MET/ERK2 pathway regulates the motility of human alveolar rhabdomyosarcoma cells

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Abstract. In alveolar rhabdomyosarcoma (ARMS) that is a highly malignant pediatric soft tissue tumor, MET, a receptor of hepatocyte growth factor (HGF), was reported to be downstream of the PAX3-FOXO1 fusion gene specific to ARMS, and a key mediator of metastatic behavior in RMS. So far, no studies have investigated the downstream signaling pathways of MET in ARMS, even though HGF and MET have been suggested to be deeply involved in the invasiveness of ARMS. In this study, we demonstrated the functions of MET signaling in ARMS *in vitro* by using three human ARMS cell lines and three human embryonal rhabdomyosarcoma (ERMS) cell lines. *MET* mRNA levels and MET protein expression in ARMS cell lines was higher than those in ERMS cell lines as detected by real-time quantitative PCR and western blotting, respectively. Based on cell growth and cell cycle analyses it was found that HGF stimulation did not enhance the proliferation of ERMS or ARMS cell lines. HGF-stimulated cell motility of ARMS cell lines was inhibited by U0126 (ERK1/2 inhibitor) but was only partially inhibited by PD98059 (ERK1 inhibitor) or rapamycin (mTOR inhibitor) as observed in wound-healing and migration assays. Western blotting revealed that ERK1/2 was dephosphorylated by U0126 to a higher extent than by PD98059 in the ARMS cells. HGF-stimulated cell motility of Rh30 cell line was inhibited not by ERK1 siRNA, but by ERK2 siRNA. Our data thus suggest that HGF/MET signaling promotes motility of ARMS cells mainly through ERK2 signaling. A specific inhibitor of ERK2 phosphorylation could therefore be a specific anticancer agent against invasiveness and metastasis in ARMS.

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Introduction

Rhabdomyosarcoma (RMS) is the most common malignant soft tissue sarcoma, and is histologically classified into two major subtypes; embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS). Although ERMS prognosis has improved via multidisciplinary treatment (1), prognosis for ARMS is still poor. In particular, the subgroup of ARMS which expresses the *PAX3-FOXO1* chimera gene derived from the t(2;13) translocation, especially shows invasive and metastatic behavior (2,3) resulting in extremely poor prognosis (4). Therefore, it is necessary to elucidate the pathogenesis of metastasis in ARMS to develop novel drugs and treatment methods.

PAX3 is one of the transcriptional factors that play a significant role in the appropriate development during the prenatal period. PAX3 regulates the expression of MET during limb muscle development, and is required for the proper migration of myogenic precursor to the limbs (5).

Hepatocyte growth factor (HGF) and its receptor (MET) stimulate the proliferation or migration of various cancer cell lines (6). Additionally, MET promotes motility of premyogenic cells (7), and is involved in tumorigenic processes including proliferation, metastasis and invasion in malignant tumors such as hepatocellular carcinoma, colon cancer, lung cancer, bone sarcoma, or ovarian cancer (8,9). In ARMS, PAX3-FOXO1 as well as PAX3 upregulates MET (10). In mice with RMS, in which HGF is excessively expressed and there is a deficiency of Ink4a/Arf, RMS tumors develop at high rates (11) and the invasiveness of RMS cell lines is enhanced upon forced expression of MET (12). We have already confirmed that downregulation of PAX3-FOXO1 caused reduction in levels of MET, resulting in reduced cell motility upon HGF stimulation (13). Therefore, we speculate that HGF and MET are deeply involved in tumorigenesis and invasiveness of ARMS. Previous studies have reported the inhibition of metastasis in RMS cell lines by small interfering RNA (siRNA), drugs or by the constitutive activation of MET (14-16). However, the changes in the downstream processes of RMS metastasis are unknown.

In this study, we compared the responses of ERMS and ARMS cell lines to HGF stimulation and investigated the signal transduction pathways downstream of MET. Our study

suggests that ERK2 signaling, downstream of MET is a therapeutic target to suppress the invasive and metastatic properties of ARMS.

Materials and methods

Cell culture and reagents. Human ERMS cell lines RD, CTR subclone 11 (CT11), and KP-RMS-KH (KH) (17), and human ARMS cell lines SJ-Rh30 (Rh30), SCMC-RM2 (RM2) (18), and SJ-Rh28 (Rh28) were used. HepG2 cells were used as the MET expressing control cell line. Cells were maintained in RPMI-1640 containing 10% fetal calf serum (FCS) at 37°C in a 5% CO_2 incubator. Recombinant human HGF was purchased from PeproTech Inc (Rocky Hill, NJ, USA).

Quantification real-time PCR. Total RNA was extracted from the cell lines using QIAamp RNeasy Protect Mini kit (Qiagen, Valencia, CA, USA). PAX3-FOXO1 mRNA and MET mRNA were amplified by the polymerase chain reaction (Takara Bio Inc., Siga, Japan) from the cDNA template which was prepared by using Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), and real-time RT-PCR was carried out in the 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) sing SYBR-Green 1 (Takara Bio Inc.). In order to quantify the target mRNAs level, glucose-6-phosphate dehydrogenase (GAPDH) gene was used as an internal control. The cycle threshold values (Cq) for target mRNAs and GAPDH for each sample were calculated. A normalized target value was then derived by subtracting the amount of target mRNAs by that of GAPDH (ΔCq) and calculated by the ΔΔCq method (19).

siRNA. Silencer select siRNA for ERK1 (#4390624 S11137; Ambion, Austin, TX, USA), ERK2 (#4390624 S11141; Ambion) or negative control #1 (#4390843; Ambion) was transfected in Rh30 using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's recommendation. The concentration of siRNA was 0.5 nM, and the final concentration of lipofectamine RNAiMAX was 0.1%.

Western blot analysis. Cells were seeded on 6-well plates at the concentration of $2x10^5$ cells/well in 2 ml growth medium (RPMI-1640 with 10% FCS). After overnight incubation, the medium was replaced with serum-free medium for starvation. Incubations were carried out for 30 min in the presence or absence of various inhibitors, followed by HGF addition and culture for 24 h. Whole cell lysates were separated by SDS-polyacrylamide gel electrophoresis. Analysis was performed using various antibodies as previously described (13).

Cell growth analysis. Cells were seeded on 6-well plates at the concentration of $2x10^5$ cells/35 mm-well in 2 ml of growth medium (RPMI-1640 with 10% FCS). After culturing overnight, the medium was replaced with fresh RPMI and serum-starved (0 h). Then, the cells were cultured without serum, in the presence of 10% FCS or HGF. The cells were harvested by trypsin processing after 24, 72 and 120 h and counted by Coulter counter.
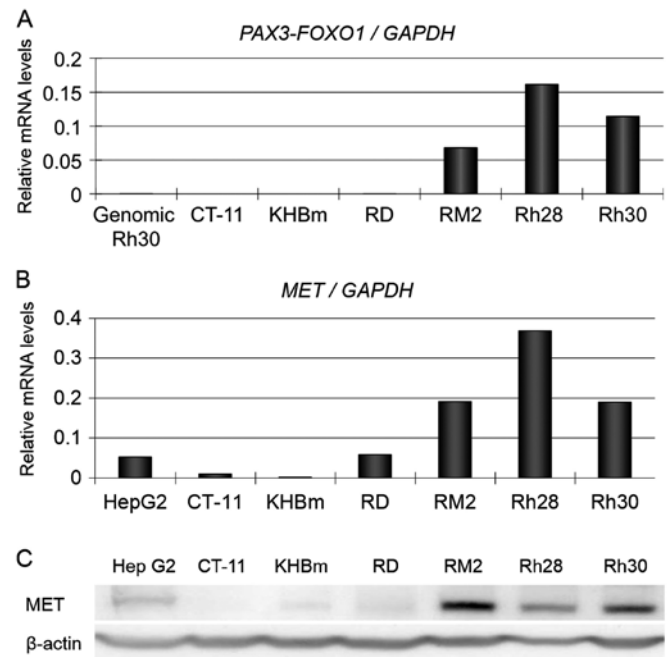

Figure 1. Expression of *PAX3-FOXO1* and *MET* on human RMS cell lines. Quantitative real-time PCR by SYBR Green I method was performed for *PAX3-FOXO1* and *MET* genes (A and B). *GAPDH*, which is a known housekeeping gene, was used as the internal control. The experiment was repeated thrice with similar results. MET protein expression in RMS cell lines was examined by western blotting (C).

Cell cycle analysis. Cells were harvested after being cultured for 24 h in the presence of nocodasol in the culture bottle. Then, these were seeded on a 100-mm dish and cultured overnight. The medium was changed to the serum free medium, and the cells were cultured for another 24 h without serum, in the presence of 10% FCS or HGF 75 ng/ml. These were harvested after trypsinization and washed once with PBS. After releasing the cells in the propidium iodide solution (50 mg/ml) to which RNase had been added and incubation for 30 min, analysis was made by flow cytometry.

Wound-healing assay. Cells were seeded on 12-well plates and cultured for 24 h. These cell layers were then scratched with a pipette tip and washed with RPMI (0 h). After incubating in serum-free medium, or with rapamycin (100 ng/ml), PD98059 (20 μM), and U0126 (20 μM) for 30 min, HGF (75 ng/ml) was added and the cells were then cultured for 24 h. Images of each well were captured at 0 and 24 h, and the distance between edges of the wound region was measured.

Migration assay. An insert (pore size 8 micrometer; Nunc A/S, Roskilde, Denmark) was placed on the wells of 12-well plates, and cells were seeded in the upper chamber of the insert ($3x10^4$ cells/insert). Various inhibitors were added to the upper chamber of the insert and treatments were carried out for 30 min. HGF (75 ng/ml) was then added to the lower chamber of the insert, and cells were cultured for 24 h. The cells that migrated were fixed and stained with Diff-Quik (Sysmex, Hyogo, Japan) and counted under the microscope.
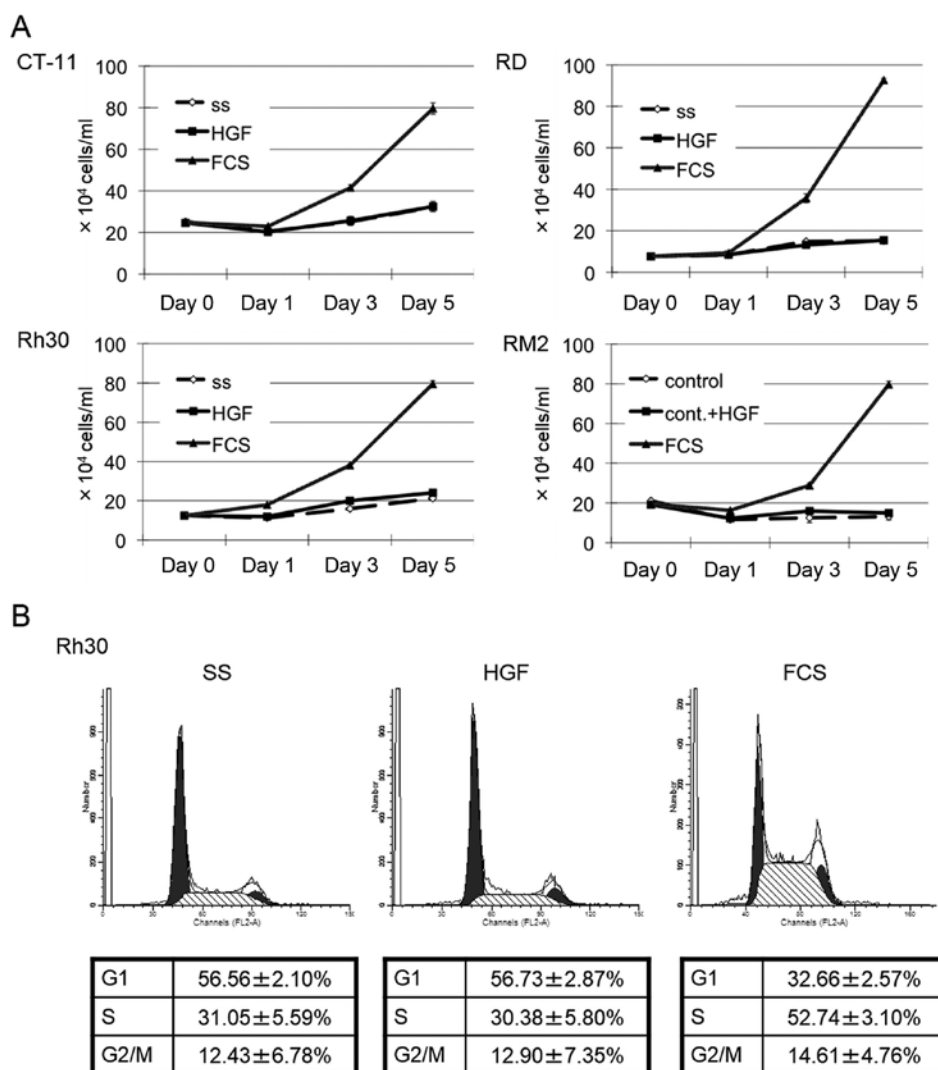
Figure 2. HGF stimulation does not influence proliferation of ARMS cell lines. HGF does not regulate cell growth of the ERMS or the ARMS cell line. The control in RM2 is 0.5% FCS (A). HGF does not stimulate cell cycle progression in the Rh30 ARMS cell line (B).

Results

MET is overexpressed in parallel with PAX3-FOXO1 expression in ARMS cells. *PAX3-FOXO1* and *MET* mRNA levels were examined in both ERMS and ARMS cells. *PAX3-FOXO1* expression was detected only in the ARMS cell lines (Fig. 1A) whereas *MET* mRNA levels in the ARMS cell lines (Rh28, Rh30, and RM2) were apparently higher than those in ERMS cells (RD, CT-11, and KH-BM) (Fig. 1B). By western blotting, we confirmed that MET expression in the three ARMS cell lines was higher than in the three ERMS cell lines that did not express *PAX3-FOXO1* fusion gene (Fig. 1C).

HGF stimulation does not influence proliferation of alveolar type Rh30 cells. We investigated the effect of HGF stimulation on ERMS and ARMS cell proliferation under serum starved conditions. Upon stimulation with HGF, there was no significant growth in the ERMS cells, RD and CT-11, or in the ARMS cells, Rh30 and RM2, whereas all four cell lines proliferated in the presence of 10% FCS (Fig. 2A). We then examined the influence of HGF on cell cycle progression in ARMS Rh30 cells. When ARMS Rh30 cells were stimulated

with HGF, no significant changes in cell cycle phase distribution were observed, whereas addition of 10% FCS apparently stimulated a decrease of cells in the G1 phase and increased the cells in the S phase indicating G1-S cell cycle progression (Fig. 2B).

U0126 inhibits HGF-stimulated motility of ARMS cell lines. The effect of HGF on the motility of the ARMS cell lines, Rh30, RM2, and Rh28 was then examined by a wound-healing assay. In 24 h after HGF stimulation, the percentage of the distance between the wound edges in comparison to unstimulated control cells were calculated to be 78.7±7.9, 100±0, and 67.3±20.0%, respectively. On the other hand, wound healing was not observed in the ERMS cell line, RD (Fig. 3A). We then studied the effects of several specific inhibitors against the potent signaling downstream of MET on the HGF-stimulated motility. Motility of the three ARMS cells upon HGF stimulation was not obviously inhibited by the mTOR inhibitor, rapamycin (100 ng/ml) (74.6±17.7, 85.0±6.6 and 80.7±9.3%, respectively). When the MEK inhibitor, PD98059 (20 μM) was used, motility was inhibited partially (47.5±2.5, 95.5±5.4 and 64.3±23.3%, respectively). However,
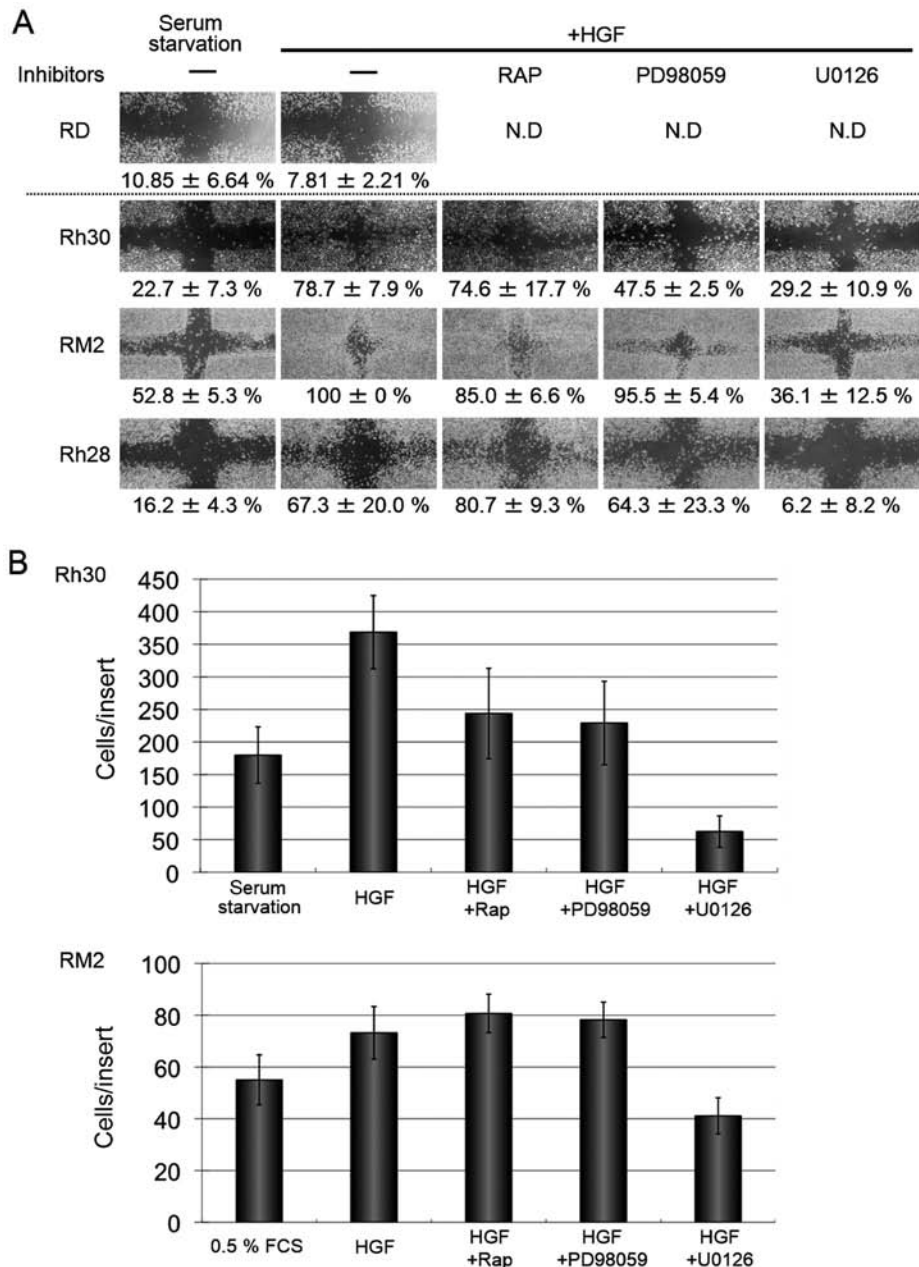
Figure 3. HGF induces motility of ARMS cell lines. Wound healing, which reflects cell motility and is induced by HGF, is not inhibited by Rapamycin, partially inhibited by PD98059, and remarkably inhibited by U0126 (A). The percentage indicated below the images represents the degree to which the wound is healed (mean ± SD, repeated 3 times). Migration assay shows that HGF induces motility of ARMS cell lines, which is inhibited by U0126, but neither by Rapamycin nor PD98059 (B).

another MEK inhibitor, U0126 at the concentration of 20 μM could remarkably inhibit the motility of the three ARMS cell lines, Rh30, RM2, and Rh28 (29.2±10.9, 36.1±12.5 and 6.2±8.2%, respectively) (Fig. 3A).

Further, we confirmed this phenomenon by a migration assay. The number of migrating cells upon HGF stimulation was increased in ARMS cell lines, Rh30 and RM2 (369±56 cells and 73±10 cells, respectively) compared with unstimulated controls (179±43 cells and 55±9 cells, respectively). HGF-stimulated migration was not completely inhibited by rapamycin (100 ng/ml) (244±69 cells and 81±7 cells, respectively) or by PD98059 (20 μM) (229±64 cells and 78±1 cells, respectively). However, it was markedly inhibited by U0126 at 20 μM (Fig. 3B) (63±24 cells and 41±7 cells, respectively).

ERK1/2 in RMS cells was completely dephosphorylated by U0126. In order to investigate differences in the inhibitory effects of MEK inhibitors PD98059 and U0126 on the motility of ARMS cells, ERK1/2 phosphorylation status was compared in the ERMS cell line RD and the ARMS cell lines Rh30, RM2, and Rh28. In all three ARMS cell lines even 20 μM of PD98059 did not completely inhibit ERK2 phosphorylation, whereas the same concentration of PD98059 completely blocked ERK2 phosphorylation (Fig. 4A) in ERMS RD cells. On the other hand, 20 μM of U0126 completely blocked ERK2 phosphorylation in ERMS as well as ARMS cells (Fig. 4A).

HGF-stimulated motility of Rh30 stimulation is inhibited by ERK2 siRNA. In order to confirm the role of ERK2 in
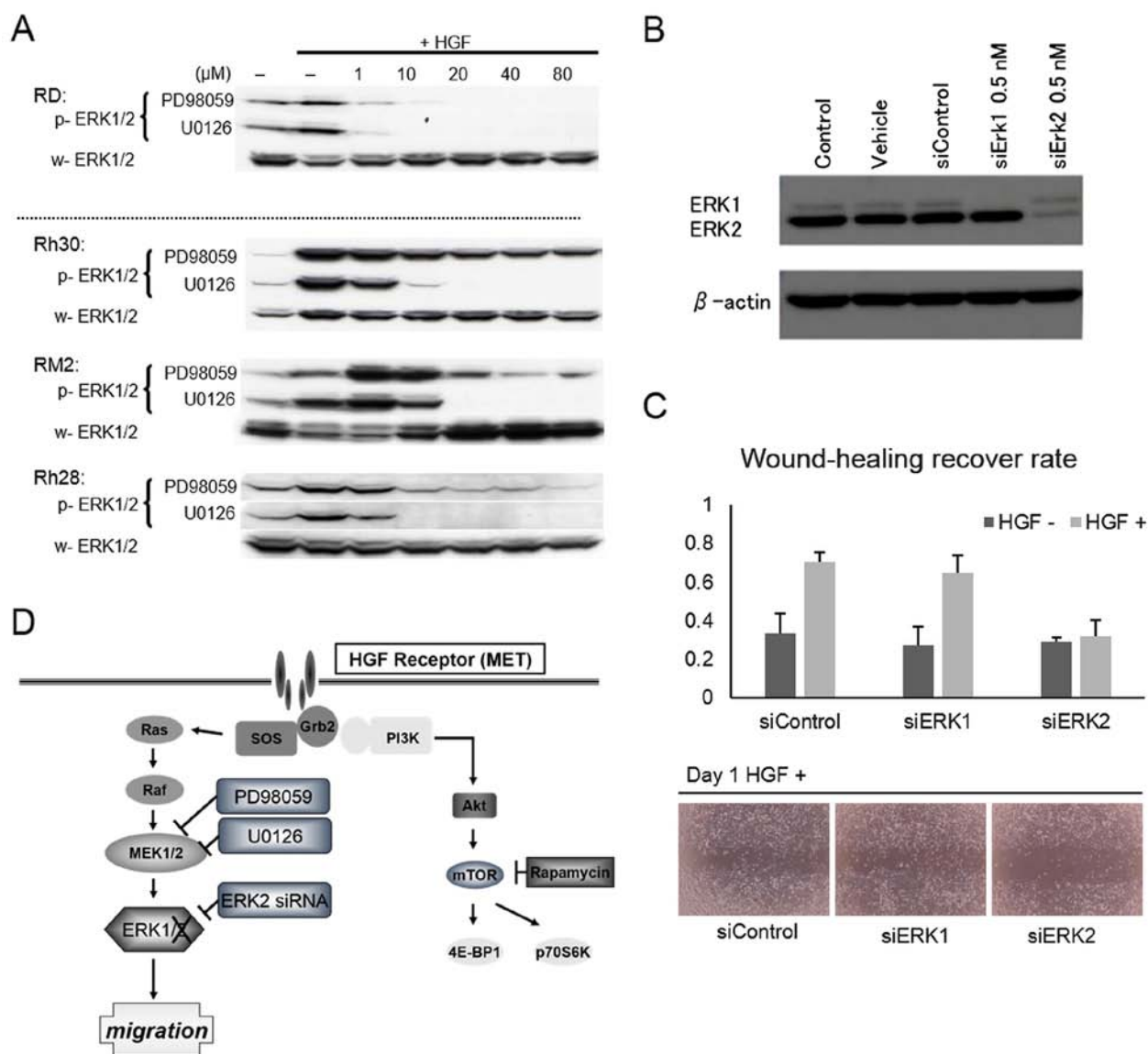
Figure 4. ERK2 regulates motility of ARMS cells. ERK1/2 in RMS cells was completely dephosphorylated not by PD98059, but by U0126 (A). Knockdown of ERK1 or ERK2 protein by respective ERK1 or ERK2 siRNA (B). Downregulation of ERK2 but not ERK1 inhibited HGF-induced motility of Rh30 ARMS cell line (C). Schematic representation of the cellular motility related signal transduction pathway regulated by HGF/MET in ARMS cell lines (D). HGF-induced motility of ARMS cell lines is mainly regulated by the Ras-Raf-ERK1/2 signaling pathway and ERK2 phosphorylation has the most important role on this effect.

promoting HGF-stimulated mobility of ARMS cells, we knocked down ERK1 or ERK2 expression using specific siRNA in the ARMS Rh30 cells. ERK1 and ERK2 protein levels were sufficiently suppressed by siRNA transfection (Fig. 4B). When the wounding assay was performed using the siRNA transfected cells, the percentages of wound-healing recovery for HGF-stimulated cells with the control siRNA, ERK1 siRNA, and ERK2 siRNA were calculated to be 70.7±4.98, 65.0±9.17, and 32.1±8.01%, respectively. This indicated that HGF-stimulated motility of Rh30 cells was not inhibited by the knockdown of ERK1, but ERK2.

Discussion

Clinically, ARMS is well known to be more aggressive than ERMS, probably due to the presence of the PAX3-FOXO1 fusion protein (20). It has been reported that MET is upregulated

in ARMS cell lines that express PAX3-FOXO1 (10). Recently it was reported that MET is regulated by the muscle-specific miR1/206 (21).

First, we confirmed that MET is more strongly expressed in the PAX3-FOXO1-positive human ARMS cell lines Rh30, RM2, and Rh28 than in the human ERMS cell lines RD, CT-11, and KH (Fig. 1). We then studied the biological significance of MET expression in ARMS cells. Unlike observations in other cancers (8,9,22), HGF stimulation did not promote RMS cell proliferation (Fig. 2). Since HGF and MET contribute to motility in colon cancer, osteosarcoma, and small cell lung cancer cell lines (23), we studied the effect of HGF on motility in RMS cell lines using the wound-healing assay and migration assays. Our findings, that HGF stimulated motility in the three ARMS cell lines, but not in the ERMS RD cells (Fig. 3), suggests that HGF/MET signaling regulates the metastatic and invasive features of ARMS. Ras-Raf-ERK1/2 and PI3K-

AKT-mTOR pathways are well known to be downstream of HGF/MET signaling (24,25). We confirmed that HGF could phosphorylate ERK1/2 that is downstream of MET, in all three ARMS cell lines suggesting that these pathways are active in ARMS.

In order to determine the pathway responsible for motility of ARMS cells, we used several inhibitors including the mTOR inhibitor, rapamycin (26), the ERK1 inhibitor, PD98059, and the ERK1/2 inhibitor, U0126. The inhibitory effects of these compounds on the HGF-stimulated motility of Rh30, Rh28, and RM2 cells, were studied by both the wound-healing and migration assay. Our finding, that the motility of all three cell lines was not inhibited by either rapamycin or by PD98059, but was completely inhibited by U0126 (Fig. 3), indicates that ERK1/2 signaling regulates motility of ARMS cells.

We next examined the HGF-stimulated phosphorylation status of ERK1/2 upon treatment with PD98059 or U0126 at the same concentrations used in the wounding and migration assays. At a concentration of 20 μM, the ERK1 inhibitor PD98059 could not completely dephosphorylate ERK2 (remaining lower band in western blotting in Fig. 4A) in all three ARMS cell lines. However, U0126, an inhibitor of both ERK1 and ERK2, completely dephosphorylated ERK1 and ERK2 at a concentration of 20 μM or higher (Fig. 4A). According to all the above results, we hypothesized that ERK2 phosphorylation was responsible for the HGF-stimulated motility in ARMS cells.

We then knocked down ERK2 expression by using a specific siRNA against ERK2. The inhibitory effect on the mobility of ARMS Rh30 cells was studied using an ERK2 siRNA designed with confirmed efficacy (Fig. 4B). Knockdown of ERK2 inhibited the wound-healing induced by HGF at a concentration which did not stimulate cell growth in Rh30 cells (Fig. 4C). Inhibition of wound-healing by ERK2 knockdown indicates that ERK2 is a key mediator for the motility of ARMS Rh30 cells. The isoform specificity of ERK1 and ERK2 has not been widely addressed. Indeed several studies have shown redundant functions for ERK1 and ERK2 (27,28), but it has been recently reported that ERK2, but not ERK1, mediates cell motility (29,30), which is consistent with our observations (Fig. 4B and C).

Our findings are summarized in Fig. 4D. PAX3-FOXO1 upregulates the HGF receptor MET, in ARMS cells. HGF stimulates motility of ARMS cells mainly through the ERK1/2 pathway. ERK2 phosphorylation particularly appears to be the most critical event in ERK1/2 signaling for cell motility in the more aggressive ARMS cells.

HGF/MET has been reported to be involved in tumorigenicity in various cell types (6). The possibility of modifying tumor metastasis by inducing angiogenesis has also been suggested (31,32). Previous studies have reported that the levels of HGF in serum, amplification of MET, or MET overexpression were clinically associated with poor prognosis for some cancers (33,34). Even in RMS, overexpression of MET as well as expression of PAX3-FOXO1 are closely associated with malignancy, progression, and invasion (13,35,36).

In addition, HGF promotes the metastatic behavior of MET-positive RMS cell lines to the bone marrow or lymph nodes and contribute to their resistance to Radio-chemotherapy (37). The existence of metastasis is an important poor prognostic factor in patients with PAX3-FOXO1 positive ARMS (38). Furthermore, the frequency of PAX3-FOXO1 positive cells in metastatic ARMS tumors is higher than that in the primary ARMS tumors in preclinical mouse models (39). These reports and our results collectively suggest that the poor outcome of ARMS results from metastasis induced by high expression of PAX3-FOXO1 and MET.

In this study, our results suggest that the MET-ERK1/2 pathway, especially the phosphorylation of ERK2 plays a major role in cell motility of ARMS. Selective inhibitors against this pathway, and particularly against ERK2 phosphorylation, might be a novel anticancer agent to regulate the invasive and metastatic characteristics of ARMS.

Acknowledgements

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