (C) Representative images of mice with invasive tumors at 8 weeks (a) and 13 weeks (b) after intravenous injection of vector-transfected control PC-3 cells. Bone metastatic lesions were observed in the neck (a,b), rib (c), and upper limb (d). Bone metastatic growth was confirmed by histopathology with hematoxylin and eosin staining (e) and X-ray (f). Arrows indicate osteolytic lesions. B, bone; T, tumor.
Bone metastatic lesions in vector-transfected control PC-3 cells were not only seen in the early stage (8 weeks after injection, shown in Figure 3C, a), but these lesions also invaded the subcutaneous and skeletal tissues, and large tumor masses were frequently observed in most of the mice 13 weeks after injection (Figure 3C, b). Surprisingly, the metastatic lesions were observed at multiple sites, including neck (Figure 3C, a and b), ribs (Figure 3C, c) and upper limb (Figure 3C, d). Experimental bone metastasis and other organ/tissue metastatic lesions were confirmed by histopathology (Figure 3C, e) and X-ray (Figure 3C, f).

**OLFM4 interacts with and reduces cathepsin D protein**

To determine the underlying mechanisms of the OLFM4 mediated inhibition of cancer growth and experimental bone metastasis, OLFM4-expressing clone 3 and vector-transfected control PC-3 cells were selected and BD PowerBlot Western Arrays were used to screen target protein alteration. Cathepsin D was one of the proteins identified that demonstrated significant reduction in expression in OLFM4-expressing clone 3 PC-3 cells compared with vector-transfected control PC-3 cells (data not shown). The reduction of the endogenous mature cathepsin D protein was further validated by western blotting in two other OLFM4-expressing clones (Figure 4A, left panel) and in other prostate cancer cell lines, such as LNCaP (Figure 4A, middle panel) and DU145 cells (Figure 4A, right panel), after transfection of OLFM4. However, a reduction of cathepsin D at the mRNA level was not observed in the OLFM4-expressing clones when compared with vector-transfected control PC-3 cells (supplementary Figure S2 is available at Carcinogenesis Online), suggesting that OLFM4 mediated reduction of endogenous mature cathepsin D occurred through posttranscriptional regulation.

![Fig. 4. OLFM4 interacts with cathepsin D and reduces cathepsin D protein.](image-url)

(A) Western blot of OLFM4 with V5 tag (anti-α-V5 antibody; top row), cathepsin D (anti-cathepsin D monoclonal antibody; middle row) and β-actin (anti-β-actin antibody; bottom row) in PC-3 cell clones (left panel), LNCaP cells (middle panel) and DU145 clones (right panel). LNCaP cells shown are parental (Control), vector-transfected control cells (Vector) or OLFM4-expressing cells (OLFM4). DU145 clones shown are vector-transfected control (V1, V2 and V3) or OLFM4-expressing (O1, O2 and O3) cells. MCF7 cell lysate as a positive control.

(B) Coimmunoprecipitation analysis. Vector-transfected control PC-3 cells and OLFM4-expressing PC-3 cells (clones 1, 2 and 3) were lysed and immunoprecipitated with anti-V5-tag antibody, then the immunoprecipitates analyzed by western blotting with cathepsin D antibody (right panel). Vector-transfected control PC-3 cells and OLFM4-expressing PC-3 cell clones were lysed and immunoprecipitated with cathepsin D antibody, then the immunoprecipitates analyzed by western blotting with anti-V5-tag antibody (left panel). (C) Representative images of immunofluorescent staining for PC-3 clone 2 with cathepsin D and V5-tag antibodies. The cells were stained with a specific polyclonal antibody against the cathepsin D 34 kD active form and with an anti-V5 monoclonal antibody against the OLFM4-V5 tag, then incubated with secondary antibodies [goat anti-rabbit conjugated to Alexa Fluor 488 (green) or goat anti-mouse IgG conjugated to Alexa Fluor 594 (red) (Invitrogen)]. Nuclei were counterstained with Hoechst (blue). (D) Western blot of LC3B (anti-LC3B monoclonal antibody; upper panel) and β-actin (anti-β-actin antibody; lower panel) in PC-3 cell clones (vector-transfected control or OLFM4-expressing clone 1, 2 or 3 cells). (E) Representative images of LC3B in PC-3 cell clones (vector-transfected control or OLFM4-expressing clone 1, 2 or 3 cells) stained with acridine orange (1 μg/ml) for 30 min. Nuclei were counterstained with Hoechst (blue). Arrows indicate autophagic cells. (F) Percentage of autophagic cells in PC-3 cell clones treated as in E. Autophagic cells were counted in images captured using a 20× objective. Data represent the mean from analysis of three individual images. The experiments were repeated three times. *P < 0.05 (analysis of variance test).
To explore how OLFM4 affects cathepsin D protein at the protein level, we performed coimmunoprecipitation and immunofluorescence-staining studies. We found a physical interaction between OLFM4 and cathepsin D proteins. An OLFM4-cathepsin D protein complex was detected in OLFM4-V5-expressing PC-3 cells immunoprecipitated with anti-cathepsin D antibody (Figure 4B, left panel) or anti-V5-tag antibody (Figure 4B, right panel). Immunofluorescence staining of OLFM4-expressing PC-3 cells for OLFM4-V5 and cathepsin D revealed that OLFM4 protein (red) colocalized with cathepsin D protein (green), displaying a merged yellow color in the cytoplasm of the OLFM4-expressing PC-3 cells (Figure 4C).

**OLFM4 expression affects cell autophagy**

We next examined whether the OLFM4 protein’s interaction with cathepsin D, which is localized in lysosomes, affects cell autophagy. Western blot analysis with an antibody recognizing LC3B, an autophagosomal marker (37), demonstrated that LC3BII protein was increased in the OLFM4-expressing PC-3 clones compared with the vector-transfected control PC-3 clone (Figure 4D). The percentage of autophagic cells was also significantly increased in OLFM4-expressing PC-3 clones compared with vector-transfected control PC-3 cells (Figure 4E and F). To further verify the increase in autophagic cells in OLFM4-expressing PC-3 clones, the cells were cultured in RPMI 1640 medium without serum for 3 days. We found that the number of autophagic cells increased ~10–20% in OLFM4-expressing PC-3 clones compared with the vector-transfected control PC-3 clone (supplementary Figure S3A and B is available at Carcinogenesis Online). These results suggest that expression of the OLFM4 gene in PC-3 cell affects cell autophagy.

**OLFM4 inhibits cathepsin D enzymatic activity and biological functions**

To investigate whether the interaction between OLFM4 and cathepsin D protein affects cathepsin D activity, we performed cathepsin D assays using human cathepsin D in the presence of purified OLFM4 protein or Pepstatin A, an inhibitor of cathepsin D used as a control (Figure 5A). Cathepsin D activity was inhibited in the presence of 2.7 nM OLFM4 protein and inhibition increased with an increasing concentration of OLFM4 protein (Figure 5A). The inhibition of cathepsin D autocatalytic activity by OLFM4 was shown to be OLFM4 dose dependent and reaction time dependent (Figure 5B). These results demonstrated that OLFM4 protein inhibits cathepsin D activity and affects cathepsin D autocatalytic activity.

To investigate the biological effects of the OLFM4 and cathepsin D genes, we next cotransfected OLFM4 and cathepsin D plasmids or OLFM4 and cathepsin D siRNA plasmids into DU145 cells. Expression of cathepsin D alone stimulated cell growth but adding OLFM4 plasmid led to growth inhibition and blocked cathepsin D-induced cell proliferation in DU145 cells (Figure 5C). In addition, knock-down of cathepsin D with cathepsin D siRNA inhibited cell growth, and cotransfection of OLFM4 and cathepsin D siRNA inhibited DU145 cell proliferation (Figure 5D). The results provided additional

---

**Fig. 5.** OLFM4 negatively regulates cathepsin D functions. (A) Effects of various concentrations of OLFM4 (2.7, 5.4 and 13.5 nM) on the enzymatic activity of human cathepsin D (hCathD, 2.4 nM). (B) Western blot analysis for cathepsin D autocatalytic activity. Cathepsin D autocatalytic assays were performed with pure human cathepsin D (0.24 μM) in an enzyme assay buffer (Sigma A3855) with or without 0.27 or 0.54 μM pure human OLFM4 protein or Pepstatin A (Pep.A, 2 μM) at 37°C for 15 or 60 min. The reaction mixtures were then subjected to western blotting with OLFM4 antibody (upper panel) or cathepsin D antibody (bottom panel). Arrow indicates fractions of cleaved cathepsin D. (C) DU145 cell growth was detected after cotransfection and G418 selection for 12 or 14 days. Cotransfections were: vector control plasmid (Vector + Vector), vector control plasmid and cathepsin D-expressing plasmid (Vector + Cath.D), OLFM4-expressing plasmid and vector control plasmid (OLF4M4 + Vector) or OLFM4-expressing and cathepsin D-expressing plasmids (OLF4M4 + Cath.D). *P < 0.05; **P < 0.01 (n = 3) (Tukey–Kramer honestly significant difference test). (D) DU145 cell growth was detected after cotransfection and G418 selection for 12 or 14 days. Cotransfections were: vector control and control RNA-expressing plasmids (Vector + cRNA), vector control and cathepsin D shRNA-expressing plasmids (Vector + shRNA), OLFM4-expressing and control RNA-expressing plasmids (OLF4M4 + cRNA) or OLFM4-expressing and cathepsin D shRNA-expressing plasmids (OLF4M4 + shRNA). *P < 0.05; **P < 0.01 (n = 3) (Tukey–Kramer honestly significant difference test).
OLFM4 interacts with SDF-1 and suppresses SDF-1-stimulated PC-3 cancer cell invasion

As described earlier, the SDF-1/CXCR4-signaling pathway has been reported to play a critical role in cancer development, progression, angiogenesis and metastasis (12–16). Gene Cards networking (www.genecards.org) also demonstrated a potential functional partnership between OLFM4 and SDF-1 with a string score of 0.905, stimulating us to pursue regulation of SDF-1 by OLFM4 and to investigate their interaction in prostate cancer growth and metastasis.

Discussion

Our study was the first to demonstrate the anticancer functions of OLFM4 in human prostate cancer. Firstly, we found that OLFM4 was highly expressed in human normal prostate tissues and normal prostate cell lines and was moderately expressed in human benign prostatic hyperplasia tissue samples but was significantly reduced or lost in human prostate cancer tissues and cancer cell lines. Secondly, increased expression of human OLFM4 in PC-3 cells led to tumor growth inhibition in vitro and in xenografts in nude mice, and, most impressively, led to the inhibition of cancer cell bone invasion and multiple organ metastasis in vivo. Thirdly, OLFM4-mediated tumor inhibition was associated with a negative interaction with cathepsin D and SDF-1.

High levels of OLFM4 mRNA expression have been described in several tumor tissues (24,26–28), and a positive correlation between OLFM4 mRNA with stomach cancer progression and disease stage has been observed (25). However, we found OLFM4 mRNA expression is significantly reduced or lost in human prostate cancer. The published database (Gene Card) also showed a similar expression pattern in human normal prostatic tissues and prostate cancer. These results suggest that the pattern of OLFM4 gene expression is dependent on tumor type. On the other hand, using an OLFM4-specific antibody, we found OLFM4 protein was highly expressed in well-differentiated tumors and was decreased in most undifferentiated colon, stomach (29,30) and prostate cancers (data not shown).

More recently, Conrotto et al. reported that OLFM4 protein was detected in only one of five prostate cancer samples tested using an OLFM4 monoclonal antibody and that fewer than 15% of the tumor cells stained positively in that positive case. They also found that 64% of colorectal tumors expressed OLFM4 protein, but no correlation with tumor cell differentiation was reported (31). Although a high level of OLFM4 mRNA was detected in several tumor tissues compared with that in normal tissues, the role of the OLFM4 gene in human cancer initiation and progression was not clear. Our previous studies have demonstrated that OLFM4 binds to cell-surface adhesion molecules, such as lectins and cadherin, facilitating cell adhesion in HEK 293 cells (20). Overexpression of OLFM4 in the HT-29 colon cancer cell lines altered filamentous actin distribution, cell shape and cell adhesion but did not inhibit cancer cell growth (30). In this study, we showed that OLFM4-expressing PC-3 cell clones reduced cell growth, invasiveness and metastasis. In contrast, a previous study
reported that overexpression of the OLFM4 gene in mouse prostate Tramp-C1 cells promoted tumor growth in syngeneic mice (32). Although these studies used different technical approaches from our studies that may account for the different results obtained, the differences in results also suggest that OLFM4 may play dual roles in tumor cell growth, depending on gene expression level and cellular context.

Cathepsin D plays a number of important roles underlying physiological and pathological conditions (38). It has been reported that cathepsin D is involved in regulating cell autophagy, a lysosomal degradation pathway that regulates many physiological and pathological processes, including cancer (39). In the present study, we demonstrated that OLFM4 protein physiologically interacts with cathepsin D and regulates cathepsin D autocalytic activity, as well as blocks cathepsin D biological functions. We also found that OLFM4-expressing PC-3 clones had significantly increased cell autophagy compared with vector-transfected control PC-3 cells. It is possible that OLFM4 may exhibit its inhibitory effects on cancer cell growth through regulating cell autophagy in OLFM4-expressing PC-3 cells. Although many factors are involved in the regulation of the autophagic pathway, we postulate that cathepsin D is one of the OLFM4 target molecules involved in the regulation of autophagy in OLFM4-expressing PC-3 cells. Our results suggest that the OLFM4 gene may be involved in the regulation of cell autophagy in human prostate cancer cells.

The participation of SDF-1/CXCR4 in prostate cancer invasion and bone metastasis has recently been documented (40,41) and neutralizing anti-CXCR4 antibody has also been reported to block metastasis and growth of PC-3 cancer cells in osseous sites (42). Moreover, it has been reported that chemokine interactions could modulate SDF-1-mediated prostate cancer progression and metastasis (43–46). In the current study, we have identified a direct binding of OLFM4 and SDF-1 and their colocalization in prostate cancer cells. We also found that the interaction between OLFM4 and SDF-1 influenced SDF-1/CXCR4 signaling, resulting in prostate cancer cell growth and bone metastasis. Our results did not completely reveal how SDF-1 interacts with intracellular or extracellular OLFM4 and other unidentified matrix components of SDF-1/CXCR4 signaling during prostate cancer progression. However, our data are sufficient to suggest that, as with its interaction with cathepsin D, OLFM4 was able to attenuate SDF-1/CXCR4 signaling-mediated tumor cell growth and bone invasion. SDF-1 and other growth factors may have multiple binding partners for modulating optimal oncogenic signaling in prostate tissues (47), but the interaction between SDF-1 and OLFM4 may have unique functions that lead to decreased sequestration of SDF-1 to the cell surface, a reduced chemokine gradient for tumor cell migration, or quaternary structures and oligomerization status of SDF-1 binding of OLFM4 that alter SDF-1 mitogenic activity. It is also possible that cell surface OLFM4 modulates SDF-1 function in vivo by forming a dimeric complex with the receptor and then inhibiting SDF-1/CXCR4 downstream signal transduction.

Taken together, this study has identified a novel anticancer function of OLFM4 by demonstrating that OLFM4 suppresses human prostate cancer cell growth and metastasis via negative interaction with cathepsin D and SDF-1. Therefore, OLFM4 may have therapeutic potential for prostate cancer therapy and metastasis prevention.

Supplementary material
Supplementary Figures S1–S4 can be found at http://carcin.oxfordjournals.org/

Funding

Acknowledgements
We thank Drs Christian A.Combs and Daniela Malide (Light Microscopy Core Facility, National Heart, Lung and Blood Institute, National Institutes of Health) for help with immunofluorescence images analysis. We acknowledge the National Institutes of Health Fellows Editorial Board for reviewing the manuscript.

Conflict of Interest Statement: None declared.

References
43. Santiago, B. et al. (2006) CXCL12 is displayed by rheumatoid endothelial cells through its basic amino-terminal motif on heparan sulfate proteoglycans. Arthritis Res. Ther., 8, R43.

Received November 9, 2010; revised February 22, 2011; accepted March 25, 2011