Inhibition of MSP-RON signaling pathway in cancer cells by a novel soluble form of RON comprising the entire sema sequence

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Abstract. The RON receptor tyrosine kinase and its ligand macrophage stimulating protein (MSP) play a role in epithelial tumorigenesis. We report here a novel RON variant that antagonizes the RON-MSP pathway in various cancer cells. The variant is an 85 kDa soluble protein from an mRNA transcript with an insertion of 49 nucleotides between exons 5 and 6. The insertion created a stop codon leading to the formation of a RON variant consisting of the entire 35 kDa α-chain and a 45 kDa partial extracellular ß-chain. The protein was featured by a sema domain, a hinge motif and a portion of the first IPT unit (designated as RONΔ85). RONΔ85 binds directly to MSP, forms MSP-RONΔ85 complex, and inhibits RON phosphorylation. RONΔ85 disrupts RON or RONΔ160 dimerization, prevents their phosphorylation, and attenuates downstream signaling events. The action of RONΔ85 is specific to RON and has no effect on MET and EGFR. In colon and pancreatic cancer cells, RONΔ85 inhibits spontaneous or MSP-induced Erk1/2 and AKT phosphorylation, which results in impaired cell proliferation and colony formation. RONΔ85 also inhibits spontaneous and MSP-induced cell migration. We conclude that RONΔ85 is an antagonist to the MSP-RON pathway, which has potential for regulating RON/RON160-mediated tumorigenic activities.

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Abbreviations: CHO, Chinese hamster ovary; EGFR, epidermal growth factor receptor; HGF, hepatocyte growth factor; IPT, immunoglobulins, plexin, and transcription factor; MAP, mitogen-activated protein; MDCK, Madin-Darby canine kidney; MSP, macrophage-stimulating protein; PSI, plexin-semaphorin-integrin; RON, recepteur, d'origine nantais; RT, reverse transcription; RTK, receptor tyrosine kinase; sema, semaphorin; si, small interfering

Key words: receptor tyrosine kinase, truncated variant, antagonistic activity, signal transduction, epithelial cancer

Introduction

Macrophage-stimulating protein (MSP) activates the RON receptor tyrosine kinase, which regulates activities of epithelial cells including migration, proliferation, and survival (1). The MSP-RON pathway plays also a role in epithelial carcinogenesis (2). RON is overexpressed in many primary cancer samples including breast, colon, and pancreatic tumors (3). Overexpression has been associated with advanced clinical stages and poor prognosis (4). In mouse model, mammary-specific RON expression causes the formation of highly metastatic tumors (5). Transgenic expression of RON in lung epithelial cells also results in lung tumors with features of bronchiole-alveolar carcinomas (6). These pathogenic phenotypes are manifested by RON-directed signals that regulate cell dissociation, motility, and matrix invasion (7). Thus, targeting the MSP-RON pathway has therapeutic potential. Currently, various approaches including therapeutic antibodies (8), siRNA (9), and small molecule inhibitors (10), are under intensive investigation. Results from these studies are promising with prospects for future clinical evaluation (11).

MSP-induced RON activation depends on the coordinated interaction between binding domains on the MSP molecule and ligand-recognition pockets in the RON extracellular sequences. Two binding moieties on MSP have been observed. The high affinity binding moiety is located in the serine-protease-like domain in the MSP ß-chain (12). The α-chain contains a weak binding moiety, which resides in the kringle domains of the α-chain (13). Both sites are functionally required to activate RON for signaling events (12). Ligand-recognition pocket(s) on the RON extracellular sequences are currently unknown. RON extracellular sequences contain a sema domain followed by a cysteine-rich hinge (PSI), and four IPT (immunoglobulin-plexins-transcription) units (14). The

sequences coding the sema domain are a stretch of 500 amino acids that are located on both RON α- and ß-chain (14). Analysis of MET, a homologue of RON, have revealed that the sema domain contains a low affinity site for HGF binding (15). The high affinity HGF binding pocket resides in the fourth IPT of the MET ß-chain (16). Using recombinant RON fragments containing the sema domain, it has been showed that the protein prevents MSP binding to RON and inhibits MSP-induced RON activation (17), suggesting that the sema domain may bind to MSP. Thus, the sema domain of RON represents not only an important structure for ligand recognition but also a critical requirement for receptor activation and signal transduction.

The study in this report is on the biological significance of a naturally occurring soluble RON variant containing the complete sema sequence. Our purpose is to determine if the sema domain of RON plays a role in interaction with MSP and acts as a potential inhibitor of RON-mediated tumorigenic activities. Since altered RON expression exists in many cancer cells, which is characterized by the presence of various shortened, spliced, or truncated mRNA transcripts (18), we used PCR techniques to isolate RON mRNA transcripts that are not translated but possess potential regulatory activities upon recombinant expression. One mRNA transcript was isolated from breast cancer Du4475 cell line. The synthesized protein is an 85 kDa RON variant composed of a full 40 kDa α-chain and a partial extracellular 45 kDa ß-chain (designated as RONΔ85). Both chains are linked by a disulfide bond(s). Biochemical and biological analyses demonstrated that this novel RON variant directly binds to MSP and inhibits MSP-induced RON activation. It also regulated signaling events and tumorigenic activities mediated by RON and its oncogenic variant RONΔ160 in a ligand-dependent or independent manner. Our results highlight the potential inhibitory use of this novel RON variant to regulate tumorigenic phenotypes mediated by the MSP-RON pathway.

Materials and methods

Cell lines, antibodies and reagents. Human breast (Du4475, HCC1937, T-47D, MDA-MB-231, MCF-7), colon (HCT116, SW620, SW683 and HT29), and pancreatic (BxPc3 and Panc-1) cancer cell lines and CHO-k1 cell line were from ATCC (Manassas, VA). MDCK cells expressing RON (M-RON) or RONΔ160 (M-RONΔ160) were used as previous described (19). Human recombinant HGF, pro-MSP, mature MSP, MSP α-, and ß-chain were from Dr E.J. Leonard (National Cancer Institute, Bethesda, MD). Human EGF and anti-v5 antibodies were from Invitrogen (Carlsbad, CA). Cross-linker Sulfo-EGS was from Pierce (Rockford, IL). Mouse mAb Zt/g4 against the sema domain of RON and rabbit IgG antibody against RON C-terminus were used as previously described (20). Antibodies to various signaling proteins including, ERGR, MET, Erk1/2, and AKT were from Cell Signaling, Inc. (Beverly, MA).

RT-PCR and RONΔ85 cDNA construction. Total RNAs were isolated from Du4475 or other cell lines using TRIzol (Invitrogen) as described previously (21). RT-PCR was carried out using 2 μg of total RNAs with SuperScript kit (Invitrogen). RON oligo-primers that cover the RON extracellular sequences were designed as previously described (21). The cDNA fragments were sequenced at the Texas Tech University DNA Sequence Core facility. The mammalian expression vector pcDNA3.1V5/His was used for RONΔ85 expression.

Establishment of transfected cell lines and purification of RONΔ85. Transfection of CHO-k1 cells with plasmids was performed as previously described (20). Positive clones (CH-RONΔ85) were selected and expended into cell lines. To purify RONΔ85, supernatant from CH-RONΔ85 cells was concentrated and then loaded on the Ni-NTA column. The eluded proteins were analyzed on 10% of SDS-PAGE, filter-sterilized and used for further experiments.

Protein cross-linking. Cells at $1.5x10^6$ cells in a 60 mm culture dish were treated at 37˚C for 15 min with or without MSP (5 nM), RONΔ85 (35 nM) for their combination. Cross-linker Sulfo-EGS was added to the culture medium at a final concentration of 2 mM for 30 min at room temperature. Reactions were stopped by washing cells with cold PBS. Proteins from cell lysates were analyzed by Western blotting using rabbit antibodies to RON followed by ECL visualization.

Immunoprecipitation and Western blot analysis. These methods were performed as previously described (22). Individual samples (350 μg protein/sample) were immunoprecipitated by respective antibodies (2 μg/sample) coupled to protein G Sepharose beads. Western blot analysis was performed by using specific antibodies followed by ECL reaction. Results were recorded and analyzed using VersaDoc Imaging System (Bio-Rad).

Assays for cell proliferation and migration. These assays were performed as previously described (19). For proliferation, cells were cultured ($4x10^3$ cells/well) in triplicate in a 96-well plate and then treated with or without MSP (5 nM), RONΔ85 (35 nM) or their combination for 3 days. Cells were stained with Hema-3 stain kit (Fisher Scientific) and then lysed in 1% SDS buffer. Color intensity was measured at 570 nM in an ELISA plate reader. Absorbance was converted to cell number with reference to a standard curve. For migration studies, the wound healing assay was performed (19). After addition of MSP, RONΔ85 for 16 or 24 h dependent on individual cell lines, the area covered by migrated cells was examined under a microscope and photographed.

Results

Identification of a novel mRNA transcript encoding RONΔ85. To study RON mRNA transcripts that are not translated but possess regulatory activities upon recombinant expression, total RNAs from colon, breast, and pancreatic cancer cells were subjected to RT-PCR analysis. Three pairs of primers that covers the entire RON extracellular sequence (-11 to +1663, +1311 to +2315, and +1646 to +2921) were used (23). As shown in Table I and Fig. 1A, majority of cDNA fragments were previously reported and coded for the extracellular sequences of RON, RONΔ165 (deletion of exon 11), RONΔ160

Table I. Identification of various types of cDNA fragments encoding RON or its variants in colon, breast, and pancreatic cancer cells.

Cell lines	Different types of RON mRNA transcripts[a]					
	Wild-type	Deletion of exon 11	Deletion of exons 5 and 6	Deletion of exons 5, 6 and 11	Truncation at Met^{2765}	Intron Insertion
HT-29	+	+	+	+	+	-
SW620	+	+	+	-	+	-
HCT116	+	-	-	-	-	-
T-47D	+	-	-	-	-	-
Du4475	+	-	+	-	-	+
HCC1937	+	-	-	-	-	-
MDA-MB231	+	+	-	-	+	-
MCF-7	-	-	-	-	-	-
BxPC-3	+	+	-	+	+	-
Panc-1	+	+	-	-	+	-

[a]Cell lines expressing mRNA transcripts of RON or its variants are marked as +. Deletion, insertion, and truncation were observed by comparison with published RON gene sequences (23).

(deletion of exons 5 and 6), RONΔ155 (deletion of exons 5, 6, and 11), and RONΔ52 (alternatively initiated at MET^{913}) (18). Only one novel cDNA fragment (~1.3 kb) was discovered in Du4475 cells (Fig. 1A and B) (designated as DucDNA-8). To verify these results, Western blot analysis for RON expression was performed. RON variants including RONΔ165 and RONΔ160 were detected (Fig. 1C). However, proteins with molecular mass of 80 kDa were not observed. These results were confirmed by immunoprecipitation with mAb Zt/g4 in Du4475 cell lysates followed by Western blot analysis using anti-RON α-chain antibody (Fig. 1D). RON, RONΔ165, and RONΔ160 were immunoprecipitated from lysates of all four cell lines. However, proteins around 80 kDa were not observed. These result, together with Fig. 1C, demonstrated that DucDNA-8 mRNA is transcribed but not translated in Du4475 cells.

In comparison with RON cDNA sequences, DucDNA-8 had an insertion of 49 nucleotides between the last nucleotide G^{1908} of the exon 5 and the first nucleotide A^{1909} of exon 6. Inserted sequences created six new amino acids followed by a stop codon of TAA (Fig. 1A). Thus, DucDNA-8 encoded a truncated RON variant with a molecular mass of ~85 kDa (RONΔ85) (Fig. 1E), which contains the complete α-chain and a portion of extracellular sequences of the ß-chain. The structure of RONΔ85 was featured by the complete sema domain followed by a hinge and a portion of the first IPT unit.

Expression and purification of RONΔ85 from transfected CHO-k1 cells. RONΔ85 cDNA was constructed, tagged at C-terminus with V5/His, and expressed in CHO-k1 cells. Results in Fig. 2A show that RONΔ85 existed in cell lysates as a 75 kDa single-chain precursor. In contrast, pro-RONΔ85 and mature RONΔ85 (as evident by the presence of the 45 kDa partial ß-chain) were detected in the supernatant. The size of mature RONΔ85 (~85 kDa) was slightly larger than pro-RONΔ85, due to glycosylation during the release of RONΔ85 into the culture fluids. Using a Ni-NTA affinity column for His-tagged protein (Invitrogen), RONΔ85 was purified from the supernatant (Fig. 2B). Mature RONΔ85 was bound to the Ni-NTA column in amounts greater than pro-RONΔ85. Five micrograms of RONΔ85 was able to be purified from 150 ml culture supernatant.

Interaction of RONΔ85 with pro-MSP, MSP, and MSP subunits. To determine if RONΔ85 interacts with MSP, recombinant pro-MSP, MSP, MSP α-, and MSP ß-chain were individually used for co-immunoprecipitation assay followed by Western blot analysis using specific antibodies. Results in Fig. 3A show that pro-MSP or RONΔ85 was not detected by Western blot analysis in samples of anti-V5 or pro-MSP antibody immunoprecipitation, suggesting that RONΔ85 did not interact with pro-MSP. However, the formation of the protein complex between RONΔ85 and MSP was observed (Fig. 3B). In this case, rabbit anti-MSP antibody was able to detect MSP (as evident by the presence of MSP-α chain) in the anti-V5 co-immunoprecipitated samples. This result was further confirmed by Western blotting of RONΔ85 using anti-MSP antibody in mAb anti-MSP clone 2S co-immunoprecipitated samples. To determine which subunits of MSP were involved in interaction with RONΔ85, co-immunoprecipitation was performed where free MSP α- or ß-chain was incubated with RONΔ85. Results in Fig. 3C show that the MSP α-chain was not detected by Western blotting using anti-MSP α-chain antibody after anti-V5 co-immunoprecipitation, indicating that RONΔ85 does not form a complex with the MSP α-chain. However, the MSP ß-chain was detected by Western blotting of anti-V5 co-immunoprecipitated samples using rabbit antibodies to the MSP ß-chain (Fig. 3D). These results, together with Fig. 3A-C, demonstrate that RONΔ85 interacts with

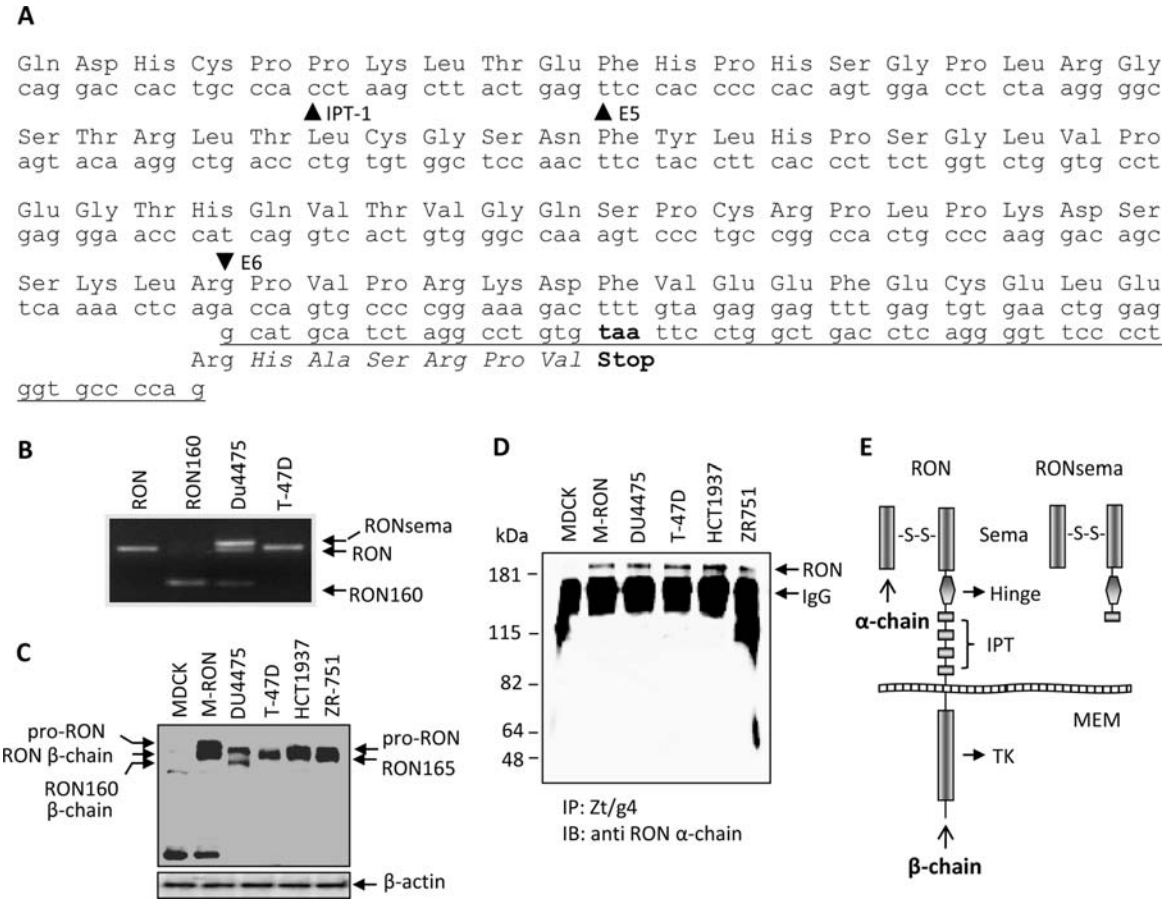

Figure 1. Identification and cloning of the RONΔ85 mRNA transcript from breast Du4475 cells: (A) Partial sequences of the cDNA fragment encoding RONΔ85. The beginning of first IPT domain is labeled. The first nucleotide for exon 5 and 6 is indicated with arrow. The inserted 49 nucleotides are underlined. The created stop codon was marked in bold. The sequences of six newly created amino acids are provided. (B) Detection of the cDNA fragment encoding RONΔ85 by RT-PCR. The RON or RONΔ160 cDNA was used as the control. The cDNA fragments corresponding to RON, RONΔ160, or RONΔ85 were amplified from RNAs derived from Du4475 and T-47D cells. (C) Expression of RON and its variants by breast cancer cells. Cellular proteins (50 μg per sample) were subjected to Western blot analysis using rabbit IgG antibody to RON. MDCK and M-RON cells were used as the control. In Du4475 cells, only pro-RONΔ160 and RONΔ160 ß-chain were detected. No proteins at molecular mass of ~80 kDa were observed. (D) Validation of RON and its variant expression by breast cancer cells. Cellular proteins (300 μg per sample) were immunoprecipitated with mAb Zt/g4 followed by Western blot analysis under non-reduced conditions. Mouse mAb to RON α-chain was used as the detecting antibody. MDCK and M-RON cells were used as the control. No proteins at molecular mass of ~80 kDa were detected in DU4475 cells. (E) Schematic representation of RON and RONΔ85. Functional domains in the extracellular sequences including sema, hinge, and IPTs are indicated.

MSP but not pro-MSP. The interaction occurred by binding to the MSP ß-chain.

Effect of RONΔ85 on MSP-induced RON dimerization and activation. The effect of RONΔ85 on MSP-induced RON dimerization and activation was studied using M-RON cells as the model. MSP stimulation induced RON dimerization as evident from the appearance of a RON dimer (Fig. 4A). However, the amount of the RON dimer was significantly deceased after RONΔ85 addition. Judged by band intensities, only 5% of RON dimers remained. The effect of RONΔ85 on MSP-induced RON phosphorylation is shown in Fig. 4B. About 90% of MSP-induced RON phosphorylation was reduced when 35 nM of RONΔ85 was used. The inhibition was also dose-dependent with progressive reduction of RON phosphorylation in the presence of increasing concentrations of RONΔ85 (Fig. 4C). Moreover, RONΔ85 inhibited MSP-induced phosphorylation of downstream signaling proteins (Fig. 4D). More than 50% reduction of Erk1/2 and 70% reduction of AKT as determined by densitometry analysis were observed. Thus, RONΔ85 impaired MSP-induced RON dimerization. This impairment affected MSP-induced RON phosphorylation and subsequent activation of downstream signals.

Effect of RONΔ85 on constitutive activation of RONΔ160. RONΔ160 is a constitutively active variant with oncogenic activities (21). The effect of RONΔ85 on constitutive phosphorylation was studied using M-RONΔ160 cells as a model. Results in Fig. 5A show that RONΔ85 formed a protein complex with RONΔ160, which was immunoprecipitated using anti-V5 antibody. Such interaction affected spontaneous RONΔ160 dimerization even in the absence of MSP (Fig. 5B). The effect of RONΔ85 on constitutive RONΔ160 phosphorylation is shown in Fig. 5C. RONΔ85 treatment substantially reduced the levels of RONΔ160 phosphorylation. MSP had only marginal effect on prevention of RONΔ85-mediated inhibition. The inhibitory effect of RONΔ85 on RONΔ160

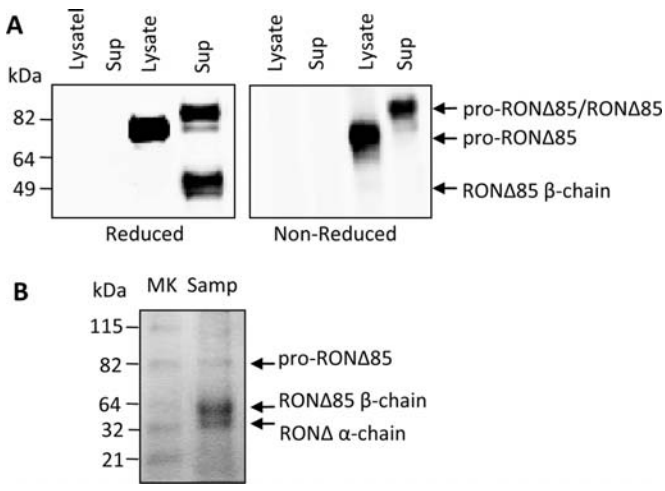

Figure 2. Expression and purification of recombinant RONΔ85 from cDNA transfected CHO-k1 cells: (A) Cell lysates and culture fluids (50 μg protein per sample) from control and transfected CH-RONΔ85 cells were analyzed by Western blot analysis under reduced or non-reduced conditions using anti-V5 antibody. Mature RONΔ85 was detected only in culture fluids from transfected CH-RONΔ85 cells. (B) Culture fluids (150 ml) of CH-RONΔ85 cells were concentrated and loaded on Ni-NTA column. This column was much better in purification of mature RONΔ85 than pro-RONΔ85. The obtained RONΔ85 (~2 μg protein/sample) was analyzed in a 10% SDS-PAGE, stained with Coomassie blue, and photographed. Both RONΔ85 α- and ß-chain were observed. The amount of pro-RONΔ85 was less than 10% of the total preparation. MK, molecular marker.

phosphorylation was dose-dependent. Maximal inhibition was seen when 35 nM of RONΔ85 was used. In addition, we observed the inhibitory effect of RONΔ85 on constitutive phosphorylation of Erk1/2 and AKT in M-RONΔ160 cells (Fig. 5D). Thus, results in Fig. 5 demonstrate that RONΔ85 directly interacts with RONΔ160. Such interactions impair spontaneous RONΔ160 dimerization, which leads to diminished tyrosine phosphorylation and attenuated downstream signaling activities.

Effect of RONΔ85 on MSP-RON signaling pathways and its specificity. Four cancer cell lines (SW837, HCT116, T-47D, BxPC-3) were used to validate the results described above. RONΔ85 treatment significantly inhibited MSP-induced RON phosphorylation in all four cell lines tested (Fig. 6A). The levels of inhibition varied among cell lines and ranged from 50 to 90%. The inhibitory effect of RONΔ85 on phosphorylation of Erk1/2 and AKT was also confirmed using BxPC-3 and HCT116 cells as model (Fig. 6B). More than 50% of reduction in MSP-dependent or independent Erk1/2 phosphorylation was observed in both cell lines. Significant inhibition by RONΔ85 on constitutive AKT phosphorylation was also observed (Fig. 6B). Taken together, these results suggest that RONΔ85 inhibited phosphorylation of RON, Erk1/2 and AKT in cancer cells.

Cross-talk between RON and MET or EGFR exists in cancer cells (24,25). We selected BxPC-3 cells as the model since they express RON, MET and EGFR. Results in Fig. 6C show that HGF-induced MET phosphorylation was not affected by the presence of RONΔ85. Similarly, EGF-induced EGFR phosphorylation was not affected by RONΔ85 (Fig. 6C). In both cases, levels of phosphorylation were comparable to those of positive controls. These results suggest that the effect of RONΔ85 is specific only to RON and not to structure-related MET or unrelated EGFR.

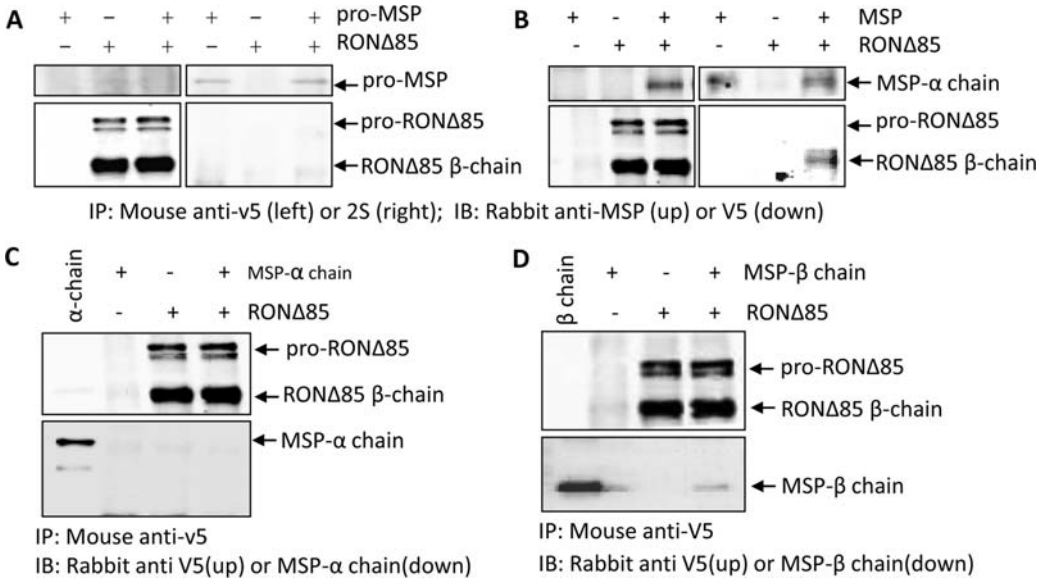

Figure 3. Direct interaction of RONΔ85 with MSP and its ß-chain: (A) RONΔ85 did not interact with pro-MSP. Purified RONΔ85 (2 μg/sample) was mixed with or without recombinant human pro-MSP (1.5 μg per sample) at 4˚C for 12 h in PBS. Samples were then immunoprecipitated with mouse mAb to V5 or to pro-MSP (clone 2S) followed by Western blot analysis using rabbit antibodies to V5 or to MSP. (B) RONΔ85 interacted with MSP. Experimental conditions were similar to those in A. MSP was mixed with RONΔ85 followed by anti-V5 or anti-MSP immunoprecipitation. (C) RONΔ85 failed to interact with the MSP α-chain. Purified RONΔ85 (2 μg/sample) were mixed in PBS with or without recombinant human MSP-α chain (2.5 μg per sample) at 4˚C for 12 h. Proteins were immunoprecipitated using mouse antibody to V5 followed by Western blot analysis using rabbit anti-V5 or anti-MSP-α chain antibodies (38). The pure MSP-α chain was used as the control. (D) RONΔ85 interacted with the MSP-ß chain. Experimental conditions were similar to those in C. The MSP-ß chain was used at 2.5 μg/sample. After anti-V5 immunoprecipitation, the presence of the MSP-ß chain was determined by Western blot analysis using rabbit antibody to V5 or to the MSP-ß chain (38).

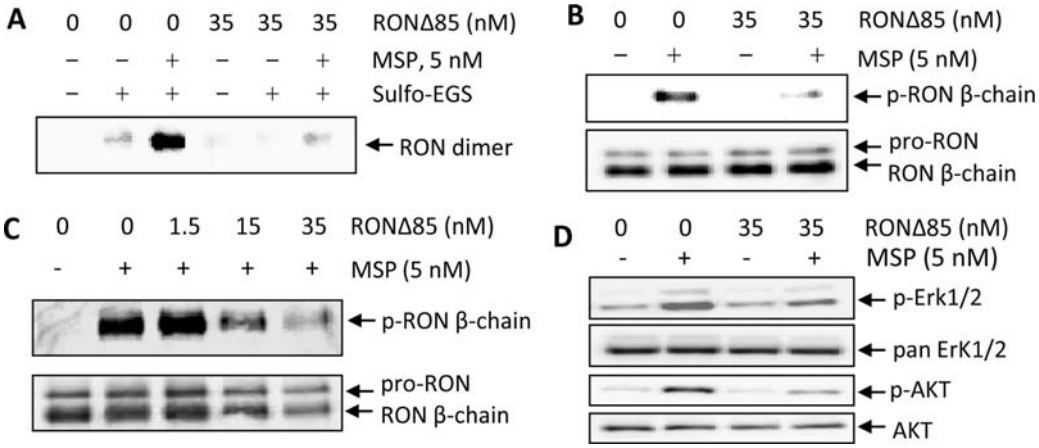

Figure 4. Inhibitory effect of RONΔ85 on MSP-induced phosphorylation of RON and downstream signaling proteins: M-RON cells were used in all experiments. (A) RONΔ85 inhibits MSP-induced RON dimerization. Cells ($2x10^6$ cells in 60 mm culture dish) were stimulated at 37˚C for 30 min with or without 5 nM of MSP in the presence or absence of 35 nM of purified RONΔ85 followed by addition of the chemical cross-linker Sulfo-EGS. The formation of the RON dimer was determined by Western blot analysis using antibodies to RON. (B) RONΔ85 inhibits MSP-induced RON phosphorylation. After MSP stimulation for 15 min in the presence or absence of RONΔ85 (35 nM), Zt/g4 immunoprecipitation was performed followed by Western blot analysis using mAb PY-100 for phosphorylated RON. The membrane was also reprobed with antibody to RON as the loading control. (C) Dose-dependent inhibition by RONΔ85 of RON phosphorylation. Experimental conditions were similar to those in B, except for difference in amount of RONΔ85 added to the cell culture. (D) Effect of RONΔ85 on RON-mediated phosphorylation of Erk1/2 and AKT. Cells stimulation and Western blot analysis were similar to those in B. Phosphorylated Erk1/2 and AKT were detected by Western blot analysis using individual antibodies. Data shown here are from one of three experiments with similar results.

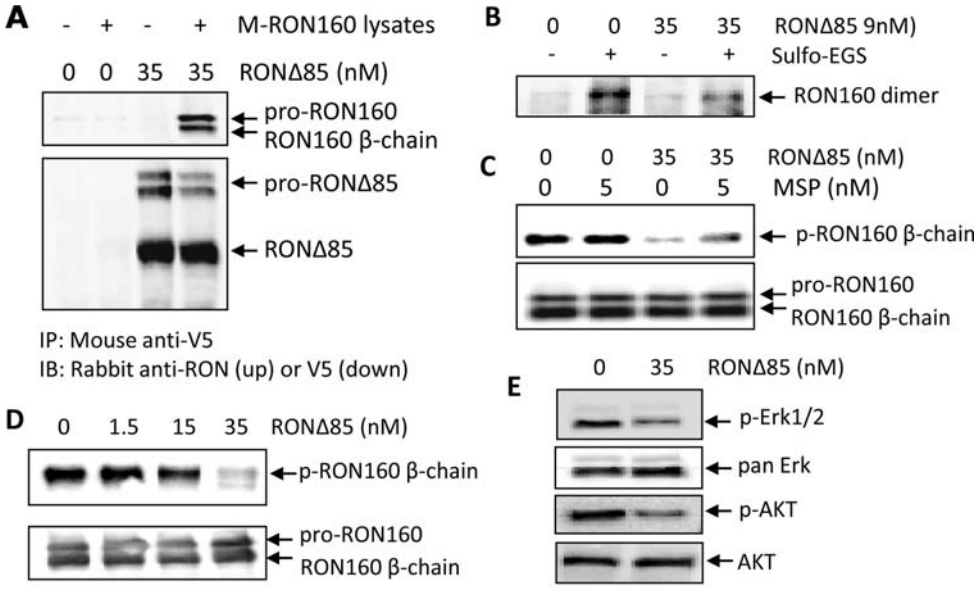

Figure 5. Inhibitory effect of RONΔ85 on MSP-independent RON phosphorylation and downstream signaling activation. M-RONΔ160 cells were used as the model in all experiments. (A) Interaction of RONΔ85 with RONΔ160. Proteins (300 μg/sample) from cell lysates were mixed with or without RONΔ85 (35 nM) at 4˚C for 12 h followed by anti-V5 immunoprecipitation. The presence of RONΔ160 or RONΔ85 was determined by Western blot analysis using rabbit anti-RON or V5 antibody, respectively. (B) Effect of RONΔ85 on RONΔ160 dimerization. Experiments were performed as detailed in Fig. 4A except that MSP is not included. The RONΔ160 dimer was determined by Western blot analysis using rabbit anti-RON antibodies. (C) Effect of RONΔ85 on constitutive RONΔ160 phosphorylation. Cell stimulation, RONΔ85 treatment, and Western blot analysis were done as described in Fig. 4B. Protein intensity was determined by densitometric analysis. (D) Dose-dependent inhibition by RONΔ85 of constitutive RONΔ160 phosphorylation. Experiments were performed as described in Fig. 4C. MSP was not included. (E) Effect of RONΔ85 on constitutive Erk1/2 and AKT phosphorylation. Cells were treated with RONΔ85 followed by Western blot analysis as described in Fig. 4D. Data shown here are from one of three experiments with similar results.

Effect of RONΔ85 on MSP-dependent or independent growth of cancer cells. MSP-dependent and independent cell growth was first studied. M-RON and M-RONΔ160 cells were used as the model. Results in Fig. 7A show that RONΔ85 moderately inhibited MSP-induced cell proliferation. This effect was also seen in M-RONΔ160 cells, in which cell proliferation is independent of MSP stimulation. The inhibition was validated in BxPC-3 and HCT116 cells (Fig. 7B). Again, only moderate inhibition was observed. The rate of inhibition was about 60% for BxPC-3 and 36% for HCT116.

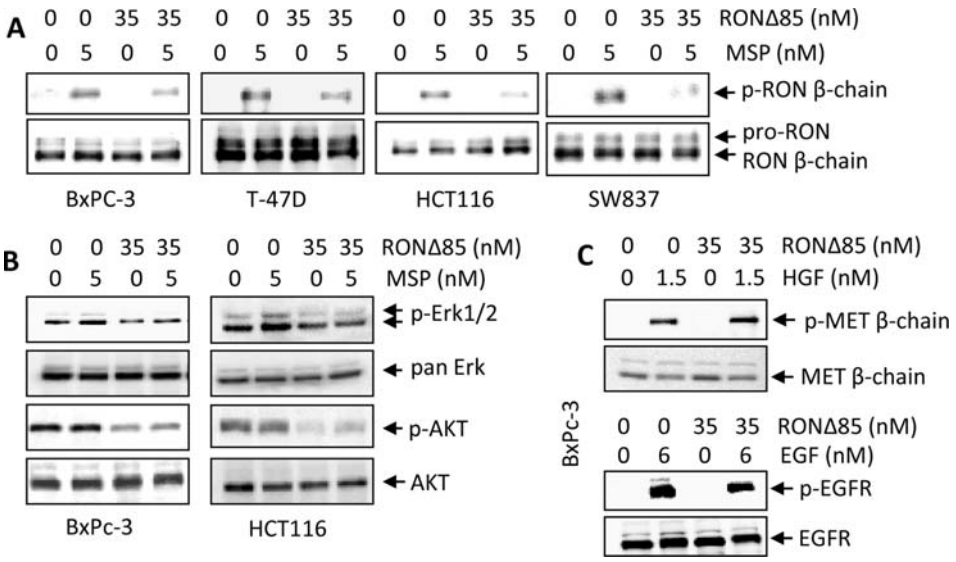

Figure 6. Effect of RONΔ85 on phosphorylation of RON, MET, EGFR, and downstream signaling proteins. (A) Inhibitory effect of RONΔ85 on RON phosphorylation in four cancer cell lines. Cells ($3x10^6$ cells per sample) were stimulated at 37˚C for 15 min by MSP in the presence or absence of RONΔ85. After Zt/g4 immunoprecipitation of cellular proteins, phosphorylation of RON was detected by Western blot analysis using mAb PY-100. Membranes were also reprobed with rabbit antibodies to RON as the loading control. (B) Effect of RONΔ85 on Erk1/2 and AKT phosphorylation. BxPC-3 and HCT116 cells were used as the model. Experimental procedures were similar to those in Fig. 4D. Protein intensity was determined by densitometric analysis. (C) Effect of RONΔ85 on phosphorylation of MET or EGFR. BxPC-3 cells were stimulated with HGF or EGF for 15 min followed by immunoprecipitation using corresponding antibodies. MET and EGFR phosphorylation was determined by Western blot analysis using PY-100. Membranes were also reprobed for MET and EGFR using corresponding antibodies, respectively, as the loading controls.

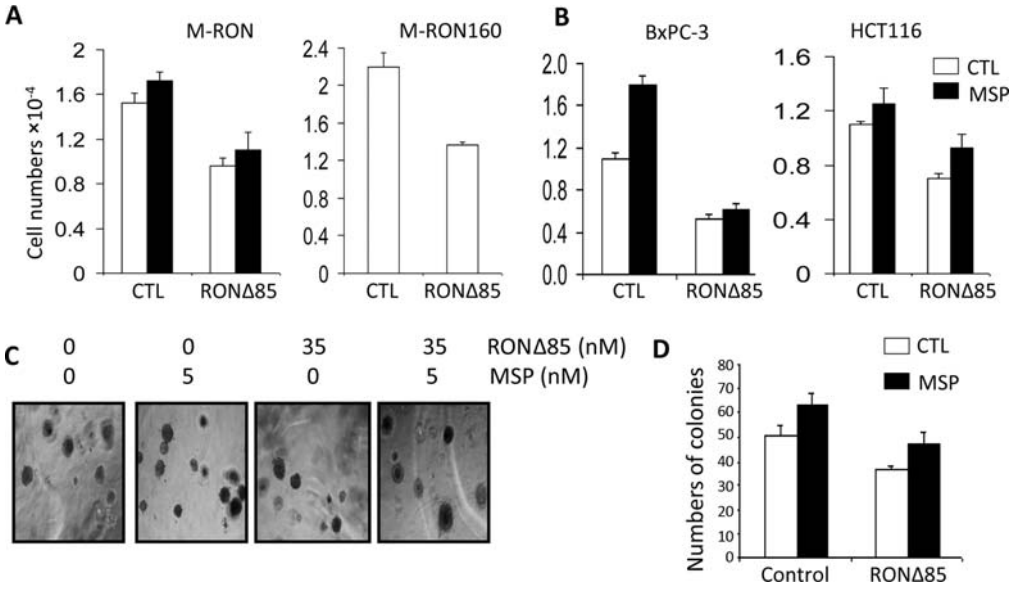

Figure 7. Effect of RONΔ85 on MSP-induced cell growth and colony formation: (A) Cells at $1x10^4$ cells/well in triplicate in a 96-well plate were cultured with or without MSP, RONΔ85, or their combination for 5 days. Cells were stained as previously described (19). Cell number was determined by comparison with standard curve. (B and C) HCT116 cells at $2x10^3$ cells/well in soft agar in duplicate were cultured for 18 days. Colonies in individual wells were photographed and counted.

The effect of RONΔ85 on anchorage-independent growth of HCT116 cells were also determined (Fig. 7C and D). Number of both spontaneous and MSP-stimulated colonies was moderately reduced in the presence of RONΔ85. Similar results were also obtained when BxPC-3 cells were used. Thus, results in Fig. 7 demonstrate that RONΔ85 moderately inhibited MSP-dependent or independent cell growth of cancer cells.

Effect of RONΔ85 on MSP-dependent or -independent cell migration. Migration of BxPC-3 and HCT116 cells was assessed by the wound healing assay. Results in Fig. 8A show that BxPC-3 cells had relatively high levels of spontaneous migration, which was enhanced further by MSP stimulation. RONΔ85 inhibited both spontaneous and MSP-induced cell migration. The percentage of space covered by migrated cells was reduced from 47 to 28% in unstimulated cells and

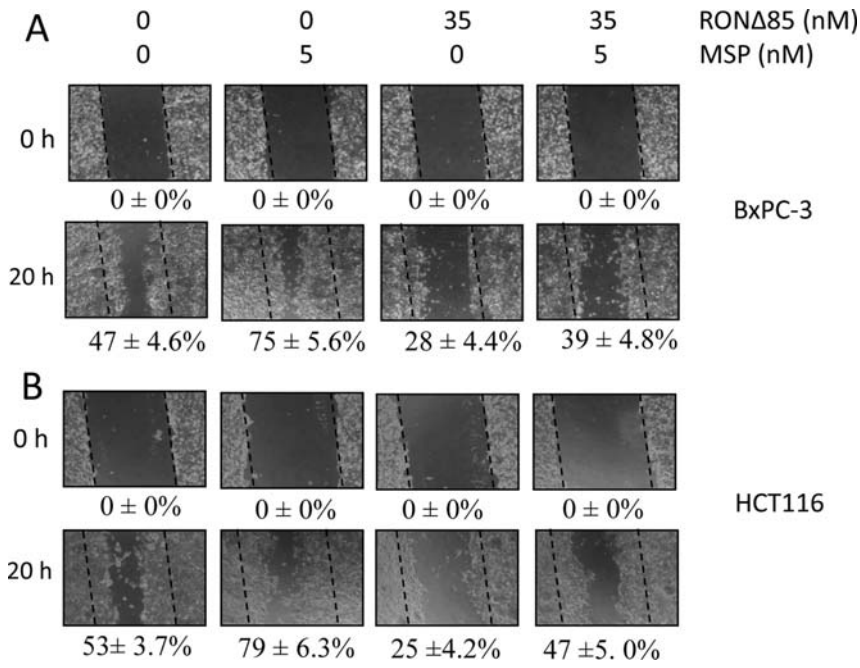

Figure 8. Effect of RONΔ85 on spontaneous or MSP-induced cell migration: The wound healing assay was performed as previously described (19). (A) BxPC-3 and (B) HCT116 cells were used as the model. The wound closure was calculated as percentages of the area covered by the migrated cells.

75 to 39% in MSP-stimulated cells, respectively. Similar patterns of inhibition were also observed in HCT116 cells (Fig. 8B). The percentage of space covered by migrated cells was reduced from 53 to 25% in MSP un-treated cells and 79 to 47% in MSP treated cells, respectively. These results suggest that RONΔ85 inhibited cell migration in MSP-dependent or -independent manner.

Discussion

The central findings of this study are the discovery of RONΔ85 and its inhibitory activities in the MSP-RON pathway in epithelial cancer cells. As indicated in Fig. 1, RONΔ85 mRNA transcript was not translated in cancer cells but displayed inhibitory activities when used as a recombinant protein. Previous studies have shown that aberration in the MSP-RON pathway contributes to tumorigenic progression in cancer cells (1). Disruption of MSP-RON signaling by RON specific mAbs (8) and small molecular inhibitors (10,26) inhibits tumorigenic activities *in vitro* and impairs tumor growth *in vivo*. These activities are mainly mediated by interaction of mAbs or agents with functional domains in the RON extracellular sequences or in the kinase domain, which impairs RON dimerization, phosphorylation, kinase activities, and subsequently inactivates downstream signaling events (10,26). Consistent with these observations, the results from this study demonstrate that RONΔ85 mRNA transcript exists in cancer cells. Upon expression as a recombinant protein, RONΔ85 acts as an antagonist to RON-mediated signaling and tumorigenic activities.

Altered RON expression exists in primary cancer samples and established cell lines (3), which is characterized by the presence of various mRNA transcripts and corresponding variants (18). A survey by PCR techniques of primary cancer samples has shown that various RON mRNA transcripts such as those from RON165, RONΔ160, and RON155 were wildly produced by different types of cancer cells (27). A recent study further revealed that different mRNA transcripts encoding known or unknown RON variants exist in human glioma samples with relatively high frequencies (28). Currently, seven RON variants have been identified and their corresponding mRNA transcripts have been cloned (18). These RON mRNA transcripts are formed mainly by aberrant splicing, insertion, alternative initiation, protein truncation, and other unknown mechanisms (18). Thus, the RON gene transcription is altered in cancer cells. Different lengths of RON mRNA transcripts are synthesized in tumor cells, which increase the complexity and diversity of the RON receptor.

Cellular translation of mRNA transcripts is a regulated process. Only certain transcripts encoding RON, RONΔ165, RONΔ160, and RONΔ155 or others are translated into proteins (18). Majority of the transcripts are degraded quickly without protein synthesis. Currently, mechanism(s) underlying the generation of various RON transcripts in cancer cells are largely unknown. Also, mechanism(s) that governs the translation of a particular RON mRNA transcript are also unknown. Nevertheless, the existence of these untranslated transcripts provides opportunities to study if they possess regulatory activities after recombinant expression. Unique RTK variants derived from cancer cells have been shown to have therapeutic effect in various tumor models (29-31). Thus, identification of RONΔ85 with antagonistic activities opens an avenue for the development of potential therapeutics for targeted cancer therapy.

Limited surveys have revealed that Du4475 cell line is the only one that harbors RONΔ85 mRNA transcript. This suggests that the frequency of this particular transcript is low and its formation may occur through a random process.

Nevertheless, the existence of such transcript with an insertion of 49 nucleotides seems not to be a normal event. A similar transcript, almost identical to the RONΔ85 mRNA, has recently been identified (28). The transcript is generated by alternative splicing that deletes exon 6. This deletion causes a reading-frame shift, which creates a stop codon after addition of three amino acids. The synthesized protein RONΔ90 inhibits MSP-induced RON phosphorylation and cell migration. Sequence comparison reveals that RONΔ90 differs from RONΔ85 only in last six amino acids. Unlike RONΔ85 mRNA found only in a single cell line, RONΔ90 mRNA has been detected in three normal brain tissues and in the majority of brain tumor samples (13/17 cases). It was also produced by colon cancer DLD-1 cells. Moreover, RONΔ90 was detected in culture supernatants from 5 glioblastoma cell lines (28). It appears that RONΔ90 mRNA transcript is wildly expressed, particularly in normal and cancerous cells of brain. In contrast, RONΔ85 mRNA expression is limited to a particular cell line only.

Functional domains in the RON extracellular sequence including sema and IPT domains are important in ligand recognition and subsequent activation (7). MSP utilizes its high affinity binding site located on the ß-chain to bind RON (12). However, the ligand-recognition pocket on the RON molecular is still largely unknown. To address the importance of the sema domain in the MSP-RON pathway, two studies have been conducted. One study showed that the soluble sema domain of RON, produced by recombinant techniques, impairs MSP binding to RON, and inhibits MSP-induced RON phosphorylation and cellular responsiveness (17). Although direct interaction has not been proved, results provide an important clue indicating that the sema domain participates in ligand binding by the full-length receptor. A recent study provided additional evidence showing that the sema domain plays a role in MSP-induced RON activation (28). Studies in MET, the homologue of RON, have shown that the sema domain in the MET receptor contains a HGF/SF-interaction pocket (32). Therefore, the sema domain of RON molecule possesses a similar function. Data from this study extend the previous findings and provide a direct evidence showing that the sema domain of RON physically forms a protein complex with MSP, which is mediated by binding to the MSP ß-chain. Since the MSP ß-chain contains a high affinity binding site, it is reasoned that the corresponding ligand-recognition pocket may reside in the sema domain of RON.

Study of RONΔ85 has revealed the following biochemical features: First, RONΔ85 directly interacts with MSP and the MSP ß-chain but not with pro-MSP or the MSP α-chain (Fig 3). This was confirmed by co-immunoprecipitation in which MSP and RONΔ85 was detected by Western blot analysis using their corresponding antibody, respectively. These results indicate that the sema domain contains a ligand-recognition pocket(s). Second, RONΔ85 inhibits MSP-induced RON dimerization and phosphorylation (Fig. 4). Both MSP-induced RON dimerization and subsequent phosphorylation were significantly diminished upon RONΔ85 treatment. These results indicate that RONΔ85 is an effective antagonist to MSP-induced RON activation. Third, RONΔ85 impairs MSP-induced phosphorylation of downstream signaling molecules (Fig. 4D). The inhibition by RONΔ85 of MSP-induced Erk1/2 and AKT phosphorylation indicates that RONΔ85 blocks the signal transduction from RON to downstream molecules. Fourth, RONΔ85 physically forms a complex with RONΔ160, inhibits its dimerization, reduces spontaneous phosphorylation, and attenuates downstream signaling activation (Fig. 5). The formation of RONΔ85-RONΔ160 complex affects spontaneous RONΔ160 dimerization, which impairs constitutive phosphorylation of RONΔ160, Erk1/2 and AKT. It is reasoned that the sema-sema interaction between RONΔ85 and RONΔ160 is the possible mechanism underlying the observed effect (17,29). Moreover, the negative effect on RONΔ160 indicates that RONΔ85 regulates MSP-independent RON activation. Fifth, the inhibitory effect of RONΔ85 acts not only in transfected cells but also in established cancer cell lines (Fig. 6). MSP-induced phosphorylation of RON, Erk1/2 and AKT in cancer cells was inhibited after RONΔ85 treatment. Finally, the effect of RONΔ85 is specific to RON and not to structure-related MET or unrelated EGFR, even though both are known to have cross-talk with RON. In summary, results from Figs. 3 to 6 demonstrate that RONΔ85 is a specific antagonist to RON-mediated signaling events. They also serve as a mechanistic mode of RONΔ85 in inhibition of the MSP-RON pathway.

Activation of the MSP-RON pathway directs a cellular program known as invasive growth characterized by increased cell replication, migration, and matrix invasion (33). Results from studying RONΔ85 in regulating tumorigenic activities revealed that RONΔ85 inhibits cell growth and colony formation in both transfected and established tumor cell lines. Both MSP-dependent and -independent cell growth were affected. These results indicate that the inhibitory effect on signaling events has biological consequences. However, the effectiveness of RONΔ85 on cell growth varied among cell lines. Significant inhibition was observed only in BxPC-3 cells in contrast to moderate inhibition in HCT116 cells. Moderate levels of inhibition were also seen in anchorage-independent growth of HCT116 cells in soft agar. Such differences in inhibition may reflect the differential dependency of cancer cells on RON-mediated signaling. The relatively weak inhibition by RONΔ85 on Erk1/2 phosphorylation could also be a reason for the modest inhibition. Regardless of the mechanisms underlying RONΔ85-mediated growth inhibition, results in Fig. 7 imply that RONΔ85 has the ability to inhibit cell growth and its effectiveness is dependent on cellular contexts of individual cell lines.

The effect of RONΔ85 on MSP-dependent or -independent cell migration is strong as compared to cell growth. More than 50% of inhibition (67% for BxPC-3 cells and 53% for HCT116 cells, respectively) was achieved in MSP-independent cell migration. Furthermore, inhibition of MSP-dependent migration was also achieved around 67% for BxPC-3 cell and 41% for HCT116 cells. Strong inhibition seems to be linked to the effect of RONΔ85 on AKT phosphorylation. RONΔ85 substantially inhibited spontaneous and MSP-induced ATK phosphorylation in both colon and pancreatic cell lines tested (Fig. 6). Activation of the PI-3 kinase-AKT pathway is involved in RON-mediated cell migration and matrix invasion (34-36). Recent studies further demonstrate that PI-3 kinase activation and AKT phosphorylation were

dramatically reduced upon siRNA-mediated silencing of the RON gene expression (37). These negative effects significantly affect *in vitro* cell migration, invasion and *in vivo* tumor metastasis (37). Considering these results, it is likely that RONΔ85-induced inhibition of AKT phosphorylation is a mechanism responsible for the inhibition of cell migration. Definitely, other pathways are also involved in inhibition of cell migration. Further studies should provide insight into the mechanisms by which RONΔ85 regulates RON signaling and its biological activities.

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