The Multifunctional Growth Factor Midkine Promotes Proliferation and Migration in Pancreatic Cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) has a devastating prognosis among solid tumors and despite increased knowledge of the molecular mechanisms contributing to progression and metastasis, minimal progress has been done in establishing new targeted therapies for this deadly disease. The expression of the multifunctional growth/differentiation factor midkine (MK) promotes a variety of cellular functions leading to increased angiogenesis, proliferation, migration, and survival. Moreover, MK is intensively discussed as a potential new-therapy target and as biomarker for cancer progression and chemotherapeutic resistance in multiple cancers. Therefore, the present study investigated the molecular role of MK in pancreatic cancer. It was found that MK is elevated in PDAC and differentially expressed in other histologic subtypes of pancreatic cancer, whereas normal pancreatic cells did not express MK, thus making it an attractive candidate for targeted therapies. As a secreted growth/differentiation factor, MK was investigated as a biomarker in clinical serum specimens using ELISA. In addition, knockdown studies of MK revealed a link to proliferation and migration status in vitro. Finally, upstream signaling pathways were analyzed, with TNF-α and EGF being the main inducers of MK expression in PDAC.

Implications: This study presents novel MK functions and new upstream signaling effectors that induce its expression to promote PDAC and therefore defines an attractive new therapeutic target in pancreatic cancer. Mol Cancer Res; 12(5); 670–80. ©2014 AACR.

Introduction

An unusual aggressiveness and early metastatic locoregional as well as distant spread of pancreatic cancer cells reflects the urgent necessity of new therapeutic options for this deadly disease as its incidence still nearly equals mortality in the Western countries (1). The clinical treatment failure of patients is often attributed to the early metastatic growth, an unmet high drug resistance to standard therapy options, and high rates of local recurrence (2–4). However, insufficient diagnostic tools and therapeutic options for pancreatic ductal adenocarcinoma (PDAC) still substantiate its ranking as fourth leading cause of cancer-related death (5). Therefore, a better understanding of newly identified and cancer-specific key molecules that promote proliferation, migration, and survival of cancer cells may lead to the development of more effective therapeutic strategies.

Previous studies have revealed that expression of midkine (MK) plays an important role in different aspects of tumor progression in diverse solid tumors (6–8). We have recently shown that high mRNA and protein expression of MK in PDAC protected cancer cells from chemotherapy-induced apoptosis. Moreover, we have identified that gemcitabine dose-dependently induced expression and secretion of MK followed by activation of Notch signaling to promote epithelial–mesenchymal transition (EMT) and intrinsic as well as acquired chemotherapy resistance through upregulation of NF-κB. In addition, our results explained for the first time how MK may trigger cancer cells to become more resistant against cell death and how it promotes cell viability (9). Recently, we and others have shown that the secretion of MK in tumor cells may serve as biomarker for gastrointestinal tumors and glioblastomas (10, 11). Therefore, the development of MK inhibitors has been considered as an attractive idea to prevent tumor growth and antiapoptosis in cancer cells (12, 13).

In this study, we addressed the question of whether MK is also expressed in other histologic subtypes of PDAC and that siRNA- and short hairpin RNA (shRNA)—mediated depletion of MK may have dramatic effects on cancer cell proliferation and migration in vitro. The analysis of MK upstream signaling may provide reasonable explanations for the observed high MK expression levels in different cancers and therefore link therapeutic benefits with targeting of the multifunctional growth factor MK.
Materials and Methods

Study design and patients
The study was approved by Ethics Committee of Chamber Physicians in Hamburg, Germany. Written informed consent was obtained from patients using tissue and serum. We analyzed 103 pancreatic cancer tissue spots and 37 serum samples for MK expression, retrospectively. As healthy controls, 148 blood bank donors were included. A tissue microarray (TMA), including 56 ductal, 28 papillary adenocarcinoma, and 19 neuroendocrine resected pancreatic cancer was stained with antibodies against human MK (Abcam).

ELISA
For quantification of human MK in serum of patients with cancer, we used MK ELISA (Antigenix America). Briefly, microtiter wells precoated with anti-human MK antibodies were incubated with patient’s serum. After washing, streptavidin–HRP (horseradish peroxidase) conjugate was added to convert substrate H₂O₂–tetramethylbenzidine.

TMA construction and immunohistochemistry
Tissue samples were fixed in 4% formalin, paraffin embedded, and used for TMA construction as described elsewhere (14). Hematoxylin-eosin–stained sections were made from selected primary tumor blocks (donor blocks) to define representative tumor regions. Immunohistochemistry (IHC) and scoring were judged by two independent board-certified pathologists (T. Rawnaq and R. Simon). For each tissue sample spot, the fraction of positive-immunostained tumor cells was recorded, and staining intensity was estimated on a four-step scale (0, 1+, 2+, and 3+). A final score was then built from these two parameters according to the following score: negative scores had absence of MK staining; weak scores had staining intensity of 1+ in ≤70% of tumor cells or staining intensity of 2+ in ≤30% of tumor cells; moderate scores had staining intensity of 1+ in >70% of tumor cells, staining intensity of 2+ in >30% but in ≤70% of tumor cells or staining intensity of 3+ in ≥30% of tumor cells; and strong scores had staining intensity of 2+ in >70% of tumor cells or staining intensity of 3+ in >30% of tumor cells.

Cell lines, conditions, and growth factors
Tumor tissues for establishment of primary chemoresistant PDAC cell line PaCa 5061 was taken from a patient who underwent total pancreaticoduodenectomy for advanced PDAC in 2010. The procedure of cell line establishment from tumor tissue was previously published (15). Primary cells were cultured in complete TUM medium (15). PANC-1 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS; Lonza), 200 IU/mL Pen-Strep (Sigma-Aldrich) at 37°C; 5% CO₂. Establishment of HPDE was described elsewhere (16). HPDE cells were cultured in K-SFM (Invitrogen) supplemented with bovine pituitary extract, 5 ng/mL EGF and 5% FCS (Lonza), and 200 IU/mL Pen-Strep (Sigma-Aldrich). All cell lines were authenticated using the STR GenePrint System (Promega) in 2012. For MK induction studies, cells were grown overnight (12–15 hours) in serum-free media and were stimulated using 10% FCS (Lonza), recombinant TNF-α (10 ng/mL), EGF (10 ng/mL), insulin growth factor I (IGF-I; 10 ng/mL), basic fibroblast growth factor (bFGF; 10 ng/mL; R&D Systems) and (all-trans-) retinoic acid (1 μmol/L; Sigma-Aldrich). For analysis/quantification of secreted MK (S-MK), we used human MK-ELISA kit (Antigenix America). Recombinant human MK (rh-MK; R&D Systems) was used at concentrations of 20 ng/mL.

siRNA/shRNA transfections and Western blot analyses
Cells were transfected with siRNAs against MK or control-siRNA (Eurofins Scientific) using Lipofectamine (Invitrogen). PANC-1 and PaCa 5061 cells were transfected with indicated siRNAs. PaCa 5061 cells were transfected using shRNA-plasmids for stable MK downregulation. Briefly, cells were transfected either with sh-control or specific sh-MK plasmids (RH5453-EG4192, Open Biosystems). After 72 hours, cells were incubated in the presence of puromycin and positive cell clones were subcloned using glass cylinders (Sigma-Aldrich). Viable cell clones were subsequently lysed and analyzed using SDS-PAGE and Western blot analysis for specific MK knockdown using anti-MK antibody. Following transfection, cells were lysed in RIPA buffer (Sigma-Aldrich) containing 1× protease inhibitor cocktail (Roche). For analyses of S-MK, cell culture media were collected and filtered (0.22 μM). Supernatants were resolved by SDS-PAGE and followed by immunoblotting as previously described (9). Western blot analyses and immunostainings were performed using MK antibodies (rab. anti-MK, Abcam; mouse anti-MK, Abnova).

Real-time reverse transcription PCR
Real-time reverse transcription PCR (real-time RT-PCR) was conducted to quantify gene expression or to verify siRNA-mediated downregulation. Of note, 1 μg of total RNA was reverse-transcribed using the Transcriptor cDNA Kit (Roche). Data were analyzed according to the comparative C_T method and were normalized for cyclophilin expression in each sample.

Immunocytochemistry and subcellular protein fractionation
Cells were grown on coverslips in 24-well plates. Following fixation using paraformaldehyde (4%), cells were blocked with 1% BSA/0.1% Triton-X. Cells were then incubated using anti-MK antibody overnight and washed extensively using PBS (Invitrogen). For visualization, Cy3-coupled secondary antibodies (Invitrogen) were used. Image acquisition was done using confocal microscopy (Leica). For analyses of subcellular MK distribution, we stepwise separated cytoplasmic, membrane, and nuclear soluble proteins.
by using the Subcellular Fractionation Kit (Pierce). As marker proteins for different compartments, we used anti-calpain, anti-calnexin, and anti-lamin antibodies (Santa Cruz Biotechnology).

Scratch and Transwell migration assay

We performed scratch-/wound healing assay by plating PaCa 5061 and PANC-1 wild-type, shControl- and sh-MK–transfected subclones to create a confluent monolayer. Monolayer was scraped in a straight line to create a “scratch” in similar size with tips. Debris was washed out using PBS. For image acquisition, we used phase-contrast microscopy (Leica) at different time points (0, 16 hours). For Transwell assay, corresponding knockdown clones of PaCa 5061 cells and PANC-1 cells were transferred to migration assay for indicated time points. Cell migration was analyzed using 24-well inserts (8 μmol/L) according to the manufacturer’s protocol (BD Biosciences). Briefly, cancer cells remaining on top-side of the membrane were carefully removed, whereas cancer cells that had migrated to lower-side of the chamber were fixed and stained with crystal violet. At least 5 randomly selected fields per insert were photographically documented and average counted.

Cell proliferation

Cell proliferation was determined by CellTiter 96 AQueous One Solution Assay according to the manufacturer’s protocol (Promega). Each experiment was performed in quadruplicate. The absorbance was measured at 490 nm. Values for control cells were considered as 100% viability. Every measurement was performed at least in three independent experiments.

Data analyses

Expression analyses and achieved data were analyzed using GCOS 1.4 and scaled to default target signal value of 150. Absolute and comparative analyses were performed using MAS 5.0 algorithm. Annotations were further analyzed with interactive query analysis. Experiments presented in figures are representative of three different repetitions. The data are presented as the mean values ± SE.

Statistical analysis

SPSS for Windows (v21.0; SPSS Inc.) was used for statistical analysis. Statistical significance was evaluated by the Mann–Whitney U test and the Kruskal–Wallis test. Significance statements refer to P values of less than 0.05.

Results

MK expression in normal pancreatic and cancer cells

First, the MK gene and protein expression were evaluated in PDAC cell lines PANC-1 and PaCa 5061, whereas HPDE cells served as control. HPDE cells were previously established from normal pancreatic tissue (16). PaCa 5061 cells were previously established from patient-derived PDAC tissue, operated in our clinic (15).

Because MK was already shown to be significantly overexpressed in cancer cells compared with corresponding normal cells (9), we investigated and quantified MK mRNA in the investigated lines using real-time RT-PCR (Fig. 1A). We observed a statistically significant mRNA overexpression (P < 0.05), whereas no expression was detected in HPDE cells. In a previously published report, we already showed that MK expression in patients with PDAC is significantly upregulated in more than 50% of the investigated samples (9). To verify that the MK mRNA expression resulted in robust protein expression, we investigated the MK protein expression in cancer cell lines and HPDE cells using specific antibodies (Fig. 1B). Because MK is a secreted growth factor involved in paracrine/autocrine regulation of growth and differentiation, we investigated whether the MK secretion is increased in PDAC compared with HPDE cells. Indeed, we

Figure 1. MK expression in normal pancreatic and cancer cells. A, differential MK mRNA expression in PaCa 5061 and PANC-1 cells compared with normal pancreatic cells (HPDE) obtained by real-time RT-PCR. MK is significantly overrepresented in PDAC cell lines, more than 5-fold compared with HPDE cells. Results are expressed as fold over control; *, P < 0.05. B, Western blot analyses showing steady-state MK expression (top) as well as S-MK levels (bottom) in PaCa 5061 and PANC-1 cells, compared with HPDE cells.
found robust released MK levels in PDAC cells, whereas no increased MK expression was detectable in HPDE cells at all (Fig. 1B).

**Elevated MK expression in pancreatic cancer tissue and serum samples**

To gain relevant insight into expression of MK in PDAC patient samples, we analyzed the MK protein expression using TMA. Because the vast majority of published information about MK expression in cancer cells resulted from evaluation of ductal adenocarcinomas, we hypothesized that MK may also be differentially expressed in other histologic subtypes. We, therefore, conducted TMA stainings from 103 patients with pancreatic cancer and 10 normal pancreatic tissue samples that were spotted on the TMA. From these 103 samples, 56 were ductal adenocarcinoma, 28 intraductal papillary-mucinous tissue samples, and 19 neuroendocrine tumors and all of them were stained with MK antibodies (Fig. 2A). Consistent with previous observations where MK was shown to be significantly overexpressed in ductal adenocarcinomas compared with normal control cells (9), we found moderate to strong MK expression in 36% (20 of 56) and 38% (21 of 56) of ductal adenocarcinomas, respectively. More interestingly, we found also strong MK expression in 64% (18 of 28) of papillary adenocarcinoma tissue samples as well as in 47% (9 of 19) of neuroendocrine patient samples (Table 1).

Because MK is a secreted growth factor and its detection may serve as biomarker, we also quantified MK expression in patient’s serum using ELISA. This analysis showed significantly increased serum MK levels in patients with PDAC compared with healthy donors (Fig. 2B; Table 2).

**MK is primarily expressed in the cytoplasm of PDAC cells**

The MK expression was previously shown to promote survival through influencing expression of apoptosis-related proteins such as Bcl-2 (17). In addition, MK was shown to localize to the nucleus and nucleolus of HepG2 cells (18), whereas other studies showed different MK localizations. The nucleolus is a known hotspot for rRNA transcription, and the authors conducted siRNA-mediated knockdown of MK, which resulted in lower rRNA transcription rates. The authors claimed that nucleolar MK expression influenced the transcription of rRNA in HepG2 cells and therefore affecting cell proliferation and apoptosis. The intracellular expression pattern of MK in cancer cells is still enigmatic and confusing. We, therefore, analyzed intracellular localization of endogenous MK in PDAC cells. First, we performed immunocytochemical stainings with PANC-1 and PaCa 5061 cells using specific MK antibodies. The subcellular localization of MK was examined using laser confocal microscope (Fig. 3A). MK was clearly expressed and localized exclusively in the cytoplasm. Second, we separated subcellular fractions from PANC-1 and PaCa 5061 cells and analyzed MK expression in comparison with expression of cell compartment–relevant protein markers. Strikingly, we found that MK is predominantly expressed in the cytoplasm.
cytoplasm of pancreatic cancer cells (Fig. 3B), which is in contrast with previously published results (18).

siRNA-mediated knockdown of MK is associated with decreased migration of pancreatic cancer cells in vitro

Early metastasis formation and invasion is a hallmark of pancreatic cancer and reflects the urgent need of new therapeutic strategies for this deadly disease. Because MK was earlier discussed to be involved in migration of embryonic neuronal cells (19), we hypothesized that MK expression is linked to the highly migratory potential of PDAC cells. To pinpoint whether overexpressed MK promotes migration of PDAC cells in vitro, we first performed transient siRNA and stable shRNA-mediated downregulation of MK in PaCa 5061 (Fig. 4A) and PANC-1 cells (Fig. 4E). Effective MK downregulation was proven by Western blotting whole-cell lysates and cell culture medium using specific antibodies (Fig. 4B and F) and real-time RT-PCR (Fig. 4C and G). In a second approach, knockdown and corresponding control cells were transferred to a scratch-induced migration assay. After PANC-1 and PaCa 5061 cells had grown for 16 hours under normal conditions, we compared the respective cell migration. Strikingly, downregulation of MK is linked to markedly impaired migratory potential of both cell lines and the injured area is nearly unchanged after 16 hours (Fig. 4D and H). On the other hand, the respective control cells showed no impairment in migration and filled the injured area. In addition, we performed Transwell assays and quantified the migrative potential of PDAC cell lines lacking MK expression, compared with corresponding control siRNA transfections (Fig. 4I). Our results clearly demonstrate that MK is indeed linked to the high migratory potential of PDAC cells in vitro.

MK depletion is linked with decreased proliferation of pancreatic cancer cells in vitro

MK is a heparin-binding growth factor and was recently shown to promote growth of various cancer cells in vitro and in vivo. The MK overexpression and/or extracellular treatment of tumor cells with recombinant MK was shown to promote and accelerate the growth of osteosarcoma cells (20), neuroblastoma (21) and gastric cancer cells (22). To examine whether MK promotes proliferation of PDAC cells in vitro, we first downregulated MK expression through

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Figure 3. MK is primarily expressed in the cytoplasm of PDAC cells. A, intracellular localization of endogenous MK (red) in PaCa 5061 and PANC-1 cells. Cells were grown on cover slips followed by fixation, blocking, and antibody treatment. Appropriate secondary Cy3-labeled antibodies were used. DAPI (4',6-diamidino-2-phenylindole) was used for costaining nuclei (blue) and immunoreactions were documented on a confocal microscope. B, subcellular fractions from PANC-1 and PaCa 5061 cells were analyzed for MK expression in comparison with expression of cell compartment–relevant protein markers. MK is predominantly expressed in cytoplasm of pancreatic cancer cells.
transient transfections of specific siRNA or control siRNA into PaCa 5061 (Fig. 5A) and PANC-1 cells (Fig. 5C). We then analyzed consequences of depleted MK and performed proliferation assays. Corresponding untransfected wild-type cells were used as independent control. Interestingly, we found that MK depletion is strongly linked with impaired growth of PaCa 5061 (Fig. 5B) and PANC-1 cells (Fig. 5D). In contrast, proliferation of control transfected cells was not

Figure 4. siRNA-mediated knockdown of MK is associated with decreased migration of pancreatic cancer cells in vitro. A, shRNA-mediated knockdown of MK in PaCa 5061 cells efficiently downregulated MK as shown by Western blotting total cell lysates of two independent cell clones, compared with control-transfected cells. Actin served as loading control. B, shRNA-mediated knockdown of MK in PaCa 5061 cells resulted in undetectable S-MK levels as shown by Western blotting cell culture medium from two knockdown cell clones, compared with control-transfected cells. C, effective MK downregulation on mRNA level was proven by real-time RT-PCR. D, PaCa 5061 wild-type, control-transfected, and two independent MK-knockdown cell clones were transferred to scratch-induced migration assay. After placing the scratch, cells were grown for additional 0 and 16 hours, respectively, and cell migration was captured using high-power field microscope. E, siRNA-mediated knockdown of MK in PANC-1 cells using two independent and specific siRNAs efficiently downregulated MK as shown by Western blotting total cell lysates. F, siRNA-mediated knockdown of MK in PANC-1 cells resulted in decreased S-MK levels as shown by Western blotting cell culture medium. G, effective MK downregulation on mRNA level was proven by real-time RT-PCR. H, PANC-1 wild-type, control-transfected, and two MK-knockdown cell clones were transferred to scratch-induced migration assay. After placing the scratch, cells were grown for additional 0 and 16 hours, respectively, and cell migration was captured using high-power field microscope. I, Transwell migration assay was performed with PaCa 5061 and PANC-1 MK knockdown cells. The graphs indicate the average number of cells per field at the indicated time points. **, P < 0.01; *, P < 0.05.
impaired. In proof-of-concept studies, we performed rescue experiments via exogenous treatment of MK-depleted PaCa 5061 and PANC-1 cells with recombinant human MK (rh-MK; 20 ng/mL; Fig. 5B and D). Here we observed substantially increased proliferation in rh-MK–treated MK-depleted cells, compared with untreated cells and proliferation reached almost similar levels as control-transfected and wild-type cells. Because proliferation of PANC-1 and PaCa 5061 cells was strongly impaired in MK-depleted cells compared with control transfected and wild-type cells, these results suggest that high expression and secretion of MK positively regulate proliferation of PDAC cells in vitro.

Interestingly, exogenous treatment of MK-depleted PANC-1 and PaCa 5061 cells with rh-MK rescued the observed impaired proliferation of MK-depleted pancreatic cancer cells.

**MK expression is inducible by EGF and TNF-α in pancreatic cancer cells in vitro**

Although much effort has been done on catching cellular MK functions during embryonic development and in neoplastic cells, very little information is available about upstream signaling that controls expression of this interesting multifunctional growth factor. A single report has investigated the role of various growth factors and cytokines that may induce MK transcription in embryonal carcinoma cell differentiation (24, 25), we also treated cells with retinoic acid. First, we serum starved PaCa 5061 and PANC-1 cells for 12 to 15 hours and treated cells with various growth factors/cytokines or serum (10%) for 24 hours or left them untreated and analyzed MK expression by Western blotting using specific antibodies (Fig. 6B and D). In a second attempt, we quantified MK expression in PaCa 5061 and PANC-1 cells following growth factor/cytokine treatment using a specific MK ELISA (Fig. 6A and C). Interestingly, we were able to induce strong MK secretion with extracellular treatment of TNF-α (10 ng/mL) and EGF (10 ng/mL) in both cell lines. Although the MK expression in PaCa 5061 cells was induced to a lesser content by bFGF (10 ng/mL) and IGF-I (10 ng/mL), we detected very faint MK expression after retinoic acid treatment (1 μmol/L) in both cell lines (Fig. 6A). In contrast, treatment of PANC-1 cells with bFGF showed undetectable MK expression levels. Moreover, serum-treated PaCa 5061 and PANC-1 cells displayed comparable MK expression levels as those resulted from single TNF-α and EGF treatments, whereas control cells showed almost no MK expression at all (Fig. 6C). These results suggested that MK is indeed inducible by TNF-α, EGF, and serum, whereas retinoic acid, IGF-I, and serum starvation (12–15 hours) failed to induce MK expression in PDAC, we treated PaCa 5061 and PANC-1 cells with serum, IGF-I, bFGF, TNF-α, and EGF or left them untreated. Because MK was first identified as a retinoic acid–inducible gene during embryonal carcinoma cell differentiation (24, 25), we also treated cells with retinoic acid.

Figure 5. MK-depletion is linked with decreased proliferation of pancreatic cancer cells in vitro. A, PaCa 5061 cells were transiently transfected with two independent siRNAs against MK or with si-control for 48 hours. Protein lysates were extracted. Actin served as loading control. B, MTT assay of PaCa 5061 wild-type (WT), control-transfected, and MK knockdown cells transfected with two independent siRNAs. In rescue experiments, MK-knockdown cells were also treated with rh-MK (20 ng/mL) for indicated time points. C, PANC-1 cells were transiently transfected with two independent siRNAs against MK or with si-control for 48 hours. Protein lysates were extracted. Actin served as loading control. D, MTT assay of PANC-1 wild-type, control-transfected, and MK-knockdown cells. In rescue experiments, MK-knockdown cells were also treated with rh-MK (20 ng/mL) for indicated time points.
Expression of PDAC cells. Similar results were also observed in Western blotting experiments, reflecting consistency (Fig. 6B and D). Moreover, it is known that the MK signaling is posttranslationally extinguished by ubiquitination and proteasomal degradation. Therefore, we examined whether exogenously applied growth factor-induced MK upregulation occurs at the mRNA level. Indeed, we found that MK mRNA expression is robustly induced by TNF-α, EGF, and serum (10%; Fig. 6E and F).

Discussion
There are still no satisfactory treatment options for pancreatic cancer. As a matter of fact, the clinical reality regarding
successful therapeutic intervention of PDAC still reflects that only very few patients benefit from current therapeutic options and the majority of patients still cannot be cured in 90% to 95% (26). PDAC is often identified in an advanced stage in which it is no longer possible to operate. This is due to rapid growth and invasive phenotypes leading to early onset metastatic spread, accompanied by frequent occurrence of chemotherapeutic resistance. Even surgical complete tumor-resected patients develop relapse disease, which leads to death despite receiving chemotherapy.

Early detection and novel therapeutic targets are urgently needed to improve the outcome. Therefore, an intensive investigation of molecular mechanisms contributing to tumor proliferation and migration of PDAC should be conducted as a basis for successful establishment of new targeted therapies. Elevated MK expression levels have been detected in various tumors (27). MK is described as a potential prognostic marker in several malignancies (10, 28–32). The present study confirmed the frequent overexpression of MK in patient-derived PDAC tissue samples. More interestingly, analyses of histologic subclasses revealed strong MK expression in the majority of patient-derived papillary adenocarcinoma and neuroendocrine tumors. Furthermore, we demonstrated that serum MK concentrations were significantly elevated in patients with PDAC compared with healthy controls. These results encourage us to examine serum MK levels as a novel prognostic tumor marker for PDAC, which can be easily detected in peripheral blood using commercially available ELISA. Moreover, downregulation of MK by shRNA and siRNA strategies resulted in substantially reduced proliferation rates compared with control cells. Interestingly, exogenous treatment of PDAC cells with rh-MK rescued proliferation deficiency, suggesting that MK may trigger extracellular signaling to accelerate proliferation of PDAC cells. In addition, MK is known to be a secreted growth factor and high expression levels may contribute to increased proliferation by autocrine/paracrine signaling.

A pronounced stromal response referred to as desmoplastic reaction is a hallmark of PDAC that is characterized by a complex interplay between the host epithelial cells, invading tumor cells, stromal fibroblasts, pancreatic stellate cells, immune cells, proliferating endothelial cells, and an altered extracellular matrix. Elevated expression and secretion of MK by the tumor might affect the surrounding stromal cells that express MK receptors in a paracrine mechanism and thereby contribute to the formation of the latter. Because it was shown that the stromal compartment in PDAC negatively affects radiation therapy (33, 34) and creates a milieu for chemotherapy resistance (35, 36), a fundamental issue in PDAC treatment, the role of MK in tumor stroma interplay and its effects on chemotherapy resistance requires further investigation.

Because several studies have already shown that MK may interact with various extracellular receptors (27), it is challenging to identify the specific receptors contributing to this effect. The list of MK interacting receptors on cell surface is still growing, since we were recently able to identify the Notch-2 receptor as a new MK interactor in PDAC cells whose activation by extracellular MK ended in a highly chemoresistant phenotype accompanied by EMT (9).

PDAC is known to have a devastating prognosis, which is primarily due to an early locoregional and distant metastatic spread. This process is supported by the ability of primary cancer cells that acquire invasive properties and gain access to blood and/or lymphatic vascular systems to establish a new deadly niche. In this study, we could clearly show that MK is involved in migration processes of PDAC cells. The migrative ability of PDAC cells in which MK is specifically and stably knocked down, revealed strong migration deficiency. The specificity of our observation was further supported by transient siRNA transfections targeting MK in other PDAC cell lines. In line with this, MK was recently shown to interact with various protein members of the TGF-β pathway in vitro, a pathway that is well accepted to be a central mediator of EMT processes and consequently navigate increased migration of cancer cells in vitro and in vivo (37).

On the other hand, subcellular distribution of MK in cancer cells is still enigmatic. Therefore, we analyzed MK localization in PDAC cells using specific antibodies and detected MK in cytoplasm of all investigated PDAC cell lines, whereas no nuclear staining was observed. Moreover, we separated subcellular fractions of cultured cells and used specific compartment markers that corroborate our findings. This is in contrast with previously shown observations in which MK was found primarily localized to nucleus and nucleolus in HepG2 cells; the latter is a known hotspot for rRNA synthesis. The authors claimed that MK might interfere with rRNA transcription and ribosomal assembly and ultimately with increased protein synthesis in vitro (38). Therefore, MK intracellular distribution may be cell context–dependent and need further attention in future studies.

The primary goal of this study was to gain relevant insight into mechanisms governing MK expression in PDAC and to further identify relevant new target structures that might interfere with MK expression. Recently, it was shown that growth factors EGF and TNF-α induced expression and secretion of MK in prostate cancer cells in vitro. Of all the cytokines and growth factors tested, TNF-α was the strongest inducer of MK expression in LNCaP cells. The authors claimed that MK expression is inducible by the NF-κB signaling pathway and elevated MK may partially inhibit TNF-α–induced apoptosis in prostate cancer cells (23). In line with this, our investigations revealed that TNF-α may also induce MK expression and secretion in all tested PDAC cell lines. Moreover, treatment with EGF resulted again in robust MK expression and secretion levels in an equal manner, suggesting that MK expression is in fact primarily inducible by TNF-α and EGF in PDAC. Because retinoic acid seems to be the main inducer of MK expression during mouse embryogenesis (39), our analyses showed almost no influence on MK expression in PDAC cells in vitro.

There are accumulating evidences in the literature reflecting that cellular MK act as a multifunctional growth factor whose expression is highly linked to proliferation, migration, survival, angiogenesis, and EMT in various neoplasms. Interestingly, several studies have already discussed the
posibility of successfully neutralizing MK by using antibodies, antisense RNA, or aptamers and thereby blocking tumor growth or reversing chemotherapeutic resistance (9). Presumably, in case of PDAC, the appliance of TNF-α antagonists such as infliximab might be conceivable to counteract elevated MK expression in general or even during chemotherapeutical treatment modalities. Another strategy might be the utilization of tyrosine kinase inhibitors such as sunitinib and axitinib that have been designed to counteract angiogenesis. This might also reflect a new strategy to interrupt MK-induced angiogenesis in salivary gland tumors (40), in which inhibition of angiogenesis may limit metastasis formation, which is, especially for PDAC, a very early and challenging clinical event and the primary cause of cancer-related death worldwide.

In conclusion, our study showed to our best knowledge for the first time that MK is, beyond PDAC, frequently upregulated in other histologic and neoplastic subtypes of the pancreas like neuroendocrine tumors or papillary adenocarcinoma. The MK expression is robustly inducible by TNF-α and EGF and high expression levels are strongly linked to the proliferative and migrative potential of PDAC cells in vitro. Therefore, it is of great interest to investigate how MK may influence proliferation, metastasis formation, and even chemotherapeutic resistance of PDAC cells in a mouse model and how currently available treatment modalities might circumvent MK expression to gain prospectively a benefit for patients suffering from this deadly disease.

Relevance of MK in Pancreatic Cancer

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Development of methodology: T. Rawnaq, L. Dietrich, C. Güngör

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Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): T. Rawnaq, G. Wolters-Eisfeld, Y.K. Vashist, K. Bachmann, C. Güngör

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