Alterations in a defined extracellular region of the RON receptor tyrosine kinase promote RON-mediated motile and invasive phenotypes in epithelial cells

KUN ZHANG[1,3], YONG-QING ZHOU[2], HANG-PING YAO[1] and MING-HAI WANG[1,3]

[1]Laboratory of Cancer Biology and Therapeutics, [2]Division of Neurosurgery, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, P.R. China; [3]Cancer Biology Center and Department of Pharmaceutical Sciences, Texas Tech University Health Sciences Center School of Pharmacy, Amarillo, TX 79106, USA

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Abstract. Cell migration followed by matrix invasion is a critical step required for epithelial cell differentiation, growth and survival. This study determined the RON receptor in regulation of motile-invasive phenotypes of epithelial cells. Two RON variants, RON165.e11p and RON165, with alterations in a defined extracellular domain were used as the model. RON165 is a splicing variant generated by an mRNA transcript with an in-frame deletion of 49 amino acids encoded by exon 11. In contrast, RON165.e11p was produced by a partial deletion of exon 11 with the elimination of the first 40 amino acids. Thus, RON165.e11p differs from RON165 with nine amino acids retained in the fourth immunoglobulin-plexin-transcription (IPT) domain. Biochemically, both RON165 and RON165.e11p exist as a single-chain protein, residing in the cytoplasm, and failed to mature into the two-chain receptor. Both RON165 and RON165.e11p spontaneously formed oligomers *in vivo* leading to constitutive phosphorylation and the activation of downstream signaling proteins. Although lacking cell-transforming activities, RON165 and RON165.e11p mediated the epithelial to mesenchymal transition with spindle-like cell morphologies, diminished E-cadherin expression, and increased N-cadherin and vimentin expression. These changes facilitated epithelial cell migration and invasion as modeled in Martin-Darby canine kidney (MDCK) cells. Moreover, expression of RON165 or RON165.e11p in breast epithelial MCF-7 cells diminished epithelial cell phenotypes and increased motile and invasive activities. Thus, alterations in the defined extracellular region result in two unique RON variants with similar biological properties. The ability of RON165 or RON165.e11p to promote motile-invasive phenotypes may represent a mechanism by which RON regulates epithelial cell phenotypes and biological activities.

Correspondence to: Dr Y.Q. Zhou, Division of Neurosurgery, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, P.R. China
E-mail: zhouyq56@hotmail.com

Dr M.H. Wang, Department of Pharmaceutical Sciences, Texas Tech University Health Sciences Center School of Pharmacy, Amarillo, TX 79106, USA
E-mail: minghai.wang@ttuhsc.edu

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Introduction

Activation of receptor tyrosine kinases such as HER2, MET, and VEGFR by their cognate growth factors in epithelial cells elicits a cascade of intracellular signals that regulate various cellular activities including morphological changes, cellular migration and matrix invasion into the extracellular space (1-3). These activities are collectively known as invasive growth (4), which is essential for epithelial cell differentiation, proliferation, and survival under various physiological conditions. Increased invasive growth is often present in transformed epithelial cancer cells and contributes to epithelial cell tumorigenesis and malignant progression (1-4). As indicated in malignancy of breast cancers, alterations in HER2 expression result in increased local tissue infiltration and distance metastasis by tumor cells (5). Thus, receptor tyrosine kinases are critical players in regulating migration and invasive properties of epithelial cells. Altered expression and activation of receptor tyrosine kinases have pathogenic implications relevant to cancer development and malignancy (4-6).

The RON (recepteur d' origine nantais) receptor tyrosine kinase is a member of the MET proto-oncogene family (7). RON is composed of a 45 kDa extracellular α-chain and a 150 kDa transmembrane ß-chain linked by a disulphide bond (7). Recent studies have uncovered a previously unrecognized link between aberrant RON expression and pathogenesis of human epithelial cancers including those from breast and colon (8-11). Immunohistochemical staining of primary cancer samples has revealed that RON is overexpressed at around 50% of primary breast cancer sampled with different histological subtypes (8,12). Overexpression associates with the diseases at any stage, correlates with the post-menopausal

status (8), and serves as an independent predictor of distant relapse in breast cancer patients (13). Biochemically, RON overexpression causes constitutive tyrosine phosphorylation, which stimulates downstream signaling pathways including MAP kinase and PI-3 kinase (8-11). These activities lead to dramatic cellular morphological changes with increased cell migration and matrix invasion (8-11). In animal models, mammary-RON expression induces highly metastatic tumors (9,10). RON activation clearly plays a role in regulating cellular invasive behavior and contributes to tumor progression towards metastatic phenotypes.

One of the biological features in RON-expressed epithelial cells is the generation of biologically active RON variants (14-16). Currently, six RON variants, RON170, RON165, RON160, RON155, RON110 and RON52, have been identified in primary cancer samples and in established cell lines (11). RON160 is an example of a constitutively active variant (11). It is produced by a RON mRNA transcript through alternative splicing that eliminates 109 amino acids in the RON extracellular domain (11). These amino acids are encoded by exons 5 and 6, which constitute the first IPT domain in the RON ß-chain (17). This deletion results in conformational changes and leads to constitutive phosphorylation (11). Another RON variant, designated as RON52, is a truncated RON isoform produced by alternative initiation at MET^{913} in the RON ß-chain extracellular sequence (15). MET^{913} is in the coding region of exon 11 (15,17). The RON52 protein lacks the majority of the extracellular sequences but retains the complete trans-membrane and tyrosine kinase domains (15). RON52 is constitutively active with autophosphorylation (15). *In vitro* experiments show that RON160 and RON52 are capable of transducing signals that regulate various cellular activities (11,15). These activities facilitate the epithelial cell morphological change, cellular migration, and matrix invasion.

The goal of the present study is to determine the biological significance of the alterations in the extracellular domain that contributes to the RON-mediated motile-invasive phenotype in epithelial cells. By studying RON variants with complete or partial deletion in exon 11, we demonstrate that alterations in the fourth IPT domain in the RON extracellular sequence results in the constitutive activation of RON with increased tyrosine phosphorylation. Expression of these RON variants causes dramatic morphological changes and increases cell migration. These changes were accompanied with loss of epithelial cellular markers and the gain of mesenchymal phenotypes. Thus, alteration in defined regions in the RON extracellular domain is a mechanism by which RON regulates motile and invasive phenotypes of epithelial cells.

Materials and methods

Cell lines and reagents. Human BC cell lines HCC-1937, MDA-MB231, T-47D, ZR-751 and MCF-7 were from ATCC (Manassas, VA). Variants of MDA-MB231 cells metastasized into bone (MB-Bo231) or brain (MB-Br231) were kindly provided by Dr T. Yoneda (University of Texas, Health Sciences Center, San Antonio, TX). MDCK, MDCK-RON and -RON160 cells were established as previously described (18). Mouse monoclonal antibodies (mAb) specific to RON (clones Zt/g4 and Zt/c1) were used as preciously described (19). PD98059 (PD), SB203580 (SB) and wortmannin (WT) were from Calbiochem (San Diego, CA). Mouse mAb specific to phosphor-tyrosine (clone PY-100), phosphor-Erk1/2, Akt, and other proteins were from Cell Signaling (Danvers, MA). Rabbit or goat IgG antibodies specific to E-cadherin, N-cadherin, vimentin, claudin-1 or ß-actin were from BD Transduction Laboratories (Lexington, KY).

RT-PCR and DNA sequence. Total RNAs were isolated from BC cell lines and primary human BC samples using TRIzol (Invitrogen, CA). RT was carried out using 2 μg of total RNAs with a SuperScript Preamplification kit (Invitrogen). PCR was conducted as previously described (20). The oligomers used to clone the RON165 or RON165.e11p cDNA fragment were designed as previously described (20). The amplified cDNA fragment was cloned into the pGEM-T-easy vector (Promega) and sequenced at the Texas Tech University DNA Sequence Core facility.

Construction of the full-length RON165 and RON165.e11p cDNA and their expression in MDCK cells. The RON165.e11p cDNA was constructed by replacing a 0.67 kb fragment in the wild-type RON cDNA with the amplified 0.64 kb fragment to create the full-length RON165.e11p cDNA. Transfection of MDCK cells with RON165.e11p cDNA, selection of stable cell lines and Western blot analysis of protein expression were conducted as previously described (20).

Immunoprecipitation and Western blot analysis. These methods were performed as detailed previously (20). Individual proteins were detected using specific antibodies. The membrane was also reprobed with anti-ß-actin antibodies to ensure the equal sample loading (20).

Immunofluorescent cell surface labeling and staining. Fluorescent cell surface/cytoplasmic labeling was performed by fixing cells either by 3% formalin-PBS (membrane not permeable) or by cold acetone (membrane permeable) and then analyzed by FACScan (19). Immunofluorescent cytostaining to determine RON165.e11p cytoplasmic localization was carried out as previously described (19). Cells were fixed by acetone. Fluorescent staining was observed under an Olympus microscope equipped with fluorescent apparatus and photographed with a digital camera. In all assays, mAb Zt/g4 was used to detect RON antibody as previously described (19).

Transepithelial electric resistance (TER) assays. Cells ($2x10^5$) were seeded on a polycarbonate filter to form a monolayer. After washing with PBS, A Millicell-ERS volt-ohm meter (Millipore, CA) was used to determine TER value in each sample as previously described (21). Final TER values were calculated by subtracting the blank value and then normalized for the area of the filter.

Cell migration assays. A 48-well migration chamber was used to determine cell migration in response to macrophage-stimulating protein (MSP) stimulation (20). Polycarbonate membranes (Neuro Probe, Gaithersburg, MD) were coated with 50 μg/ml type IV collagen. Cells were added to the top

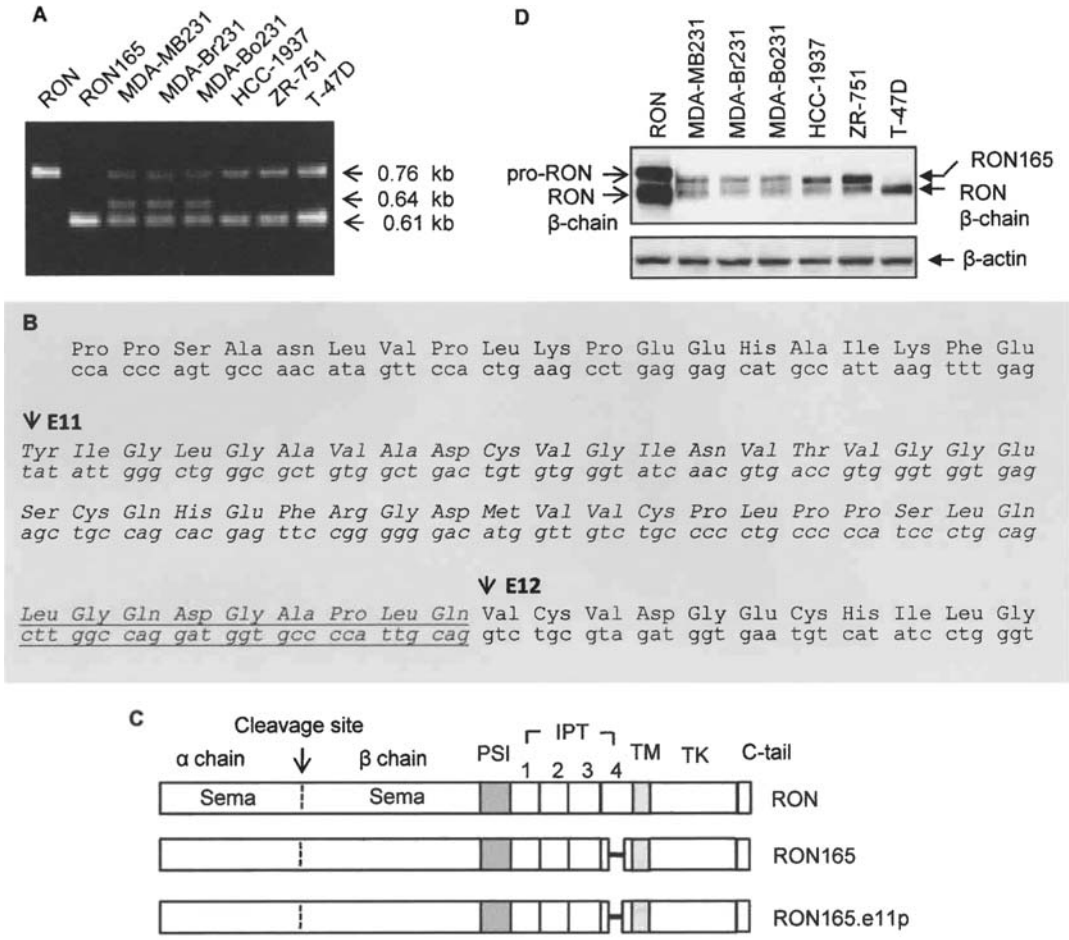

Figure 1. Identification of two RON variants in MDA-MB-231 and its derivative cells. (A) Detection of three RON mRNA transcripts by RT-PCR. Total RNA from six breast cancer cells was reverse-transcribed followed by PCR analysis using a pair of primers covering exons 7-12 of the RON mRNA. Total RNA from 3T3-RON or 3T3-RON165 cells served as the positive control. (B) Partial sequences of the cloned RON cDNA fragment. The cDNA sequences containing the exons 10, 11 and 12 were shown. The beginning of exon 11 or 12 is marked with an arrow. The complete deletion of 49 amino acids coded by exon 11 was italicized (as detected in RON165 cDNA). The partial deletion with retained the 9 amino acid sequence from exon 11 was underlined (as detected in RON165.e11p cDNA). (C) Schematic representation of wild-type RON, RON165 and RON165.e11p. RON contains a sema domain (localized in both α- and ß-chains) followed by a PSI motif and four IPT units. The partial deletion of exon 11 occurs in the fourth IPT unit. TM, transmembrane domain; TK, tyrosine kinase domain. (D) Detection of RON and its variant expression in a panel of breast cancer cell lines. Cellular proteins (50 μg/lane) from lysates of MDA-MB231, MDA-Br231, MDA-Bo231, HCC-1937, ZR-751 and T-47D were subjected to Western blot analysis using the rabbit IgG antibodies to the RON C-terminal peptide. 3T3-RON cells were used as the positive control. The membrane was reprobed for ß-actin as the loading control.

wells. The bottom wells were filled with 2 nM of MSP. Migrated cells were determined after cells were incubated for 8 h. The wound-healing assay was used to determine the ability of cells to cover the open space (21). After MSP stimulation for 12 or 24 h, the percentages of open spaces covered by migrated cells were determined.

Bioassays for cell-invasive activity (branch-like structure formation in collagen gel). The assay was performed as previously described (22). Briefly, MDCK-RON165 or -RON165.e11p cells were mixed with DMEM with 5% FBS supplemented with collagen gel (final concentration 1 mg/ml) and then placed at $1.5x10^4$ cells/well in a 24-well plate. MSP (2 nM) was added simultaneously after initiation of cell culture. After a 7-day incubation, cell invasiveness as judged by the formation of branch-like structures in the collagen gel were determined and photographed. The assays were repeated twice to ensure the accuracy of the data.

Results

Molecular cloning of RON165 and RON165.e11p from breast MDA-MB-231 cells. To determine if any mRNA transcripts encoding novel RON variants exist in breast cancer cells, total RNA isolated from a panel of six established human BC cell lines were subjected to RT-PCR analysis. The amplified regions were on the RON ß-chain extracellular sequences covering exon 7 to exon 12. Results in Fig. 1A show RON specific cDNA fragments amplified from individual mRNA samples. The 0.76 kb fragment was amplified from all six BC cell lines. The size was matched with the positive control from the wild-type RON mRNA. The 0.61 kb fragment was also present in BC cell lines and its size was identical to the samples from the RON165 mRNA. An interesting finding was the presence of a 0.64 kb fragment detected only in MDA-MB-231 cells and their organ-specific variants. Thus, it was reasoned that this is a novel RON mRNA transcript.

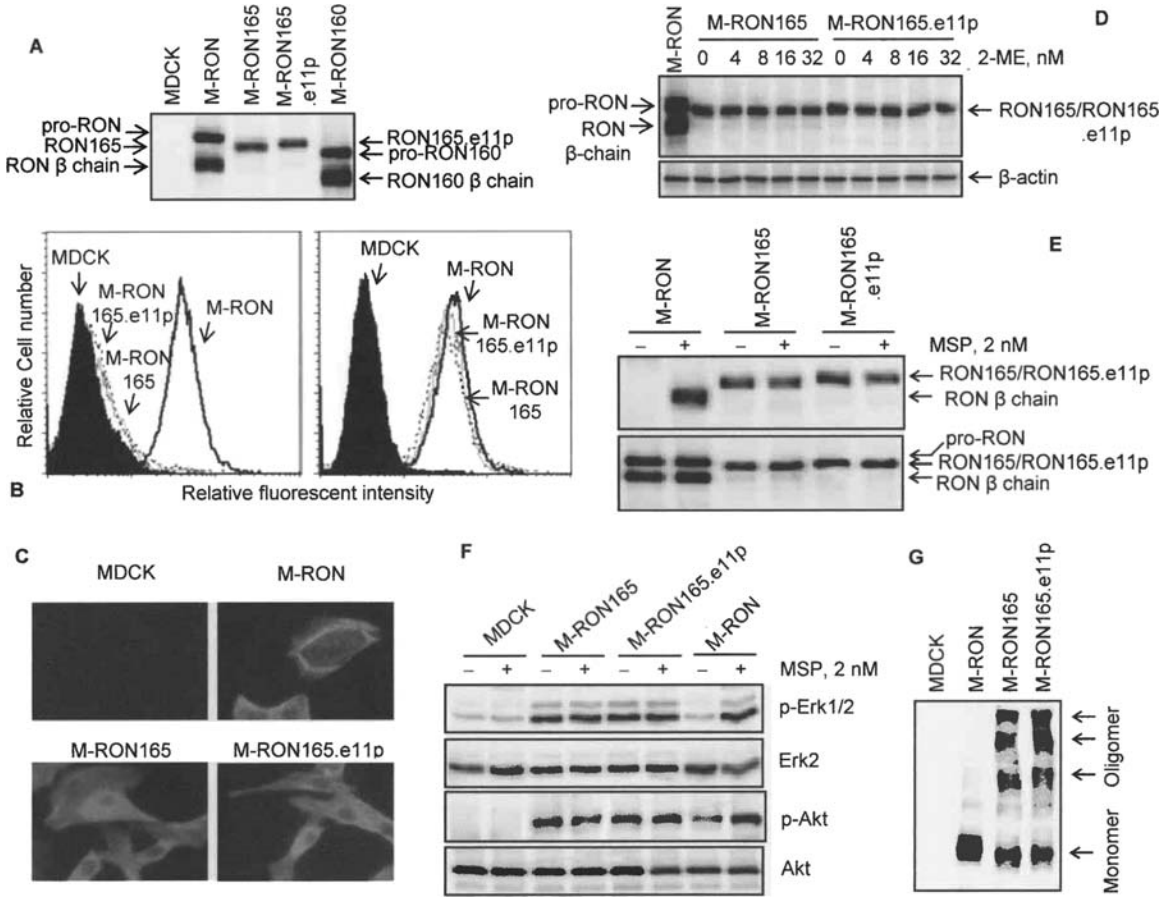

Figure 2. Expression, localization and activation of RON165 or RON165.e11p in MDCK cells. (A) Expression of RON165 or RON165.e11p in MDCK cells. MDCK cells expressing RON, RON165, or RON165.e11p were established as detailed previously (20). Western blot analysis of RON165 or RON165.e11p was performed as detailed in Fig. 1A. (B) Immunofluorescent analysis of RON165 or RON165.e11p expression. Cells ($1x10^5$ cells/sample) were fixed either by 3% formalin in PBS (left panel, cell membrane not permeable) or by cold acetone (right panel, cell membrane permeable) and then incubated with mAb Zt/g4 (1 μg/ml) specific to the RON extracellular domain. Goat-anti-mouse IgG coupled with FITC was used as the second antibody. Fluorescent intensities of individual samples were analyzed in FACScan. (C) Fluorescent localization of RON165 or RON165.e11p in cytoplasm. Cells attached on coverslip were fixed with cold acetone. Mouse mAb Zt/g4 was incubated with cells followed by FITC-coupled goat anti-mouse IgG antibodies. Immunofluorescence was determined by the BK40 Olympus microscope equipped with a fluorescent apparatus. (D) Effect of 2-ME treatment on proteolytic maturation of RON165 or RON165.e11p. MDCK-RON165 or -RON165.e11p cells ($3x10^6$ cells/sample in DMEM + 10% FBS) were treated with different amounts of 2-ME for 4 h as previously described (16). Western blot analysis using the rabbit IgG antibodies to the RON C-terminal sequence were used to determine the maturation of RON165 or RON165.e11p. The membrane was reprobed for ß-actin as the loading control. (E) Spontaneous phosphorylation of RON165 or RON165.e11p in MDCK cells. Cells ($3x10^6$ cells/sample) were stimulated with or without MSP (2 nM) for 10 min. Cellular proteins were immunoprecipitated with mAb Zt/g4 (1 μg/ml) followed by Western blot analysis using mAb PY-100. The membrane was also reprobed with the rabbit anti-RON IgG antibodies as the loading control. (F) Effect of RON165 or RON165.e11p expression on phosphorylation of downstream signaling proteins. Cell stimulation and Western blot analysis using antibodies to regular or phosphorylated Erk1/2 and AKT were carried out as previously described (20). (G) Spontaneous oligomerization of RON165 or RON165.e11p *in vivo*. Proteins (50 μg/samples) from cell lysates were separated in 6% SDS-PAGE under non-reduced conditions followed by Western blot analysis using the rabbit anti-RON IgG antibodies. Protein bands with high molecular masses are indicated with arrows. Data shown here are from one of three experiments with similar results.

All three cDNA fragments were subjected to DNA sequence analysis. The sequence of the 0.76 kb fragment was found to be identical to those from the wild-type RON mRNA. The 0.61 kb fragment contained a deletion of 147 nucleotides coded by exon 11, confirming that the RON165 mRNA transcript exists in MDA-MB-231 cells and the other four BC cell lines. The 0.64 kb fragment is a distinct RON cDNA fragment (Fig. 1B) and it missed 120 nucleotides from T^{2678} to C^{2798} in the RON ß-chain extracellular sequence. Such changes resulted in an in-frame deletion of the first 40 amino acids encoded by exon 11. However, the remaining 27 nucleotides were retained as the coding sequence, which encodes 9 amino acids. Schematic representations of the complete or partial exon 11 deletion and the corresponding RON variant (designated as RON165 and RON165.e11p) are shown in Fig. 1C. From sequence analysis, the deletion occurs in the region of the fourth IPT domain. Thus, three specific mRNA transcripts encoding wild-type RON, RON165 and RON165.e11p exist in MDA-MB-231 and their derivative cells. RON165.e11p is clearly a novel variant from a unique RON mRNA transcript, which has not been previously reported.

To study if RON165 and RON165.e11p mRNA transcript exists in primary breast cancer samples and other breast cancer cell lines, RT-PCR was performed on total RNA from 10 fresh human breast cancer samples and an additional five BC cell lines. The cDNA fragments corresponding to wild-type RON (all samples positive) or RON165 (8 out of 10 primary BC

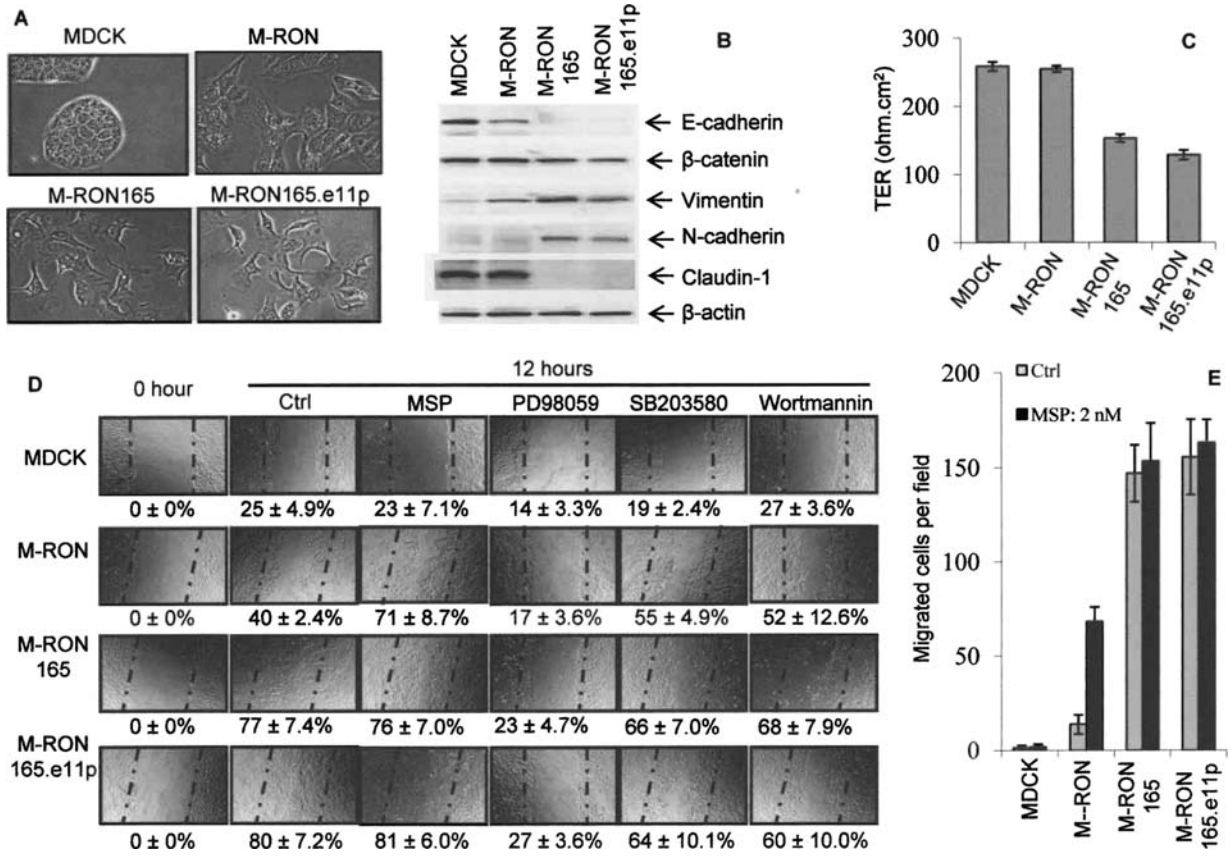

Figure 3. Effect of RON165 or RON165.e11p expression on phenotypic changes of MDCK cells. (A) RON165 or RON165.e11p expression results in MDCK morphological changes. MDCK-RON165 or -RON165.e11p, and other cells ($1x10^4$ cells/dish) were cultured in DMEM + 10% FBS for 48 h. Parental MDCK or MDCK-RON cells were used as the control. Morphological changes were observed by a microscope and photographed (magnification, x200). (B) Diminished expression of epithelial cellular makers in MDCK-RON165, or -RON165.e11p cells. Western blot analysis of individual proteins was performed using specific antibodies as detailed in Fig. 1A. The level of ß-actin was used as the loading control. (C) Reduced TER in MDCK cells expressing RON165 or RON165.e11p. Cells ($2x10^5$ cell/dish) in DMEM + 10% FBS were grown into monolayer. TER was measured as previously described (21). (D) Effect of RON165 or RON165.e11p expression on MDCK cell migration on plastic surface. Experiments were performed as previously described (20). Individual chemical inhibitors were added immediately after a wound has been created. After a 12 h incubation, areas that covered by migrated cells from the edge was photographed and calculated. (E) Effect of RON165 or RON165.e11p expression on trans-membrane migration of MDCK cells. The assays were performed using a 48-well migration chamber as previously described (20). Spontaneous or MSP-induced cell migration was measured. Migrated cells attached on the low side of the membrane were counted after a 5-h incubation.

samples and 4 out of 5 cell lines positive) were frequently amplified. The fragment containing the partial deletions of exon 11 was not observed (data not shown). These results suggest that alternative splicing such as the deletion of exon 11 is a common event in primary BC cells. However, the partial deletion of exon 11 is a rare event and occurred only in MDA-MB-231 cells and their variants.

To determine if variants of RON corresponding to the isolated mRNA transcripts were expressed in BC cells, cellular proteins from the above BC cell lines were subjected to Western blot analysis using antibodies specific to the RON C-terminal peptide (Fig. 1D). The mature RON ß-chain was detected in MDA-MB-231, MDA-Br231, MDA-Bo231, HCC1937, ZR-751, and T-47D cells with variable levels. In addition, a protein band with molecular mass of 165 kDa, similar to RON165 variant, was also detected in five cell lines except T-47D cells. These results were consistent with the RON165 mRNA expression in Fig. 1A and confirmed that RON165 is synthesized and expressed in these BC cell lines. However, since the molecular mass of the novel variant encoded by the RON165.e11p mRNA is almost identical to that of RON165, we were unable to confirm if RON165.e11p was expressed in MDA-MB-231 cells or its derivatives.

Biochemical characterization of RON165 and RON165.e11p expressed in MDCK cells. To study the biological significance of the exon 11 deletions, RON cDNA containing the complete or partial deletion of exon 11 was constructed (designated as RON165 or RON165.e11p cDNA). This was achieved by replacement of the NheI-SanDI fragment in the wild-type RON cDNA with the cloned 0.64 or 0.61 kb PCR fragment. The cDNA was inserted into the pDR-2 mammalian expression vector and then stably transfected into MDCK cells. Results from Western blot analysis showed that either RON165 or RON165.e11p was expressed at the expected molecular mass as a single-chain precursor, which illustrated the failure of cleavage into the two-chain mature protein (Fig. 2A). Immunofluorescent cell surface analysis using mAb Zt/g4 specific to the RON extracellular sequences showed that neither RON165 nor RON165.e11p were expressed on the cell surface. Upon

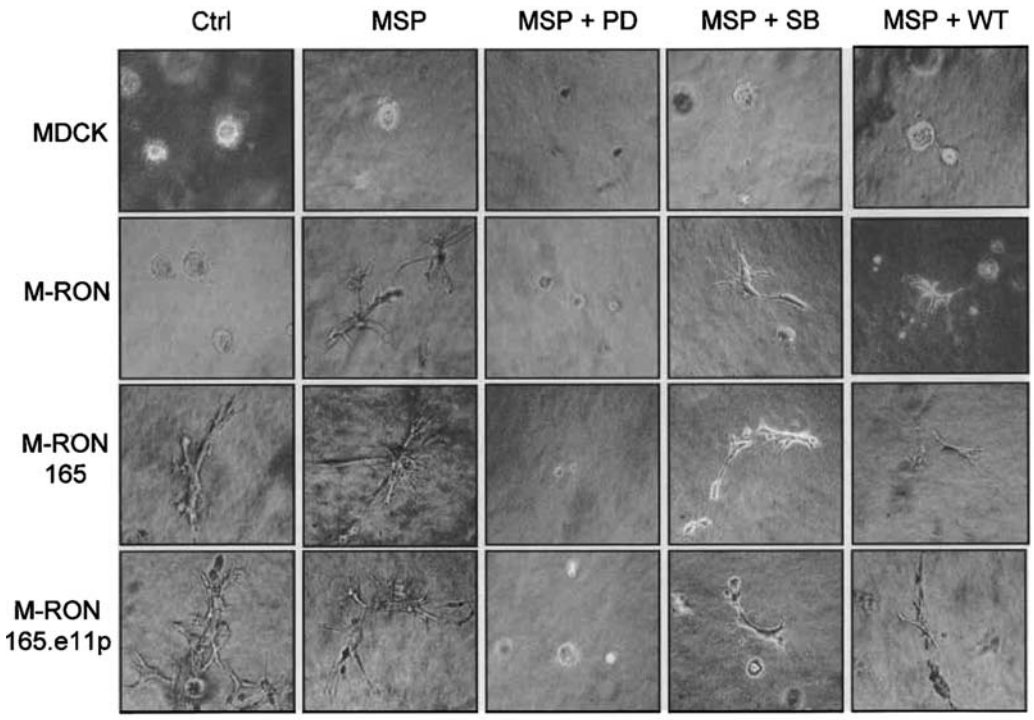

Figure 4. Effect of RON165 or RON165.e11p expression on formation of branch-like structures by MDCK cells in collagen gel. Experiments were carried out as detailed previously (22). MSP (2 nM), PD98059, SB203580, wortmannin, and their different combinations were added after initiation of cell culture. Cells were incubated for 7 days and then photographed. Branch-like structures were observed in MDCK-RON (MSP-stimulated), MDCK-RON165, or -RON165.e11p cells. PD98059 completely prevented the formation of branch-like structures by all types of cells. However, SB203580 and wortmannin displayed only partially preventive effect.

permeablization of cell membrane, RON165.e11p was readily detected by Zt/g4 (Fig. 2B). Cytoplasmic localization of RON165 or RON165.e11p was confirmed by fluorescent cytostaining in which RON165 and RON165.e11p were found to be accumulated in the cytoplasm (Fig. 2C). To determine if 2-ME treatment facilitated the proteolytic process of the single-chain RON165 or RON165.e11p into the two-chain form, MDCK-RON165 or -RON165.e11p cells were treated with 2-ME and the conversion of RON165 or RON165.e11p was determined by Western blot analysis. To our surprise, proteolytic conversion of RON165 or RON165.e11p was not observed in cells treated with various concentrations of 2-ME (Fig. 2D). Prolonged treatment up to 24 h did not result in any conversion (data not shown). These results demonstrated that RON165 and RON165.e11p were synthesized and maintained as a single-chain protein that resides in the cytoplasm.

In order to determine if complete or partial deletion of exon 11 affects the RON protein phosphorylation, cells were stimulated with or without MSP followed by Zt/g4 immunoprecipitation. Results in Fig. 2E showed that both RON165 and RON165.e11p were highly and constitutively phosphorylated at tyrosine residues. Because of its intracellular localization, MSP stimulation had no effect on its phosphorylation status. In contrast, phosphorylation of RON required MSP stimulation. We also studied if downstream signaling proteins were phosphorylated in RON165 and RON165.e11p cells. Results in Fig. 2F showed that RON165 or RON165.e11p expression resulted in constitutive phosphorylation of Erk1/2 in MDCK cells. Although the levels were relatively low, a slight increase in AKT phosphorylation was also observed in MDCK-RON165 or -RON165.e11p cells after MSP stimulation. The effect of RON165.e11p expression on phosphorylation of p38 MAP kinase was also studied. However, no phosphorylation was observed (data not shown). Thus, RON165 and RON165.e11p were constitutively active RON variants and had the ability to selectively activate downstream signaling proteins such as Erk1/2.

To determine if the constitutive phosphorylation of RON165 or RON165.e11p is related to the formation of oligomers *in vivo*, RON165 and RON165.e11p were immunoprecipitated from cell lysates by Zt/g4 and separated in 6% SDA-PAGE under non-reduced conditions. The oligomerization of RON165 was detected as bands with high molecular masses (Fig. 2G), which is consistent with a previous report (16). Similarly, protein bands with high molecular masses were also found for RON165.e11p. In contrast, RON was detected as a monomer at molecular mass of 180 kDa in quiescent MDCK cells. These results suggested that spontaneous oligomerization of RON165 and RON165.e11p occurred in transfected MDCK cells, which could be a mechanism responsible for constitutive phosphorylation.

Effect of RON165 or RON165.e11p expression on epithelial to mesenchymal transition in MDCK cells. Expression of RON165 or RON165.e11p in MDCK cells caused dramatic cellular morphological changes with spindle-like appearances (Fig. 3A). Morphological changes at a lesser degree were also seen in MDCK-RON cells. These properties resemble epithelial to mesenchymal transition (EMT), a process required for cell migration and invasion (23). To determine if EMT

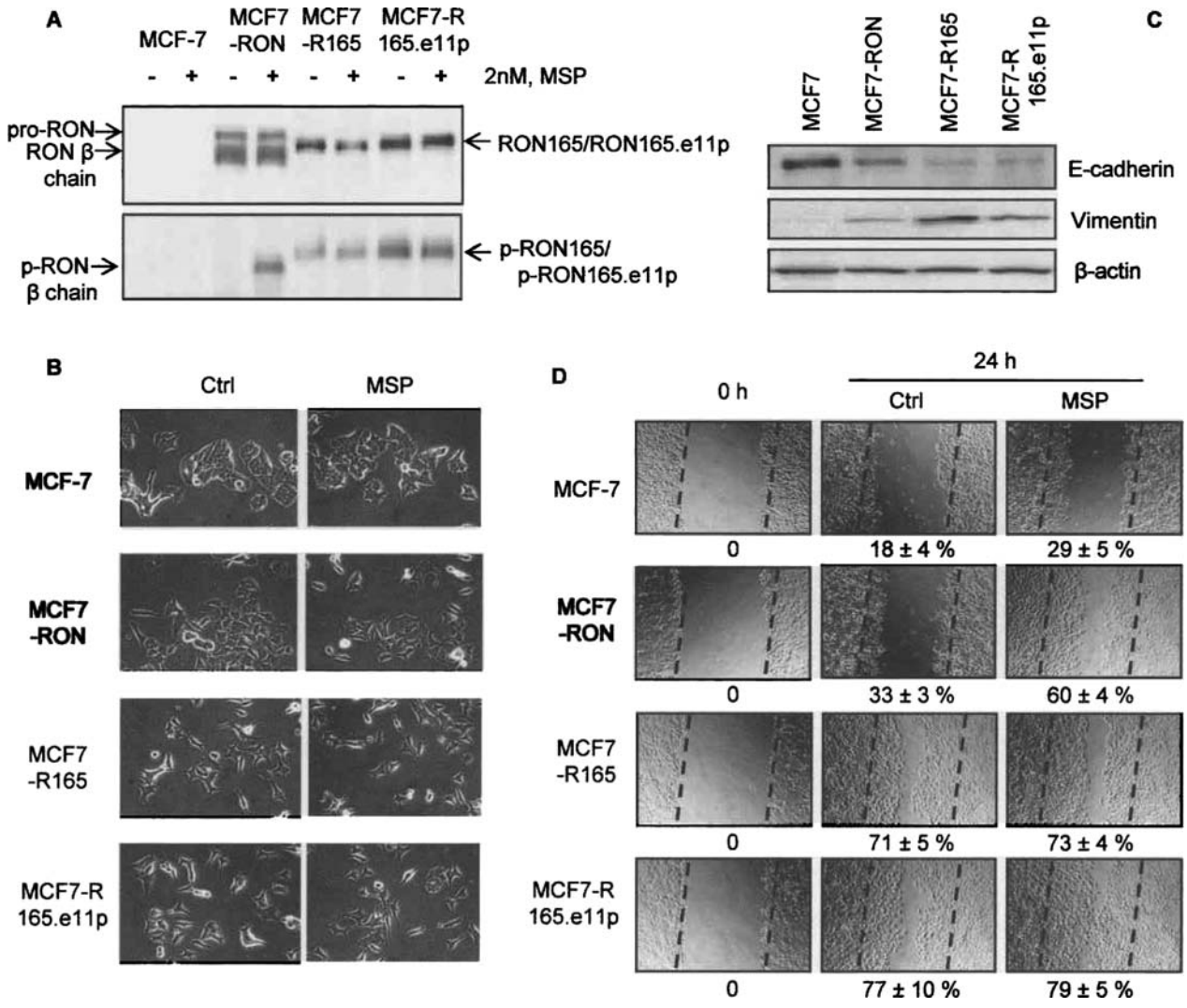

Figure 5. Effect of RON165 or RON165.e11p expression on phenotypes of breast epithelial MCF-7 cells. (A) Expression and phosphorylation of RON165 or RON165.e11p in MCF-7 cells. Levels of RON, RON165, or RON165.e11p expressed in established MCF-7 cells were determined by Western blot analysis (top panel) as detailed in Fig. 1A. MSP-dependent or independent phosphorylation of RON165 or RON165.e11p was determined as described in Fig. 2E. (B) Effect of RON165 or RON165.e11p expression on morphological changes of MCF-7 cells. The experimental conditions were similar to those shown in Fig. 3A. MSP (2 nM) was added after initiation of cell culture. (C) Effect of RON165 or RON165.e11p on E-cadherin and vimentin expression. Lysates of MCF-7 cells expressing RON, RON165, or RON.e11p were subjected to Western blot analysis using antibodies specific to E-cadherin or vitamin. Actin was used as the loading controls. (D) Effect of RON165 or RON165.e11p expression on migration of MCF-7 cells. Experiments were conducted as detailed in Fig. 3D with slight modifications. Migration was determined after cells were cultured for 24 h. Results shown here are from one of three experiments with similar results.

indeed occurred in MDCK-RON165 or -RON165.e11p cells, cellular proteins representing epithelial or mesenchymal markers were analyzed by Western blot analysis. The epithelial cellular marker E-cadherin was dramatically diminished in MDCK-RON165 and -RON165.e11p cells (Fig. 3B). Along with the loss of the epithelial cell marker was the increased expression of two mesenchymal proteins vimentin and N-cadherin in RON165 and RON165.e11p expressing cells. The patterns of changes in MDCK-RON165.e11p cells were similar to those in MDCK-RON165 cells. This suggested that RON165.e11p displayed similar effects as RON165 in the induction of EMT in MDCK cells.

To determine if RON165.e11p affected the expression of other junction proteins, we measured tight junction protein claudin-1 expression. As shown in Fig. 3B, claudin-1 expression was completely diminished in MDCK-RON165 and -RON165.e11p cells. Analysis of transepithelial electric resistance (TER), a functional measure of tight junction (24,25) revealed that TER was significantly reduced in both MDCK-RON165 and -RON165.e11p cells (Fig. 3C). These results, together with those in Fig. 3A and B, suggested that epithelial markers are diminished and mesenchymal proteins are expressed in MDCK-RON156 and -RON165.e11p cells. Thus, expression of RON165 or RON165.e11p affected protein expression not only in the adherens junction but also in the tight junction.

EMT is featured by increased cell migration and invasiveness (23,26). To determine if RON165 or RON165.e11p expression resulted in increased cell motility, two methods were employed. In assays measuring cell movement towards an open space in the surface of a culture dish, it was observed that RON165 or RON165.e11p expression spontaneously

increased MDCK movement towards the open space. More than 75% of the open space was covered by migrated MDCK-RON165 or RON165.e11p cells. Again, this effect was not seen in parental MDCK cells. In the migration chamber assay, MDCK-RON165 or -RON165.e11p cells showed increased spontaneous migration through the collagen-coated membrane. Again, this effect was not seen in parental MDCK cells. The levels of MDCK-RON165 cell migration were comparable to those of MDCK-RON165.e11p cells. Because of intracellular expression, further enhancement of cell migration was not observed after MSP stimulation.

In an effort to determine the potential signaling events involved in cell migration, we found that RON165 or RON165.e11p-mediated cell migration was sensitive to the MAP kinase inhibitor PD98059, but resistant to the PI-3 kinase inhibitor wortmannin or the p38 MAP kinase inhibitor SB203580 (Fig. 3D). Thus, RON165 or RON165.e11p expression had the ability to increase MDCK cell migration, which was sensitive to the MAP kinase inhibitor PD98059.

Effect of RON165 or RON165.e11p on MDCK cell tubular formation in collagen gel. Persistent activation of RON by MSP resulted in the formation of branch-like structures when MDCK cells were cultured in collagen gel (Fig. 4) (18). These effects were also seen in MDCK-RON165 or -RON165.e11p cells. Unlike MDCK-RON cells that required MSP stimulation to form branch-like structures, RON165.e11p expression resulted in formation of branch-like structures by MDCK cells in collagen gel. Again, MSP stimulation had no enhancing effect on RON165.e11p-mediated branch-like formation due to the intracellular localization of the variant. To determine signaling proteins involved in these events, individual chemical inhibitors were added to cells stimulated with MSP. PD98059 prevented the MDCK branch-like structure formation mediated by activated RON, RON165 and RON165.e11p. Preventive effects of SB203580 and wortmannin were also seen although their activities were moderate. In these cases, the sizes of the branch-like structures were relatively smaller than those seen in untreated MDCK-RON165 or -RON165.e11p cells. Thus, RON165 or RON165.e11p expression had the ability to cause MDCK cells to form branch-like structures and the process was highly sensitive to PD98059 and partially prevented by wortmannin or SB203580.

Increased migratory phenotypes in BC MCF-7 cells expressing RON165.e11p. Results from transfected MDCK cells prompted us to determine if RON165 or RON165.e11p would exert similar effects on breast epithelial cells. MCF-7 cells were chosen as the model because they did not express RON as evident in Western blot analysis (Fig. 5A) and had low migratory activities as indicated in the cell migration assays (Fig. 5D). Expression of RON165 or RON165.e11p in MCF-7 cells was established along with MCF-7 cells that expressed wild-type RON. Consistent with results from MDCK cells, RON165 or RON165.e11p was constitutively phosphorylated at tyrosine residing (Fig. 5A, low panel) and localized in cytoplasmic compartments (data not shown). Constitutive phosphorylation of Erk1/2 and AKT by RON165 or RON165.e11p expression in MCF-7 cells was also observed (data not shown). Assessment of morphological changes revealed that RON165 or RON165.e11p expression resulted in scatter-like morphological changes in MCF-7 cells even in the absence of MSP (Fig. 5B). Similar effects were also seen in MCF7-RON165 cells. The morphological changes were accompanied with diminished E-cadherin expression and increased vimentin expression (Fig. 5C). This suggested a conversion of epithelial cell appearance to mesenchymal phenotypes.

Function analysis showed that RON165 or RON165.e11p expression caused increased cell migration. As presented in Fig. 5D, the spontaneous migration of MCF-7 cells was significantly increased upon RON165 or RON165.e11p expression, which was not regulated by MSP. In contrast, RON-mediated cell migration relied on MSP stimulation. These results suggested that the expression and phosphorylation of RON165.e11p was required for the initiation of the motile machinery of MCF-7 cells.

Discussion

The findings in this study illustrate that the alterations in the fourth IPT domain in the extracellular sequences result in the formation of two RON variants with similar biological activities. This suggests that exon 11 is subject to unique splicing regulation, which has a biological consequence on cellular behavior. Generation of RON variants is an intriguing event. Currently, six RON variants, RON170, RON165, RON160, RON155, RON115 and RON52 have been identified (11). They are produced either by alternative mRNA splicing, proteolytic modification, or by alternative initiation (11,15,16). Unlike wild-type RON that requires ligand stimulation for phosphorylation and biological activities, RON variants are often constitutively active (except RON170 which is a kinase-dead variant) (11). They possess various biological activities such as morphological changes, cellular transformation, matrix invasiveness, and tumor growth *in vivo* (11,15,16). These activities are implicated in regulating epithelial cell behavior and tumorigenic phenotypes (11). The cloning of RON165.e11p expands the numbers of RON variants to seven. To our knowledge, this is the first example showing the partial deletion in exon 11 in the RON extracellular sequence. Considering the roles of RON165.e11p in mediating EMT in MDCK cells and in regulating MCF-7 cell motile-invasive phenotypes, it is believed that this variant may play a role in regulating cellular activities in certain epithelial cells.

RON165 and RON165.e11p are produced by two different mRNA transcripts created by the aberrant mRNA splicing process as evident in sequence analysis. The aberrant splicing events act selectively on exon 11, which contains the coding sequences for 49 amino acids that were completely or partially eliminated. It is known that exon 11 encodes 49 amino acids and belongs to the fourth IPT domain in the RON ß-chain extracellular sequences (17). Among exons coding for the RON extracellular domains in the RON gene, exon 11 is frequently targeted for deletion (14,16). RON165, the first RON variant, also known as ΔRON (16), is featured by the complete deletion of exon 11 (16). This is seen in breast cancer samples and cell lines as described in our results. The consequence of this deletion is the production of a protein that differs significantly from wild-type RON (16). For example,

RON165 remains as the single-chain precursor after its synthesis and is not cleaved into the two-chain mature form (Fig. 2A) (16). The protein resides in the cytoplasmic compartment and is unable to be transported onto the cell surface (Fig. 2B and C) (16). Due to conformational changes, RON165 spontaneously forms oligomers *in vivo*, which lead to the constitutive tyrosine phosphorylation with increased kinase activities (Fig. 2E and G) (16). Thus, amino acids coded by exon 11 are critically important in controlling the RON precursor maturation, directing the cell surface localization, and modulating phosphorylation status. The identification of RON165.e11p provides additional evidence that supports the importance of exon 11 in the biology of the RON protein. Moreover, it demonstrates that the deletion of the first 40 amino acids encoded by exon 11 is sufficient to cause the conformational changes that block RON precursor maturation, prevent cell surface localization, and activate receptor tyrosine phosphorylation. As shown in Figs. 1 and 2, RON165.e11p displayed various biochemical features similar to those of RON165. It is maintained as a single-chain precursor, resides exclusively in the cytoplasm, forms oligomers, and is constitutively active in the tyrosine residues. Thus, the complete or partial deletion of exon 11 has a profound impact on functional aspects of the RON protein.

The frequency of RON165 and RON165.e11p occurring in breast epithelial cells is currently unknown. Results from analysis of a limited number of breast cancer samples and cell lines seem to indicate that the formation of RON165.e11p mRNA is not a common event in breast epithelial cells. At present, MDA-MB231 cells and their variants are the only cells that harbor RON165.e11p mRNA. In contrast, the mRNA transcript for RON165 was commonly observed in breast epithelial cells. As shown in Fig. 1A and our survey of primary BC samples and BC cell lines, the complete deletion of exon 11 is readily detected in the majority of tumor samples and cell lines. Analysis of published articles also reveals that the complete deletion of exon 11 commonly occurs not only in tumor samples, but also in normal epithelial cells such as cell of colonic mucosa (14,27). However, the synthesis of the RON165 protein occurs mainly in cancerous samples but not in normal epithelial cells (14,27). The inability of normal epithelial cells to produce RON165 even in the presence of naturally occurring RON165 mRNA transcript seems to be a mechanism responsible for maintaining the integrity of the normal epithelial layer. On the other hand, RON165 is often synthesized and overexpressed in cancerous cells (14,16). Although its biological significance is unknown, it is likely that the generation of RNA165 mRNA transcript is a physiological event. Production of RON165.e11p is currently unknown. Analysis of limited numbers of primary breast cancer samples and established cell lines illustrates that RON165.e11p mRNA exists only in MDA-MB231 cells and its variants. Considering biological potentials of RON165.e11p, it will be interesting to study the frequency of RON165.e11p expressed in BC or other epithelial cancers and to determine its role in regulating tumorigenic phenotypes of breast cancer cells *in vivo*.

The generation of different RON variants is implicated as a mechanism contributing to epithelial cell homeostasis and malignant progression (11). In colorectal cancers, increased RON expression is accompanied with the generation of oncogenic variant RON160 and RON155 and results in increased motile-invasive phenotypes (20,27). Similar effects are also seen in breast tumors, in which RON165 expression promotes aggressive behavior of cancerous cells (14,16). The biological activities of RON165.e11p as manifested in MDCK and MCF-7 cells are similar to those of RON165. This is not surprising since RON165.e11p is almost identical to RON165 with only a 9-amino acid difference at the protein sequence level. As shown in Fig. 1, both RON165.e11p and RON165 remain as the single-chain precursor and fail to be processed into the two-chain form. This suggests that RON165.e11p displays a similar conformation to RON165, which prevents enzymes to assess the proteolytic cleavage site required for generating the RON α- and ß-chain. The absence of a precursor conversion leads to both proteins residing in the cytoplasmic compartment. Phosphorylation studies demonstrate that RON165.e11p is constitutively active as RON165 *in vivo*. A similar mechanism is likely involved in this event because the patterns of oligomer formation between RON165 and RON165.e11p are almost identical as indicated in Fig. 2G. In analyzing RON-mediated EMT, it was found that unlike RON that causes a partial EMT as illustrated in the MDCK or MCF-7 cell models, RON165 or RON165.e11p has the ability to mediate a complete EMT with dramatic morphology change, complete loss of E-cadherin, and increased expression of vimentin and N-cadherin. Expression of RON165 and RON165.e11p also disrupts tight junction formation as evident in diminished claudin-1 expression and impairs transepithelial electric resistance. It is believed that these changes originate from an identical biochemical process existing in both RON165 and RON165.e11p expressing cells. Functional evaluation of MDCK cell migration and invasiveness (branch-like structure formation) in collagen gel further demonstrates that RON165.e11p has the ability to drive cell migration and invasiveness at the level similar to that of RON165. These activities are also reproducible in breast cancer MCF-7 cells, suggesting that similar signaling pathways are elicited in both transfected cells. In conclusion, analysis of RON165.e11p reinforces our concept on the significance of exon 11 in RON biology. It also provides evidence indicating that RON165 and RON165.e11p have the ability to regulate epithelial phenotypes that may have implications in certain pathogenic conditions.

Different mechanisms including alternative pre-mRNA splicing, proteolytic conversion and alternative initiation, have been implicated in the production of RON variants (11). The majority of RON variants are produced by alternative mRNA splicing (11). Deletion of exons 5, 6, 11 and 19, results in the formation of RON160, 155, 165 and 170 (11). RON165.e11p is the product of an aberrant mRNA splicing process. However, the mechanisms underlying RON165.e11p production are unknown. A previous study about detailed splicing mechanisms underlying RON165 production (14) may provide insight into how RON165.e11p is generated. Exon 11 splicing is controlled by a silencer and an enhancer located in exon 12 (14). The identified exon splicing enhancer (ESE) contains a purine-rich sequence that matches the consensus-binding motif for splicing factor (SF) 2 (29). SF2 belongs to a group of highly conserved serine/arginine rich (SR) proteins and is essential for constitutive splicing as well as regulation of alternative

splicing (29). In tumor cells expressing RON165, the levels of SF2 parallel the activities of ESE and the amount of RON165 mRNA transcript. Molecular analysis confirmed that SF2 binds directly to the ESE element but not the mutated sequence. In addition, the binding is specific only to SF2 and no other SR family members (14). Overexpression of SF2 in cells naturally expressing RON regulates alternative exon 11 splicing and promotes the production of the RON165 mRNA transcript (14). Since RON165.e11p is formed through targeting exon 11, it is believed that some of the mechanisms responsible for the generation of the RON165 mRNA transcript are participating in the RON165.e11p mRNA generation. However, further aberrations either in the splicing factors or in the splicing process are needed to cause the formation of the RON165. e11p mRNA transcript. Experiments are currently underway to determine potential mechanisms responsible for the partial deletion of exon 11 in breast epithelial MDA-MB-231 cells.

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