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Screening of novel Midkine binding protein by BioID2-based proximity labeling

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ABSTRACT

Midkine (MK), a heparin-binding growth factor, is associated with the poor prognosis of the pediatric tumor, neuroblastoma. MK would be a druggable target as many studies showed inhibition of its function in various cancers suppressed tumor developments. To establish the therapy targeting MK, identification of its binding partners, and elucidation of its intracellular signaling are needed. It was reported that exogenous MK induced phosphorylation of ribosomal protein S6 (RPS6) downstream of mTOR signaling. Using RPS6 phosphorylation as a marker of MK response, we searched for MK reactive cell lines. We found that MK cell lines expressing less MK tended to respond better to MK. Next, using an MK reactive neuroblastoma cell line, MK-knocked down SH-SY5Y cells, we employed a proximity-dependent biotin identification method, which was invented to evaluate protein-protein interactions by biotinylation. We confirmed that secreted MK fused to the biotin ligase BioID2 (MK-BioID2) was able to biotinylate proteins from the cells. Biotinylated proteins were identified by liquid chromatography-mass spectrometry analyses. Twenty five proteins were found to be overlapped after three independent experiments, among which insulin-like growth binding protein 2 (IGFBP2) was further analyzed. IGFBP2 was indeed detected with immunoblotting after streptavidin pull down of MK-BioID2 labeled cell extract of MK-knocked down SH-SY5Y cells. Our study suggests that the BioID2 method is useful to identify binding partners of growth factors.

Keywords: MK, BioID2, IGFBP2

Abbreviations:
MK: midkine
RPS6: ribosomal protein S6
BioID: proximity-dependent biotin identification
LC-MS/MS: liquid chromatography-mass spectrometry
IGF: insulin-like growth factor
IGFBP2: insulin-like growth factor binding protein 2

INTRODUCTION

Midkine (MK) is a heparin-binding growth factor, which is associated with the poor prognosis of the pediatric tumor, neuroblastoma.[1,2] A study for pancreatic cancer suggested that MK was involved in the migration and proliferation of cancer cells *in vitro*.[3] Another study for non-small cell lung carcinoma suggested that MK was involved in epithelial-mesenchymal transition.[4] Secreted MK from cancer cells also promoted migration and angiogenesis of endothelial cells in tumor microenvironments.[4] Furthermore, inhibiting MK function suppressed the growth of several tumors in vivo.[4-7] In a study using neuroblastoma xenograft models, RNA aptamer targeting MK suppressed tumor growth.[5] A study for osteosarcoma demonstrated that MK neutralizing antibody treatment reduced its proliferation and metastasis.[6] Therefore, MK would be a druggable target for new cancer therapy. To develop the therapy targeting MK, identification of its binding partners, and elucidation of its intracellular signaling are needed. Although MK receptors have been reported,[8-15] the biological significance of the relationship between MK and these receptors was inconsistent or ambiguous. Thus, there may be another novel MK receptor involved in MK's multiple functions[16] or interacting proteins which is required to transmit intracellular signaling through putative receptors.

To identify novel MK binding partners, any types of bindings including transient or weak protein-protein interaction need to be extensively detected. The BioID method has been developed to observe protein-protein interactions by biotin ligase fused to a protein of interest.[17,18] This biotin ligase-fused protein biotinylates proximal proteins promiscuously within the radius of 10 nm. Since the biotinylation is covalent binding, the modification of proximal proteins can remain even though their interactions are transient or proximal proteins are moved out from the biotin ligase fused-protein.[18] By the combination of this method and liquid chromatography-mass spectrometry (LC-MS/MS) analysis, we sought to identify novel MK binding partners.

MATERIALS AND METHODS

Cell culture

SK-N-BE(2), SK-N-DZ, and SH-SY5Y Wild type (WT) cells were purchased from ATCC (USA). SH-SY5Y with MK knockdown (SH-SY5Y MK-K/D) cell line was generated as follows: briefly, SH-SY5Y (WT) cells were infected with the lentivirus carrying an shRNA targeting human MDK 3'UTR (TRCN0000303918; Sigma-Aldrich Co.), and they were selected by puromycin (anti-pr; InvivoGen) at the final concentration of 2.0 μg/mL. They were cultured in Minimum Essential Medium Eagle (MEM) (M4655-500ML, Sigma-Aldrich Co.)/Ham's F-12 (11765-054; Thermo Fisher Scientific Inc.) supplemented with 1% sodium pyruvate (S8636-100ML; Sigma-Aldrich Co.) and 1% MEM non-essential amino acids (1140-050; Thermo Fisher Scientific Inc.). SK-N-DZ cells were cultured in MEM supplemented with 1% MEM non-essential amino acids. LA-N-5 cells were obtained from the Childhood Cancer Repository (USA). KELLY cells were provided by Dr. N. Hattori (National Cancer Center Research Institute, Tokyo, Japan). SH-EP cells were provided by Prof. M. Schwab from the German Cancer Research Center (Germany) and CHP134 cells were purchased from RIKEN BRC Cell Bank (Japan). They were maintained in RPMI-1640 (R8758-500ML, Sigma-Aldrich Co.). IMR-32 cells were purchased from the JCRB Cell Bank (Japan) and cultured in MEM supplemented with 1% non-essential amino acids, 1% sodium pyruvate, and 1% MEM non-essential amino acids. NB1 cells were purchased from RIKEN BRC Cell Bank (Japan) and cultured in MEM/RPMI-1640. All media were supplemented with 10% heat-inactivated fetal bovine serum (FB-1285/500; Biosera). All

cell lines were maintained in a humidified 5% CO_2 incubator at 37°C.

MK administration experiment

3.0×10^5 cells of SH-SY5Y WT and MK-K/D, and 1.0×10^5 cells of SH-EP were seeded on 6-well plates. 4.0×10^5 cells of SK-N-DZ and 5.0×10^5 cells of LA-N-5 were seeded on collagen-coated 6-well plates. After two days, SH-SY5Y WT, MK-K/D, and SK-N-DZ were starved for 12 hours, SH-EP were starved for 24 hours and LA-N-5 were starved for 6 hours. After the starvation, recombinant human MK (rhMK) derived from *E.coli* (450-16; PeproTech), rhMK derived from yeast,[19] at the final concentration of 5.0 μg/mL and 1×PBS (−) as control were administrated. These plates were mildly shaken, and then incubated for 20 minutes. Cells were rinsed with 1×PBS (−) and lysed by 1×sample buffer (62.5 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 1.55% dithiothreitol, and 0.01% bromophenol blue).

Plasmids

MCS-BioID2-HA[20] was a gift from Kyle Roux (#74224; Addgene). MK-BioID2-HA was prepared as follows. Human MK (variant 3; NM_002391) cDNA was obtained from SH-SY5Y cells. The coding sequence of human MK was amplified by a primer set containing NheI and BamHI restriction sites, and subcloned into a multiple cloning site of MCS-BioID2-HA.

Preparation of MK-BioID2 conditioned medium

The biotin in fetal bovine serum was depleted by streptavidin-conjugated agarose beads (20349; Thermo Fisher Scientific Inc. Inc.) at the final concentration of 0.5% (slurry: 50%) and shaken overnight at 4°C, and then filtered using PES membrane (pore size: 0.22 μm) (SLGPR33RB; Merck Millipore Ltd.). It was preserved at 4°C.

Human embryonic kidney 293T (HEK293T) cells were obtained from IFOM (Italy). 15 cm dishes were coated by Collagen Type 1 (08-115; Merck Millipore Ltd.) dissolved in 1×PBS (−) (approximately 3.0 μg/cm²). $1.0–1.5 \times 10^7$ cells of HEK293T were seed on the dishes. HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (D5796-500ML; Sigma-Aldrich Co.) supplemented with 10% biotin-depleted fetal bovine serum. After 2 days, the culture medium was replaced with DMEM supplemented with 1% Insulin-Transferrin-Selenium-A (51300-044; Thermo Fisher Scientific Inc.), and HEK293T cells were transfected with MK- or MCS-BioID2 plasmid using poly-ethyleneimine-Max (pH 7.2) (24765-1; Polyscience Inc). Briefly, 100 μg (in 100 μL) of poly-ethyleneimine-Max and 850 μL of DMEM were vigorously mixed and incubated for 5 minutes at room temperature. Then 50 μg (in 50 μL) of the plasmid were added into the mixture, vortexed and then incubated for 20 minutes. It was added into the cultured cells dropwisely. After 1 day, the culture medium was changed with DMEM supplemented with 1% insulin-transferrin-sodium-selenium-A and 0.5% penicillin-streptomycin, and further cultured for 2 days. Culture supernatant was collected in a protein low binding tube, and preserved at −80°C.

Biotinylation assay

For in tube biotinylation assay, MK-BioID2 conditioned medium was supplemented with 80 μg/mL biotin (B4639; SIGMA) dissolved in DMSO, 0.5 mM ATP (A6419; SIGMA) and 1.5 mM magnesium acetate (63052; SIGMA).[21] Then, they were incubated for 24 hours at 37°C with agitating at 1500 rpm using ThermoMixer C (Eppendorf). The reaction mixture was collected by 2×sample buffer.

For biotinylation assay to cells, SH-SY5Y MK-K/D cells were cultured for 3 days in DMEM supplemented with 10% biotin-depleted fetal bovine serum and 0.5% penicillin-streptomycin. The culture medium was replaced with DMEM supplemented with 1% insulin-transferrin-sodium-

selenium-A and 0.5% penicillin-streptomycin to deplete residual biotin for 24 hours. Then, the culture medium was changed with MK-BioID2 conditioned medium supplemented with 80 μg/mL biotin dissolved in DMSO, 0.5 mM ATP, and 1.5 mM magnesium acetate, and then cultured for 24 hours. Cells were lysed with a 1×sample buffer.

Pull down of biotinylated proteins using streptavidin agarose beads

9.0×10^5 cells of SH-SY5Y MK-K/D per one dish (×10) were seeded in each condition. Cells were rinsed with 1×TBS buffer (317-90371; Nippon Gene) twice, and lysed with 1.0 mL TBS lysis buffer containing 0.4% NP-40 and 2% protease inhibitor cocktail (04080-24; Nacalai Tesque). They were placed on ice for 30 minutes with occasional mixing, harvested to a 1.5 mL tube, centrifuged at 10000 g at 4°C for 1 minute, and sonicated. This procedure was repeated three times. Then, these samples were centrifuged at 20000 g at 4°C for 30 minutes to remove insoluble materials. 850 μL of supernatants were transferred to protein low binding tubes containing 150 μL streptavidin-conjugated agarose beads (slurry: 50%), and rotated overnight at 4°C. Residual supernatants were collected as inputs. The beads were centrifuged and washed with the same TBS buffer containing 2.0 M urea five times. Tubes were changed whenever the beads were washed, and at the final wash, a part of their suspensions was collected as streptavidin-pulled down samples to confirm biotinylated proteins by silver staining and immunoblotting. The beads were eluted with 150 μL of 8.0 M Guanidine-HCl (pH 1.5) for 30 minutes at room temperature. The beads were centrifuged, and supernatants were transferred to 1.5 mL tubes and neutralized by the addition of 1/10 volume of 3.0 M Tris-HCl (pH 8.5). Sample preparation for LC-MS/MS was performed three times independently.

LC-MS/MS analysis

The eluted proteins were digested by trypsin for 16 hours at 37°C after reduction and alkylation. The peptides were analyzed by LC-MS using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific Inc. Inc.) coupled to an UltiMate3000 RSLCnano LC system (Dionex Co.) using a nano HPLC capillary column, 150 mm × 75 μm i.d (Nikkyo Technos Co.) via a nanoelectrospray ion source. Reversed-phase chromatography was performed with a linear gradient (0 min, 5% B; 100 min, 40% B) of solvent A (2% acetonitrile with 0.1% formic acid) and solvent B (95% acetonitrile with 0.1% formic acid) at an estimated flow rate of 300 nL/min. A precursor ion scan was carried out using a 400–1600 mass to charge ratio (m/z) before MS/MS analysis. Tandem MS was performed by isolation at 0.8 Th (Thomson: the unit of charge ratio) with the quadrupole, HCD fragmentation with a normalized collision energy of 30%, and rapid scan MS analysis in the ion trap. Only those precursors with charge states 2–6 were sampled for MS2. The dynamic exclusion duration was set to 15 seconds with a 10 ppm tolerance. The instrument was run in top speed mode with 3 seconds cycles.

For data analysis, the raw data were processed using either Proteome Discoverer 1.4 (Thermo Fisher Scientific Inc. Inc.) in conjunction with the MASCOT search engine, version 2.6.0 (Matrix Science Inc.) for protein identification. Peptides and proteins were identified against the human protein database in UniProt (release 2020_04), with a precursor mass tolerance of 10 ppm, a fragment ion mass tolerance of 0.8 Da. A Fixed modification was set to carbamidomethylation of cysteine, and variable modifications were set to oxidation of methionine and biotinylation of lysine. Two missed cleavages by trypsin were allowed.

LC-MS/MS data filtration

Detected proteins from LC-MS/MS analyses were further filtered as follows: 1. Keratins and keratinocyte proline-rich protein were removed. 2. The proteins whose score of CRAPome version

2.0[22] were less than 100 (/716) were applied. 3. The proteins whose biotin (+) MASCOT score was more than 0 and its proportion of biotin (+/−) was more than 2.0 were applied. Using the filtrated data, the proteins detected in all three independent analyses became candidates.

Immunoblotting blotting

Cultured cells were lysed with a 1×sample buffer. Conditioned medium, cultured medium and the samples lysed with TBS buffer were mixed with a 2×sample buffer and boiled for 5 min. Samples were separated by SDS-PAGE and transferred to nitrocellulose membranes (10600001; Cytiva). The following antibodies were used: anti-ß-actin (C-15) mouse monoclonal antibody (mAb) (A5441; Merck), MK (A-9) mouse mAb (sc-46701; Santa Cruz Biotechnology), RPS6 (5G10) rabbit mAb (#2217; CST), phospho-RPS6 (D57.2.2E) XP rabbit mAb (#4858; CST), insulin-like growth factor binding protein 2 (IGFBP2) (EPR18012-257) rabbit mAb (ab188200; Abcam). ALK (C26G7) rabbit mAb (#3333; CST), ApoER2 (EPR3326) rabbit mAb (ab108208; Abcam), Syndecan-2 rabbit antibody (#36-6200; Invitrogen), Horseradish Peroxidase-conjugated streptavidin (434323; Invitrogen) and peroxidase-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG (315-035-048 or 111-035-144; Jackson ImmunoResearch). Chemiluminescence was developed using the Immobilon Classico or Forte substrate (WBLUC0100 or WBLUF0100; Merck) and detected on the Amersham Imager 680 (Cytiva). Silver staining was conducted using PierceTM Silver Stain Kit (24612; Thermo Fisher Scientific Inc. Inc.) according to manufactures protocol.

RESULTS

MK low expression cell lines show RPS6 phosphorylation by MK administration

We sought to determine MK reactive cell lines. We firstly speculated that the degree of endogenous MK expression in each cell line would be a factor influencing the response to MK. We prepared various neuroblastoma cell lines and evaluated the MK protein expression by immunoblotting. In addition, as a positive control of MK low expression, we also prepared SH-SY5Y with shRNA-mediated MK knockdown (hereafter: SH-SY5Y MK-K/D) to compare the MK expression in other neuroblastoma cell lines. MK is highly expressed in SH-SY5Y wild type (WT), NB1, and SK-N-DZ cells (Fig. 1A). SH-SY5Y MK-K/D cells showed the lowest MK expression, followed by SH-EP, SK-N-BE(2), and LA-N-5 cells (Fig. 1A). As we wondered whether there was a correlation between MK expression and its putative receptors, we evaluated the expressions of ALK, ApoER2, and Syndecan-2. ALK was highly expressed in NB1 cells, but was not in SK-N-DZ and SH-SY5Y WT cells, even though both two cell lines showed MK high expression (Fig. 1A). For the other receptors, there was little correlation between the expression of ApoER2 and Syndecan-2 and that of MK (Fig. 1A).

Olmeda et al reported that the conditioned medium from MK expressing melanoma cells induced phosphorylation of ribosomal protein S6 (RPS6) downstream of mTOR signaling in human lymphatic endothelial cells.[23] Therefore, we wondered whether MK also induced PRS6 phosphorylation in neuroblastoma cells. We used a commercially available MK derived from E. coli and a house-made MK derived from yeast.[19] Considering that MK concentration in plasma ranged from 23 pg/ml to 1.1 μg/mL in neuroblastoma patients,[2] it is expected that MK concentration is higher than this range in tumor tissues. We determined that the minimum concentration of MK required for RPS6 phosphorylation was 1.25 μg/mL (data not shown). Thus, we performed this MK administration experiment at 5.0 μg/mL MK to induce RPS6 phosphorylation. MK could induce RPS6 phosphorylation clearly at 20 minutes in MK low expression cell lines,

SH-SY5Y MK-K/D, SH-EP, and LA-N-5 (Fig. 1B). In contrast, in MK high expression cell lines, SH-SY5Y WT and SK-N-DZ, PRS6 phosphorylation was not induced (Fig. 1C). Taken together, MK induced RPS6 phosphorylation in neuroblastoma cells that have little or no MK expression, which indicated that these cells could be a good model to investigate MK receptor or binding partners.
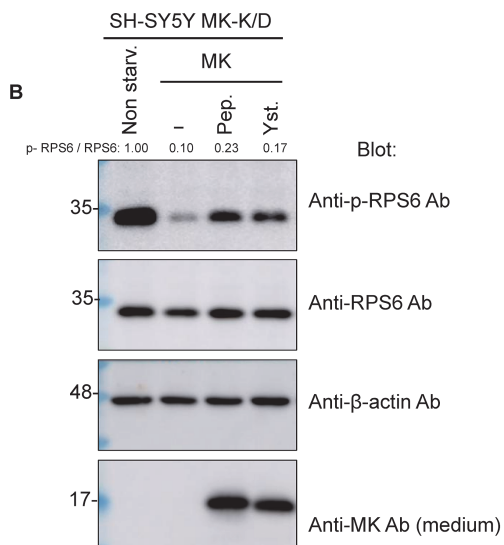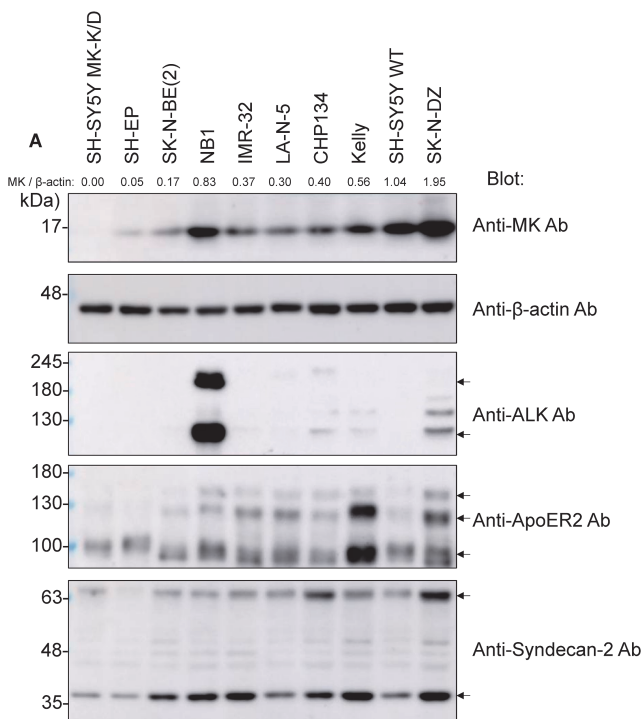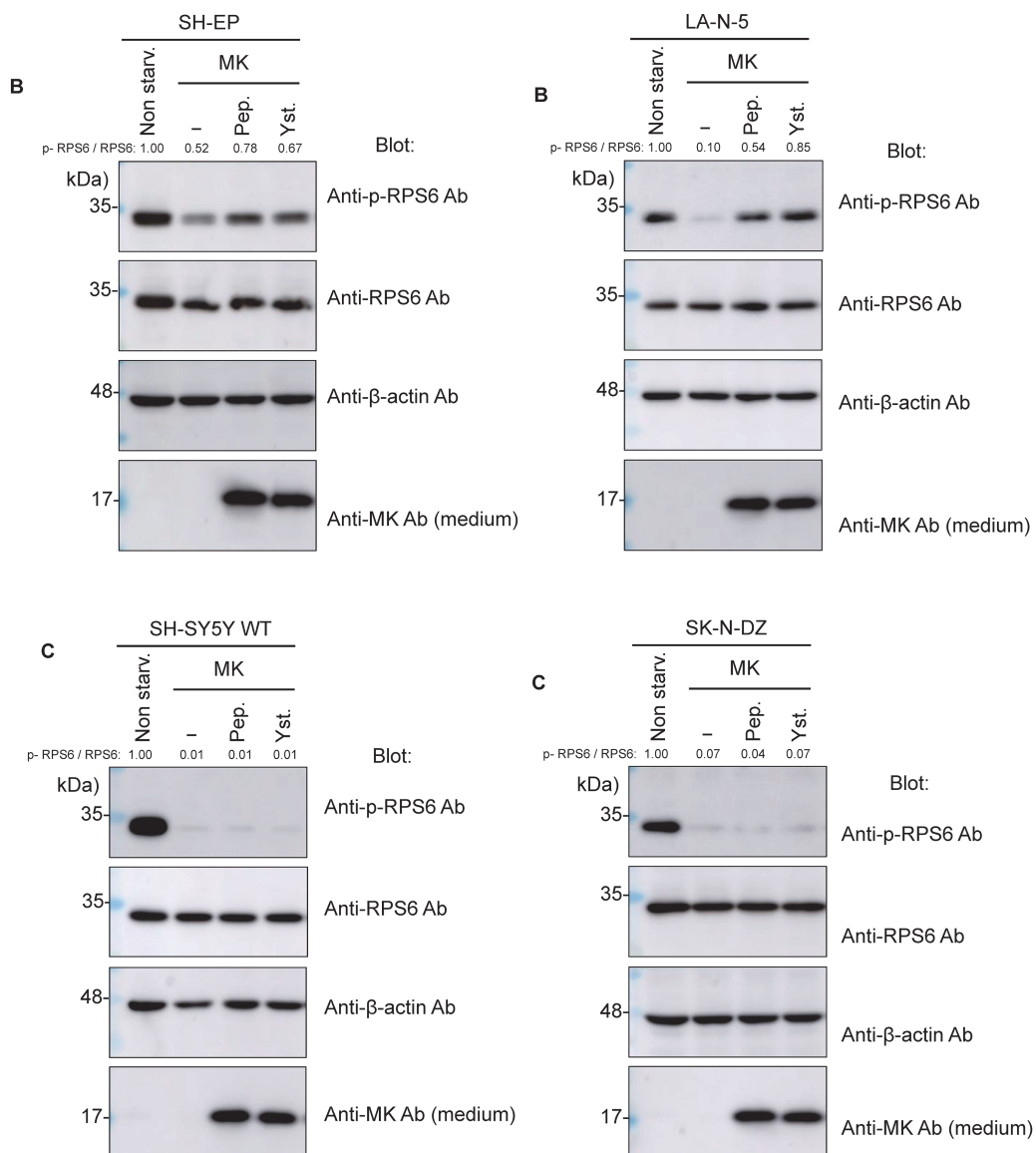
Fig. 1 Evaluation of MK expression and determination of MK reactive cell lines

Fig. 1A: Evaluation of MK and its putative receptors expression in cancer cell lines. Arrows indicate the expected size of proteins.

Fig. 1B: MK administration induced RPS6 phosphorylation in MK low expression neuroblastoma cell lines.

Fig. 1C: MK administration little induced RPS6 phosphorylation in MK high expression neuroblastoma cell lines.

No starv: No starvation

Pep: rhMK from *E. coli* purchased from PeproTech

yst: rhMK from yeast

Both MK proteins were administrated at the final concentration of 5.0 μg/mL.

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Secreted MK-BioID2 were able to biotinylate proteins

To identify a novel MK binding partner, we employed the BioID method.[17] We inserted the MK coding sequence into the N-terminus of BioID2 plasmid[20] and transfected it into HEK293T cells. Since MK is a secreted protein, we collected it as MK-BioID2 protein in the conditioned medium (Fig. 2A). The concentration of MK-BioID2 protein in conditioned medium was determined by dilution series of rhMK concentration (Fig. 2B), and we used the conditioned medium whose MK-BioID2 protein concentration was more than 1.25 μg/mL for biotinylation experiments.

It was reported that ATP was required for biotinylation.[17,20] Therefore, we set the conditions with or without ATP in the tube. After 24 hours reaction, biotinylated proteins in MK-BioID2 conditioned medium were detected only in the condition containing both ATP and biotin (Fig. 2C left). It suggested that secreted bioID2 protein had a biotinylation activity.

We also found that MK-BioID2 protein was able to biotinylate proteins from SH-SY5Y MK-K/D cells (Fig. 2C right). In a previous study, magnesium acetate was used to promote biotinylation activity in TurboID, one of the promiscuous biotin ligase[21]; however, the addition of magnesium acetate did not promote the activity (Fig. 2C).
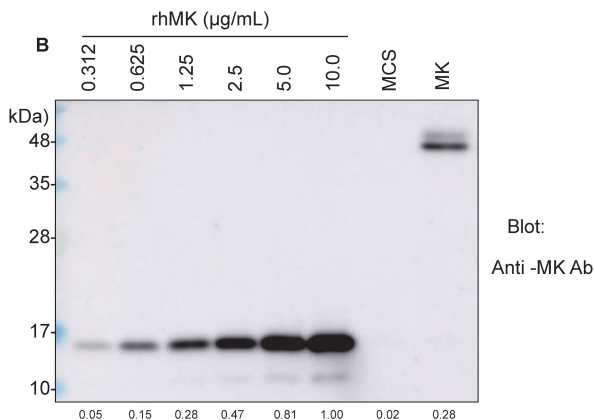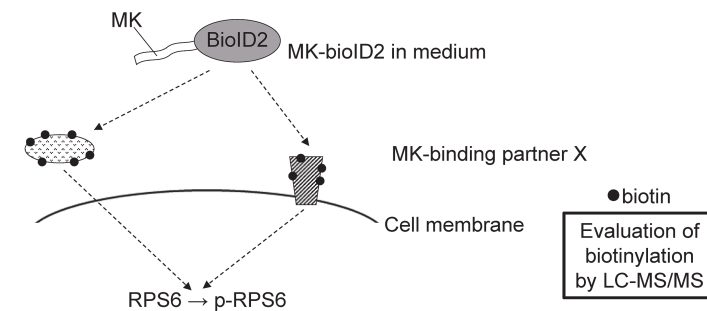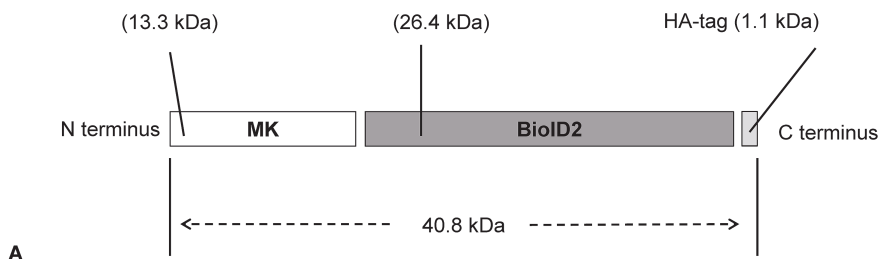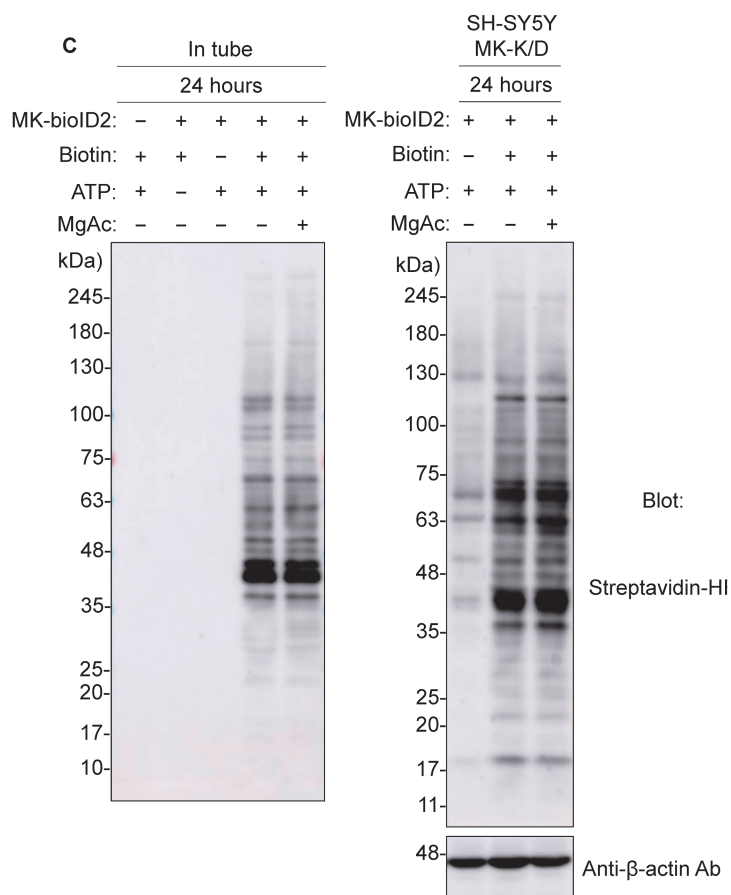
Fig. 2 The strategy for identification of MK novel binding partners using MK-BioID2 method, and the evaluation of MK-BioID2 biotinylation activity

Fig. 2A: A part of the construct of MK-BioID2 expression plasmid, and biotinylation using MK-BioID2 protein in conditioned medium and LC-MS/MS analysis.

Fig. 2B: Evaluation of the concentration of MK-BioID2 protein in conditioned medium.
The concentration of MK-BioID2 was calculated by the signal intensity of serially-diluted rhMK (yeast) samples in immunoblotting. The concentration was calculated by the equivalent concentration of rhMK. MCS conditioned medium was produced by HEK293T cells transfected with MCS-BioID2-HA (as a negative control).

Fig. 2C: MK-BioID2 protein was able to biotinylate proteins contained in its conditioned medium (left) and from SH-SY5Y MK-K/D (right). After 24 hours incubation, they were collected and immunoblotted using HRP-conjugated streptavidin. MgAc stands for magnesium acetate. MCS stands for multiple cloning sites.

Three independent LC-MS/MS analyses detected 25 novel MK-binding candidates

To search for the novel MK binding partner, we next performed biotinylation of proteins on SH-SY5Y MK-K/D cells with MK-BioID2 conditioned medium, followed by LC-MS/MS analyses. Silver staining and immunoblotting for streptavidin-pulled down samples revealed that the combination of MK-BioID2 and biotin biotinylated proteins most abundantly (Fig. 3A). Next, we performed three independent experiments, and LC-MS/MS analyses were performed

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for streptavidin-pulled down samples. We finally obtained 25 candidates overlapping the three independent data (Fig. 3B). Investigating the list of the overlapped proteins, IGFBP2 and insulin-like growth factor 2 (IGF2) were identified. IGF2 is involved in insulin-like growth factor 1 receptor and insulin receptor (IR) signaling, and it is in the upstream of PI3K/Akt signaling[24,25] and RPS6. IGFBP2 modulates these receptors signaling by binding IGFs. In addition, MK was also included, which was consistent with the previous reports that MK formed homodimers[26] (Table 1). The detection of pleiotrophin, a member of the MK family,[27] suggested that pleiotrophin formed heterodimers with MK (Table 1).

We focused on IGFBP2 because it was ranked within the fifth in each experiment. We performed immunoblotting using a part of streptavidin-pulled down samples, and confirmed that IGFBP2 was pulled down by streptavidin-conjugated agarose beads (Fig. 3C).
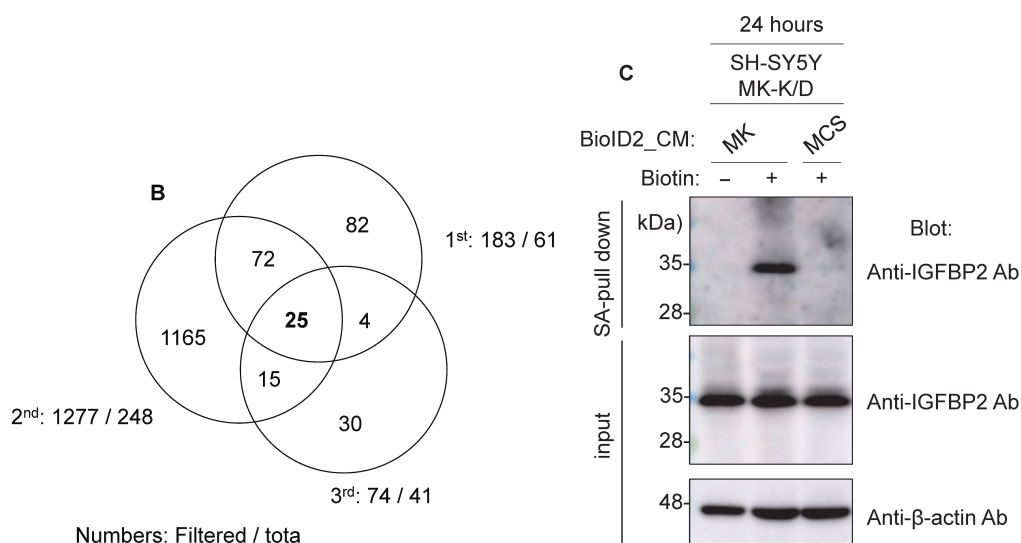
Fig. 3 MK-BioID2 method and LC-MS/MS detected 25 candidates

Fig. 3A: Silver staining and streptavidin blot for evaluation of proteins pulled down by streptavidin. After 24 hours biotinylation by MK-bioID2, biotinylated proteins were pulled down by streptavidin-conjugated agarose beads followed by LC-MS/MS analysis.

Fig. 3B: 25 overlapped proteins were detected in three independent LC-MS/MS analyses. Proteins in raw data were filtered by the criteria described in the material and methods.

Fig. 3C: IGFBP2 protein from lysates prepared for LC-MS/MS was detected only when MK-bioID2 and biotin existed.

DISCUSSION

This study provides results suggesting that the BioID2 method could be used for the identification of binding partners for growth factors. Successful biotinylation may be dependent on the concentration of MK-BioID2 protein in a conditioned medium. Indeed, we observed no biotinylated proteins from cell lysate when we used a conditioned medium containing a low concentration of MK-BioID2 protein (data not shown). Thus, the evaluation of the concentration of the protein should be performed each time the conditioned medium is prepared.

IGFBP2 belongs to the IGFBP family, and they can bind to IGFs and modulate IGF1R, IR or hybrid IGF1R/IR signaling.[25,28] Given IGF2 was also detected in our LC-MS/MS data analysis (Table 1), we speculate that MK may inhibit IGFBP2 binding to IGF2, free IGF2 may transmit these receptor signalings, and then RPS6 is phosphorylated. This hypothesis remains to be verified in future studies.

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Table 1 The overlapped 25 protein names and their MASCOT scores

Protein name	1st			2nd			3rd			CRAPome ver. 2.0
	rank	biotin +	biotin −	rank	biotin +	biotin −	rank	biotin +	biotin −	
Insulin-like growth factor-binding protein 2 [GN=GFBP2]	1	1733.44		3	1157.85		5	286.10		1 / 716
Alpha-2-HS-glycoprotein [GN=AHSG]	2	801.47		4	1019.13		6	249.50		12 / 716
Insulin-like growth factor-binding protein 5 [GN=IGFBP5]	3	549.98		8	399.82		20	33.86		1 / 716
Neurotrypsin [GN=PRSS12]	4	293.84		1	5826.63		9	137.63		1 / 716
28S ribosomal protein S26, mitochondrial [GN=MRPS26]	5	184.65		14	182.12		12	95.27		51 / 716
Signal recognition particle 19 kDa protein [GN=SRP19]	6	161.25		17	127.93		17	56.75		94 / 716
Mitochondrial import inner membrane translocase subunit TIM14 [GN=DNAJC19]	7	48.41		9	377.65		22	30.15		22 / 716
Ribosome biogenesis regulatory protein homolog [GN=RRS1]	8	47.23		2	1357.40		15	77.48		93 / 716
BUB3-interacting and GLEBS motif-containing protein ZNF207 [GN=ZNF207]	9	37.86		13	200.93		13	86.26		92 / 716
Nucleolar protein 12 [GN=NOL12]	10	33.81		12	228.66		16	60.58		12 / 716
60S ribosomal protein L37 [GN=RPL37]	11	25.23		21	520.45	22.86	19	50.09		70 / 716
Pleiotrophin [GN=PTN]	12	22.80		7	557.15		24	20.17		1 / 716
Calmodulin OS=Homo sapiens [GN=CALM1]	13	20.70		18	44.29	73.25	14	81.04		no data
Zinc finger protein 787 [GN=ZNF787]	14	20.67		24	452.98		8	153.28		6 / 716
Insulin-like growth factor II [GN=IGF2]	15	393.77	25.92	11	261.11		10	105.07		0 / 716
Tissue factor pathway inhibitor 2 [GN=TFPI2]	16	760.88	89.60	19	3668.71	65.12	2	456.79		1 / 716
Midkine OS=Homo sapiens [GN=MDK]	17	623.54	117.26	23	1785.97	134.19	7	166.95	29.83	7 / 716
28S ribosomal protein S14, mitochondrial [GN=MRPS14]	18	389.03	95.28	25	331.39	62.22	4	385.55		52 / 716
Hemoglobin subunit alpha [GN=HBA1]	19	226.30	62.86	22	1116.55	55.68	3	418.46		90 / 716
40S ribosomal protein S17-like [GN=RPS17L]	20	955.10	303.10	20	3836.18	150.00	1	982.64		no data
BUD13 homolog OS=Homo sapiens [GN=BUD13]	21	67.64	23.03	15	169.80		23	27.09		35 / 716
Growth arrest and DNA damage-inducible proteins-interacting protein 1 [GN=GADD45GIP1]	22	109.19	39.68	6	652.19		18	55.49		57 / 716
Coiled-coil domain-containing protein 137 [GN=CCDC137]	23	144.65	54.11	10	266.70		11	97.53		45 / 716
Cellular nucleic acid-binding protein [GN=CNBP]	24	73.73	29.28	16	138.73		21	30.60		37 / 716
MAP7 domain-containing protein 1 [GN=MAP7D1]	25	71.97	30.04	5	986.09		25	19.60		60 / 716

DISCLOSURE STATEMENT

The authors have no conflict of interest.

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