UNIVERSITÀ DEGLI STUDI DI TORINO

Ron Kinase Transphosphorylation Sustains MET Oncogene Addiction.

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Abstract

Receptors for the scatter factors HGF and MSP that are encoded by the MET and RON oncogenes are key players in invasive growth. Receptor cross-talk between Met and Ron occurs. Amplification of the MET oncogene results in kinase activation, deregulated expression of an invasive growth phenotype, and addiction to MET oncogene signaling (i.e., dependency on sustained Met signaling for survival and proliferation). Here we show that cancer cells addicted to MET also display constitutive activation of the Ron kinase. In human cancer cell lines coexpressing the 2 oncogenes, Ron is specifically transphosphorylated by activated Met. In contrast, Ron phosphorylation is not triggered in cells harboring constitutively active kinase receptors other than Met, including Egfr or Her2. Furthermore, Ron phosphorylation is suppressed by Met-specific kinase inhibitors (PHA-665752 or JNJ-38877605). Last, Ron phosphorylation is quenched by reducing cell surface expression of Met proteins by antibody-induced shedding. In MET-addicted cancer cells, short hairpin RNA–mediated silencing of RON expression resulted in decreased proliferation and clonogenic activity in vitro and tumorigenicity in vivo. Our findings establish that oncogene addiction to MET involves Ron transactivation, pointing to Ron kinase as a target for combinatorial cancer therapy. Cancer Res; 71(5); 1945–55. ©2011 AACR.

1. Introduction

The RON oncogene, also known as “stem-cell derived tyrosine kinase” in mice, belongs to the receptors family of which MET is the prototype. RON gene, located on chromosome 3p21.3, is made of 20 exons encoding a 185-kDa transmembrane heterodimer, formed by a 35-kDa α chain and a 150-kDa β chain. The α chain is completely extracellular and includes a SEMA domain endowed of binding activity for the ligand macrophage stimulating protein (MSP; ref. 1), whereas the β chain, crossing the plasma membrane, encompasses a juxtamembrane, a tyrosine kinase, and a C-terminal domain (2). Ron displays with its sibling receptor Met 25% homology in the extracellular region and 63% within the tyrosine kinase domain (3). Ron signals through mechanisms analogous to Met, the receptor for hepatocyte growth factor (HGF), and generates similar biological responses, as reviewed by Benvenuti and Comoglio (4). Similar to Met, Ron plays a central role in embryo development (5). In addition it is expressed in adult epithelial cells and macrophages, where it orchestrates a complex genetic program known as “invasive growth” that includes control of cellular proliferation, adhesion, motility, and protection from apoptosis (6).

Scattered observations suggest that RON is overexpressed and aberrantly activated in a number of human cancers, including intestinal (7), pancreatic (8), gastric (9), pulmonary (10), mammary (11), ovarian (12), hepatocellular (13), urinary (14), and renal carcinomas (15) where it plays a role in tumor progression, possibly being involved in the metastatic spread (16). Moreover, RON overexpression correlates with an unfavorable clinical outcome in bladder (14) and breast (17) cancers. The Ron oncogenic potential can be unleashed by overexpression both in vitro (11) and in vivo (18) where it induces an increase in cellular proliferation, motility, and invasion and drives tumourigenesis. Ron synergizes with other oncogenes, that is, polyoma virus middle T (19) and RAS (20), enhancing their oncogenic

potential. From an opposite but complementary perspective Ron downregulation by siRNA in a panel of colon cancer cells impairs proliferation and motility and induces apoptosis (21). A human anti-Ron monoclonal antibody inhibits growth of a panel of cell lines xenografted in nude mice (22).

Oncogene addiction—the “Achilles' heel” of cancer—indicates the dependence for survival and proliferation upon an overactive oncogene or its downstream pathway. Consequently, the disruption of that oncogene/pathway leads to growth arrest and programmed cell death, even in the presence of concomitant multiple genetic lesions (23). The oncogene addiction concept represents a major expectation, as it would provide a significant improvement for strategies of targeted therapy (24). This concept applies to certain cancer cells displaying MET amplification, as already shown in gastric carcinomas (25), rabdomyosarcomas (26) and non–small cell lung cancers (NSCLC; ref. 27). In this study, we show that Ron transphosphorylation is critical to sustain the transformed phenotype of MET-addicted cells, offering a second target for therapy of sensitive cancers.

2. Material and methods

Inducible short hairpin RNA vectors

Ron short hairpin RNA (shRNA) sequence was as follows: GATCCCCGCTGGCTCTCATTGGTATCATTTCAAGAGAATGATACCAATGAGAGCCAGCTTTTTGGAAA; CTR sequence was as follows:

GATCCCCGCTGGCTCCCATTGGTATCATTTCAAGAGAATGATACCAATGAGAGCCAGCTTTTTGGAAA. The 2 sequences were cloned into the inducible lentiviral vectors as previously described (28). RON cDNA insensitive to shRNA was produced by insertion of 3 point mutations (G3460C, C3466A, and T3472C) by QuikChange II XL Site-directed Mutagenesis Kit (Stratagene), according to manufacturer's instructions. All mutations were verified by DNA sequencing.

Cell lines, lentiviral infections, and reagents

A549 (lung carcinoma), NCI-H1993 (NSCLC), SW620 (colorectal adenocarcinoma), T47D (breast carcinoma), and Cos-7 (African Green Monkey SV40-transf'd kidney fibroblast) cell lines were purchased from American Type Culture Collection. EBC1 (NSCLC) and MKN45 (stomach cancer, metastasis to the liver) were acquired from Health Science Research Resources Bank; GTL16 is a laboratory batch obtained from limiting dilutions of MKN45 (29). All cells were kept in culture for less than 8 weeks and used between passage 2 and 20. Cells were grown in recommended media (Invitrogen Carlsbad) supplemented with 50 units/mL penicillin (Sigma Aldrich), 50 mg/mL streptomycin (Sigma Aldrich), and 2% or 10% FBS (Sigma Aldrich) as indicated. Cells were maintained at 37°C, in 5% CO2 atmosphere. When indicated, cells were treated with doxycycline (1 μg/mL). Viral particles were produced by transient cotransfection of 293T cells with vectors of interest in combination with 3 μg of envelop plasmid (pMD2-VSV-G), 5 μg of core packaging plasmid (pMDLg/pRRE), and 2.5 μg of pRSV-REV as previously described (30). Cells were transduced with the lentiviral particles for 6 to 8 hours in the presence of Polybrene (Sigma-Aldrich). Particle concentration was estimated by viral p24 antigen by HIV-1 p24 Core profile ELISA (NEN Life Science Products). PHA-665752 was from Tocris Bioscience; JNJ-38877605 was produced by Janssen Pharmaceutica/Ortho Biotech (Supplementary Fig. S1).

Molecular biology

PCR primers designed to amplify RON (Gene Bank ID: NC_000003 referring to NM_002447) coding sequence—exons 1 to 20—are listed in Supplementary Table S1. Genomic DNA extractions and PCR amplification reactions were carried out as previously described (31). Cycle sequencing was carried out using BigDie Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were purified using CleanSeq (Agencourt Bioscience, Beckman Coulter) and analyzed on a 3730 DNA Analyzer, ABI capillary electrophoresis system (Applied Biosystems). Real-time

quantitative PCR (qPCR) was done as previously described (31). TaqMan probes for genomic DNA and mRNA were from Applied Biosystems.

Biochemical analysis

Cells lysates were run as total extracts or immunoprecipitated using anti-Ron (C-20 Santa Cruz Biotechnology); anti Met (DL-21 produced in our laboratory (32); anti-Egfr (Upstate Biotechnology), and anti-Her2 -trastuzumab (Roche) antibodies. Equal amounts of proteins were loaded on to SDS-PAGE gels and transferred to nitrocellulose membrane supports (Hybond C+; Amersham). The membranes were decorated with the following antibodies: anti-Met DL-21, and anti-actin (Santa Cruz Biotechnology), anti-phospho Tyr (P-Tyr) (Upstate Biotechnology), and anti-phospho Met Y1234/Y1235 (Cell Signaling Technology). Antimouse, antirabbit, antigoat, and protA-peroxidase conjugated secondary antibodies were from Amersham. Signal detection was done using ECL system (GE Healthcare).

Biological assays

Cell proliferation rates were checked every other day for 7 days using Cell Titre Glo Luminescence (Promega Corporation). All the experiments were done at least 2 times in triplicates.

Anchorage-independent growth was carried out plating 3 × 103 cells in 0.5% low melting agarose. The overlay medium was substituted every 3 days. After 2 weeks, cell viability was assessed by Alamar blue staining (Resazurin sodium salt, Sigma-Aldrich), and then colonies were visualized using 0.02% iodonitrotetrazolium chloride (0.02% in PBS, Sigma-Aldrich).

Xenograft transplantation experiments

A total of 5 × 105 lentiviral-transduced GTL16 or 1×106 lentiviral-transduced EBC1 cells were injected s.c. in the posterior flanks of 6-week-old immunodeficient nu-/- female mice on a CD-1 background (Charles River Laboratories). Tumor volume was calculated as previously described (33). At the end of the experiment immunohistochemical staining was conducted on paraffin-embedded sections of the tumors using standard techniques (33). Experiments were carried out on groups of 12 animals per point. All animal procedures were approved by the Ethical Commission of the University of Torino (Italy) and by the Italian Ministry of Health.

3. Results

Ron is transphosphorylated in Met-addicted cells

We investigated Ron expression levels and tyrosine phosphorylation in 4 MET-addicted human cancer cells of different histotypes: GTL16 and MKN45 (gastric adenocarcinomas), NCI-H1993 and EBC1 (NSCLCs). All 4 cell lines harbor increased MET copies in their genome, being MET-amplified >6 times (Fig. 2), and produce an excess of Met protein at the cell membrane resulting in constitutive receptor activation (34). In these cells, MET oncogenic addiction was confirmed by treatment with the specific inhibitors PHA-665752 (25, 35) and JNJ-38877605 (Supplementary Fig. S1), resulting in growth arrest in G0 (36). Ron expression was investigated by qPCR (data not shown) and confirmed by Western blotting; Ron was found to be highly expressed in all cells (Fig. 1A). We then checked Ron activation status in basal conditions (e.g., in absence of exogenous ligand -MSP- stimulation) and showed that the receptor was strongly phosphorylated in all examined cells (Fig. 1A). No phosphorylation was observed, on the contrary, in a panel of cells expressing various levels of Ron, but not addicted to MET (Fig. 1B).

Figure 1.

Ron expression and basal activation. A, Western blot analysis of Ron protein in 4 MET-addicted (EBC1, GTL16, MKN45 and NCI-H1993) and (B) in 4 not addicted (DU145, T47D, SW620 and A549) cell lines; 800 μg of lysates were immunoprecipitated with anti-Ron antibody and probed with anti–P-Tyr and Ron antibodies. Ron is expressed in all cells but is constitutively active only in the MET-addicted cells.

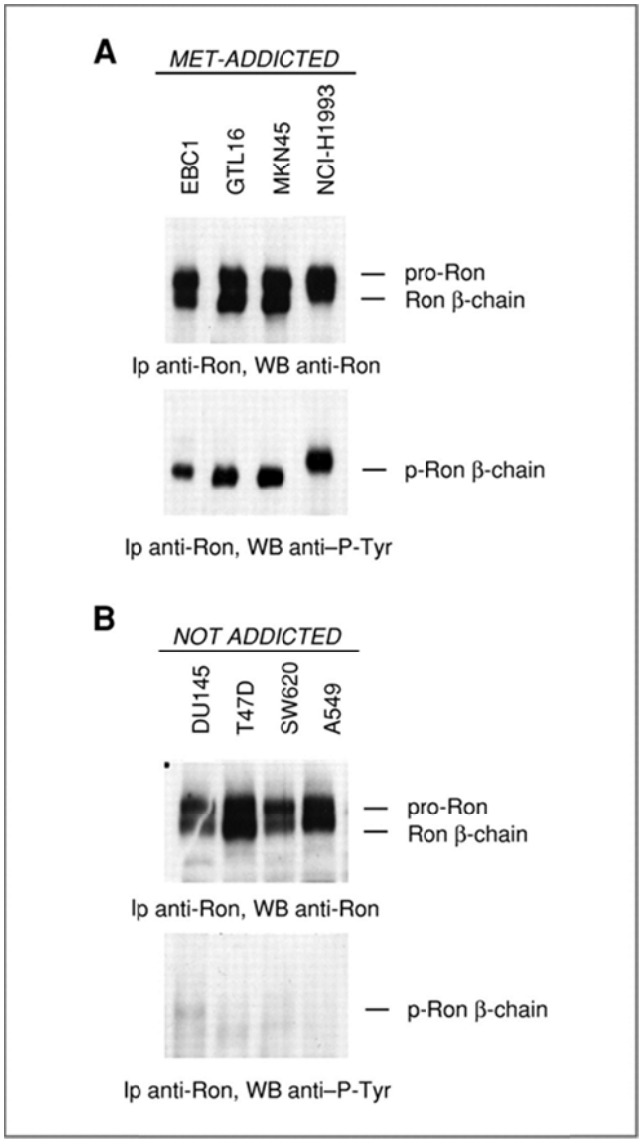
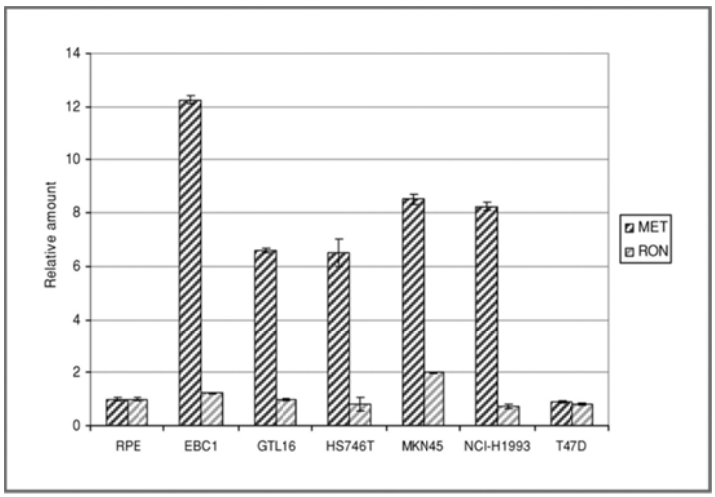


Figure 2.

Gene copy number analysis. MET (black dashed bars) and RON (gray dashed bars) amplification status was investigated by qPCR. Results were normalized on RNaseP and calculated with the ΔΔct method (31). MET is amplified in EBC1 (12.2 times), GTL16 (6.6 times), NCI-H1993 (8.2 times), and MKN45 (8.5 times). RPE, a diploid cell line, was used for normalization, and T47D as control. RON was not amplified in any of the analyzed cell lines.



It has been described that constitutive activation of kinase receptors can be achieved in 3 different ways: (i) with establishment of ligand/receptor autocrine loops; (ii) via receptor amplification and consequent overexpression; and (iii) in the presence of activating point mutations. We therefore explored the mechanism leading to Ron constitutive activation in the panel of MET-addicted cells. First, we ruled out the presence of an autocrine circuit ligand/receptor (MSP/Ron) conducting qPCR on mRNAs obtained from each cell line (data not shown). We next examined if Ron constitutive activation was the result of gene amplification carrying out a gene copy number analysis. To normalize variations in copy number we used genomic DNA extracted from nonmalignant retinal pigmental epithelial cells (RPE) that are notably diploid. We proved that every cell line contains 2 copies of the RON gene; as a control, we confirmed that MET was amplified (>6 times) in all analyzed cells (Fig. 2). It should be noted that there is not a linear correlation between MET gene amplification and protein levels; this is likely due to transcriptional and posttranscriptional events such as mRNA stability or protein degradation. We finally explored the possibility that Ron basal phosphorylation resulted from genetic lesions affecting the RON gene itself. To this end we conducted mutational analysis on genomic DNA extracted from EBC1, GTL16, NCI-H1993, and MKN45 cells as previously described (31). PCR primers were designed to amplify the 20 exons encompassing RON's entire coding sequence (listed in Supplementary Table S1). A total of 104 PCR products were generated and subjected to direct sequencing. No activating mutations were found. Only the NCI-H1993 line displayed 2 nucleotide changes corresponding to the amino acid substitutions: Q523R in exon 4 and R1335G in exon 20; the first is a single nucleotide polymorphism reported on public databases, whereas the latter does not affect amino acids involved in the kinase activity. We therefore concluded that Ron constitutive activation in MET-addicted cells was not due to the presence of either gene amplification or somatic mutations.

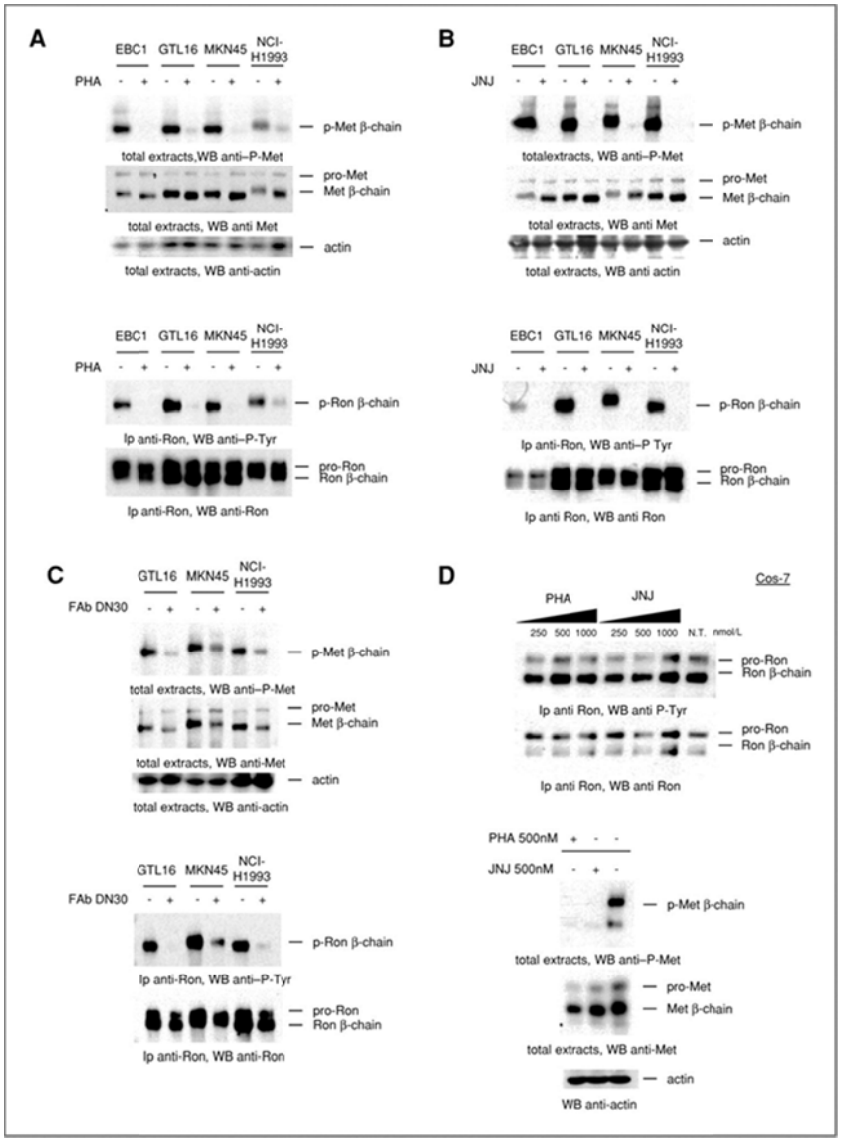
It has been previously shown in our laboratory that—while specifically activated by their ligands—the 2 tyrosine kinase receptors Ron and Met cross-talk. Ligand-induced activation of Met results in transphosphorylation of Ron and vice versa (37). The experiments were conducted using the “kinase dead” mutants of the 2 receptors (RonKD and MetKD), which are devoid of kinase activity. Overexpression of wild-type Ron (RonWT) or Met (MetWT) in Cos-7 cells resulted in their ligand-independent phosphorylation, whereas the corresponding “kinase dead” receptors were inactive. However, RonKD and MetKD displayed tyrosine phosphorylation when coexpressed with RonWT and MetWT, respectively. This indicates that through the formation of Ron/Met heterodimers reciprocal phosphorylation on tyrosines occurs.

We therefore verified if Ron basal activation resulted from specific transphosphorylation by the active cognate receptor Met in a scenario of endogenous MET oncogene addiction. We treated for 2 hours EBC1, GTL16, NCI-H1993, and MKN45 with 2 Met-selective inhibitors: PHA-665752 and JNJ-38877605 at the standard dose of 500 nmol/L (36). As expected we showed that both inhibitors caused a significant reduction of Met phosphorylation but notably of Ron as well (Fig. 3A, 3B). Unlike JNJ-38877605, PHA-665752 treatment did not abolish completely Met phosphorylation in NCI-H1993 cells; accordingly neither Ron was completely inhibited. To rule out the unlike possibility that the 2 compounds could inhibit Ron directly, we overexpressed Ron in Cos-7 cells, under conditions resulting in its constitutive phosphorylation. Treatment with increasing concentrations of PHA-665752 or JNJ-38877605 (250, 500, and 1,000 nmol/L) for 2 hours was ineffective on Ron phosphorylation status, confirming Met specificity (Fig. 3D). Afterward, we strengthened the data obtained by means of Met specific inhibitors showing that Ron phosphorylation was quenched also reducing the number of Met receptors from the cellular surface using specific antibodies. It has been shown that the monoclonal antibody DN30, an IgG2A directed against the extracellular moiety of the human Met, acts as inhibitor of Met signaling and biological responses through a mechanism of receptor “shedding” (38). The latter results from proteolytic generation of a soluble extracellular fragment that removes the receptors from the cell surface and forms inactive heterodimers with the residual intact molecules (38). MET-addicted cells (GTL16, NCI-H1993, and MKN45) were plated in low serum (2%) and treated with the FAb of DN30 (28 μg/mL) or PBS for 72 hours. As reported in detail elsewhere (38), we showed that upon antibody treatment the amount of Met protein was reduced and its phosphorylation strongly quenched (Fig. 3D). In agreement with the working hypothesis Met antibody treatment resulted in diminished Ron

phosphorylation, without substantial reduction of the total number of Ron receptors. Incidentally, EBC1 cells were shown to be extremely sensitive to Fab of DN30 not enduring 72 hours treatment; this is not surprising as they harbor the highest MET amplification—12.20 times—and are the most sensitive to MET addiction (Fig. 2).

Figure 3.

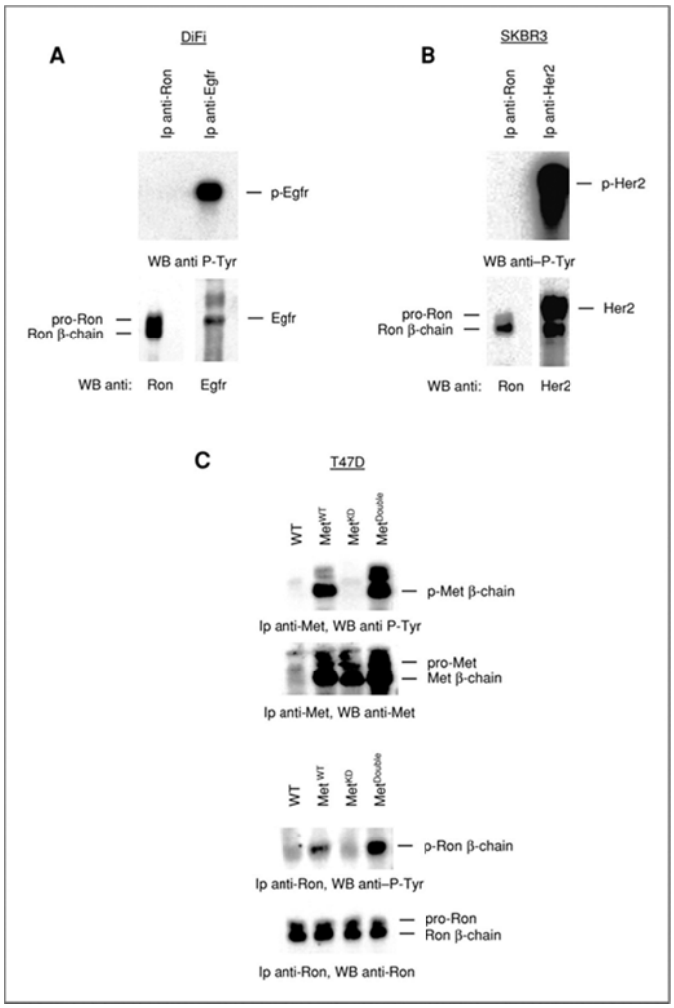
Ron phosphorylation is specifically driven by Met. EBC1, GTL16, MKN45, and NCI-H1993 were cultured in complete medium supplemented with 10% serum and treated for 2 hours with Met-selective inhibitors. A, PHA-665752 (PHA) and B, JNJ-3887760 (JNJ) at the standard dose: 500 nmol/L. C, cells cultured in 2% serum were treated for 48 hours with Fab of DN30 (28 μg/mL). Cell lysates were either run as total extracts (30 μg) and Met phosphorylation status was checked probing the membranes with a specific anti–phospho-Met (P-Met) antibody or immunoprecipitated with Ron antibody (800 μg), and receptor phosphorylation status was checked probing the membranes with anti–P-Tyr antibody. Membranes were then reprobed with anti-Met and anti-Ron antibodies, respectively. Actin was used as loading control when total lysates were run. Treatment with PHA-665752, JNJ-3887760, and Fab DN30 caused a significant reduction of Met phosphorylation but notably of Ron as well. D, PHA-665752 and JNJ-3887760 specificity were assessed in Cos-7 cells overexpressing Ron. Cells were treated with increasing concentrations of the 2 inhibitors (250, 500, and 1,000 nmol/L) for 2 hours and lysates processed as described above. Ron phosphorylation was not affected at any of the doses used, confirming specificity. Inhibitors' activity was verified in Cos-7 cells overexpressing Met (bottom). Anti-Ron and anti-Met antibodies' specificity has been checked (Supplementary Fig. S4).



To analyze whether Ron constitutive phosphorylation is restricted to MET-addicted cells or is a common feature of cells addicted to any tyrosine kinase, we extended the analysis to cells harboring constitutive activation of Egfr or Her2. Two models were investigated: DiFi, a human colorectal carcinoma–derived cell line that displays constitutive active Egfr, being the receptor amplified in the range of 60 to 80 copies/cell (39), and SKBR3 cells, a human breast tumor line in which HER2 is amplified >10 copies/cell (40). We unambiguously showed that, in both lines, although expressed at high levels, Ron was not basally phosphorylated (Fig. 4A, 4B).

Figure 4.

Ron phophorylation is Met-specific and does not depend on downstream transducers. To analyze whether Ron constitutive phosphorylation is restricted to MET-addicted cells or is a common feature of cells addicted to any tyrosine kinase, the following were investigated: A, DiFi, a human colon carcinoma–derived cell line that displays amplified EGFR; and B, SKBR3 cells, a human breast tumor line in which HER2 is amplified >10 copies/cell. Cell lysates were immunoprecipitated with Ron, Egfr and Her2 antibodies (800 μg). Receptor phosphorylation status was checked probing the membranes with anti–P-Tyr antibody. Ron, although highly expressed in both cells, was not basally phosphorylated. C, 3 different Met constructs were overexpressed in T47D cells: Met wild type (METWT), Met kinase dead (MetKD, devoid of kinase activity), and the receptor mutated in its docking site (MetDouble, and therefore unable to recruit signal transducers, but still retaining its kinase activity). Met and Ron were immunoprecipitated (800 μg) with specific antibodies and phosphorylation status checked using anti–P-Tyr antibodies. Membranes were then reprobed with anti-Met and anti-Ron antibodies, respectively. MetDouble expression resulted in Ron phosphorylation proving incontrovertibly that Met is able to directly transphosphorylate Ron.



To further prove that Met-driven Ron transphosphorylation is a direct event not dependent on downstream molecules we overexpressed 3 different Met constructs in T47D cells that notably express good levels of unphosphorylated Ron, and do not express the cognate receptor Met. The 3 constructs were as follows: Met wild type (MetWT), Met kinase dead (MetKD), and the receptor mutated in its docking site (MetDouble) and therefore unable to recruit signal transducers, but still retaining its kinase activity. As expected MetWT-induced Ron transphosphorylation, MetKD did not and, notably, MetDouble did proving incontrovertibly that Met directly transphosphorylates Ron without the need of downstream transducers (Fig. 4C).

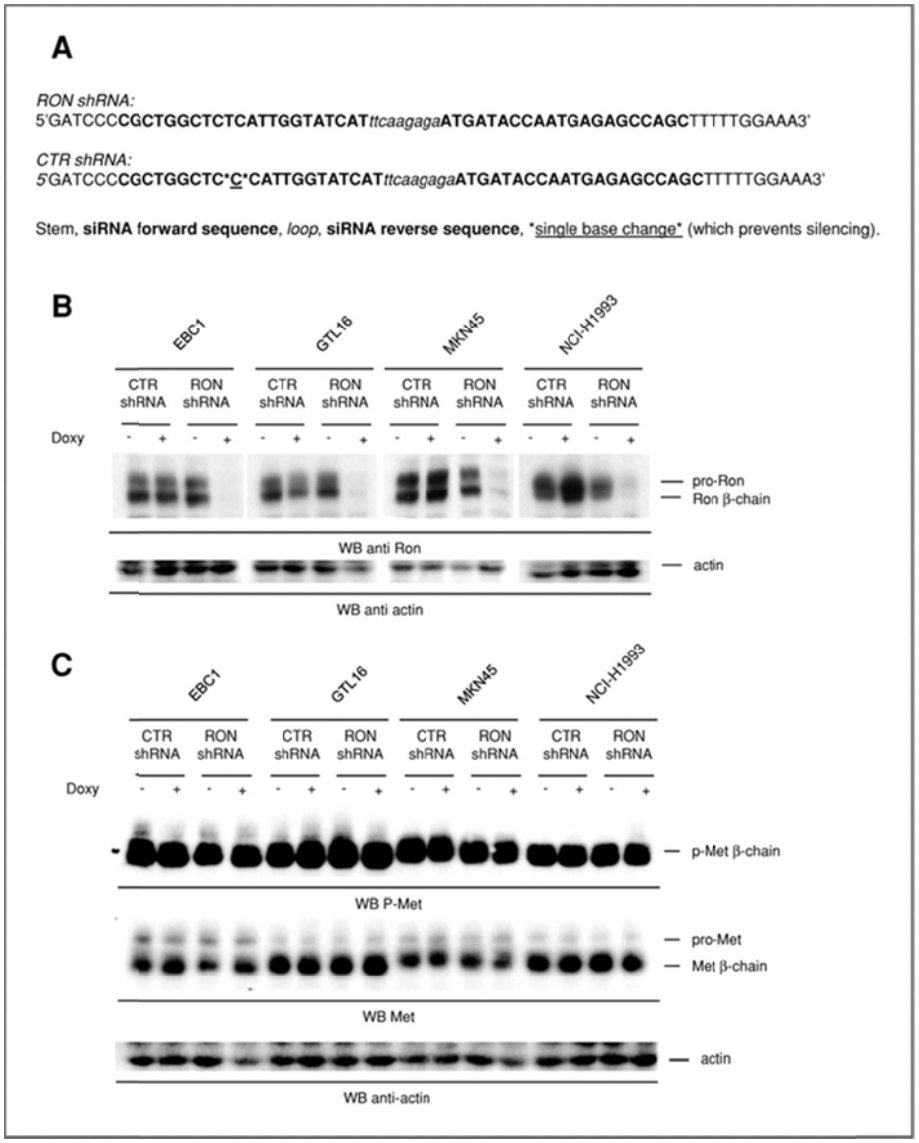
It should be noted that within the contest of MET-oncogene addiction in the absence of active Met (achieved by pharmacological inhibition) the loss of Ron phosphorylation is not reverted by its ligand MSP (Supplementary Fig. S2). This finding is not surprising since homodimerization of Ron receptors in Met-addicted cells is impaired by heterodimerization with the (chemically inhibited) Met, which is overexpressed at an exceedingly high stoichiometry.

Ron silencing abrogates the transformed phenotype of Met-addicted cells

To asses the functional contribution of the constitutive phosphorylation of Ron in the contest of MET-addiction, we established stable cell lines in which Ron expression could be knocked down by inducible shRNAs. Briefly, as previously described (28), shRNAs were cloned into inducible lentiviral vectors in which the transgene expression was under doxycycline control; vectors were then used to generate cell lines in which doxycycline treatment (1 μg/mL) modulates Ron silencing. To discriminate for unwanted “off-target” effects, we engineered control shRNA (CTR shRNA) displaying a single-base substitution (10 T>C) abrogating Ron silencing (Fig. 5A). We transduced the panel of MET-addicted cells and produced pools to circumvent clonal variation. After transduction we checked effective Ron silencing both by qPCR (data not shown) and Western blotting (Fig. 5B). We showed that the receptor's expression was almost completely abolished by RON-specific shRNA, the inhibition being 72 hours after silencing induction, as high as 74% in MKN45, 77% in NCI-H1993, 81% in GTL16 and 86% in EBC1. As expected Ron protein levels were not altered in cells transduced with the CTR shRNA upon antibiotic administration. We checked total Met and phospho-Met levels in the 4 cell lines upon Ron knockdown and showed that in none of them neither Met total amount nor its phosphorylation status changed upon Ron silencing (Fig. 5C).

Figure 5.

Inducible shRNA system. A, sequences of RON and CTR shRNAs. Asterisks designate the base change that abrogates silencing. B, Western blot analysis of total cell lysates of EBC1, GTL16, MKN45 and NCI-H1993 cells transduced either with RON or with CTR shRNA viruses. As shown, 72 hours after doxycycline treatment (1 μg/mL) induced a dramatic Ron silencing as high as 74% in MKN45, 77% in NCI-H1993, 81% in GTL16, and 86% in EBC1 (densitometry values were normalized over actin and compared with doxycycline untreated cells). As expected Ron protein levels were not altered in cells transduced with the CTR shRNA upon drug administration. Actin was used as loading control. C, total Met and phospho-Met levels were checked upon Ron knockdown. In none of the 4 cell lines neither Met total amount nor its phosphorylation status were altered upon Ron silencing.

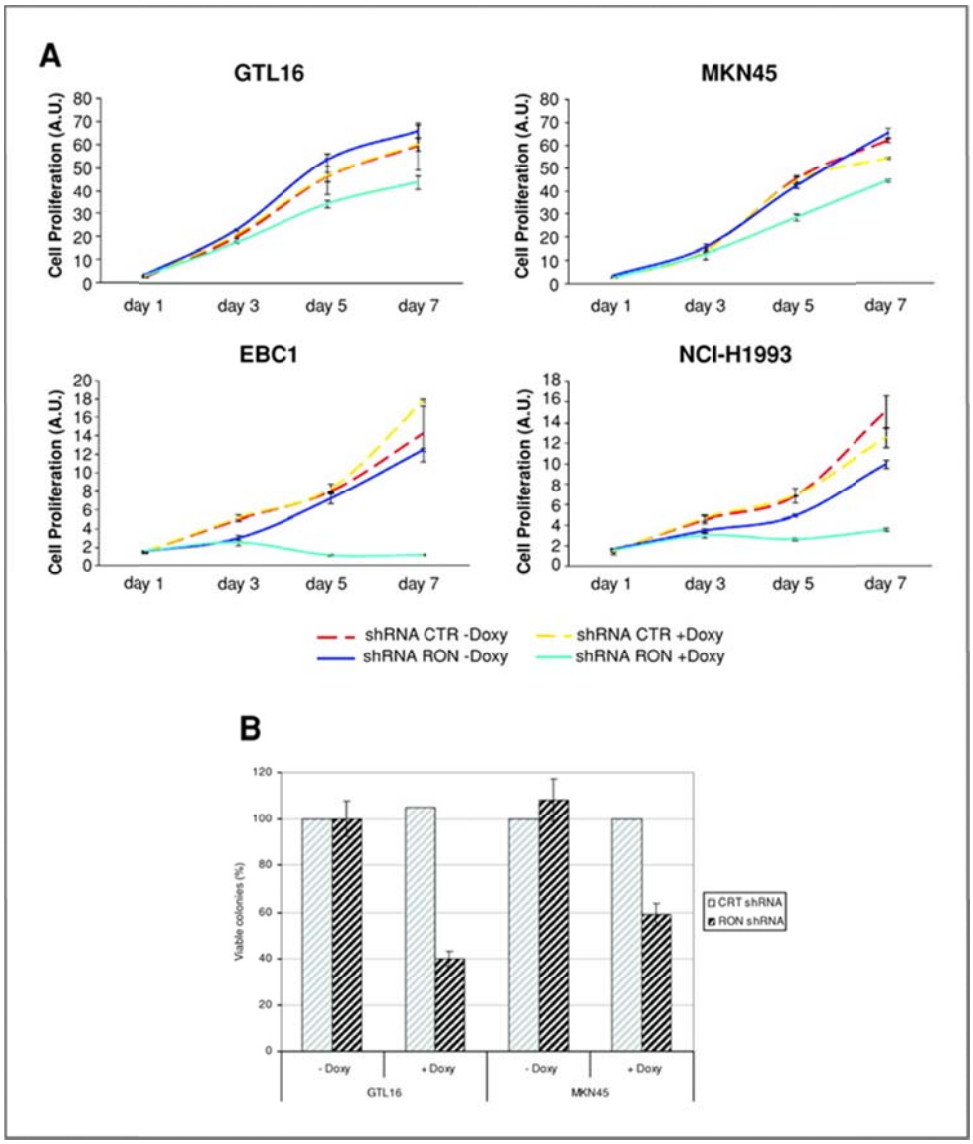


Ron silencing effects were then tested in 2 in vitro assays. Proliferation was deeply affected upon RON somatic knockdown: GTL16 and MKN45 showed a 30% reduction in proliferation 7 days after silencing induction. NCI-H1993 and EBC1 displayed an even stronger response reaching 63% and 90% inhibition, respectively. As expected, no effects were registered in cells transduced with the CTR shRNA either in presence or absence of doxycycline (Fig. 6A). The stronger effect detected upon RON silencing in the 2 NSCLC cells (EBC1 and NCI-H1993), compared with the 2 gastric cells (GTL16 and MKN45), suggests that Ron is essential to support the oncogenic phenotype of cancer cells displaying MET amplification at different extents according to the tissue histotype. We expanded the analysis and conducted an anchorage-independent growth assay to study the ability of MET-addicted cells to grow in soft agar upon Ron deprivation. As EBC1 and NCI-H1993 do not grow in soft agar, only GTL16 and MKN45 (engineered as described above) were tested. Anchorage-independent growth was strongly impaired: 60% and 40%, respectively. No reduction was registered in cells transduced with CTR shRNA, in presence or absence of the antibiotic (Fig. 6B). As control we transduced T47D that express high amount of Ron without it being constitutively phosphorylated. As expected silencing did not result in inhibition of cellular proliferation and did not impair the clonogenic activity (data not shown).

Figure 6.

In vitro effects of Ron silencing. A, growth curves of EBC1, GTL16, MKN45, and NCI-H1993 transduced either with RON or with CTR shRNAs and treated or not with doxycycline (1 μg/mL). In all analyzed cells proliferation was deeply affected upon Ron somatic knockdown: GTL16 and MKN45 showed a 30% reduction in proliferation 7 days after silencing

induction; NCI-H1993 and EBC1 showed 63% and 90% inhibition, respectively. As expected, no effects were registered in cells transduced with the CTR shRNA either in presence or absence of doxycycline. B, anchorage-independent growth in soft agar assay. GTL16 and MKN45 cells were grown for 2 weeks. Upon Ron silencing anchorage-independent growth was strongly impaired: 60% and 40%, respectively (black dashed bars). No reduction was registered in cells transduced with CTR shRNA (gray dashed bars) in presence or absence of the antibiotic. Error bars report standard deviations of 2 experiments done in quadruplicate.



To further control for unwanted off-target effects while silencing Ron expression a “rescue” experiment was done by expressing a RON cDNA (triple Ron) harboring 3 silent mutations which render the receptor refractory to specific shRNA silencing (Supplementary Fig. S3A). Mutations, introduced by site directed mutagenesis, were checked by direct sequencing; in addition the entire Ron coding sequence was sequenced to rule out the presence of additional unwanted changes. Indeed re-establishment of Ron expression in EBC1 cells expressing RON shRNA in presence of doxycycline was followed by restoration of cellular proliferation proving unequivocally that the in vitro phenotypes described above are specifically due to Ron silencing (Supplementary Fig. S3B), analogous results were obtained in GTL16 (data not shown).

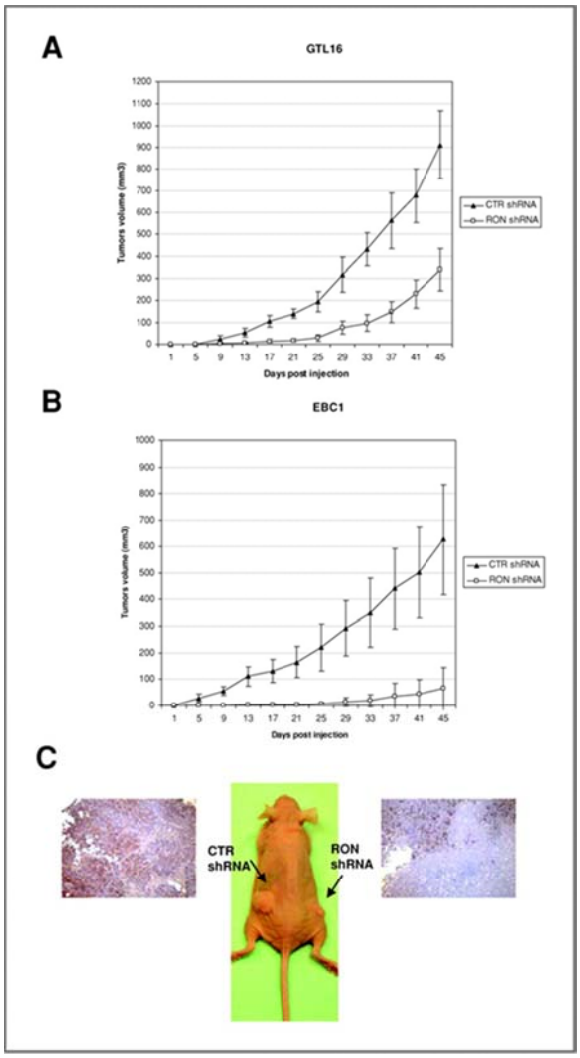
Ron silencing impairs growth of Met-addicted tumors

To evaluate whether Ron expression is required to sustain tumorigeniesis of MET-addicted cells, we tested the effect of RON knockdown in nude mouse xenografts. GTL16 and EBC1 stably expressing the 2 inducible shRNAs described

above (RON shRNA and CTR shRNA) were implanted s.c. in nude mice; Ron downregulation was induced the day after injection and maintained for the whole experiment by administration of doxycycline in drinking water. In each mouse RON shRNA cells were implanted on one flank and CTR shRNA cells on the other, so that each mouse was carrying its own control. Tumor growth was monitored twice a week and tumor weight was determined after sacrifice. To verify the actual amount of Ron expression, we performed immunohistochemical staining of serial sections of paraffin-embedded tumors explanted at the end of the experiments (Fig. 7C). As expected shRNA tumors displayed an overall strong decrease in Ron expression when compared with controls. It should be noted, however, that the RON-silenced tumors contained small areas of cells displaying residual Ron expression: This is probably due to the outgrowth of a subpopulation of untransduced cells counterselected in vivo for their advantage in survival. In spite of this, Ron downregulation in the bulk of transplanted cells resulted in a severe inhibition of tumor growth that reached 66% in GTL16 and 95% in EBC1, proved by reduction of tumor burden (Fig. 7A, 7B) and tumor weight (data not shown).

Figure 7.

Ron silencing results in growth inhibition of tumor xenografts in nude mice. GTL16 and EBC1 cells transduced either with inducible RON (squares) or CTR (triangles) shRNAs were implanted subcutaneously in nude mice. Ron downregulation was induced the day after injection and maintained by administration of doxycycline in drinking water. In each mouse RON shRNA cells were implanted on 1 flank and CTR shRNA cells on the other. A, tumor volume was measured at the indicated days in GTL16 B, and EBC1. As shown Ron downregulation in the bulk of transplanted cells resulted in a severe inhibition of tumor growth that reached 66% in GTL16 and 95% in EBC1. C, Ron successful silencing was verified by immunohistochemical staining of serial sections of paraffin embedded tumors explanted at the end of the experiments. Error bars report standard errors.



4. Discussion

It has been established that, due to their documented genomic instability, cancer cells accumulate an exceedingly high number of mutations, some affecting proteins involved in growth control. In this discouraging scenario, the pharmacologic inhibition of the whole spectrum of mutated proteins is the therapeutic counterpart of the myth of Sisyphus. Growing evidence points out that cancers arising as a result of a given oncogenic lesion remain dependent on the continued expression of that oncogene (reviewed in ref. 41). The phenomenon, known as “oncogene addiction,” has been documented in cells harboring genetic alterations of EGFR (42), HER2 (43), PDGFR (44, 45), ALK (46), BRAF (47) and, last but not least, MET (25), an oncogene implicated in a number of malignant diseases (reviewed in ref. 6). The mechanistic explanation of MET oncogene addiction is still elusive (30). In this study, we show that the RON oncogene, encoding the MET's sibling tyrosine kinase receptor, is involved. Analyzing 4 cancer cell lines in which MET is constitutively active as a consequence of gene amplification, we showed that Ron is transphosphorylated by Met. Extending the previous data reporting Ron and Met cross-talk, we observed in an artificial cellular system in which both receptors were exogenously overexpressed (37), we provided direct evidence that Ron transphosphorylation also occurs in cells naturally coexpressing the 2 oncogenes. In addition, we have shown that Ron transphosphorylation is specifically driven by Met, as it is suppressed both by means of Met-specific chemical inhibitors and by reducing the number of Met proteins from the surface by antibody-induced shedding. Moreover, Ron is not phosphorylated in cells harboring constitutively activated kinase receptors other than Met, such as Egfr or Her2. The consequence of the MET-driven transphosphorylation likely results in enhanced signal transduction activity. In fact, Ron is a weak kinase. We previously showed that, swapping kinase domains between Met and Ron, by genetically engineering the TPR chimeras, Ron catalytic efficiency is 5 times lower than that of Met. Moreover, Ron activation triggers the downstream MAP kinase signaling approximately 3 times less than Met (48). We thus suggest that Ron, although a weak kinase per se, behaves as an amplifying platform for Met signaling. In a contest of MET oncogene addiction, this signal amplification may be critical to reach the equilibrium that sustains tumor growth and survival. An analogous phenomenon has already been described for Her3. It was shown that within Her2/Her3 heterodimers Her2 transphosphorylates Her3 but not vice versa, being Her3 devoid of kinase activity (49). The interesting observation is that, although kinase death, within this frame, Her3 is necessary to sustain Her2-mediated transformation in Her2-positive breast cancer cells (50).

By means of somatic knockdown, via inducible lentiviral shRNAs, we have shown that Ron deprivation in 4 different MET-addicted cells has functional relevance and results in an impaired–transformed phenotype: decreased proliferation rates, clonogenic potential in vitro, and tumorigenicity in vivo. Relevant clinical implications can be drawn from the knowledge that Met and Ron form a functional oncogenic unit in addicted cells. First of all, Met inhibitors, especially those already in clinical trials, could be used in combination with Ron inhibitors to potentiate the therapeutic response. Alternatively, dual-specific inhibitors, acting on both members of the Met family, may turn out to be more effective than specific ones. In either case, the finding that oncogenic addiction to MET involves Ron transactivation brings to light the potential advantage of combinatorial therapies designed at blocking simultaneously Met and Ron.

In summary, we demonstrate that Ron is essential to support the oncogenic phenotype of cancer cells displaying amplification of Met kinase and suggest that Met-addicted tumors exhibit a “non-oncogene” addiction to Ron. Our results show on one hand the complexity of the signaling networks regulated by addictive oncogenes and, on the other reveal their “Achilles' heel.” Interfering with one single component of the network (here we suggest the weak oncogene Ron) seems to be sufficient to attenuate the transformed phenotype sustained by the altered oncogene (Met).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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