Midkine Enhances Soft-Tissue Sarcoma Growth: A Possible Novel Therapeutic Target

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Abstract

Purpose: New therapeutic targets for soft-tissue sarcoma (STS) treatment are critically needed. Midkine (MK), a multifunctional cytokine, is expressed during midgestation but is highly restricted in normal adult tissues. Renewed MK expression was shown in several malignancies where protumorigenic properties were described. We evaluated the expression and function of MK in STS.

Experimental Design: Immunohistochemistry, reverse transcription-PCR, and Western blotting (WB) evaluated MK expression in human STS tissues and cell lines. WB and flow cytometry analyzed MK receptor expression. Cell growth assays evaluated the effect of MK on STS cell growth, and WB assessed MK downstream signaling. MK knock-in and knockout experiments further evaluated MK function. The growth of parental versus MK-transfected human fibrosarcoma cells was studied in vivo.

Results: MK was found to be overexpressed in a variety of human STS histologies. Using a rhabdomyosarcoma (RMS) tissue microarray, cytoplasmic and nuclear MK was identified; nuclear MK expression was significantly increased in metastases. Similarly, several STS cell lines expressed and secreted MK; RMS cells exhibited nuclear MK. STS cells also expressed the MK receptors protein tyrosine phosphatase ζ and lipoprotein receptor-related protein. MK significantly enhanced STS cell growth potentially via the Src and extracellular signal-regulated kinase pathways. STS cells stably transfected with MK exhibited increased growth in vitro and in vivo. MK-expressing human STS xenografts showed increased tumor-associated vasculature. Furthermore, MK knockdown resulted in decreased STS cell growth, especially in RMS cells.

Conclusion: MK enhances STS tumor growth; our results support further investigation of MK and its receptors as therapeutic targets for human STS.

Soft-tissue sarcomas (STS) are mesenchymal-originating malignancies diverse in presentation and outcome. STS, as a group, have distinctive features including chemoresistance, persistent local recurrence, and/or propensity for pulmonary metastases, the major cause of STS mortality (1, 2). STS 5-year overall survival has stagnated at 50% for several decades. Current systemic STS chemotherapy regimens have modest response rates; nonresponders incur major toxicities without deriving therapeutic benefit. Most importantly, nearly all patients with metastatic STS will eventually become resistant to all standard therapeutic chemotherapy regimens; therefore, new approaches are needed to significantly improve STS outcomes. In this era of increasingly successful targeted cancer therapy, it is essential to identify molecules that are critical for STS growth and/or metastasis, are highly and/or differentially expressed by STS cells versus normal cells, and consequently may present therapeutically relevant targets.

Midkine (MK) is a basic cysteine-rich heparin-binding growth factor (3, 4) normally expressed in the neuroectoderm during midgestation where it plays a role in neural migration and also neural differentiation, maturation, and survival (5, 6). MK expression is highly restricted at very low or even undetectable levels in normal human adult tissues and is reexpressed in inflammatory processes and wound healing (4, 7, 8) where it functions as a proinflammatory cytokine promoting the migration of inflammatory leukocytes, neutrophils, and macrophages (9). Increased MK serum and tumor expression has been identified in several human malignancies including brain, breast, ovarian, and gastrointestinal cancers (10–14) where it may be involved in oncogenesis and tumor progression. Protumorigenic activities such transforming NIH3T3 cells, promoting cell growth and angiogenesis, and inducing tumor cell survival and migration are all described (15–19), although the mechanisms of these MK actions are incompletely understood. Although MK function has been studied in epithelial-originating tumors, little is known about its role in STS. The protumorigenic effect of MK in NIH3T3 cells eludes to its potential role in mesenchymal originating malignancies (16). Recently, we have identified increased MK expression in alveolar
soft part sarcoma, a STS histologic subtype (20). Similarly, increased MK expression was identified previously in malignant peripheral nerve sheath tumors and gastrointestinal stromal tumors, two additional STS subtypes (13, 18). These initial findings led us to further investigate MK expression and function in human STS.

Materials and Methods

Human STS specimens, cell lines, and reagents. Frozen and paraffin blocks of human STS specimens were selected from The University of Texas M. D. Anderson Institutional Review Board–approved STS tissue bank. H&E-stained slides of all selected tumors were re-reviewed by an expert STS pathologist (A.I.L.); presence of tumor and histologic subtype were confirmed. A preconstructed RMS tissue microarray (TMA), including 105 human RMS specimens, was obtained for immunohistochemical analysis; complete data were available for 75 samples, which were included in the statistical analysis (21). The SKLMS1 (leiomysarcoma), HT1080 and SW684 (fibrosarcoma), RD, RH30, HS729 [rhabdomyosarcoma (RMS)], SW872 (liposarcoma), and A204 (unclassified sarcoma) human STS cell lines as well as THP1 (human acute monocytic leukemia) cell line and human umbilical vein endothelial cells were obtained from the American Type Culture Collection. Primary cultures of normal human fibroblasts were obtained from Cambrex. Cells were cultured in DMEM (A204 in McCoy’s 5A), supplemented with 10% FCS (Life Technologies).

Recombinant MK was purchased from R&D Systems and reconstituted in water for stimulation experiments. The MEK inhibitor UO126 was purchased from Cell Signaling. Commercially available antibodies were used to detect MK (Antigenix America); protein tyrosine phosphatase ζ (PTPζ; R&D Systems); lipoprotein receptor-related protein (LRP; Fitzgerald Industries); phospho-Src and Src, phospho-extracellular signal-regulated kinase (ERK) and ERK, and phospho-AKT and AKT (Cell Signaling Technology); proliferating cell nuclear antigen (PCNA); and CD31 and β-actin (Santa Cruz Biotechnology). Secondary antibodies included horseradish peroxidase–conjugated (Universal Kit HRP; Biocare Medical) and fluorescent secondary antibodies (anti-rabbit Alexa 488 and anti-mouse Alexa 594; Jackson ImmunoResearch). Other reagents included CytoFQ FC Receptor block (Innovex Bioscience), Hoechst 33342 (Polysciences), and propyl gallate (ACROS Organics).

Immunohistochemical analysis. Immunohistochemistry was done using 5-μm-thick sections of STS tumor blocks (human samples and human xenograft tumors) and TMA sections as described previously (22). Briefly, paraffin sections were dewaxed and rehydrated before antigen retrieval. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol before blocking with horse serum. The sectioned tissue was subjected to microwave antigen retrieval. Primary and secondary antibodies described above were used. For MK staining, anti-MK antibody (1:100) was used as primary antibody followed by biotinylated anti-rabbit secondary antibody (1:200) before ABC peroxidase system application (Vectastain ABCComplex; Vector Laboratories), DAB color development (Sigma), and Mayer’s hematoxylin counterstaining. For immunofluorescence staining, fluorescence-conjugated secondary antibodies were used followed by nuclear staining with Hoechst. Staining distribution and intensity was evaluated and scored by two independent reviewers (B.K. and A.I.L.). Photographs were obtained using a Leica DM4000B microscope and a Leica HCXPL-S-APo 40 ×/0.75 numeric aperture objective lens (Leica Microsystems). Images were captured using Spot digital camera (Diagnostic Instruments) and processed using Spot advanced acquisition software (Diagnostic Instruments).

Reverse transcription-PCR. Reverse transcription-PCR (RT-PCR) was done as described previously (23). Briefly, total RNA was isolated from cultured STS cells or frozen specimens using TRIzol reagent (Invitrogen) per manufacturer’s instructions. Total RNA (1 μg) was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) and 2 μl of the product were used as templates for multiplex PCR containing both target MK and glyceraldehyde-3-phosphate dehydrogenase or β-actin primers for normalization. PCR primers were designed using primer 3 software: MK 5'-ATGCAAGACCGCAGGGCTTCT-3' and 5'-TTCCCTGGCTTCCTGCTTGA-3', glyceraldehyde-3-phosphate dehydrogenase 5'-GACGACATCTCGCTACACG-3' and 5'-CTTCCTACGGTCACCCACCC-3', and actin 5'-AGAAAATCTGGGACCAACC-3' and 5'-CTCCCTAATGTCACCCAGCA-3'. The PCR reaction mixture contained 2× PCR Master Mix (Promega), 0.2 μmol/L of primers (each 0.5 μmol/L), and 2 μl cDNA. PCR consisted of a first step of denaturation for 3 min at 94°C, 30 cycles of denaturation for 40 s at 94°C, annealing for 40 s at 58°C, and an extension for 50 s at 72°C. PCR cycles were terminated by an extension at 72°C for 7 min and products were resolved on a 2% agarose gel.

Quantitative RT-PCR. Total RNA was isolated from STS cell lines and reverse transcription reactions with random primers were carried out (Promega) as described above. cDNA (1 μl) and EvaGreen (2.5 μl; Biotium) were added to 50 μl reaction mixture (Promega). The expression of MK mRNA was analyzed using Mastercycler ep realplex (Eppendorf) real-time RT-PCR. The levels of gene expression were normalized using glyceraldehyde-3-phosphate dehydrogenase levels based on comparative threshold cycle method.

Western immunoblotting. Western blotting (WB) was done by standard methods. Briefly, proteins (25-50 μg) extracted from cultured cells (for some experiments, nuclear and cytoplasmic protein fractions were isolated) or frozen human samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were then blocked and blotted with relevant antibodies. Horseradish peroxidase–conjugated secondary antibodies were detected by enhanced chemiluminescence (Amersham Biosciences). IRdye680- and IRdye800-conjugated secondary antibodies (Molecular Probes) were detected using Odyssey Imaging (LI-COR Biosciences).

ELISA. MK levels were measured in STS cell collected conditioned medium using ELISA. The assay was constructed and done following the manufacturer’s instructions (Antigenix America).

Measurement of cell proliferation. Cell growth assays were done using CellTiter96 Aqueous Nonradioactive Cell Proliferation Assay kit (Promega) per manufacturer’s instructions. STS cell lines stably transduced to express MK or after MK knockdown with small interfering RNA (siRNA) were plated at concentrations of 1.5 × 10^4 to 5 × 10^4 cells per well in 96-well plates. The growth rate was analyzed after 24. For MK stimulation experiments, cells were cultured in serum-free medium overnight and MK (in increasing doses of 1-100 nmol/L) was added for an additional 24 h; when applicable, U0126 was added to medium for 2 h before MK. Absorbance was measured at a wavelength of 490 nm, and the absorbance values of treated cells were presented as a percentage of the absorbance of untreated controls.

Flow cytometric analysis for LRP expression. Cell suspensions were prepared in PBS-2% FCS (fluorescence-activated cell sorting buffer). For flow cytometry, cells were incubated with the primary antibody at concentrations of 2 μg/10^6 cells for 30 min at 4°C and washed in fluorescence-activated cell sorting buffer before incubation with FITC-conjugated anti-mouse antibodies for 30 min at 4°C. Unstained cells, cells stained with secondary antibody only, and cells stained with isotype control only were used as controls. After washing in fluorescence-activated cell sorting buffer, data acquisition and analysis were done with a BD FACSCalibur (Coulter) flow cytometer. For each condition, 1 × 10^5 cells were counted.

Stable MK transfection. Total RNA extracted from RD cells was used for MK plasmid construction. Total RNA (1 μg) was used to synthesize cDNA with InvPrim-II reverse transcription system (Promega). The pcDNA3.1-MK plasmid was constructed by directional PCR cloning following the instructions of the manufacturer (directional TOPO expression kit, Invitrogen). Sequences of the
cloning primers were as follows: forward 5’-CACCATGCAGCACC-GAGGCTTCCT-3’ and reverse 5’-CTAGTCCTTTCCCTTCTTTCTT-GGCTT-3’. The plasmid was used to transform Escherichia coli (DH-5α) enabling its use for transfection. Before transfection, the sequence and inserted direction of MK in pcDNA3.1 plasmid were confirmed. pcDNA3.1-MK and pcDNA3.1 plasmid (2 μg/well in a six-well plate) were used to transfect HT1080 cells (natively expressing low MK levels).

Fig. 1. MK is commonly expressed in human STS. A, 27 of 30 randomly selected STS patient samples showed MK expression by immunohistochemistry; representative cases are shown (inset, staining control, secondary antibody only). Immunohistochemistry was done as per Materials and Methods. a, primary malignant fibrous histiocytoma; b, metastatic malignant fibrous histiocytoma; c, primary leiomyosarcoma; d, recurrent leiomyosarcoma; e, primary liposarcoma; f, metastatic liposarcoma; g, primary RMS; h, recurrent RMS; i, primary alveolar soft part sarcoma; j, recurrent alveolar soft part sarcoma. Asterisk, adjacent and intratumoral normal tissue, no MK expression was seen. B, increased MK expression was shown via RT-PCR (top) and WB (bottom) using RNA and protein extracted from human STS samples (same tumors were used for both assays when available); a correlation between only minimal MK levels were observed in paired normal tissues. FS, fibrosarcoma; SS, synovial sarcoma; LPS, leiomyosarcoma; T, tumor; N, paired normal tissue. C, RMS TMA MK staining (left). Three separate spots showing negative expression (left), cytoplasmic expression (middle), and nuclear expression (right). A, original magnification, ×200 (a–h, including insets), ×40 (i), and ×100 (j); C, original magnification, ×1 (slide), ×40 (TMA cores), and ×400 (insets).
FuGene 6 (Roche) was used for transfection per manufacturer’s instructions. Selection was conducted using G418; clones were screened by RT-PCR and WB for MK expression.

**siRNA knockdown of MK.** RD and SW872 cells (5 × 10⁴ per well) were plated in a six-well plate and incubated overnight at 37°C. The following morning SmartPool MK siRNA or nontargeting siRNA constructs (Dharmacon) were transfected using LipofectAMINE 2000 (Invitrogen) reagents according to the manufacturer’s instructions. Mock-transfected cells were treated with LipofectAMINE 2000 only. Incubation time for transfection reagents was 24 h, at which time medium was replaced with fresh regular medium containing. The next day, cells were harvested for WB and cell growth assays.

In vivo animal model. All animal procedures and care was approved by the Institutional Animal Care and Usage Committee of the University of Texas M. D. Anderson Cancer Center. Animals received humane care as per the Animal Welfare Act and the NIH “Guide for the Care and Use of Laboratory Animals.” Trypan blue staining-confirmed viable HT1080, HT1080/pCDNA, and HT1080/MK pool and clones 1, 2, and 3 cells (5 × 10⁵/0.1 mL HBSS/mouse) were injected s.c. into the flank of 6-week-old female nude/nude mice (National Cancer Institute/NIH; n = 10/group). S.c. tumors were followed for size as was measured twice weekly with a caliper; tumor volume was calculated as V = L × W 2 × π / 6, where V is the volume, L is the length, and W is the width. Mice were sacrificed when any group of tumors reached an average of 1.5 cm in largest dimension. Tumor was resected, weighed, and frozen and fixed in formalin and paraffin embedded for further immunohistochemical studies.

**Statistical analysis.** Cell culture based assays were repeated at least three times and mean ± SD was calculated. Cell lines were examined separately. For outcomes that were measured at a single time point, two-sample t tests were used to assess the differences. Differences in xenograft growth in vivo were assessed using a two-tailed Student’s t test. Significance was set at P ≤ 0.05.

For TMA, patient characteristics and staining data were summarized using median (range) for continuous variables and frequency (percentage) for categorical variables. Fisher’s exact test was used to assess the association between staining intensity and other categorical variables. Kruskal-Wallis test or Wilcoxon rank-sum test was used to compare the percent of area staining between groups of patients. P values < 0.05 were deemed as statistically significant. All statistical analyses were carried out in Splus (24).

**Results**

MK is highly expressed in human STS, especially RMS. Recently, we have identified MK overexpression in alveolar soft part sarcoma (20). Expanding this initial observation, we evaluated whether MK expression could be identified in other STS subtypes by immunostaining a panel of 30 human primary, recurrent, and metastatic STS of different histologic subtype and location using a specific anti-MK antibody (Fig. 1A). MK expression at varying levels was identified in 27 tumor sections (low/moderate = 11; high = 16); only minimal expression was observed in adjacent normal tissues (Fig. 1A). Increased MK expression in human STS was further confirmed using RT-PCR and WB (Fig. 1B); minimal MK expression was observed in several paired normal tissues. Immunostaining revealed that MK was mostly expressed in tumor-associated stroma and tumor cell cytoplasm (Fig. 1A); however, nuclear MK expression was observed in two RMS. This RMS finding prompted further consideration of MK expression using a large preconstructed TMA consisting of primary (n = 34), recurrent (n = 19), and metastatic (n = 22) RMS specimens retrieved from 75 adult patients. Positive staining was shown in 92% (69 of 75) of samples (21) at expression levels that varied in intensity, distribution, and subcellular localization (Fig. 1C). Intensity was scored as low in 28, moderate in 32, and high in 9. MK expression was observed in <30% of tumor cells in 27 samples, 30% to 70% in 6 samples, and >70% in 36 samples. Cytoplasmic/stromal staining was evident in 95.3% of MK-expressing tumors, whereas nuclear staining was shown in...
53.3%. No correlation was found between MK expression and patient demographics such as age or sex or with tumor-related parameters such as histology (pleomorphic, embryonal, and alveolar), site, or size. However, nuclear MK expression intensity significantly correlated with the tumor source: whereas only 14.7% (5 of 34) of primary lesions expressed moderate/high nuclear MK levels, 54.5% of metastases (11 of 22) exhibited increased nuclear MK \((P = 0.006)\). Taken together, these findings suggest that MK is reexpressed in mesenchymal-originating STS such as RMS and that enhanced STS nuclear MK expression is observed more frequently in metastatic RMS, justifying further examination of its role in STS progression.

MK and its potential receptors are highly expressed in human STS cell lines, especially RMS. To study the function of MK in STS, we first evaluated whether the increased MK expression observed in STS specimens could be recapitulated in human STS cells growing in culture. MK mRNA expression was detected at varying levels in several STS cell lines tested (quantitative RT-PCR; Fig. 2A); minimal expression was detected in normal human mesenchymal cells (fibroblasts and human umbilical vein endothelial cells; Fig. 2A). WB showed increased MK protein expression in concordance with mRNA levels in several STS cell lines, with only minimal expression in normal cells (Fig. 2A). MK is a secreted cytokine, so MK expression was further evaluated using conditioned medium from the same panel of cell lines (Fig. 2A). ELISA and WB results were concordant, showing increased MK levels in conditioned medium of MK-positive cell lines. Interestingly, RMS cell lines (RD, RH30, and HS) exhibited the highest MK expression of all STS cell lines tested (Fig. 2B); increased nuclear MK expression was identified in the two RMS cell lines compared with the other two MK-positive STS cell lines tested (Fig. 2C), similar to observations in human RMS tumors. However, this cell line sample is too small to make definite conclusions regarding the correlation between STS histologic subtype and MK nuclear localization.

Next, we evaluated MK receptor expression in STS. Several potential cell surface MK receptors have been suggested, although their exact mechanism of action is not certain (25–27). We evaluated the expression of two of these proposed receptors: receptor PTPz and low-density LRP. WB showed increased PTPz expression in protein from human STS specimens (Fig. 3A) and human STS cell lines; LRP expression was identified in several human STS cell lines (fluorescence-activated cell sorting analysis; Fig. 3B). Taken together, these data suggest that MK and its potential receptors are highly expressed in a broad range of human STS, especially in RMS, and support further studying the autocrine function of MK in these tumors.

**MK contributes to STS cell growth.** MK can elicit an array of protumorigenic effects in a variety of epithelial-originating tumor cells (14, 28). To examine whether MK has proproliferative effects in STS cells, we incubated HT1080 and SW684 human STS cell lines with recombinant human MK (Fig. 4A). These cells express low levels of endogenous MK but do express the MK receptors PTPz and/or LRP. Exogenous MK significantly \((P < 0.05; \text{Fig. } 4A)\) increased the proliferation of HT1080 and SW684 in a dose-dependent manner (no additional increase in cell proliferation was observed using doses >10 nmol/L). To further identify possible MK-induced intracellular signaling contributing to MK-mediated proproliferative effects, we analyzed the effect of exogenous MK on the Src kinase, ERK, and AKT signaling pathways in STS cells (Fig. 4B). WB showed a rapid MK-induced decrease (within 1 min) in phospho-Src (Y527), suggesting an increase in Src kinase activation and pointing toward an effect of MK-activated PTPz. Furthermore, MK induced an increase in phospho-ERK (Fig. 4B).
An unexpected decrease in AKT phosphorylation was observed within 30 min after MK stimulation (Fig. 4B). Pretreatment of HT1080 cells with the MEK inhibitor UO126 (10 μmol/L) diminished the proliferative effect of MK (Fig. 4C). These results offer initial insight into possible MK-induced intracellular signaling mechanisms of action.

To further confirm observed MK-induced STS proliferative effects, we transfected HT1080 cells to stably express MK (HT-MK). Figure 5A shows the expression of MK in selected HT-MK clones (WB). MTS assays showed significantly increased proliferation of MK-transfected HT1080 clones compared with HT1080 parental or pcDNA-transfected control cells \( (P < 0.05) \). The increase in proliferation rate corresponded to the level of MK expression identified by WB, with clones 2 and 4 exhibiting a lower level of MK expression as well as a more modest but still statistically significant increase in proliferation.

Next, we evaluated whether MK inhibition has functional significance in STS cells. Smartpool siRNA directed against human MK was used to knockdown its endogenous expression. RD and SW872 cells were mock transfected, transfected with nontargeting siRNA, or transfected with MK Smartpool siRNA. Figure 5B shows that MK knockdown resulted in significant cell death and growth inhibition \( (P < 0.05) \), which was more pronounced in RD \( (~ 80\%) \) compared with SW872 \( (~ 60\%) \). It is possible that the pronounced effect observed in RD cells is due to their RMS origin and/or their MK expression in the nuclear subcellular compartment.

Taken together, these data indicate that MK enhances STS cell growth. STS cell surface MK receptor presence suggests a possible MK autocrine function. Moreover, MK inhibition significantly inhibited STS cell proliferation \textit{in vitro}, especially RMS cells, supporting further consideration of MK as a possible STS therapeutic target.

**Endogenous MK enhances human STS xenograft growth in nude mice.** To investigate if MK effects on STS cell growth \textit{in vitro} could be recapitulated \textit{in vivo}, studies were conducted comparing the growth of MK-expressing HT1080 clones with pcDNA-transfected HT1080 cells after s.c. injection in nude mice (Fig. 6A). MK-expressing xenografts had shorter latency periods: palpable tumors appeared within 1 week after injection compared with 2 weeks in controls \( (P < 0.05) \). Furthermore, MK-expressing tumors showed faster tumor growth and significantly increased volumes at study termination \((3.26 \pm 0.48 \text{ and } 2.6 \pm 0.37 \text{ g} \text{ in MK-expressing xenografts versus } 0.46 \pm 0.12 \text{ g} \text{ in control tumors}; \ P < 0.05) \). Immunohistochemical studies confirmed increased MK expression in tumors originating from HT1080 MK clones, whereas no MK expression was shown in control tumors (Fig. 6B, c and d). MK-expressing tumors also expressed increased levels of proliferating cell nuclear antigen (a nuclear marker for proliferation; Fig. 6B, e and f) and CD31 (a marker of angiogenesis; Fig. 6B, g and h). Taken together, these data suggest that MK expression contributes to growth and angiogenesis of STS xenografts \textit{in vivo}.

**Discussion**

Numerous growth factors are involved in the development, progression, and dissemination of cancer. Consequently, growth factors and their receptors are being examined as potential targets for therapy of cancer, including STS. This report focuses on the novel and not yet extensively studied growth factor MK. MK expression is limited and restricted in adult human tissues under normal conditions but is highly expressed in a diverse group of malignancies \((10–14)\). Most investigations have considered MK expression and function in epithelial origin tumors, whereas little is known of its status in...
STS. To the best of our knowledge, this report is the first to identify MK expression in human STS cell lines and tumor samples. Increased MK expression in mesenchymal-originating STS might be mimicking the physiologic increase of MK in normal mesenchymal cells that occurs during tissue remodeling (8, 29, 30). Our results show that MK expression potentially enhances STS cell growth in vitro and in vivo; the molecular mechanisms underlying STS MK overexpression are currently being investigated.

Our findings support a role for MK in STS; however, as these tumors represent a diverse cluster of malignancies with vastly different biology, further investigation to evaluate whether MK is a “universal” STS-associated cytokine versus a STS histologic subtype-specific marker are needed. Although not the main focus of our current studies, results presented here do suggest a potential prominent oncogenic role for MK in RMS.

MK has broad protumorigenic capacities. It is known to be a secreted cytokine; we have identified MK expression in the conditioned medium of STS cells. STS secreted MK might possibly have both autocrine and paracrine effects, thus affecting both STS cells and the tumor-associated normal cell microenvironment. The significantly increased tumor cell proliferation and tumor-associated blood vessel density we observed in high MK-expressing human STS cell xenografts versus low MK-expressing controls supports this hypothesis and confirms the previously described MK role as an angiogenic factor (18, 31).

The exact mechanisms of MK action are not yet established and merit further elucidation. As an extracellular protein, MK is believed to exert its effects via binding to cell surface receptors. To date, MK has been shown to bind to several such receptors, thereby inducing downstream signaling with functional consequences. Suggested MK receptors include PTP(125, 26), LRP (27), integrin α5β1 and α6β1 (32), syndecan (5), and laminin-binding protein (34). It is possible that these molecules act as a multifunctional receptor complex (32) and as such enhance MK downstream signaling. Another possibility is that these receptors individually are differentially

Fig. 5. MK overexpression results in increased STS cell proliferation, whereas MK knockdown inhibits STS cell growth. A, HT1080 cells were transfected to stably express MK; clones were selected (WB, top). MTS assay showing significant increase (P < 0.05, t test) in proliferation of HT1080/MK clones compared with parental and pcDNA-transfected cells (bottom). B, WB confirming MK knockdown with anti-MK Smartpool siRNA in RD cells. MK knockdown resulted in increased cell death as shown by images captured via Q Color 5 digital camera (Olympus) through an inverted microscope (×400 magnification) and decreased cell proliferation (MTS assay). C, similar increase in cell death, albeit to a lesser degree, was observed in SW872 cells (MK knockdown was confirmed by WB and RT-PCR).

**Midkine Contributes to STS Cell Proliferation**
MK is considered a secreted cytokine that functions through receptor binding and downstream signaling, there is increasing evidence suggesting that MK can be localized to the nuclear subcellular compartment in some cells (34, 47). Nuclear MK was found to be of biological importance, contributing to MK antiapoptotic activity (47), although the exact functions of MK within the nucleus are yet to be elucidated. In our study, we have identified the expression of nuclear MK in the RMS subset of STS. Nuclear MK was observed in human RMS specimens where an increased level was more pronounced in metastatic lesions, possibly suggesting a functional importance for nuclear MK. Furthermore, we have shown nuclear MK expression in two human RMS cell lines tested. Although the growth of both cytoplasmic MK-expressing STS cell lines and nuclear MK-expressing cell lines (RMS) was
significantly inhibited on MK knockdown, this effect was more pronounced in the latter, further suggesting a potential role for nuclear MK. MK was found to internalize into cells after binding to LRP (27, 47). LRP, a member of the LDL receptor family, is a cell membrane protein known to induce endocytosis of several ligands. Ligand-receptor internalization has traditionally been regarded as part of the cellular de novo-to-sis system; however, emerging evidence suggests that in some contexts internalization of extracellular signaling molecules is an initial step toward their nuclear targeting, which in turn may be critical to their biological activities. Here, we show that some STS cells, including RMS, express LRP. MK-LRP binding is possibly the initial step for MK internalization into STS cells but is probably not sufficient for nuclear localization. We have shown that not all LRP-expressing cells exhibit MK nuclear localization, suggesting that other factors are needed for nuclear transport. MK does not have an apparent nuclear localization sequence, thus after being internalized into the cell it possibly binds to a nucleocytoplasmic shuttle protein for further nuclear transport. One protein that has been identified as important for MK nuclear translocation is nucleolin (47). Nuclear localization sequence-deficient nucleolin was found to trap MK in the cytoplasm and inhibit MK nuclear localization. Our studies have found nucleolin to be expressed in all STS cell lines studied to date (data not shown), including cells that do not express nuclear MK, suggesting that (as is the case for LRP) although nucleolin may be necessary for MK nuclear localization, it is not sufficient for this transport. Studies to identify the mechanism and function of nuclear MK in RMS are currently being conducted.

The results presented here support further investigation of the role of MK in human STS and the efficacy of anti-MK approaches for therapy of STS, especially RMS. However, a few limitations of the described studies warrant consideration, especially the fact that MK forced expression in vitro and in vivo, although a commonly used technique, represents an artificial model. Future studies will try to use human tumor xenografts naturally expressing varying levels of MK as well as anti-MK therapies to further determine the role of MK as a potential target. Commercial anti-MK therapies are not yet available; however, several modalities such as anti-MK siRNA and anti-MK antibodies have shown effects in preclinical studies (48) and merit further consideration. A recent report showed that MK is overexpressed in chemoresistant tumor cells and perhaps participates in development of resistance to conventional chemotherapy (49). Combining anti-MK strategies with chemotherapy in high MK-expressing malignancies might result in improved therapeutic efficacy, a potential benefit that is needed in STS where current systemic therapies are particularly limited.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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