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Invited Review

Pathogenesis of RON receptor tyrosine kinase in cancer cells: activation mechanism, functional crosstalk, and signaling addiction

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Abstract

The RON receptor tyrosine kinase, a member of the MET proto-oncogene family, is a pathogenic factor implicated in tumor malignancy. Specifically, aberrations in RON signaling result in increased cancer cell growth, survival, invasion, angiogenesis, and drug resistance. Biochemical events such as ligand binding, receptor overexpression, generation of structure-defected variants, and point mutations in the kinase domain contribute to RON signaling activation. Recently, functional crosstalk between RON and signaling proteins such as MET and EFGR has emerged as an additional mechanism for RON activation, which is critical for tumorigenic development. The RON signaling crosstalk acts either as a regulatory feedback loop that strengthens or enhances tumorigenic phenotype of cancer cells or serves as a signaling compensatory pathway providing a growth/survival advantage for cancer cells to escape targeted therapy. Moreover, viral oncoproteins derived from Friend leukemia or Epstein-Barr viruses interact with RON to drive viral oncogenesis. In cancer cells, RON signaling is integrated into cellular signaling network essential for cancer cell growth and survival. These activities provide the molecular basis of targeting RON for cancer treatment. In this review, we will discuss recent data that uncover the mechanisms of RON activation in cancer cells, review evidence of RON signaling crosstalk relevant to cancer malignancy, and emphasize the significance of the RON signaling addiction by cancer cells for tumor therapy. Understanding aberrant RON signaling will not only provide insight into the mechanisms of tumor pathogenesis, but also lead to the development of novel strategies for molecularly targeted cancer treatment.

Keywords: Receptor tyrosine kinase (RON), signaling pathway, activation mechanism, signaling crosstalk, oncogene addiction, tumorigenesis

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INTRODUCTION

Discoveries of *recepteur d´origine nantais* (RON) occurred in 1993[1]. Molecular cloning of the human RON cDNA revealed that RON is a receptor protein tyrosine kinase (RTK) belonging to the C-MET proto-oncogene family (***Fig. 1A***)[2,3]. Shortly thereafter in 1994, the cDNA coding the mouse homology of RON was cloned and named as stem-cell derived tyrosine kinase receptor[4]. Human RON gene resides in the chromosome 3p21 region[1] and is highly conserved in different species including human, mouse, feline, chicken, zebrafish, and xenopus[1,4-11]. Interestingly, in avian erythroblastosis retrovirus S13 that causes chicken sarcoma, erythroblastosis, and anemia, a viral oncoprotein namely V-SEA was identified (***Fig. 1A***)[12,13]. V-SEA is a hybrid protein containing the chicken SEA kinase domain fused with viral envelope sequences[9,12,13]. The chicken SEA is a homolog of human RON[10]. These findings indicate that RON is evolutionally preserved in different species. In addition, various RON variants have been identified in cancer cells (***Fig. 1B***). In 1994, macrophage-stimulating protein (MSP, also known as hepatocyte growth factor (HGF)-like protein) was identified as the ligand of RON[14-16]. This finding establishes the MSP-RON signaling axis.

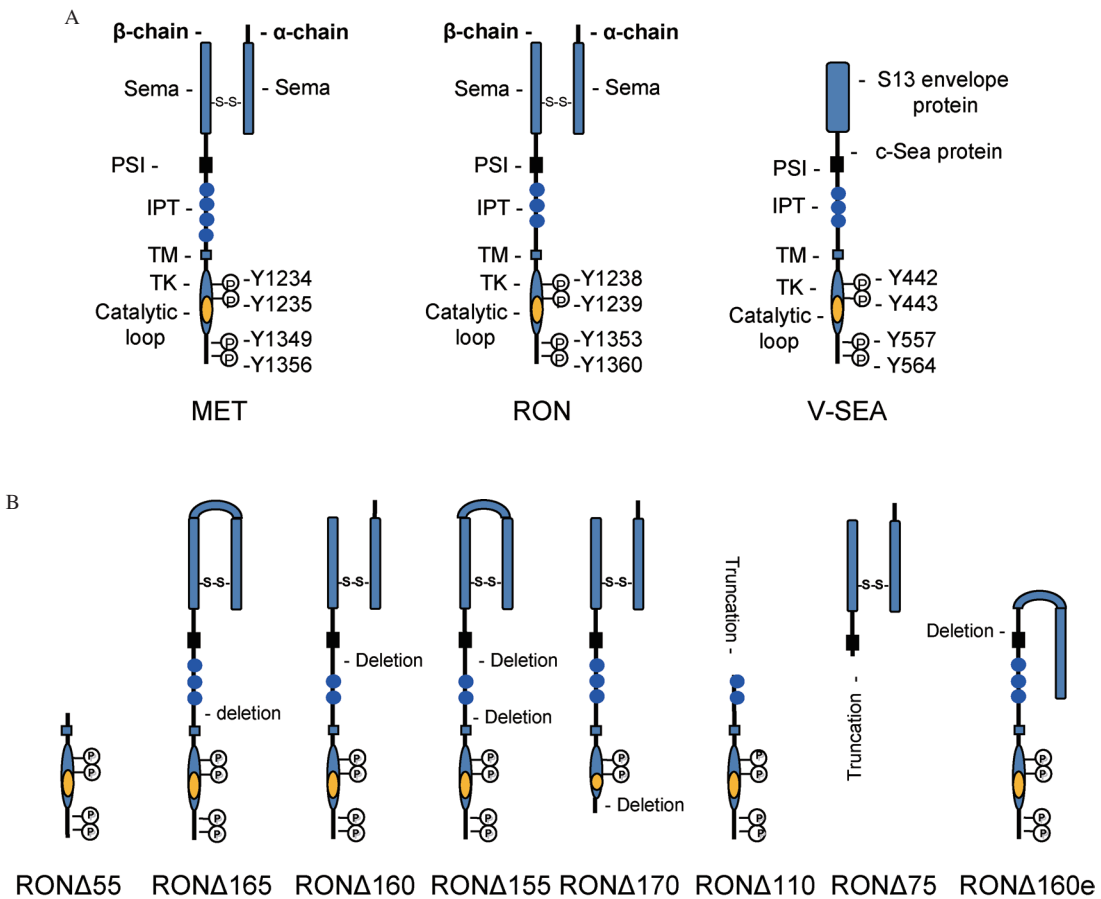

Fig. 1 **Schematic representation of RON and RON variant.** A: General features of MET, RON, and V-SEA. MET is the classical example of this family. Mature RON consists of a 35 kDa α-chain and a 145 kDa β-chain linked by a disulfide bond. The α-chain resides extracellularly and contains a portion of Semaphorin (Sema). The β-chain comprises a large extracellular domain, a short transmembrane (TM) segment, and a cytoplasmic portion harboring a tyrosine kinase (TK) domain and a C-terminal tail. The Sema domain harbors a ligand-binding pocket for the MSP β-chain. Regulatory tyrosine residues Tyr1238 and Tyr1239 in the TK domain and Tyr1353 and Tyr1360 in the C-terminal tail are marked. V-SEA is an oncogenic protein fused by the avian S13 retroviral envelope protein with the chicken SEA sequences. PSI, Plexins-Semaphorins-Integrins; IPT, immunoglobulin-plexin-transcription. B: Different RON variants. RONΔ55 is derived from alternative initiation at Met913. RONΔ165 is formed by deletion of exon 11 coding 49 amino acids. RONΔ160 has a deletion of exons 5 and 6 coding 109 amino acids. RONΔ155 has a combined deletion of exons 5, 6 and 11. RONΔ170 is derived from deletion of exon 19 in the kinase domain. RONΔ110 is formed by *N*-terminal truncation at Arg631. RONΔ85 is a free variant with C-terminal truncation at Asp634 caused by insertion. RONΔ160e is derived by deletion of exon 2.

RON signaling in tumorigenesis and therapy has gained steady attention over the last 20 years. Aberrant RON activation, featured by overexpression[17-24], isoform generation[25-35], and persistent activation of downstream signaling pathways[17-35], has been found in various types of cancers[17-35]. Moreover, functional crosstalk between RON and signaling proteins contributes to tumorigenic progression and malignancy[36-43]. The finding that RON signaling is abnormal in cancer cells provides a rationale for development of RON-targeted cancer therapy. Currently, small molecule inhibitors and therapeutic antibodies are under clinical trials (www.clinicaltrials.gov). Here, we discuss our current knowledge about mechanisms of RON activation, discuss the emerging roles of RON signaling crosstalk in cancer malignancy, and summarize the significance of RON signaling addiction by cancer cells for potential cancer therapy.

MECHANISMS OF RON ACTIVATION

Dimerization of RON in the cell surface is the first step required for RON activation[1,15,16]. Four biochemical events are known to activate RON (***Fig. 2***): specific ligand binding[44,45], receptor overexpression[17-23], generation of oncogenic variants[25,27], and point mutations in the kinase domain[46,47]. The feature of RON activation is autophosphorylation at Tyr1238 and Tyr1239 at the A-loop (Phe1227-Pro1250) in the kinase domain[1,48-50]. Phosphorylation of these regulatory residues then activates the tyrosine kinase leading to further phosphorylation of Tyr1353 and Tyr1360 in the C-terminal docking site (***Fig. 1 and 2***)[48-50]. The docking site interacts with downstream signaling proteins triggering classical RAS-MAPK and PI-3K-AKT pathways[28,34,51-57] (***Fig. 2***). These pathways are responsible for increased proliferation/ survival[58], epithelial to mesenchymal transition (EMT)[20,59,60], motile-invasive activity[51,59,61], and chemoresistance[62,63].

Ligand-induced activation

Ligand-induced activation: The binding of MSP to RON is the classical mode to induce RON dimerization leading to signaling activation (***Fig. 2***)[44,45]. MSP is the only known physiological ligand that specifically activates RON[15,16]. As a protein belonging to the HGF family[64-66], MSP is a product of hepatocyte, which circulates in blood as a biologically inactive single-chain precursor[64,66]. Proteolytic conversion results in biologically active/mature MSP[67-70], which gains the receptor binding capability[44,45].

The MSP molecule possesses two-receptor binding sites[44,45]. The high affinity-binding site is in the MSP β-chain, which binds to an interface in the RON extracellular Sema domain[44,45,71]. The MSP α-chain harbors a low affinity-binding site[45]. The location of the corresponding interface in the RON extracellular domain is unknown. Binding by both MSP α- and β-chains is required to activate RON[44,45]. Crystal structure analysis reveals that a central cleft harboring three residues in the putative catalytic site in the MSP β-chain is essential for the β-chain binding to the RON Sema domain[71]. The binding follows an enzyme-substrate mode conserved in HGF-related growth factors and proteases of the blood clotting pathway[72,73].

Structural analysis under protein crystal packing reveals that individual molecules of the MSP β-chain do not interact with each other to form a receptor binding moiety[72]. Instead, the central cleft in the single MSP β-chain directly binds to the RON Sema homodimer[72]. This suggests that dimerization of the MSP β-chain is not required for RON activation. In contrast, RON Sema molecules form a homodimer, which creates a ligand-binding interface by two Sema domains[71]. Thus, the interface created by the RON Sema dimer appears to be the high affinity binding pocket for the MSP β-chain.

In light of these discoveries, we propose a model of one MSP molecule interacting with two RON receptors for dimerization. This model depicts that as a monomeric form, MSP uses its high affinity-binding site in the β-chain to bind to the interface in the Sema domain formed the RON homodimer. The binding causes receptor conformational changes and exposes a currently unknown binding pocket in the RON extracellular domain for the low affinity-bind site in the MSP α-chain. The sequential binding of the MSP β- and α-chains initiates triggers autophosphoryla- tion of regulatory tyrosine residues in the RON kinase domain followed by activation of the tyrosine kinase and creation of the C-terminal multifunctional docking site[48-50].

Isoform-mediated activation

Generation of constitutively active RON variants is another mechanism activating RON (***Fig. 2***). Currently, at least eight RON variants have been identified (***Fig. 1B***), which include RONΔ170[74], RONΔ165[25], RONΔ165.e11p[77], RONΔ160[27], RONE5/6in[30], RONΔ155[27], RON110[76], RONΔ85[29,78,79], and RONΔ55[1,33,34]. Alternative mRNA splicing is primarily responsible for generation of RON variants[25,27,29,30,32,35,74], although protein truncation and alternative transcription also play a role[34,75,76]. RON variants are either constitutively active, oncogenic, or biologically inactive due to defects in various regions[25-35]. RON variants also display different cellular localizations either on the cell surface or in the intracellular compartments[25,27].

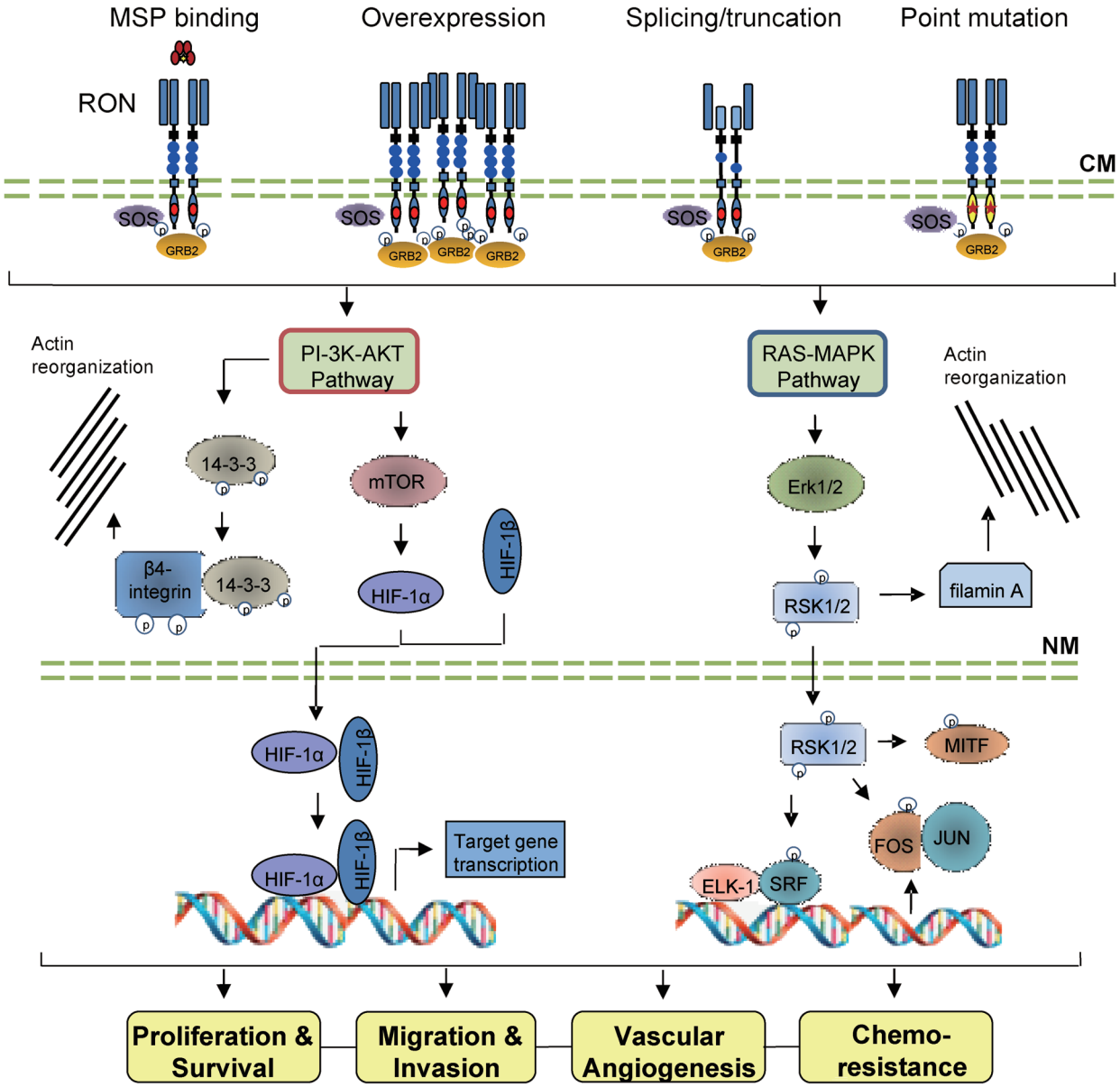

***Fig. 2* RON activation mechanisms and classical signaling pathways.** Activation of RON is mediated by MSP binding, overexpression, splicing/truncation, and point mutations. Upon activation, the C-terminal docking site recruits cytoplasmic molecules son of sevenless (SOS) and growth factor receptor-bound protein (GRB2) to initiate two classical signaling pathways, Ras-MAPK and PI-3K-AKT. The RAS-MAPK pathway regulates RON-mediated cell growth, survival, and invasiveness. Activated Erk1/2 also stimulates p90 ribosomal S6 kinase (RSK)-2 to regulate gene transcription and cytoskeleton reorganization to cause EMT. The PI-3K-AKT pathway regulates RON-mediated cell shape change, migration and matrix invasion. It also stimulates mTOR signaling to promote HIF-1α activation for gene transcription. AKT also stimulates 14-3-3 phosphorylation, which regulates α6β4 integrin for cell motility. CM, cell membrane; ELK-1, ETS domain-containing protein-1; Erk, extracellular signal-regulated kinase; MITF, microphthalmia-associated transcription factor; mTOR, mammalian target of rapamycin; NM nuclear membrane, SRF, specific response factors.

The biochemical events that control RON variant activation are largely unknown. Conformational changes due to deletion of amino acids or a particular domain in the RON protein appear to cause spontaneous tyrosine phosphorylation[25,30,35]. In the case of RONΔ160, a splicing variant with an in-frame deletion of 109 amino acids coded by exons 5 and 6 for the first IPT motif in the RON extracellular domains[1,27], deletion results in unbalanced cysteine residues in the extracellular sequences, which leads to spontaneous dimerization of the RON protein[27,35]. Moreover, deletion converts wild-type RON into an oncogenic

variant that transforms cell in vitro and causes tumor growth in vivo[27]. Thus, generation of constitutively active RON variants is a mechanism of RON activation, which has pathological implications in cell transformation and subsequent tumor progression.

Overexpression-induced activation

Overexpression of RON exists in various types of cancers and has prognostic values for patient survival[17-24,80-86]. Overexpression is characterized by abnormal accumulation of RON and RON variant proteins at high levels and their constitutive phosphorylation in cancer cells (***Fig. 2***). The cause of overexpression is complex and still under investigation. Increased RON protein stability and resistance to endocytosis, intracellular proteolysis, and degradation are possible mechanisms leading to RON overexpression[30]. Impairment in the intracellular proteasome degradation pathway in cancer cells is another mechanism resulting in RON accumulation[87]. Moreover, genetic aberrations in the RON gene can lead to RON overexpression[22]. In gastroesophageal adenocarcinoma, the RON gene is highly amplified (22), suggesting that increased gene copy number could be a mechanism of RON overexpression. Finally, cellular conditions surrounding cancer cells such as hypoxia affects RON expression and accumulation[88]. The RON gene transcription is dramatically increased through hypoxia-inducible factor (HIF)-1α in acute hypoxic cancer cells[88]. Thus, overexpression of RON is manifested at various cellular and molecular levels in cancer cells.

Overexpression-induced RON activation appears to be mediated by homodimer of two RON molecules under the condensed conditions[71]. Analysis of RON-RON interaction under crystal packing confirms that the RON Sema domains form homodimer[71]. In cancer cells, abnormal accumulation of RON in the cell surface or in the cytoplasm creates an environment with high density of RON. Such increased density is sufficient to cause formation of the RON homodimer.

Point mutation-mediated activation

Experimental mutation of certain critical residues such as Asp1232 and Met1254 in the RON kinase domain results in RON activation (***Fig. 2***)[46,47]. This constitutes the fourth types of RON activation. Asp1232 and Met1254 are two critical residues highly conserved in the kinase domain of RTKs[89,90]. The same mutations in KIT and RET cause two human malignancies, mastocytosis and multiple endocrine neoplasia type 2B, respectively[91,92]. In cell lines, Asp1232Val or Met1254Thr substitution in the RON kinase domain is sufficient to convert RON into an oncogenic agent[46,47]. Moreover, substitution overcomes the requirement for the multifunctional docking site in induction of tumor formation[46,47].Substitution of Met1254 with Thr in the RON kinase domain causes a conformational rearrangement, which stabilizes a specific open region in the a-loop in the kinase domain[93]. The rearrangement also facilitates the regulatory residue Tyr1238 moving into a position usually reserved for the substrate tyrosine. The localization in the substrate-like position allows the intramolecular or cis phosphorylation of Tyr1238, which eventually activates RON[93]. This mode of intramolecular/cis autophosphorylation provides an insight into the molecular mechanism of RON activation.

CLASSICAL RON SIGNALING PATHWAYS

RON signaling is conventionally transduced by the RAS-MAPK cascade and the PI-3K-AKT pathway (***Fig. 2***)[16,18,94,51,54,95]. This pattern is similar to that activated by MET[96]. Interaction of RON with adaptor proteins including Grb2 and β-arrestin-1 is the first step bridging RON activation with downstream signaling cascades[95,97,98]. Various cytoplasmic effector molecules such as PLC-γ[48], PI-3 kinase[53], Src[98], 14-3-3[57], c-Cbl[87,99], Hsc70[99], protein phosphatase 1[100], plectin[95], and integrin-β4[57,98] interact with RON through the C-terminal docking site. The differential and selective interactions under different conditions may determine the specificity of RON-mediated signaling in a cell context-dependent manner.

Among tumorigenic activities mediated by RON signaling, the coordinated activation of the RAS-MAPK and PI-3K-AKT pathways is critical for EMT with increased cellular motility[20,34,50,51,59,95]. In the MDCK cell model, RON-mediated EMT, featured by spindle morphologies, diminished expression of E-cadherin, and increased appearance of vimentin, is mediated by the RAS-MAPK pathway[59,101]. Ribosomal protein S6 kinase (RSK)-2, a downstream signaling protein of the MAPK pathway[102,103], is the principal molecule linking RON signaling to EMT (***Fig. 2***)[101]. Genetic studies confirm that RSK-2 functions as a molecular switch to confer promotile/invasive phenotypes in epithelial cells[102,103]. The invasive growth is further regulated by RON-mediated PI-3K-AKT signaling, which increases in vitro epithelial cell adhesion, migration, matrix invasion, and in vivo tumor cell invasion, and distant metastasis[50,51].

CROSSTALK BETWEEN RON AND SIGNALING PROTEINS

At the cell surface, RON is engaged in active cross–

talk with other RTKs such as EGFR, MET, and IGF-1R (***Fig. 3***)[36-43]. RON also crosstalks with viral oncoproteins derived from Friend leukemia virus (FLV)[104-106], Jaagsiekte sheep retrovirus (JSRV)[107,108], and Epstein-Barr virus (EBV) (***Fig. 3***)[109]. Such crosstalk has emerged as a mechanism for regulating tumorigenic phenotype and chemoresistance[36-43,104-112].

The crosstalk between RON and MET is evident by the presences of RON-MET heterodimer on the cell surface[36,37]. RON also directly associates with EGFR, irrespective of ligand stimulation[38,42]. HGF-induced MET activation results in transphosphorylation of RON at Tyr1238 and Tyr1239 residues. Similarly, MSP stimulation causes MET transphosphorylation at Tyr1234 and Tyr1235[36,37]. Such transphosphorylation up-regulates the kinase activity of RON and MET, respectively. Similarly, transphosphorylation also occurs between RON and EGFR or PDGFR[38,39,42,43].

As a signaling regulatory feedback loop, the crosstalk between RON and MET enhances or attenuates MET and RON-mediated tumorigenic activity. In cancer cells, kinase-inactive RON impairs MET-mediated cellular-transforming activity[36,37]. Moreover, RON kinase transphosphorylation is able to sustain MET oncogene addiction with increased tumorigenic activities[37]. The similar effect also is observed be-

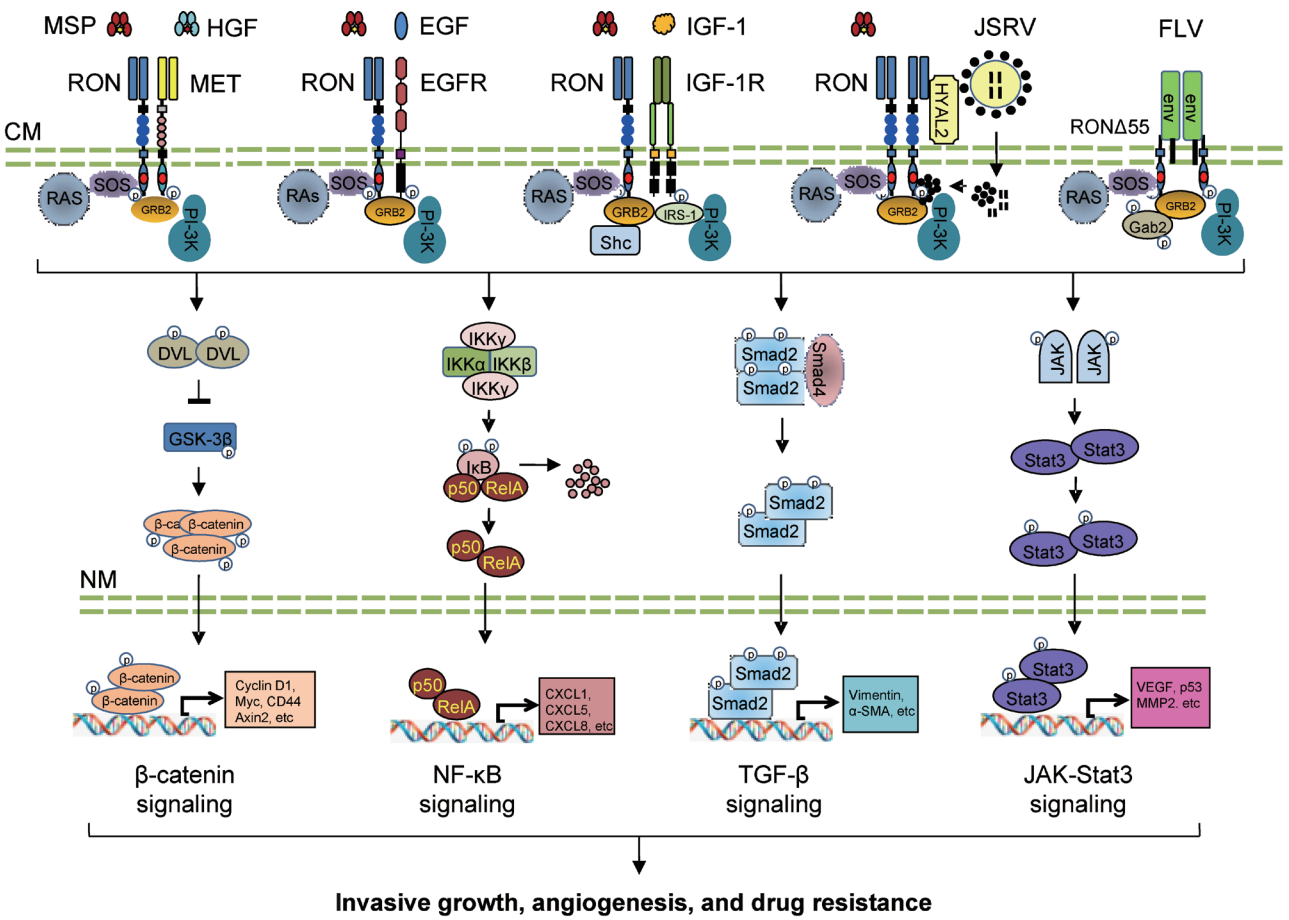

Fig. 3 **Functional crosstalk between RON and signaling protein.** The crosstalk of RON with MET, EGFR, and IGF-1R occurs in various cancer cells and cause increased tumorigenic activity. RON also crosstalks with viral envelope oncoproteins derived from JSRV and FLV to cell transformation and proliferation. At least four signaling pathways are activated upon crosstalking. The β-catenin pathway is stimulated through RON-mediated PI-3K-AKT pathway that activates protein dishevel (DVL) and inactivates glycogen synthase kinase (GSK)-3β leading to cytoplasmic β-catenin accumulation and nuclear translocation. The crosstalk between RON and the NF-κB pathway causes cancer cell growth, angiogenesis, and survival. NF-κB also directly binds the RON promoter, increases RON transcription, and enhances RON-mediated cancer cell migration. In epithelial cells, RON crosstalks with TGF-β signaling to induce EMT for cancer cell invasiveness. Moreover, RONΔ55 binds the FLV envelope protein and interacts with the JAK-Stat3 pathway to induce erythropoietin-independent proliferation of erythroid cells. CM, cell membrane; CXCL, Chemokine (C-X-C motif) ligand; Gab, GRB2-associated-binding protein; IKK, IκB Kinase; IRS-1, insulin receptor substrate-1; JAK, Janus kinase; MMP, matrix metallopeptidase; NM nuclear membrane. SMA, smooth muscle actin; Smad, mothers against decapentaplegic homolog; Stat, signal transducer and activator of transcription; and VEGF, vascular endothelial growth factor.

tween RON and EGFR[38,428,43]. Considering the fact that various RON variants are expressed in various types of cancer cells, the crosstalk between RON/RON variants and other types of RTKs should have a significant regulatory effect on tumorigenic signaling and their associated biological activities.

Crosstalk between RON and signaling proteins also serves as a signaling-compensatory mechanism (***Fig. 3***). In sarcoma cells with acquired resistance to IGF-1R targeted therapeutics antibodies, RON expression/activation has emerged as a survival mechanism[40]. In these sarcoma cells, RON is unusually expressed at high levels. Inhibition of RON expression impairs activation of ribosomal protein S6, a critical IGF-1R signaling component for acquired resistance. Furthermore, knockdown of RON expression by specific siRNA restores sensitivities of drug-resistant cells in response to IGF-1R kinase inhibitor BMS-536924. Thus, the crosstalk between RON and IGF-1R represents an escaping strategy for tumor cells in response to IGF-1R targeted cancer therapy.

RON signaling crosstalk also is manifested for viral oncogenesis (***Fig. 3***)[104-110]. In B cell transformation induced by the latent membrane protein (LMP)-1 of EBV, the crosstalk between LMP-1-induced NF-κB and RON expression promotes the growth of transformed lymphoblastoid cells[108]. In JSRV envelope protein-induced sheep lung adenocarcinoma, which is morphologically similar to human bronchioloalveolar carcinoma[111], RON is found to be directly associated with the JSRV envelope protein[107,108]. The interaction appears to be RON specific because EGFR or CD4 does not form complex with JSRV envelope protein[1073,108]. In addition, association of RON with hyaluronidase (HYAL)-2, a cell surface protein serving as the entry receptor for JSRV[111,112], also is reported[107]. In FLV-infected cells, RONΔ55 covalently interacts with the FLV viral protein to activate downstream signaling pathways[110]. These findings strongly suggest that RON signaling crosstalk is vital for virus-mediated cell transformation and tumorigenic activity.

RON SIGNALING ADDICTION BY CANCER CELLS

Involvement of RON signaling in cancer pathogenesis raises a critical question: are cancer cells fully addicted to RON signaling for growth/survival or is RON only been utilized for tumorigenic activities? The answer to this question is important to establish RON signaling in cancer biology and to provide a rationale for RON-targeted cancer therapy.

The accepted notion from various in vitro studies is that certain cancer cells are addicted to RON signaling for growth and survival[20,51,54,113]. First, knockdown of RON expression by specific siRNA causes phenotypic changes in cancer cells with decreased cell proliferation, significant cell cycle arrest, reduced cell motility, and increased apoptosis[20,51,63,54,113]. One report even finds it impossible to establish a RON-deficient pancreatic cancer BxPC-3 cell line after stable expression of RON specific shRNA[63]. However, in most cases, RON-specific siRNA-mediated activity only exerts the partial inhibitory effect or affects a small fraction of cancer cells[20,51,54,63,113,114]. Studies from in vivo tumor xenograft models also confirms that tumor growth induced by colon HT-29 and pancreatic FG cells with stable RON-specific siRNA was only partially reduced based on measuring tumor volumes[63,114]. Second, small molecule inhibitors such as PHA665752, compound-I, and BMS-777607 targeting RON/MET are able to block RON-mediated activities leading to increased growth inhibition and cell apoptosis[114-119]. Third, specific RON targeting antibodies is able to inhibit or reduce tumor growth caused by cancer cells that overexpress RON[120,121]. Again, only partial growth inhibition or reduction of tumor volume is observed from these animal work[120,121]. Thus, RON signaling appears to be integrated at certain levels into the cellular signaling network for cell growth, survival, and motility.

Cancer cells addicted to RON signaling display interesting patterns of gene expression relevant to advanced tumorigenic phenotypes[20,,51,54,55,62,63,122,113]. Global gene expression patterns indicate that RON signaling mediates a unique transcriptional program with increased expression of genes for growth, survival, and malignancy[63]. Consistent with these observations, stress-induced RON nuclear localization directly binds and regulates various gene transcription known to participate in tress-response network including p53, c-JUK, and PI-3K-AKT[43]. Activation of these stress-related signaling pathways facilitates tumor cell growth and survival under hostile environments[43]. Moreover, cancer cells addicted to RON signaling often show strong crosstalk with other signaling pathways to strengthen their malignant progression[123-125]. One example is RON signaling in crosstalk with the β-catenin pathway in colon and breast cancer cells[54,123-125]. Thus, RON-mediated gene transcription in addicted cancer cells could be a unique molecule marker determining tumorigenic and drug-resistant phenotypes.

PERSPECTIVES

Studies accumulated from the last decade have allowed us to assess the pathogenic roles of RON sig–

naling in epithelial carcinogenesis. Although lacking evidence as a cancer-causative agent, aberrant RON expression/activation is a pathogenic factor associated with tumorigenic behavior and chemoresistance. At present, our studies of RON pathogenesis in cancer have advanced into translational and clinical phases. The knowledge of RON signaling activation, crosstalk, and addiction by cancer cells provides the mechanistic insight for validating RON as a prognostic biomarker and drug target. With continued advanced in this field, the value of aberrant RON expression/activation will be established by successful application of targeted RON therapy for cancer treatment.

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