Therapeutic approaches targeting midkine suppress tumor growth and lung metastasis in osteosarcoma.

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Abstract

Midkine (MK) plays important roles in tumorigenesis, however, the biological function of MK and whether MK can be a therapeutic target in osteosarcoma are unclear. Here, we found that osteosarcoma tissues showed high MK expression. MK knockdown by small interfering RNA significantly induced apoptosis in osteosarcoma cells, whereas recombinant MK increased cell proliferation. Inhibition of MK signaling by anti-MK monoclonal antibody (anti-MK mAb) suppressed growth of osteosarcoma cells both *in vitro* and in *vivo*. Moreover, inhibition of MK function significantly suppressed lung metastasis in xenograft transplantation model. Targeting MK by anti-MK mAb may have value in the treatment of osteosarcoma.

1. Introduction

Osteosarcoma is the most common type of solid bone cancer and occurs mainly in children and young adults. The annual incidence of 65 cases per year represents 5% of all childhood cancers (1). Osteosarcomas most commonly develop in the long bones, particularly the distal femur and proximal tibia. These sarcomas are often very aggressive (high-grade tumors), and about 20% of patients present with metastases. Recent advances in chemotherapy have dramatically improved long-term survival rates for osteosarcoma patients who have no detectable metastases at diagnosis, with up to 70% survival in patients with localized disease (2, 3). However, patients with metastasis or disease recurrence have a long-term survival rate of less than 20% (2, 3). Therefore, we urgently need to understand the biology of osteosarcoma progression and metastasis to develop new therapeutic approaches.

Expression of midkine (MK), a multifunctional growth factor discovered in 1988, is intense during midgestation but has a generally weak expression in adults (4, 5). In various target cells, MK promotes cell growth, survival, and migration and gene expression (6-9). MK is also involved in reproduction and repair of damaged tissues, as well as the pathogenesis of many diseases (10-12). In particular, MK serves an important function in tumorigenesis and is more highly expressed in various epithelial malignancies than in normal tissues (13-16). We previously found that the MK concentration in blood may become a useful marker for predicting the prognosis of patients with oral squamous cell carcinoma or breast cancer (17, 18). In addition, a few recent reports have shown the involvement of MK in several types of sarcoma (19-21). However, the role of MK in those tumors and the possible use of MK as a therapeutic target still need clarification.

In the present study, we focused on MK and determined its involvement in the pathogenesis of osteosarcoma. We analyzed MK expression levels in several kinds of bone and soft tissue sarcomas. In addition, we clarified the role of MK in osteosarcoma by using MK siRNA or anti-MK monoclonal antibody (anti-MK mAb) and evaluated whether targeting MK may be useful for osteosarcoma treatment.

2. Materials and Methods

2.1. Patients and tissue specimens. Specimens of bone and soft tissue tumors were obtained from 34 patients who underwent surgery at the Department of Orthopaedic Surgery of Kumamoto University Hospital between 2000 and 2008. Tumors included osteosarcoma (n = 16), Ewing sarcoma (n = 5), chondrosarcoma (n = 6), and synovial sarcoma (n = 7). The 34 patients included 16 males and 18 females, with a mean age

of 33.9 ± 21.9 years (median, 24 years; range, 12–79 years). The study protocol was approved by the Human Ethics Review Committee of Kumamoto University, and a signed informed consent form was obtained from all patients or patients' family members for the biopsies.

2.2. Immunohistochemistry. To detect MK in osteosarcoma samples, we performed an immunohistochemical analysis with anti-MK mAb (Cellmid Limited, Sydney, Australia). Sections (4-μm-thick) were embedded in paraffin, deparaffinized in xylene, and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked by immersing the sections in 0.3% hydrogen peroxide in methanol for 30 min, and antigens were retrieved by a 15-min microwave pretreatment in citrate buffer (pH 6.0). After sections were incubated with Protein Block Serum-Free Reagent (Dako, Glostrup, Denmark), they were incubated overnight at 4°C with anti-MK mAb at 1:100 dilution. Incubation of sections with anti-mouse labeled polymer (EnVision System HRP; Dako) proceeded for 30 min at room temperature, after which 3,30-diaminobenzidine was used as the chromogen. Sections were then counterstained with hematoxylin to enhance nuclear detection. We evaluated the percentage of MK-positive cells according to the following scale: –, negative staining; +, <30% of the cells; ++, >30% of the cells.

2.3. Cell line and cell cultures. A human osteosarcoma cell line, 143B, a subline derived from the TE85 osteosarcoma line transformed with Ki-ras, was obtained from RIKEN Gene Bank (Tsukuba, Japan). We used 143B cell line in this study because of its high tumorigenicity and spontaneous metastatic potential (22). Cells were maintained in minimum essential medium (MEM) (Gibco, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and were grown in 5% CO_2 at 37°C.

2.4. Transfection with small interfering RNA (siRNA). 143B cells (1×10^5 cells) were transfected with MK siRNA (50 pmol) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. After a 72-h incubation, total RNA was isolated. 143B cells transiently transfected with control siRNA (50 pmol) were used as controls. Silencer Negative Control no. 1 siRNA (Applied Biosystems, Foster City, CA) was used as a control. MK siRNA sequence was sense strand: 5′-GGGAUUCUGGGAAGCUUGAtt-3′ and antisense strand: 5′-UCAAGCUUCCCAGAAUCCCtt-3′ (Applied Biosystems).

2.5. RNA isolation and real-time PCR. Total RNA was extracted by using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was quantified via the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA (0.5 μg) in samples was reverse transcribed to cDNA by using the ExScript RT reagent kit (Takara Bio Inc., Otsu, Japan) according to the

manufacturer's protocol. All PCR reactions were performed via the LightCycler System (Roche Diagnostics, Basel, Switzerland) with SYBR Premix DimerEraser (Takara Bio Inc.). For each sample, a reaction mixture was added to 2.0 μl of cDNA. The reaction mixture comprised 6.0 μl of water, 1 μl of forward primer (0.5 μM), 1 μl of reverse primer (0.5 μM), and 10 μl of 2× Master mix. The primers used for real-time PCR were as follows: MK forward: 5′-AGATGCAGCACCGAGGCT-3′, MK reverse: 5′-CTTTCTTTTTGGCGACCG-3′; β-actin forward: 5'-TGGCACCCAGCACAATGAA-3', β-actin reverse: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'; cyclin E1 forward: 5'-GATTGGTTAATGGAGGTGTGTGAA-3', cyclin E1 reverse: 5'-CGCCATATACCGGTCAAAGAA-3'; p21 forward: 5'-TCACTGTCTTGTACCCTTGTGC-3', p21 reverse: 5'-GGCGTTTGGAGTGGTAGAAA-3'. The β-actin gene was chosen for normalization of data. Each reaction was performed with these conditions: initialization for 10 s at 95°C followed by 45 cycles of amplification, with 5 s at 95°C for denaturation and 20 s at 60°C for annealing and elongation. After amplification, for assessment of fluorescence and thereby determination of the melting curve, the temperature was slowly raised to above the melting temperature of the PCR product.

2.6. Western blotting. Cells were washed once in ice-cold PBS and were then lysed by adding CelLytic M Cell Lysis/Extraction Reagent (Sigma, St. Louis, MO) containing a freshly added protease inhibitor cocktail (Sigma), 50 mM NaF, and 1 mM Na_3VO_4. After incubation for 15 min on a shaker on ice, cell lysate was removed from the dishes and centrifuged at 15,000 × g for 15 min to remove insoluble material. Supernatants were stored at −70°C until use. The protein concentration was determined by using the BCA kit (Pierce Chemical Co., Rockford, IL). Equal amounts of protein were fractionated via SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). These membranes were blocked by incubation with 5% skim milk in PBS containing 0.1% Tween-20. Primary antibodies included a monoclonal rabbit anti-Akt antibody (Cell Signaling Technology Inc., Danvers, MA), diluted 1:1,000; a monoclonal rabbit anti-phosphorylated Akt (pAkt, Ser473) antibody (Cell Signaling Technology Inc.), diluted 1:2,000; and a monoclonal mouse anti-β-actin antibody (Sigma), diluted 1:5,000. Secondary antibodies were anti-rabbit IgG-conjugated horseradish peroxidase (HRP; Dako), diluted 1:1,000; and anti-mouse IgG-conjugated HRP (Dako), diluted 1:1,000. Blots were visualized by using ECL Plus Western Blotting Detection Reagents (Amersham, life science, Arlington Heights, IL) according to the manufacturer's instructions.

2.7. Proliferation assay. The effect of MK on proliferation of 143B cells *in vitro* was measured by means of the MTS assay using CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI), as described previously (23). Briefly, cells (1,000/well) were incubated, in triplicate, in a 96-well plate in the presence or absence of indicated concentrations of MK siRNA or recombinant MK in a final volume of 100 μL for 48 to 96 h. Then, 20 μL of CellTiter 96 AQueous One Solution Reagent was added to each well. After 4 h of incubation, absorbance was measured at 490 nm via the EMax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.8. Cell cycle analysis. 143B cells (1.5×10^5/well) were seeded in 6-well plates. After cells were transfected with 50 nM MK siRNA for 96 h, they were harvested, washed twice with ice-cold PBS, and fixed in 70% ethanol at 4°C for 24 h. After two washes with PBS, 50 μg/mL propidium iodide (Sigma) and 1 mg/mL RNaseA (Sigma) were added to the cells, and they were incubated at room temperature for 30 min. Their DNA content was analyzed by using a BD FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA).

2.9. Apoptosis assays. Cells were incubated at a density of 5×10^5/mL and were transfected with 50 nM siRNA. After 96 h, cells were harvested for apoptosis analysis by using Annexin V-PE/7-AAD apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions. Briefly, after two washes with ice-cold PBS, cells were resuspended in binding buffer and were then incubated with 5 μL of phycoerythrin (PE)-conjugated annexin-V and 5 μL of 7-Amino-ActinomycinD (7-AAD) in the dark for 15 min at room temperature. Samples were immediately analyzed with the FACSCalibur Flow Cytometer (BD Biosciences).

2.10. Tumor xenograft generation, tail vein injection, and anti-MK mAb treatment. Male CB17/ICR-scid/scid mice (SCID mice), each 8 weeks old and weighing 20–25 g, were obtained from CLEA Japan, Inc. (Tokyo, Japan). They were maintained in a specific pathogen-free environment at the Center for Animal Resources and Development of Kumamoto University. 143B cells were trypsinized, washed with serum-free MEM, and resuspended in PBS, after which their concentration was adjusted to 2×10^4 cells/100 μL in PBS. To generate orthotopic xenograft models, these cell suspensions were injected into the anterior tibialis muscle of the SCID mice. After 24 h, these mice received intraperitoneal injections of 100 μL of PBS containing 100 μg of anti-MK mAb (Cellmid Limited) ($n = 5$) or PBS alone ($n = 5$); the mice received this same treatment every 5 days for 42 days. Tumor development in individual animals ($n = 5$/group) was assessed every 7 days by means of sequential measurements of the bilateral difference in calf circumferences. Mice were killed 42 days after injections of

cells, and the tumors and lungs were harvested. To assess the occurrence of lung metastasis, all lung tissue samples were immediately fixed in 10% neutral buffered formalin followed by immunohistochemical staining with anti-Ki-67 antibody. Ki-67 positive colony number in lung tissue was counted to evaluate the incidence of lung metastasis. For the model of settlement of tumor cells to lung, cells transfected with MK siRNA were trypsinized, washed, and resuspended in PBS, and their concentration was adjusted to a concentration of 1×10^5 cells/100 μL in PBS. Cell suspensions were then injected into the tail vein of SCID mice, followed by analysis of settlement of tumor cells to lung after 2 weeks.

2.11. Statistical analysis. Student's *t* test was used to assess differences between experimental groups. All statistical analyses were conducted by using JMP software Version 5.1 for Windows (SAS Institute Japan, Tokyo, Japan). *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Expression of MK protein in osteosarcomas and other bone and soft tissue sarcomas

We first performed immunohistochemical analysis to investigate MK protein expression in tissue specimens of osteosarcomas and other bone and soft tissue sarcomas. Several kinds of these sarcomas evidenced high expression of MK, which was localized in the cell cytoplasm (Fig. 1A–D). Of 14 osteosarcoma specimens, 11 (78.6%) were immunoreactive (Fig. 1E).

3.2. Effect of MK knockdown on osteosarcoma cell proliferation

We next investigated the role of MK in osteosarcoma by means of MK knockdown with siRNA. Real-time PCR analysis confirmed the presence of MK expression in osteosarcoma cells and its significant downregulation by MK siRNA (Fig. 2A). As Fig. 2B shows, MK knockdown by siRNA inhibited 143B cell proliferation in a dose- and time-dependent manner. To examine whether exogenous MK has pro-proliferative effects on osteosarcoma cells, we incubated 143B cells with recombinant human MK. Figure 2C demonstrates that recombinant human MK significantly increased proliferation of 143B cells, at least from the concentration of 0.01 ng/ml, and this increase depended on both dose and time ($P < 0.05$).

3.3. Induction by MK knockdown of cell cycle arrest and apoptosis in 143B cells

To elucidate how MK regulates proliferation of 143B cells, we used flow cytometry to examine the effect of MK knockdown on cell cycle characteristics. In 143B cells transfected with MK siRNA, 81.6% of cells were detected in G_0/G_1 phase, whereas 66.5% of cells transfected with control siRNA were in the same phase, a significant difference (Fig. 3A). However, MK knockdown significantly reduced the percentage of cells in S phase. The percentages of cells in G_2/M phase did not differ significantly in both groups of cells (Fig. 3A).

We next examined transcription of genes related to the cell cycle. Real-time PCR analysis revealed that MK knockdown prevented transcription of cyclin E1, an accelerator of the G_1/S phase, and upregulated the expression of p21, a decelerator of the G_1/S phase (Fig. 3B). In addition, as Fig. 3C illustrates, control siRNA-transfected cells showed an early apoptosis rate of 26.9%, whereas that for MK siRNA-transfected cells was 78.8%, which indicates that MK knockdown increased the early apoptosis rate of 143B cells. MK knockdown also significantly increased the late apoptosis rate (Fig. 3C).

Because previous studies reported that the phosphatidylinositol-3 kinase/Akt pathway regulated growth and survival in various cells and that MK induced growth of

ameloblastoma by means of Akt pathways (24-26), we studied MK-regulated intracellular signaling contributing to proliferative effects by assessing the effect of MK knockdown on the Akt signaling pathway in 143B cells. As expected, we found that downregulation of MK reduced Akt phosphorylation in these cells (Fig. 3D).

3.4. Effects of anti-MK mAb on 143B tumor growth

We investigated whether inhibition of MK activity by anti-MK mAb against C-domain of MK, which effectively blocks MK functions, suppressed osteosarcoma growth. We first confirmed that anti-MK mAb suppressed proliferation of 143B cells *in vitro* (Fig. 4A). We then evaluated the effect of anti-MK mAb on tumor growth by measuring the bilateral difference in calf circumferences in the xenograft model. Figure 4B shows that anti-MK mAb markedly reduced tumor growth in SCID mice. The mean tumor size in control SCID mice was about 39.1 mm at 6 weeks after transplantation, whereas that in SCID mice injected with anti-MK mAb was merely 13.8 mm ($P < 0.05$).

3.5. Effect of MK on lung metastasis of osteosarcoma

Because lung metastasis is well documented as the most important prognostic factor for osteosarcoma patients (2, 3, 27), we studied whether inhibition of MK affected lung metastasis of osteosarcoma *in vivo*. We initially evaluated the rate of settlement of tumor cells to lung after injection of 143B cells transiently transfected with MK siRNA into the tail vein of SCID mice. The MK knockdown group had a lower incidence of settlement of 143B cells to the lung than the control group (Fig. 5A and B). Only one case in the MK knockdown group showed a colony formation, and the one colony found was smaller compared with control colonies. Furthermore, we treated mice orthotopically inoculated by 143B cells with anti-MK mAb as described above and evaluated lung metastasis by assessing the number of Ki-67-positive colonies. As shown in Fig. 5C and D, the number of colonies was significantly reduced (mean: 5.0) in the group treated with anti-MK mAb compared with that in the control group (mean: 20.6), and the size of each colony in the treated group was smaller than that in the control group. These data indicate that inhibition of MK suppressed lung metastasis of osteosarcoma cells.

4. Discussion

In the present study, we provided evidence that MK plays important roles in the pathogenesis of osteosarcoma. We also demonstrated that targeting MK by using anti-MK mAb may have therapeutic value for osteosarcoma. Although adjuvant chemotherapy has improved the overall survival rates of patients with osteosarcoma

compared with surgery alone, fatality rates have remained unchanged for more than 20 years. More than 30% of patients with this sarcoma die of metastasis to the lung within 5 years of diagnosis (27). Therefore, new therapeutic approaches and greater understanding of genetic and molecular mechanisms that contribute to osteosarcoma growth and metastasis, especially to the lung, are essential. This work may thus allow development of new osteosarcoma treatments.

Muramatsu reported overexpression of MK in many malignant tumors, including hepatocellular, gastric, colon, lung, urinary bladder, and prostate carcinomas, neuroblastoma, and astrocytoma (28). About 80% of these tumors demonstrated MK overexpression. In our study, as in Fig. 1 shows, MK was highly expressed in most osteosarcoma tissues (78.6%), similar to the ratio of other carcinomas reported previously, which suggests that MK may have a role in osteosarcoma development and progression. The pathogenesis of osteosarcoma involves various pathways, and those related to the cell cycle and apoptosis have crucial functions in tumorigenesis (29-33). That MK promotes activities related to oncogenesis including proliferation and angiogenesis, as well as inhibiting apoptosis, in the pathogenesis and development of different solid tumors has been well documented (34–37). In addition, recent reports have shown the involvement of MK in various types of sarcomas (19-21). However, the role of MK in those tumors and the possible use of MK as a therapeutic target still need clarification.

In the present study, downregulation of MK reduced osteosarcoma cell proliferation via inducing cell cycle arrest and apoptosis (Fig. 3). Cyclin E1 reportedly promotes progression from G_1 phase to S phase (29, 30). The cyclin kinase inhibitor p21 induces G_1 arrest and blocks entry into the S phase by inactivating cyclin-dependent kinases or by inhibiting the actions of proliferating cell nuclear antigen. Overexpression of p21 thus resulted in G_1 arrest and was shown to effectively suppress tumor growth (31-33). As Fig. 3B shows, we confirmed that MK knockdown prevented cyclin E1 transcription and upregulated p21 expression, which may have arrested the cell cycle and induced apoptosis. We also found that downregulation of MK reduced Akt phosphorylation in 143B cells (Fig. 3D), as reported previously (26). Moreover, as a step toward new therapeutic approaches targeting MK, we showed that inhibition of MK signaling by anti-MK mAb suppressed osteosarcoma growth both *in vitro* and *in vivo* (Fig. 4). Together, these results clearly indicate that approaches utilizing anti-MK mAb may be effective for osteosarcoma treatment by inhibiting tumor growth. Future studies should focus on elucidating in detail the mechanisms underlying anti-MK mAb-mediated growth inhibition of osteosarcoma and the

effectiveness of this inhibition.

Although lung metastasis is one of the most important prognostic factors for osteosarcoma patients, its underlying molecular mechanisms remain unclear. In the present study, we found, by means of tail vein injection and a xenograft transplantation model, that inhibition of MK suppressed lung metastasis. We confirmed that inhibition of MK function reduced the incidence of lung metastasis, which suggests that MK regulates tumor cell release in blood, engraftment, and proliferation in the lung. Furthermore, consistent with this finding, we confirmed that anti-MK mAb treatment significantly suppressed lung metastasis in the orthotopic xenograft model (Fig. 5), which suggests that MK is a useful therapeutic target for suppression of lung metastasis of osteosarcoma. Increased MK concentrations in blood were previously reported in patients with several kinds of malignancy and reflected MK expression in tumors (37-40). In our clinical study, the relationship between MK and metastasis was not apparent because of the small number of patients (Fig. 1E). However, our finding that anti-MK mAb suppressed lung metastasis *in vivo* in the orthotopic xenograft model points to the importance of secreted MK. Additional investigations are required to elucidate the involvement of MK in lung metastasis, by using clinical samples, as well as the precise molecular mechanisms underlying MK-mediated lung metastasis.

In conclusion, we demonstrated that MK was highly expressed in human osteosarcoma and that inhibition of MK suppressed osteosarcoma cell growth and metastasis. In view of our previous study showing that the MK concentration in blood may become a useful tumor marker for predicting the prognosis of patients with oral squamous cell carcinoma or breast cancer (17, 18) and the study of Maehara et al., who found significantly higher MK expression in patients with a poor prognosis (20), our present data suggest that MK should serve as a valuable prognostic marker for osteosarcoma patients and as a potential therapeutic target in osteosarcoma.

Conflict of interest: Potential conflicts do not exist.

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Figure legends

Fig. 1. Immunohistochemical analysis of MK protein expression in specimens of bone and soft tissue sarcomas. A, osteosarcoma (24-year-old woman); B, Ewing sarcoma (20-year-old woman); C, chondrosarcoma (53-year-old man); D, synovial sarcoma (42-year-old man). Scale bars indicate 200 μm (A and C) and 400 E, percentage of MK-positive cells in bone and soft tissue sarcomas. Black, hatched, and white columns reflect the scale as defined in Materials and Methods: ++, +, and –, respectively.

Fig. 2. Effect of MK knockdown on osteosarcoma cell proliferation. A, efficacy of siRNA targeting MK in 143B cells. Total RNA was extracted from 143B cells 72 h after transfection, and the effect of MK knockdown by siRNA was confirmed by using real-time PCR. $^{*}P < 0.005$. B and C, 143B cells were transfected with the indicated concentrations of MK siRNA or control siRNA and were then incubated for an additional 96 h (B, left panel). B, right panel, 143B cells transfected with 50 nM MK siRNA were incubated for the indicated times. C, left panel, 143B cells were incubated with the indicated concentrations of recombinant human MK for 96 h). C, right panel, 143B cells were incubated for the indicated times with 100 ng/mL MK. Cells were examined for viability via the MTS assay. The dashed lines represent the control group value at each time point, set at 100%. Data are means ± SD ($n = 3$). $^{*}P < 0.005$; $^{\dagger}P < 0.01$ versus control.

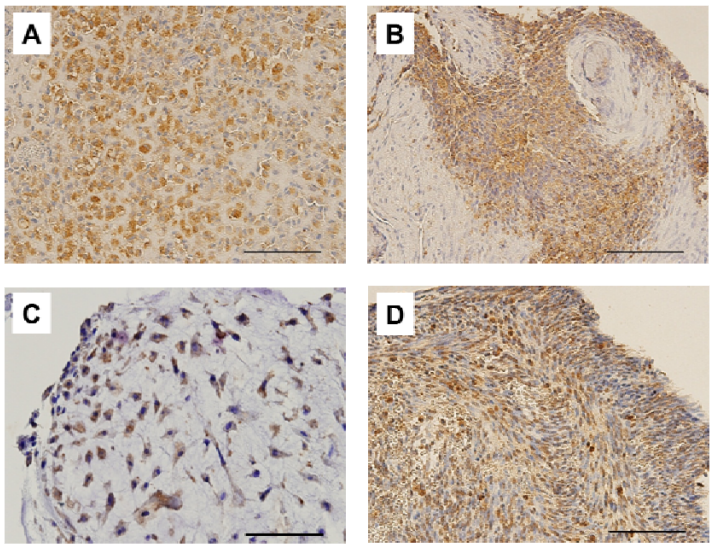
Fig. 3. Induction of cell cycle arrest and apoptosis by MK knockdown in 143B cells. A, FACS cell cycle analysis of 143B cells transfected with control siRNA or MK siRNA after 72 h of culture. $^{*}P < 0.01$. B, real-time PCR was performed to quantify mRNA expression levels of cyclin E1 (left panel) and p21 (right panel) 72 h after transfection with MK siRNA. $^{*}P < 0.01$; $^{\dagger}P < 0.0001$. C, FACS analysis of early and late apoptosis rates in 143B cells transfected with control siRNA or MK siRNA after 72 h of culture. $^{\dagger}P < 0.0001$; $^{\S}P < 0.05$. Data are means ± SD ($n = 3$). D, effect of MK knockdown on Akt phosphorylation in 143B cells. Lysates of 143B cells transfected with control siRNA or MK siRNA were immunoblotted with anti-pAkt, anti-Akt, or anti-β-actin antibody.

Fig. 4. Effect of anti-MK mAb on growth of 143B cells. A, 143B cells were incubated with 100 μg/mL anti-MK mAb for 96 h before analysis of cell viability with the MTS assay. Data, representing three independent experiments, are means ± SD ($n = 8$). B,

mice having had tumor cell injections mice received intraperitoneal injections of 100 μL of PBS containing 100 μg of anti-MK mAb or PBS alone; the animals received this same treatment every 5 days for 42 days. The graph shows the mean tumor growth rates ± SD for five animals per experimental condition. $^{*}P < 0.05$; $^{\dagger}P < 0.01$.

Fig. 5. Effect of MK on lung metastasis of 143B cells. A and B, the incidence of settlement of tumor cells to lung was evaluated after injection of 143B cells into the tail vein of SCID mice. A, representative photomicrographs of lung sections for each experimental condition; the right panel shows only one case with a colony formation in the MK knockdown group. Arrows indicate Ki-67-positive colonies. Scale bars, 2.0 mm. B, assessment of settlement of tumor cells to lung by means of the presence or absence of Ki-67-positive colonies. C and D, evaluation of lung metastasis in orthotopic xenograft model mice that had received 143B tumor cells. C, sections of lungs obtained from tumor cell-injected mice treated with PBS or anti-MK mAb were stained with an antibody against Ki-67. Arrows indicate Ki-67-positive colonies. Scale bars, 500 μm. D, lung metastasis was evaluated via assay of the number of Ki-67-positive colonies. Data are means ± SD ($n = 5$). $^{*}P < 0.05$.

Fig. 1


A
B
C
D

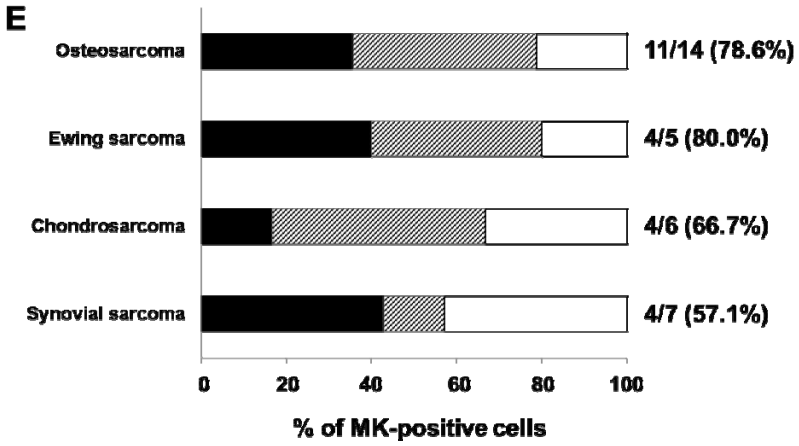

E
Osteosarcoma
Ewing sarcoma
Chondrosarcoma
Synovial sarcoma
11/14 (78.6%)
4/5 (80.0%)
4/6 (66.7%)
4/7 (57.1%)
0
20
40
60
80
100
% of MK-positive cells

Fig. 2

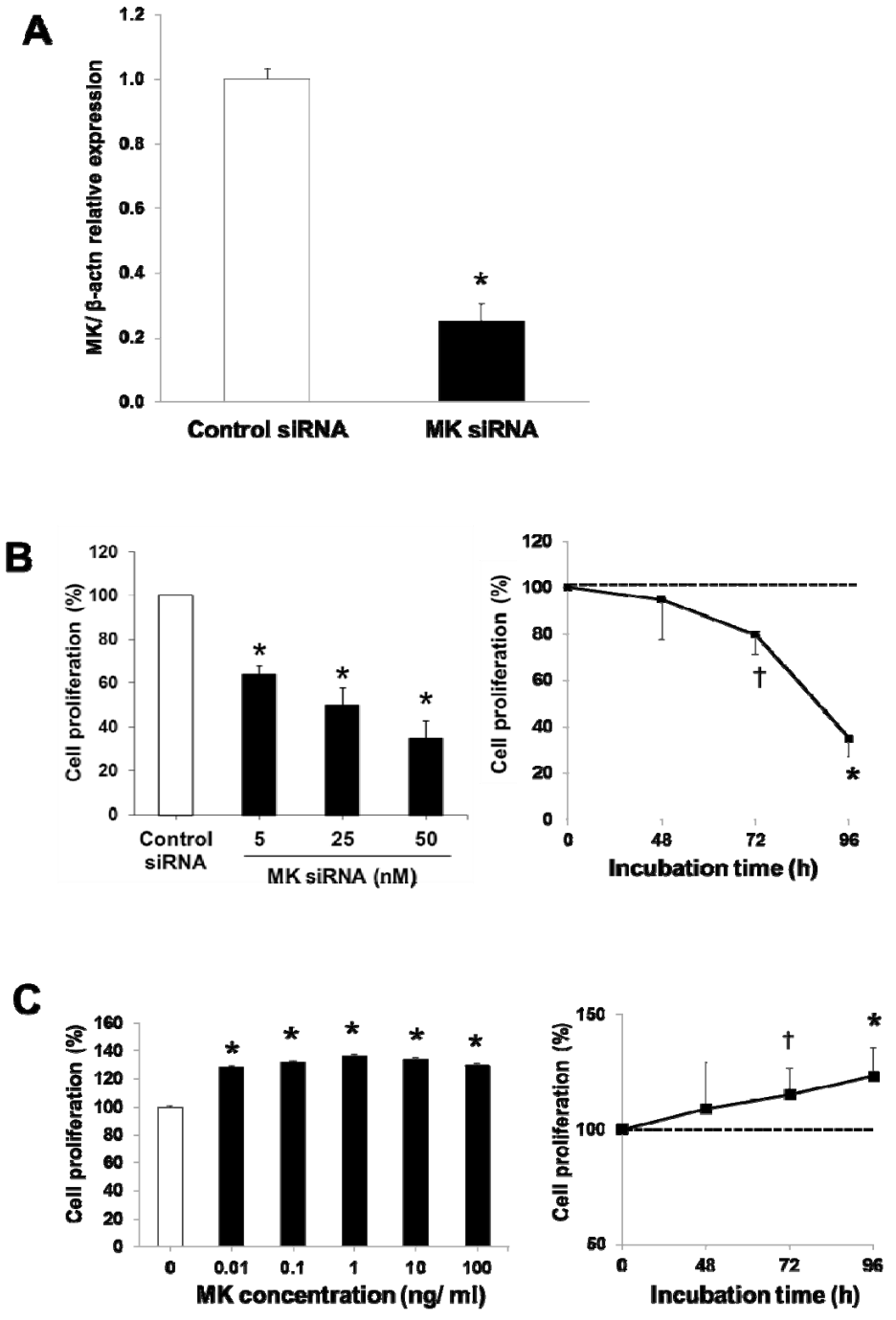
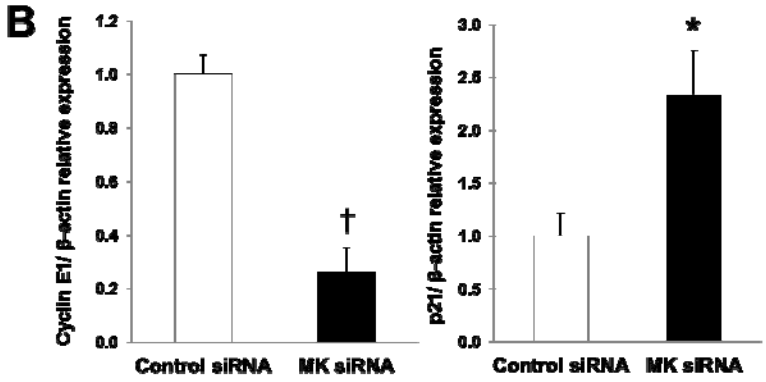
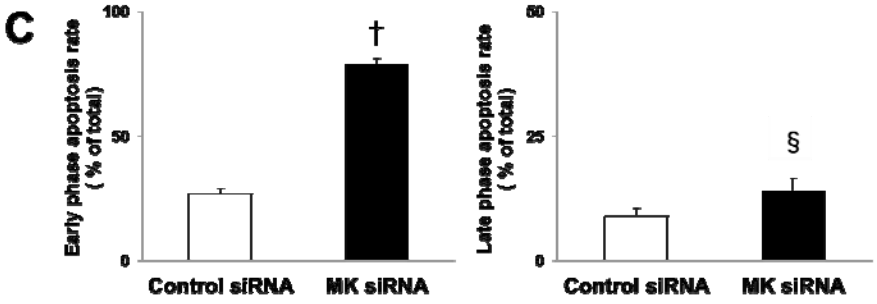

A
MK/ β-actn relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control siRNA
MK siRNA
B
Cell proliferation (%)
120
100
80
60
40
20
0
*
*
*
Control siRNA
5
25
50
MK siRNA (nM)
Cell proliferation (%)
120
100
80
60
40
20
0
†
*
0
48
72
96
Incubation time (h)
C
Cell proliferation (%)
160
140
120
100
80
60
40
20
0
*
*
*
*
*
0
0.01
0.1
1
10
100
MK concentration (ng/ ml)
Cell proliferation (%)
150
100
50
†
*
0
48
72
96
Incubation time (h)

Fig. 3

A

	Cell Cycle Phase		
	G0/G1	S	G2/M
Control siRNA	66.50 ± 3.69	26.92 ± 6.04	6.57 ± 247
MK siRNA	81.61 ± 3.71*	13.05 ± 0.98*	5.33 ± 4.62





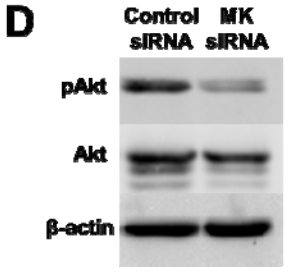


Fig. 4

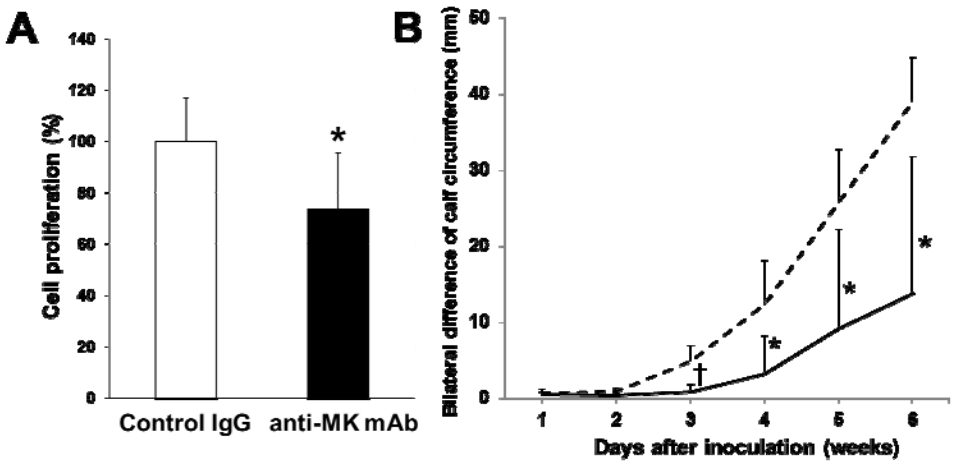
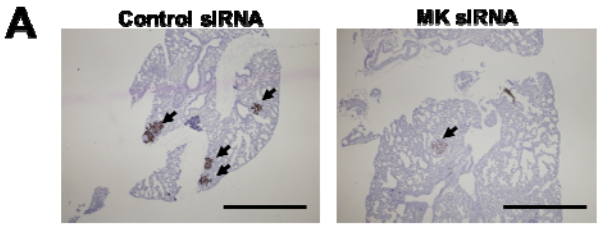

A
Cell proliferation (%)
140
120
100
80
60
40
20
0
*
Control IgG
anti-MK mAb
B
Bilateral difference of calf circumference (mm)
50
40
30
20
10
0
†
*
*
*
1
2
3
4
5
6
Days after inoculation (weeks)

Fig. 5



B

Incidence of lung metastasis (%)	
Control siRNA	3/ 6 (50%)
MK siRNA	1/ 6 (17%)