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Discovery and Function of the HGF/MET and the MSP/RON Kinase Signaling Pathways in Cancer

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1.1 Introduction

MET and RON oncogenes encoding two related tyrosine kinase receptors are among the most important genes involved in the control of the invasive growth genetic program. Under physiological conditions, such as embryonic development and organ regeneration, the invasive growth program controls the normal tissue development by coordinating, in time and space, several biological events including cellular proliferation, disruption of intercellular junctions, migration through the extracellular matrix (ECM), and protection from programmed cell death (*apoptosis*). In transformed tissues, MET or RON deregulation results in cancer formation and metastatic dissemination. Upon either ligand stimulation or constitutive receptor activation, cancer cells are induced to leave the primary tumor, degrade the basal membrane, move towards different organs and generate metastasis (1,2). The two sibling receptors exert a dual role: they are necessary oncogenes for those tumors that rely on MET activity for growth and survival (*oncogene addiction*) and adjuvant, pro-metastatic genes for other tumors, where MET activation is a secondary event that exacerbates the malignant properties of already transformed cells (*oncogene expedience*). In this complex scenario, MET and RON become very attractive candidates for targeted therapeutic intervention.

1.2 MET Tyrosine Kinase Receptor and its Ligand HGF: Structure

MET oncogene, positioned on chromosome 7q21-31, is composed of 21 exons encoding a transmembrane tyrosine kinase receptor made of a disulphide-linked heterodimer (190 kDa), which originates from the proteolytic cleavage, in the post-Golgi compartment, of a single chain precursor. The heterodimer is formed by a single-pass transmembrane β chain (145 kDa) and a completely extracellular α chain (45 kDa). The extracellular portion contains a SEMA (semaphorin) domain, an atypical motif made by over 500 amino acids, which has a low affinity binding activity for the ligand and is involved in receptor

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dimerization; a plexin, SEMA and integrin cysteine-rich (PSI) domain, which encompasses about 50 residues and contains 4 disulphide bonds; and 4 immunoglobulin-plexin-transcription structures (IPT domain), a characteristic protein-protein interaction region. A single pass hydrophobic membrane-spanning domain is followed by the intracellular portion made of a juxtamembrane section followed by a catalytic site and a C-terminal regulatory tail (Figure 1.1). The juxtamembrane segment is essential for receptor down-regulation (2). It contains a serine residue (Ser985) that, upon phosphorylation, is responsible for inhibition of receptor kinase activity, and a tyrosine (Tyr1003) capable of binding the E3-ubiquiting ligase CBL (cellular homologue of Cas NS-1 oncogene), that promotes receptor degradation (3,4). The catalytic site contains two tyrosines (Tyr1234 and Tyr1235) that regulate the enzymatic activity. Finally, the C-terminal tail encompasses two tyrosines (Tyr1349 and Tyr1356) that, when phosphorylated, generate a docking site able to recruit a vast cohort of intracellular molecules and adaptor proteins responsible for transducing the signaling triggered by the ligand-receptor interaction (5). The latter two tyrosines have shown to be essential and sufficient to execute MET physiological functions (5), and to elicit MET oncogenic potential (6).

MET high affinity ligand is known as the scatter factor (SF) or hepatocyte growth factor (HGF). SF is a factor capable of inducing scatter of epithelial cells, a complex phenomenon that consists of a first step in which cells dissociate one from another and a second phase in which the released cells begin to move (7,8). While HGF is a potent growth stimulator for primary hepatocytes kept in culture (9), the two molecules were later shown to be identical (10). SF/HGF belongs to the plasminogen family of peptidases; it contains an amino terminal hairpin loop (HL), followed by four Kringle domains, flanked by an activation portion and a serine-protease domain (SPH) devoid of proteolytic activity (Figure 1.1). This ligand, synthesized and secreted as a single chain inactive precursor (pro-HGF) by stromal cells (i.e. fibroblasts), is present in the extracellular environment of almost all tissues. Its activation occurs locally upon proteolytic cleavage by proteases that cleave the bond between Arg494 and Val495.

To date, several proteases (present either in the serum or within cells) have been proposed as HGF/SF activators, including HGF activator (HGFA) (11), plasma kallikrein and coagulation factors XIIa and XIa (12), matriptase and hepsin (13,14), TMPRSS2 (15), TMPRSS13 (16), urokinase-type plasminogen activator (uPA), and tissue-type plasminogen activator (tPA) (17). Among them, HGFA and matriptase, synthesized in turn as inactive precursors, show the most efficient pro-HGF/SF processing activity (18). Mature HGF is a heterodimer made of a 69 kDa α chain and a 34 kDa β chain linked by a disulfide bond. HGF contains two binding sites with differential affinity for the MET receptor: a high-affinity site located within the α chain and a low affinity site in the β chain. The low affinity site in the β chain becomes accessible only after pro-HGF activation, which is essential for receptor dimerization and subsequent activation. Cells of mesenchymal origin are the primary producers and source of HGF in the pericellular environment, which acts on cells expressing the MET receptor (cells of epithelial origin) in a paracrine manner.

1.2.1 The *Invasive growth* Program

Cancer is a multistep process that results from the accumulation of somatic genetic alterations, which either inactivate tumor suppressor genes (i.e. p53, pRB or APC) or

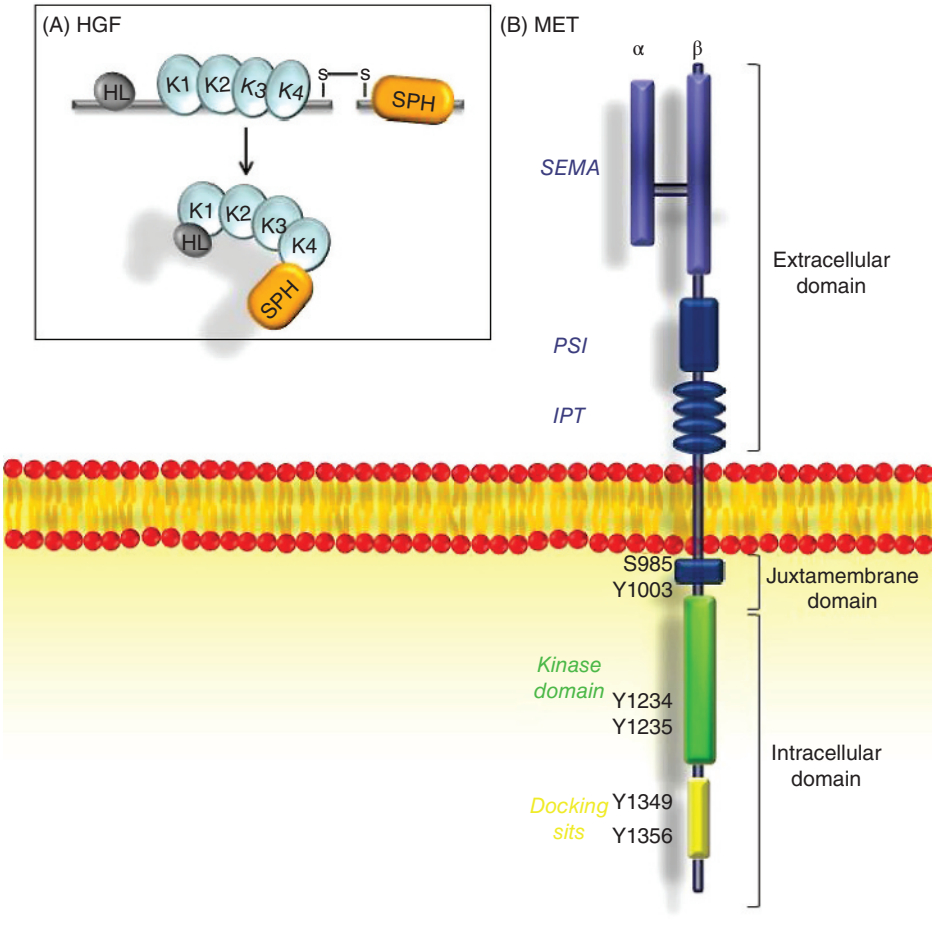

Figure 1.1 MET tyrosine kinase receptor and its ligand HGF: structure.
MET is a transmembrane tyrosine kinase receptor made of a disulphide-linked heterodimer formed by a single-pass transmembrane β chain and a completely extracellular α chain. The extracellular portion contains a SEMA domain, involved in ligand binding and receptors dimerization; a PSI domain, encompassing four disulphide bonds; and four IPT domains, a protein–protein interaction region. A single pass transmembrane domain is followed by the intracellular portion made of a juxtamembrane section, a catalytic site and a C-terminal regulatory tail. The juxtamembrane segment contains a serine (serine 985) and a tyrosine (tyrosine 1003) responsible to inhibit receptor kinase activity and promote receptor down-regulation. The catalytic site contains the 'catalytic' tyrosines 1234 and 1235 that regulate the enzymatic activity, while the C-terminal tail encompasses the 'docking' tyrosines 1349 and 1356 that, upon phosphorylation, generate a docking site able to recruit a vast cohort of intracellular adaptors and molecules responsible of triggering the signal transduction cascade.
HGF: hepatocyte growth factor; HL: hairpin loop; IPT: immunoglobulin-plexin transcription domain; K: kringle; PSI: plexin-semaphorin-integrin domain; SEMA: semaphorin domain; SPH: serine-protease domain.

activate dominant proto-oncogenes (i.e. RAS or PI3K) (19,20). These aberrant events release cells from proliferative control and allow primary tumor formation. The initial tumor growth is followed by invasive dissemination and ultimately metastasis, which is the cause of almost all cancer-related deaths. The ability of neoplastic cells to invade the surrounding tissues, survive in foreign environments, and settle at distant sites, defines a genetic program known as *invasive growth*. The invasive growth program also occurs under physiological conditions. Throughout embryogenesis, invasive growth orchestrates complex events such as gastrulation (responsible of originating the mesoderm from the embryonic epithelium), morphogenesis of epithelia, angiogenesis, nervous system formation and myoblasts migration (21). In adult life, invasive growth is necessary in normal tissues during acute injury repair (23,24) when cells at the wound edge reprogram themselves and start rapidly dividing prior to migrating towards the cut edge to regenerate the lacking tissue.

The invasive growth program consists of several stages, each of them occurring in a specific time and place, all harmoniously orchestrated to allow germ layers in the embryo, and tissues in the adult, to re-organize. All these events require cells to proliferate, migrate, overcome apoptosis, invade the surrounding tissues and re-organize themselves into new three-dimensional structures. Epithelial-mesenchymal transition (EMT) is the mechanism behind the earlier phases of the invasive growth program. During EMT, cells release junctions that maintain the epithelial monolayer structure, change their polarity by means of cytoskeleton rearrangements and attain the ability to move within the extracellular environment. Ultimately, the cells lose their epithelial phenotype to acquire a mesenchymal one. All these events, necessary during embryogenesis for correct embryo development and in adult tissues to overcome injuries, contribute to tumor formation and metastatic spread when aberrantly regulated. MET oncogene in conjunction with its ligand HGF, is one of the key players in the control of the invasive growth program.

1.2.2 MET Mediated Signaling

Under normal circumstances, MET kinase activation and its signaling cascade occurs upon ligand binding. The HGF/MET protein–protein interaction results in:

1) receptor dimerization;
2) auto-phosphorylation of the 'catalytic' residues, Tyr1234 and Tyr1235, located within the kinase activation loop and necessary to switch on receptor activity; and
3) trans-phosphorylation of the 'docking' residues, Tyr1349 and Tyr1356, located within the docking site (Figure 1.1).

Upon phosphorylation, the latter tyrosines recruit several intracellular signaling proteins and adaptors by means of their SRC homology 2 (SH2) domains (22) and trigger the broad spectrum of MET-mediated biological responses. Downstream signaling proteins include the p85 regulatory subunit of phosphatidyl inositol 3-kinase (PI3K), phospholipase Cγ (PLCγ) (22), SRC homology 2 domain containing transforming protein (SHC) (23), the adaptor growth factor receptor-bound protein 2 (GRB2) (24), the transcription factor signal transducer and activator of transcription 3 (STAT3) (25),

the v-crk sarcoma virus CT10 oncogene homolog (CRK) (26), and SRC homology domain-containing 5' inositol phosphatase (SHP-2) (27). In addition, MET associates with the scaffolding protein GRB2-associated binding protein 1 (GAB1) (28), either directly or indirectly through GRB2. GAB1 lacks intrinsic enzymatic activity. However, with the receptor interaction, GAB1 becomes phosphorylated and provides binding sites for several proteins involved in the MET signaling cascade (2). The different signaling proteins and adaptors are responsible for generating MET-specific biological activities and their harmonic coordination in time and space results in unique biological responses.

Activated MET recruits and activates RAS (rat sarcoma small GTPase) through the specific guanine nucleotide exchange factor SOS (son of sevenless) (31) which, in turn, is engaged by GRB2 and SHC. RAS, in turn, recruits and activates v-raf murine sarcoma viral oncogene homolog B1 (BRAF). BRAF sequentially activates mitogen-activated protein kinase effector kinase (MEK) then extracellular signal-regulated kinase (ERK), Jun N-terminal protein kinase (Janus kinase 1 JNK) and p38 MAPK, which translocate into the nucleus. Next, p38 modulates the activity of a number of transcription factors to promote cellular proliferation, transformation and differentiation (32). The RAS signaling is also positively reinforced by SHP2, recruited through GAB1, and is responsible for prolonging MAPK phosphorylation (29) (Figure 1.2).

GAB1 is used as a scaffolding protein to recruit, among others, the adaptor CRK. MET-GAB1-CRK complex results in JNK activation as demonstrated by a loss-of-function mutant of CRK where the activation of the JNK pathway by MET is severely impaired. In addition, JNK, through an AP-1 element in the promoter region, controls the transcription of matrix metalloproteinase-1 (MMP-1) gene (26). Indeed, the MET-GAB1-CRK signaling complex (via JNK) is a crucial event in regulating the tumorigenic phenotype of MET-transformed cells (Figure 1.2).

In a parallel signaling pathway, MET recruits p85 regulatory subunit of PI3K, directly or indirectly through GAB1, and catalyses the formation of phosphatidylinositol (3–5)-triphosphate (PtdIns(3–5)P_3). PtdIns(3–5)P_3 constitutes a docking site for AKT (AKT8 virus oncogene cellular homolog). Upon recruitment to the inner side of the plasma membrane, AKT inactivates (by phosphorylation) glycogen synthase kinase 3β (GSK3β), which antagonizes the expression of positive cell cycle regulators. AKT activation also results in protection from apoptosis through either inactivation of pro-apoptotic protein BCL-2 antagonist of cell death (BAD) or activation of E3 ubiquitin-protein ligase MDM2 (murine double minute 2) that induces degradation of the pro-apoptotic protein p53. Finally, AKT activates mammalian target of rapamycin (mTOR), which stimulates protein synthesis and physical cell enlargement (30).

Activated MET receptors also recruit and phosphorylate STAT3 monomers which, upon phosphorylation, homodimerize and translocate into the nucleus and act as transcription factors to regulate cellular proliferation, (25) transformation and tubulogenesis. Tubulogenesis is the formation of branched tubular structures in epithelial cells (25) (Figure 1.2).

Some of the biological processes regulated by HGF/MET, including cellular adhesion and migration, require regulation of cell-matrix interactions. The effect of HGF on the two major focal adhesion proteins, focal adhesion kinase (FAK) and paxillin, has been

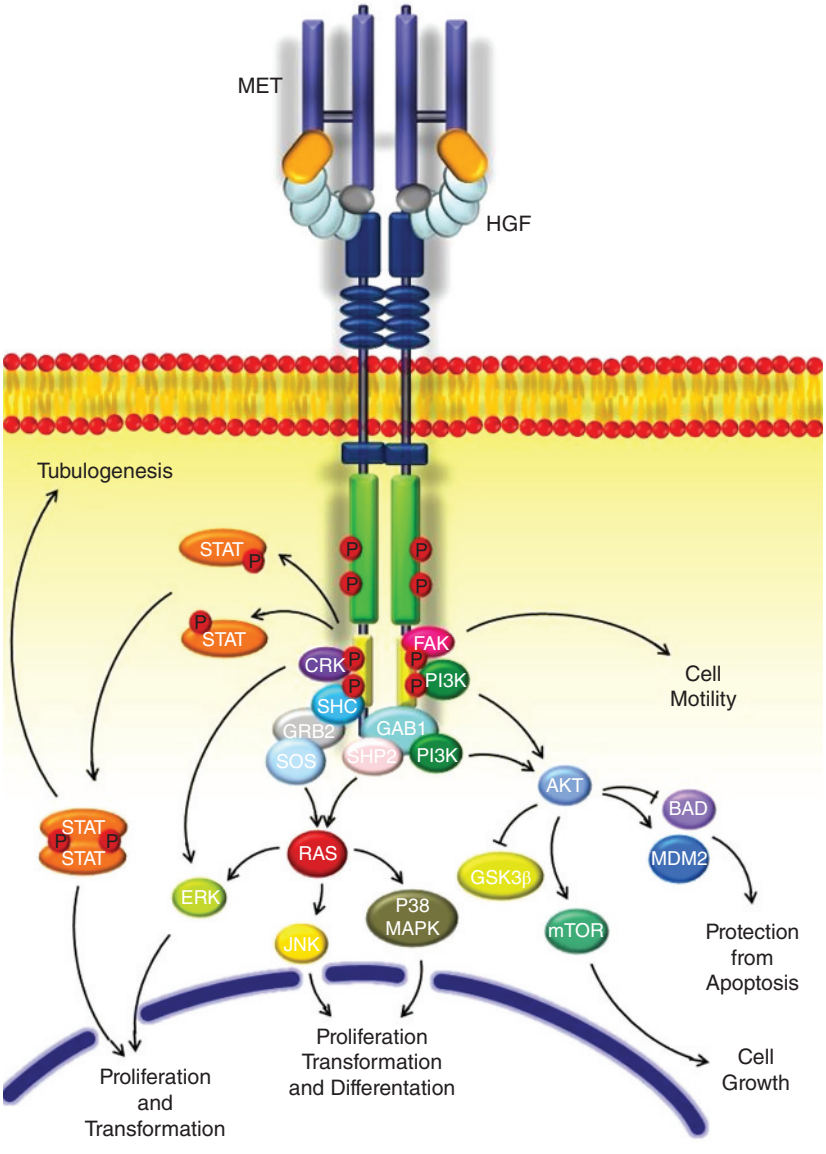

Figure 1.2 MET-driven signaling and biological activities.
HGF/MET interaction results in receptors dimerization, activation and phosphorylation of the 'docking' tyrosines. Once phosphorylated, the latter tyrosines recruit several intracellular signaling proteins or adaptors responsible for generating MET-specific biological activities and their harmonic coordination in time and space results in unique biological responses including: cell growth, differentiation, motility, proliferation, survival, transformation and tubulogenesis.
AKT: AKT8 virus oncogene cellular homolog; BAD: BCL-2 antagonist of cell death; CRK: v-crk sarcoma virus CT10 oncogene homolog; ERK: extracellular signal-regulated kinase; FAK: focal adhesion kinase; GAB1: GRB2-associated binding protein 1; GRB2: growth factor receptor-bound protein 2; GSK3β: glycogen synthase kinase 3β; HGF: hepatocyte growth factor; JNK: Jun N-terminal protein kinase; MAPK: mitogen-activated protein kinase; MDM2: murine double minute 2; mTOR: mammalian target of rapamycin; PI3K: phosphatidyl inositol 3-kinase; RAS: rat sarcoma small GTPase; SHC: SRC homology 2 domain containing transforming protein; SHP-2: SRC homology domain-containing 5′ inositol phosphatase; SOS: son of sevenless; STAT3: signal transducer and activator of transcription 3.

investigated in epithelial cells. Liu *et al*. found that HGF increased serine/threonine phosphorylation of paxillin, resulting in the recruitment and activation of FAK and subsequent enhancement of cell spreading and adhesion (31).

Finally, HGF/MET pairing stimulates NF-κB DNA binding and transcriptional activation through phosphorylation of nuclear factor-κB inhibitor-a-kinase (IKK), which in turn phosphorylates nuclear factor-κB inhibitor-a (IKB). Upon IKB's phosphorylation, the nuclear factor-κB (NF-κB) released is free to translocate into the nucleus and stimulate the transcription of various genes, including mitogenic (32) and pro-survival regulators (33).

1.2.2.1 MET Down-regulation

In non-transformed cells, MET activation is tightly regulated and receptors are switched off through diverse mechanisms. In one instance, CBL, an E3-ubiquitin ligase, is recruited to Tyr1003 within the juxtamembrane domain, and mediates ubiquitin transfer to MET, which is subsequently internalized by endocytosis and degraded (4). In another instance, tyrosine specific phosphatases, including the non-receptor protein-tyrosine phosphatase 1B (PTP1B), T-cell protein tyrosine phosphatase (TCPT/PTPN2) (34), leukocyte common antigen-related molecule (LAR/PTPrF) (35), and density enhanced protein tyrosine phosphatase-1 (DEP-1/PTPRJ) (36), are involved in MET shutdown, consequently triggering de-phosphorylation of either the 'catalytic' (in the case of PTP1B and TCPT) or the 'docking' tyrosines (DEP-1). Furthermore, recruitment of PLCγ results in activation of protein kinase C (PKC) that negatively regulates MET phosphorylation and activity (37,38).

Receptor activation is also controlled upstream through regulation of pro-HGF proteolytic processing into mature HGF in the extracellular environment by proteases, as previously discussed (18,39).

1.2.3 Cross-talk between MET and Other Receptors

Since MET is a transmembrane receptor exposed on the phospholipidic cellular membrane, MET interacts in a dynamic way with other cellular surface receptors, and the output signal originates from the combination and integration of this complex network. Ultimately, the cross-talk with other receptors generates signals that differ in length and magnitude and produce diverse biological outputs. Many different molecules have been demonstrated to be MET partners, among them integrin α6β4, the adhesive molecules CD44, the plexins B family, FAS and, lastly, several other tyrosine kinase receptors such as RON, EGFR and HER2.

MET is constitutively associated with integrin α6β4in a HGF-dependent manner: upon ligand binding and receptor activation, the integrin becomes phosphorylated, recruits intracellular signal transducers (i.e. SHC, SHP2 and PI3K) and generates a platform necessary to promote the receptor invasive growth program (40). In addition, MET and integrin interact through FAK upon MET induced phosphorylation (41).

MET is also associated with CD44, the transmembrane receptor for hyaluronic acid, responsible for connecting ECM components to the cytoskeleton. It has been described that some CD44 isoforms, generated by alternative splicing, can trigger or enhance MET activation. CD44v3, which contains the alternatively spliced exon 3, binds HGF

with high affinity and is responsible for: (i) concentrating the ligand at the cellular surface; and (ii) presenting it in multimerized complexes that result in receptor over-activation. In addition, a CD44 isoform containing the exon 6 sequence (CD44v6) is strictly required for ligand dependent MET activation, as it promotes HGF-MET interaction through its extracellular domain. It certainly has been demonstrated that CD44v6-deficient tumor cells were unable to activate MET unless they were transfected with a CD44v6 isoform. Moreover, signal transduction from activated MET to MEK and ERK required the presence of CD44v6 portion, including a binding motif for ERM proteins (45). ERM is a protein family that consists of three closely related members, ezrin, radixin and moesin, which are responsible for cross-linking actin filaments with plasma membranes and involved in signal transfer. In summary, the interaction between MET and CD44 results in an efficient functional cooperation, which generates tumor growth and metastatic spread.

MET also interacts with Plexins B. Plexins are transmembrane receptors for semaphorins, a large family of both soluble and membrane-bound ligands, which were originally identified as axon guidance cues in the nervous system (42). It has been shown that stimulation of Plexin B1 with its natural ligand SEMA4D induces plexin clustering as well as HGF-independent MET activation, resulting in an enhanced invasive growth response (43).

MET can also associate with death receptor FAS. This interaction with MET prevents FAS homo-oligomerization and clustering and ultimately results in protection for apoptosis (44).

Finally, other tyrosine kinase receptors can be MET partners. It was initially shown that MET interacts with RON, a member of the same family of tyrosine kinase receptors (discussed extensively below). It was confirmed that ligand-induced MET activation results in RON trans-phosphorylation and *vice versa*. The trans-phosphorylation occurs in a direct way, as it does not need the C-terminal docking site of either receptor and a kinase-dead RON is sufficient to block MET transforming activity (45). More recently, it was shown that in cancer cell lines displaying MET amplification, RON is specifically trans-phosphorylated by the sibling receptor and sustains MET-driven proliferation and clonogenic activity *in vitro* and tumorigenicity *in vivo* (46). These data show that, while specific for their ligands, scatter factor receptors cross-talk and combine forces to trigger specific intracellular signaling cascade (47). Similarly, it was shown that MET interacts with the orphan receptor ROR1 and is responsible for its trans-phosphorylation (48), highlighting the complexity of these signaling networks regulated by oncogene receptors. This result suggests that multiple targets are likely targeted during combinatorial therapies.

Similarly, although a direct interaction between MET and HER2 has not been described, it has been shown that the two receptors co-operate to enhance the malignant phenotype, promoting cell–cell junction breakdown and boosting invasion. This is particularly significant in cancers where HER2 is over-expressed and HGF is a physiological growth factor found in the stroma (49), such as breast cancer.

Finally, a functional link between MET and EGFR (frequently co-expressed in human cancers) has been shown: MET can be trans-activated following EGFR activation in the absence of its ligand and when concomitantly expressed the two receptors exert a synergistic effect on the activation of the downstream signaling cascade enhancing proliferation and motility (50). Moreover, it has been shown that over-expression of HGF is a

mechanism of resistance against EGFR inhibitors: HGF induces resistance to Gefitinib of lung adenocarcinoma cells displaying EGFR-activating mutations (51) by restoring the PI3K/Akt signaling pathway via phosphorylation of MET, but not EGFR or ErbB3. Similar findings have been described in breast cancers (52).

1.2.4 MET Activation in Human Cancers

More than 30 years ago, Cooper *et al.* identified the TPR-MET chimera in a cell line treated with a chemical carcinogen (53). This chimeric protein was encoded from the gene fusion originating from the chromosomal rearrangement between the translocated promoter region (TPR) and MET tyrosine kinase domain in a human osteosarcoma-derived cell line that was chemically transformed using N-methyl-N'-nitro-N-nitroso-guanidine (MNNG). The TPR portion led to constitutive dimerization and activation of the MET kinase domain and was responsible for its oncogenic behaviour *in vitro*. A few years later, Liang *et al.* showed that expression of TPR-MET in transgenic mice resulted in the development of mammary tumors and several other malignancies of epithelial origin suggesting that deregulated MET was involved in carcinogenesis (54). Since then, several MET genetic alterations have been reported in human cancers and a growing body of evidence suggests that, in an aberrant cellular environment without spatial and temporal regulation, MET is involved in tumor onset, progression and metastatic dissemination. Certainly, MET activation is implicated both in neoplastic transformation and malignant spread, as a result of its growth-promoting activity, enhancement of cell motility and protection from apoptosis. Cells that over-express either MET or HGF are tumorigenic and metastatic when implanted into immunocompromised nude mice (55). Furthermore, transgenic mice for either increased expression of MET or HGF, develop metastatic tumors (56) while, contrarily, endogenously expressing cancer cells become less aggressive when MET is switched off. Accordingly, it was demonstrated that short hairpin RNA (shRNA) mediated MET knock-down in rhabdomyosarcomas-derived cell lines results in a robust inhibition of cell proliferation, survival and invasion both *in vitro* and *in vivo* (57). Similar results were obtained in lung cancer cell lines harboring MET amplification where receptor silencing induced a significant inhibition of growth rates (58).

Constitutive receptor activation can occur through different mechanisms:

1) HGF-dependent activation with establishment of autocrine or paracrine circuits that release cells from the need of growth factors (59); or
2) HGF-independent mechanisms.

The latter can indeed take place:

1) through transactivation by other transmembrane receptors (among others: CD44, integrins, RON and EGFR, as discussed earlier);
2) by receptor over-expression, which triggers receptor oligomerization and reciprocal activation even in absence of ligands; and
3) as a consequence of somatic genetic lesions (including translocations, gene amplifications and activating mutations), which generate constitutively active receptors.

Although MET mutations are uncommon, occurring in 3–4% of unselected primary solid cancers (http://cancer.sanger.ac.uk), they have been described in several human

cancers and can hit different MET domains. Activating point mutations occurring within the tyrosine kinase domain have been originally described in patients who suffer from hereditary and sporadic papillary renal-cell carcinomas (RCC) (60) and childhood hepatocellular carcinoma (HCC) (61). Instead, alterations inside the juxtamembrane region were mainly found in human gastric carcinoma (62) and more recently in lung cancers and pleural mesothelioma (63), as well as in melanoma (64). Notably, cells displaying mutated MET receptors seem to be selected during progression of head and neck carcinomas, as they are more frequent in secondary lesions than in the matched primary tumors (65). More recently, an uncommonly high incidence of MET mutations was described in Cancers of Unknown Primary origin (CUPs), where mutational incidence (30%) was significantly higher than expected (4%), in the absence of high mutational background (66). Remarkably, these mutations affected both the catalytic and the SEMA (a protein–protein interaction motif) domains of the receptor suggesting, for the first time, that the non-catalytic domain of the receptor could be somehow involved in tumor progression by interfering with either the ligand binding or the three-dimensional structure of the receptors.

MET activation in human cancers is mostly a consequence of over-expression, which usually occurs at a transcriptional level or, more rarely, is an effect caused by increased gene copy number. Enhanced MET expression has been described in numerous solid tumors such as breast (71), colon (72), bladder (73) and ovarian cancers (74), osteosarcoma (63), gliomas (75) renal (76), hepatocellular and non-small cell lung carcinomas (77). Elevated MET is also found in tumors of the upper gastrointestinal tract, such as esophageal (78), gastric (79) and oral squamous cell carcinomas (80); pancreatic (81) prostatic cancers (82), and multiple myeloma (83), where receptor enhanced expression always correlates with poor prognosis. In the last few years, the transcriptional mechanisms responsible for increased MET expression and activity have been extensively investigated and some of them have been elucidated. Usually MET up-regulation is driven by adverse environmental conditions, such as hypoxia, a condition of oxygen deficit that can be found in the inner portion of growing tumors (67) or ionizing radiations (68), as discussed later.

MET over-expression as a consequence of gene amplification, was initially described in gastric cancers (69,70), tumors of the upper digestive tract such as biliary tract (71) and esophageal carcinomas (72). Afterwards, an increased MET copy number has also been reported in lung cancers (58,73) and metastatic colorectal cancers where it is associated with acquired resistance that arises upon targeted therapy against EGFR (74). Functional studies demonstrate that MET activation confers resistance to anti-EGFR treatment (by means of monoclonal antibodies) both *in vitro* and *in vivo:* notably, in patient-derived colorectal cancer xenografts, MET amplification correlates with resistance to EGFR blockade, and can be overcome by concomitant MET inhibition (74).

1.2.4.1 MET, Hypoxia and Ionizing Radiations

Development of human cancers is not only due to the sequential accumulation of somatic genetic alterations but also from the dynamic cross-talk between cancer cells and the tumor microenvironment, which consists of ECM, blood vessels, inflammatory cells and fibroblasts (67,75,76). It has been shown that in solid tumors, MET expression (and activity) can be transcriptionally induced by signals present in the tumor reactive

stroma, such as inflammatory cytokines and pro-angiogenic factors and by exogenous stress stimuli, such as hypoxia (67) or ionizing radiations (68).

Hypoxia, via the transcription factor hypoxia inducible factor 1α (HF1α), which itself is regulated by the concentration of intracellular oxygen, activates transcription of the MET oncogene. MET over-expression results in larger numbers of receptors being exposed on the cell surface and, additionally, amplifies HGF signaling in both promoting cell migration and invasion (67).

As previously mentioned, leading the invasive growth program, MET not only triggers proliferative signals, but also exerts an anti-*apoptotic* function and protects cells from DNA damaging agents such as ionizing radiation. Mechanistically, ionizing radiation induces transcriptional up-regulation and catalytic activation of the receptor; increased MET activity delivers anti-apoptotic signals that prevent cell death induced by irradiation (68). Ionizing radiation exerts this effect on MET expression and activity through the ATM and NF-κB signaling pathway. In parallel, MET inhibition increases tumor cell radiosensitivity and prevents radiation-induced invasiveness. In this situation, MET up-regulation provides both pro-survival and pro-invasive advantages that intensify the tumor malignant phenotype, a phenomenon known as oncogene expedience, as discussed below (77).

1.2.4.2 MET Expression in Cancer Stem Cells: a Paradigm of Inherence

The hypothesis that MET is implicated in stem cells was formulated by Kmiecik *et al.* more than 20 years ago (78), where they showed that MET and its ligand, HGF, are prerequisites to stimulate colony formation of hematopoietic progenitor cells *in vitro*. More recently, Boccaccio *et al.* demonstrated that MET is essential to maintain the stem cell phenotype in glioblastoma cancer stem cells (79). Similarly, MET signaling is required in prostate cancer stem cells for self-renewal (80). Likewise, it was shown that MET plays a role in breast cancer stem cells, where the receptor is expressed in the luminal progenitor subpopulation and prevents differentiation towards the mature luminal phenotype (81). Furthermore, it has been proposed that MET expression in tumors (often over-expression) is a paradigm of *inherence*: cancer stem cells inherit MET expression from their normal counterpart (stem and progenitor cells) committed to exert the invasive growth program as part of their physiological phenotype (82,83), and exploit it for cancer progression and metastatic spread.

1.2.4.3 Oncogene Addiction and Oncogene Expedience

Human cancer is a complex, multistep process that arises from several different genetic alterations, which ultimately are responsible for activating oncogenes and inactivating tumor suppressor genes. Nevertheless, not all the genetic changes exhibit the same significance within the tumor. Some lesions are more important than others, and tumors depend on the activity of a single or few mutated genes. This concept, formulated in the late 1990s and known as oncogene addiction (84), indicates the dependence of cancer cells on an over-active gene or pathway for survival and proliferation. Accordingly, disrupting that gene/event is sufficient to induce growth arrest, provoke massive apoptosis and, in principle, eradicate the tumor. The oncogene addiction theory represented a milestone in cancer therapy because it proposed that simply identifying and turning off the major driving gene or set of genes is sufficient to destroy any

cancer. It has been recently shown that several cancer cell lines displaying increased gene copy number of different tyrosine kinase receptors, such as EGFR (85) or HER2 (86,87), depend on that particular gene for both growth and survival *in vitro* and this dependence or 'addiction' shown *in vitro* is also replicated by tumor behaviour observed *in vivo*. Indeed, tumors treated with anti-EGFR (Cetuximab or Panitumumab) or anti-HER2 (Trastuzumab) targeted therapies display a remarkable response (measured as robust inhibition of tumor growth) whenever they display genomic amplification of EGFR or HER2 loci.

Similarly, a decade ago, it was demonstrated that certain human cancers such as gastric cancers, rabdomyosarcomas and lung cancers are addicted to MET, because MET is an absolute requirement for their proliferation and maintenance. Initially, it was shown that gastric cancer cell lines displaying a high grade of MET amplification (which results in receptor over-expression and constitutive activation) are exquisitely sensitive to MET inhibition – attained with kinase inhibitor PHA-665752 – both *in vitro* and *in vivo*. Indeed, the anti-MET specific compound induces massive apoptosis exclusively in MET amplified cell lines without affecting those lacking receptor amplification (88). Equivalent results were obtained in the two major histological subtypes of rabdomyosarcomas, embryonal and alveolar (61) and gastric carcinoma cells (106), where MET silencing (in MET amplified cells) resulted in abrogation of the full invasive growth program both *in vitro* and *in vivo*.

In following studies, it was observed that many cell lines are sensitive to MET inhibition, irrespective of the presence of MET genetic alterations. This fact can be explained by the unique biological characteristics of MET. Indeed, the physiological anti-apoptotic and pro-invasive activities of MET confer to neoplastic cells a greater benefit, helping them to overcome the selective barriers along cancer progression. Therefore, in various tumor types, activation of MET is a secondary event that exacerbates the malignant properties of already transformed cells. In these cases, aberrant MET activation usually occurs through transcriptional up-regulation and is known as oncogene expedience. In contrast to *addiction*, the inappropriate activation of MET resulting in *expedience* is the consequence rather than the cause of the transformed phenotype (77).

1.2.5 Targeting HGF/MET as a Therapeutic Approach in Human Cancer

In the last few years, oncology has been moving aggressively in the direction of personalized precision medicine. Treatments are tailored to hit specific molecules or pathways altered in each individual patient. In this scenario, tyrosine kinase receptors are ideal targets as they often sustain tumor formation and disease progression. Within this family, MET has been implicated in a number of human malignancies, including renal, liver, head and neck, gastrointestinal and breast cancers, among others. Compelling evidence strongly confirms MET as a good pharmacological target in anti-cancer therapy. Receptor inactivation would benefit both:

1) a small number of MET-*addicted* tumors, in which MET is aberrantly regulated as consequence of increased gene copy number (*addiction*); and
2) a much wider spectrum of advanced tumors, where MET is activated as a secondary event and intensifies the malignant phenotype of already transformed cells (*expedience*) fostering local invasion and distant spreading (77).

Presently, as a testament to the validation of MET as a promising target, several therapeutic agents have been developed and approved for cancer therapy. These therapeutic agents were designed to target the MET receptor, to target MET's ligand HGF, and to inhibit the downstream signaling cascade. Multiple others are currently in different phases of clinical trials or are promising in preclinical settings. The challenge remains about how to identify tumors most likely to respond to MET inhibition. Anti-MET drugs include neutralizing antibodies directed against either the receptor MET (Chapter 7) or its ligand HGF (Chapter 6), designed to prevent MET/HGF interaction and therefore block the downstream signaling cascade, and small molecule inhibitors designed to interact with receptor active sites inhibiting phosphorylation and recruitment of intracellular signal transducers. Moreover, kinase-domain directed inhibitors can be classified into three sub-groups: class I inhibitors are ATP competitors and interact with Tyr1230; class II inhibitors are equally ATP competitors but interact with a wider aminoacidic region within the kinase domain; and, lastly, non-ATP competitors. The only member of the latter group is Tivantinib that binds to inactive receptor and stabilizes it in its auto-inhibited conformation (Figure 1.3).

1.2.5.1 HGF Antagonists

HGF antagonists are molecules created to bind with high affinity to the extracellular domain of the MET receptor, yet are unable to activate both the intracellular kinase domain and downstream signal transducers. One of the earliest MET ligand-based antagonists to be developed was NK4, a synthetic truncated form of HGF bearing only the α chain. This polypeptide competes with pro-HGF and activated HGF for receptor binding but fails to activate it, thereby blocking the signaling transduction cascade and the biological outcomes. In addition, NK4 was shown to strongly prevent angiogenesis. As expected, the macromolecule, when used in experimental mouse tumor models, either administered in a conventional manner or delivered by gene transfer (89), effectively impaired tumor growth, invasion, metastasis and angiogenesis (90) (Figure 1.3).

Uncleavable HGF is another non-actionable form of HGF classified as a ligand antagonist. Michieli *et al*. engineered the ligand introducing a single amino-acid substitution in the proteolytic site, which prevents the maturation of the molecule and generates a new protein capable of blocking all MET induced biological responses. The compound acts in a dual manner. First, it competes with endogenous pro-HGF for the catalytic domain of the enzymes (HGFA, matriptase, TMPRSS2 and hepsin) responsible for its proteolytic cleavage and activation (11,13–15), thus inhibiting endogenous pro-HGF processing and maturation. Second, it binds to the MET receptor with high affinity displacing the mature ligand HGF, thus impairing HGF-mediated activation. This latter mechanism is possible in a scenario in which the ligand precursors bind MET, thus forming quiescent complexes that become active only upon pro-HGF cleavage. In their work, the authors provide evidence that both local and systemic expression of uncleavable HGF inhibits tumor growth, impairs angiogenesis and, notably, prevents metastatic spread (91) (Figure 1.3).

Neutralizing anti-HGF antibodies are also classified as ligand competitors. Pioneering work demonstrated that a minimum of three antibodies, each one acting on different HGF epitopes, was required to prevent MET tyrosine kinase activation (92). Subsequently, several papers describe the development of monoclonal antibodies

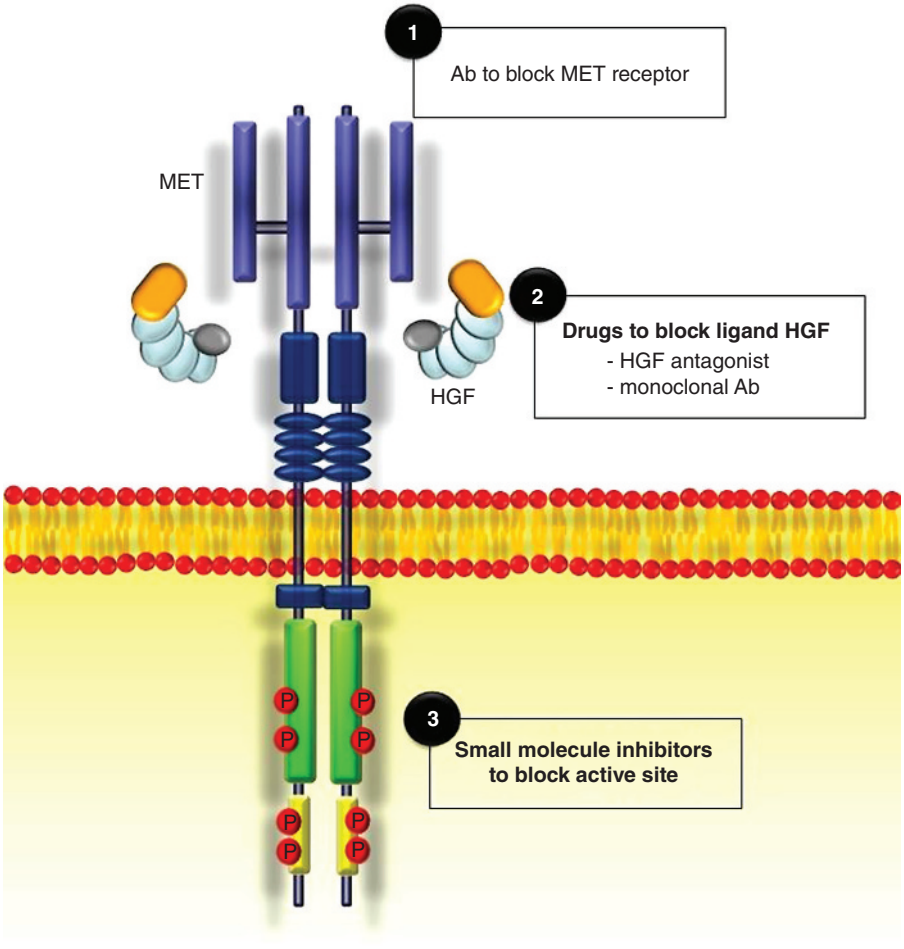

Figure 1.3 Agents targeting MET/HGF. Schematic representation of the different levels at which MET pharmacological inhibition can be attained.

that can individually bind and neutralize human HGF. These antibodies are capable of binding HGF at subnanomolar concentrations, blocking ligand-mediated receptor phosphorylation and inhibiting the downstream biological activities both *in vitro* and *in vivo* (93). A number of human monoclonal antibodies against HGF have been reported and shown to exhibit therapeutic effects in xenografts of human glioma featuring a MET/HGF autocrine loop (94). Accordingly, Kim *et al.* showed that blocking the HGF/MET interaction with systemically administered anti-HGF monoclonal antibodies results in a striking antitumor effect, even within the central nervous system (95) (Figure 1.3). Rilotumumab is a humanized monoclonal antibody directed against HGF, which has been investigated in phase II and III clinical trials in patients with advanced gastric or gastroesophageal junction adenocarcinomas, gastric adenocarcinomas, colorectal cancers, glioblastoma and advanced or metastatic renal cancers.

The efficacy results reported thus far from different clinical trials are equivocal and have elucidated the underlying need to define stringent criteria in identifying the patient population most likely to benefit from anti-MET therapy (i.e. patients with high MET expressing tumors). Another monoclonal antibody directed against HGF, named Ficlatuzumab, is currently being investigated in phase II clinical trials as treatment for lung adenocarcinomas (96).

In an alternative approach, Janetka *et al.* have formulated a novel strategy to prevent MET receptor activation by blocking the conversion of inactive single-chain pro-HGF ligand to the active two-chain HGF ligand (97). To this end, they have identified the first small molecule inhibitors of HGF (Chapter 9), which act in a similar fashion to the endogenous polypeptide inhibitors of HGF-Activation, HAI-1 and HAI-2. By inhibiting the proteolytic processing enzymes, HGF-Activator (HGFA), matriptase, hepsin and TMPRSS2, and blocking the formation of active two-chain HGF, the activation of both the ligand HGF and the receptor MET is prevented. Notably, the endogenous inhibitors HAI-1 and HAI-2 are often downregulated in cancer and decreased levels equate with elevated invasiveness in tumors and risk of disease progression. Interestingly, these same proteases function to activate MSP, the RON kinase ligand as well, which is also implicated in cancer. These inhibitors have been shown to have anticancer effects in breast (98), prostate (97,99) and colon (100,101) cancer. This innovative approach utilizes small molecules that mimic the biological function of HAIs, by targeting all proteases that are selectively inhibited by these regulators of HGF and MSP. This differs from the HGF and MET antibodies, as well as the HGF antagonist decoys discussed above.

1.2.5.2 Tyrosine Kinase Inhibitors

The most well-developed strategy to block tyrosine kinases (and other Ser/Thr kinases) utilizes small molecule kinase inhibitors directed at the intracellular kinase phosphorylation domain. These inhibitors are typically low molecular weight heterocyclic compounds that target the ATP binding site of the kinase domain and directly compete with ATP. In this fashion, the inhibitors prevent receptor transphosphorylation and subsequent signaling events via recruitment of the downstream effectors. The first reported small molecule MET kinase inhibitors were K252a, PHA-665752, and SU11274 followed by JNJ-38877605. The staurosporine analog, K252a, is a potent yet promiscuous inhibitor of all receptor tyrosine kinases (RTKs). Interestingly, K252a is more effective when MET displays the mutation Met1268Thr, typical of papillary carcinoma of the kidney (102). PHA-665752 competitively inhibits the catalytic activity of MET kinase with an IC_{50} of 9 nM and with a relatively high specificity (>50-fold) compared to other tyrosine and serine-threonine kinases. *In vitro* studies showed that this compound strongly represses both HGF-dependent and constitutive receptor phosphorylation, resulting in abolition of the main biological phenotypes elicited by the receptor (103). More recently, it was shown that gastric cancer cells displaying a high-level MET amplification were exclusively susceptible to PHA-665752, where the inhibitor was shown to trigger massive apoptosis in MET-positive cells with no effects on MET-negative cells (88). SU11274 is a potent and selective inhibitor of MET (IC_{50} 10 nM), which can effectively inhibit two mutant forms of MET, Met1268Thr and His1112Tyr, but not two other variants (104).

Finally, JNJ-38877605, an ATP-competitive inhibitor of MET belonging to class I, displays extremely high affinity for the receptor (IC_{50} of 4 nM) and greater than 600-fold

selectivity for MET compared with more than 200 other tyrosine and serine-threonine kinases. JNJ-38877605 has been shown to potently affect a significant reduction of constitutive receptors phosphorylation in a subset of MET-addicted cells. MET inhibition by JNJ-38877605 results in proliferation rates reduction *in vitro* and tumor xenografts growth *in vivo* (46,68). Due to the generation of species-specific insoluble metabolites by aldehyde oxidase activity, a mild, although recurrent, renal toxicity (not observed in preclinical studies) has been described, even at subtherapeutic doses in a phase I trial. This trial was discontinued and the compound was withdrawn from clinic (105).

More recently, new inhibitors have been developed. Some of the inhibitors are currently undergoing pre-clinical studies, others are being evaluated in clinical trials, and several have been approved for clinical use. These new drugs include Crizotinib, Cabozantinib, Foretinib and Tivantinib.

Crizotinib (Xalkori; PF-02341066) is a multi-targeted tyrosine kinase receptor inhibitor, which potently inhibits both ALK (anaplastic lymphoma kinase) and MET with IC_{50}s in cell based assays of 11 nM and 24 nM, respectively. It was initially approved for treatment of non-small-cell lung cancer (NSCLC) patients who have a chromosomal rearrangement that generates a fusion gene between EML4 (echinoderm microtubule-associated protein-like 4) and ALK. This fusion results in a constitutively active protein kinase (106). A patient with advanced squamous cell carcinoma (SCC) harboring a MET increased copy number experienced a major clinical response after Crizotinib monotherapy in the absence of ALK alterations (107). Thus, Crizotinib demonstrated its role as potent anti-MET inhibitor both *in vitro* and *in vivo*.

Cabozantinib (XL184; BMS-907351) is also a potent multi-targeted kinase inhibitor that inhibits a variety of cellular receptors, including VEGF receptors, MET, AXL, RET, FLT3, KIT and ROS1 (108,109). Similar to other kinase inhibitors, it is a reversible ATP-competitor. Initially, Cabozantinib, a potent inhibitor of MET with an IC_{50} of 1.4 nM, was reported to exert powerful antitumor activity in tumor xenografts harboring constitutively phosphorylated MET.

Foretinib (GSK1363089; XL880) is a relatively selective multi-kinase inhibitor that most potently inhibits MET (IC_{50} 0.4 nM) and KDR (IC_{50} 0.9 nM), in addition to VEGFR, RON, FLT1/3/4, PDGFRα/β, TIE-2 and AXL. Although it has been demonstrated in preclinical studies to inhibit growth of gastric cancer cells by efficiently blocking inter-receptor tyrosine kinase networks (110), Foretinib was not able to improve survival of first line patients with advanced gastric cancers (111).

Tivantinib (ARQ197), a staurosporine derivative that binds to the dephosphorylated MET kinase *in vitro,* is the first non-ATP-competitive small molecule inhibitor targeting MET (K_i of 0.355 μM). However, it still remains to be explicitly proven that Tivantinib is exclusively targeting MET. Originally, Tivantinib treatment was shown to result in inhibition of cellular proliferation of MET expressing cancer cell lines as well as induction of caspase-dependent apoptosis in cell lines with constitutive MET activation. These results were further validated *in vivo* where the drug induced growth inhibition of human tumors (130). An initial phase II study in patients with advanced unresectable hepatocellular carcinoma, who had disease progression after systemic first line therapy, confirmed that Tivantinib could provide an option for second-line treatment typically for patients with high MET expressing tumors (112). However, soon after, a second publication suggested that Tivantinib displayed its cytotoxic activity via molecular

mechanisms, molecular mechanisms that are independent from its ability to bind MET. In this work, authors analyzed the activity of Tivantinib in several models. The first utilizes cells harboring MET amplification and, therefore, addicted to MET signaling. Another, where cells are diploid for MET locus, thus not relaying on MET for proliferation and survival. A model with cells not expressing MET and, finally, employing engineered cells in which MET ATP-binding pocket was deleted by homologous recombination. Taken together, these findings demonstrated that Tivantinib displays a universal cytotoxic activity, independently of MET gene copy number, regardless of the presence or absence of MET (113). Similar results were obtained in another paper, which showed that Tivantinib exerted its anti-tumor activity in both MET-addicted and non-addicted cells, irrespective of MET status (114).

1.2.5.3 Anti-MET Monoclonal Antibodies

A different strategy to inhibit MET signaling utilizes monoclonal antibodies, with many of them either undergoing pre-clinical characterization or being tested in clinical trials. It is noteworthy that antibodies directed against the receptor, as opposed to the antibodies designed to block the ligand, have the great potential to block both HGF-dependent and constitutive receptor activation. Initially, the bivalent nature of the antibodies made it very complicated to target MET, as they mimic HGF action by inducing receptor dimerization and consequent activation. To overcome this limitation, a number of monovalent monoclonal antibodies have been rationally developed.

Petrelli *et al.* described the first ground-breaking monoclonal antibody (mAb) directed against the extracellular portion of MET (DN30). It was shown that DN30 was capable of both preventing MET activation and abrogating its biological activity, thus promoting significant down-regulation of MET. The mechanism through which DN30 efficiently down-regulates MET is via proteolytic cleavage of the extracellular portion, resulting in *shedding* of the ectodomain and formation of a soluble extracellular fragment that:

1) removes the receptors from the cell surface;
2) forms inactive heterodimers with the residual intact molecules; and
3) sequesters the ligand from the extracellular environment.

Subsequently, the intracellular domain is cleaved and degraded by the proteasome machinery (115,116). However, DN30 acts as partial agonist where its binding to MET results in *partial* activation of the kinase due to antibody-mediated receptor dimerization. To safely harness the therapeutic potential of DN30, Pacchiana *et al.* dissociated its shedding activity from its agonistic activity generating a monovalent fragment (DN30 Fab). Indeed, DN30 Fab maintains high affinity MET binding, elicits efficient receptor shedding and down-regulation (which results in impaired receptor activity both *in vitro* and *in vivo*), yet is completely devoid of agonistic activity (117,118).

Onartuzumab (MetMAb) is another humanized and affinity-matured monovalent monoclonal antibody directed against MET. It was generated using the knob-into-hole technology that enables the antibody to engage the receptor in a monovalent one-armed fashion. MetMAb potently blocks ligand binding, impairing HGF mediated receptor phosphorylation and signaling cascade resulting in antitumor activity (119,120). Ornatumumab has been tested in combination with Erlotinib in NSCLC,

where MET activation has been described in resistance to anti-EGFR therapy. In this scenario, a phase II trial in second and third line NSCLC showed that Ornatuzumab plus Erlotinib, prolonged by two-fold the progression free survival and by three-fold the overall survival, compared to Erlotinib plus placebo in tumors expressing high MET levels (121).

1.2.5.4 Alternative MET Blocking Strategies

It has been extensively established that the extracellular SEMA domain is the effector domain involved in ligand binding and receptor dimerization (122,123). Thus, another way to neutralize the receptor activity would be to develop soluble recombinant SEMA proteins. As expected, these macromolecules do produce a reduction of the downstream signaling triggered by the receptor, either in presence or absence of the ligand (122). Analogous results were obtained by engineering a soluble MET receptor (decoy MET) capable of preventing both ligand binding and receptor homodimerization (124). Accordingly, decoy MET expression resulted in impaired cell proliferation and survival in a variety of human xenografts; decreased angiogenesis and prevention of spontaneous metastases.

Alternatively, at least two other strategies have been pursued to specifically block the receptor:

- peptides competing with the intracellular transducers for the receptors docking sites, and therefore blocking the downstream signaling cascade (125); and
- reduction of the number of receptor molecules exposed on the cellular surface using either shRNA technology or adenovirus vectors carrying small-interfering RNA (siRNA) constructs (126).

First, Shinomiya *et al.* drastically reduced MET expression in a subset of mouse, canine and human tumor cell lines. This decrease in MET resulted in impaired cell proliferation and viability, inhibition of scattering and invasion *in vitro*, and a substantial reduction of tumor growth *in vivo* (126). More recently, MET was silenced in rabdomyosarcoma-derived cell lines using shRNAs expressed in lentiviral vectors under an inducible promoter. Consequently, MET down-regulation significantly affected cell growth, survival and invasion *in vitro* and promoted a considerable decrease in tumor growth in xenograft models (57).

1.2.6 Primary and Secondary Resistance

The major problems of targeted therapies, including the ones targeting tyrosine kinase receptors, are primary and secondary (also known as acquired) resistance. During primary resistance to targeted therapy, tumors do not respond to treatment from the onset. On the other hand, secondary (acquired) resistance can occur after an initial response (measured as tumor shrinkage or growth inhibition) when tumors stop responding to treatment. When tumors stop responding to drugs, it has been determined that only one or a few clones emerge and are able to grow out of control. Acquired resistance inevitably occurs; either originating from mutated cells that were already present within the tumor before treatment started or as *ex novo* mutations that have been positively selected throughout therapy. Several mechanisms have been described to drive acquired resistance (a few of them will be examined in the next section) but many others remain to be discovered. Our knowledge regarding resistance is still limited and future studies should

identify its molecular basis and develop therapeutic strategies to prevent it. Certainly, successful clinical responses will be attained using combinatorial therapies (massive attack), in which more than one lesion is hit at the same time.

1.2.6.1 MET Role in Resistance to Anticancer Agents

Several recent publications have described MET or HGF as a mechanism of resistance to targeted therapies, including EGFR, HER2, VEGFR, BRAF, and even MET inhibitors. Mechanisms of resistance occurring upon EGFR targeted therapy are well known, especially in NSCLC patients. Aside from the secondary Thr790Met mutation in EGFR kinase domain, activation of the MET pathway, as a consequence of either receptor gene amplification or up-regulation of ligand expression, has been described (51,127,128). Altogether, these data provide the rationale for treatments targeting MET in patients who progress on EGFR therapy and who also display an over-active MET pathway. Moreover, as it was shown that the subpopulation of MET-amplified cells was already present before anti EGFR therapy, upfront co-treatment is strongly recommended (148). Analogous results have been more recently obtained in colorectal cancers where MET amplification is associated with resistance to either Cetuximab or Panitumumab treatment (91). This once again provides a strong rationale for inhibiting MET to overcome acquired resistance to EGFR therapies.

There is also evidence that increased production of HGF is a key mediator of this resistance to EGFR targeted therapies in colon (100), breast (52) and lung cancer (51). For example, Klampfer *et al.* have recently demonstrated that treatment of colon cancer cells with the small molecule triplex HGFA, matriptase, hepsin inhibitor of HGF activation, SRI31215 overcomes primary resistance to both the EGFR antibody cetuximab and small molecule kinase inhibitor gefitinib (100).

Equally, a role of MET in resistance to HER2 targeted therapies has been suggested in breast (129) and gastric cancers (130), where Trastuzumab-resistant HER2-positive cell lines and primary tumors displayed increased MET and/or HGF expression. In this scenario, MET inhibition sensitized cells to anti-HER2 treatment blocking ERK and AKT phosphorylation (129).

Several works have also revealed that MET is involved in resistance to anti-VEGFR therapies. In glioblastoma that acquire resistance to Bevacizumab (a recombinant humanized monoclonal antibody that blocks angiogenesis by inhibiting VEGFR), MET is the most up-regulated gene as shown by gene expression profiling of untreated versus treated tumors. Accordingly, MET down-regulation in resistant tumors results in reduced cell invasion and proliferation (131).

MET activation has been associated also with resistance to Vemurafenib, a BRAF inhibitor that specifically targets the Val600Glu activating mutation (132).

In this case, combinatorial-targeted therapies inhibiting MET and other molecules (i.e. EGFR, HER2, VEGFR and BRAF) are currently investigated in clinical trials to overcome acquired resistance.

1.2.6.2 Mechanism of Resistance to MET Inhibitors

From an opposite but complementary viewpoint, potential mechanisms of resistance to anti-MET targeted therapy might also occur. Although present clinical data on anti-MET resistance are scarce, it is being investigated in preclinical settings. Several studies conducted in gastric carcinoma cell lines, which are exquisitely dependent on

MET for growth and survival, examined how cancers may become resistant to MET inhibitors.

It was originally shown that GTL16 gastric cell lines exposed to increasing doses of two different MET inhibitors (PHA-665752 and JNJ38877605) become resistant to MET inhibition. The mechanism of acquired resistance was either KRAS amplification (133) or activation of HER family members, including EGFR, HER3 and downstream signaling pathways common to MET and HER families (134). Qi *et al.*, exploiting the highly sensitive gastric carcinoma cell line SNU638 and two MET inhibitors (PHA-665752 and PF-2341066), described two different mechanisms of resistance that arose simultaneously. The first resistance mechanism was due to the acquisition of a MET mutation within the activation loop of the kinase domain (Tyr1230His/Cys); this alteration causes a conformational change that destabilizes the MET auto-inhibitory conformation, leading to continuous phosphorylation. The second mechanism is in response to and driven by EGFR activation after enhanced expression of its ligand TGFα. Once again, this emphasizes that a single cancer can simultaneously develop several different mechanisms of resistance, while also highlighting the great efforts that are necessary to prevent and overcome resistance (155).

Recently, it was reported that resistance to anti-MET targeted therapies could be achieved through instauration of an autocrine loop via increased expression of the MET ligand HGF in gastric cancer cells (156).

Indeed, since a number of clinical trials with anti-MET drugs are currently ongoing, it is necessary to investigate the molecular basis of resistance (that inevitably occurs) to design the best possible therapeutic strategies for preventing resistance.

1.2.6.3 Combinatorial Therapeutic Strategies

As previously described, through its multifunctional docking site, MET recruits diverse intracellular transducers that ultimately trigger MET-driven biological responses. The downstream pathways are part of a complex and redundant molecular network shared with other receptors and, when receptors or pathways are individually inhibited, compensatory mechanisms occur and result in increased activity of the other receptors. In this scenario, growing evidence suggests a promising role for combinatorial therapies designed to simultaneously block more than one kinase receptor. As previously discussed, cross-talk between MET and EGFR has been described in a number of human tumors. In particular, MET or HGF amplification has been described as a mechanism of acquired resistance to Erlotinib or Gefitinib treatments. Therefore, several studies combining MET and EGFR inhibitors are currently ongoing. Among others, a phase III trial combining Tivantinib with Erlotinib versus placebo with Erlotinib in patients with locally advanced or metastatic, non-squamous, non-small-cell lung cancer (NSCLC) suggests that the former significantly improves both progression free and overall survival compared with placebo plus Erlotinib in the subset of patients displaying MET over-expression (157,158). Furthermore, in absence of either qualitative or quantitative alterations of EGFR, it was recently shown that in MET-amplified cells, MET inhibitors induced only tumor growth inhibition, whereas dual MET/EGFR inhibition led to complete tumor regression and prevented the onset of resistance (139).

The contribution of the HGF/MET pathway to angiogenesis has been demonstrated extensively. For example, Lu *et al*. verified that VEGF directly and negatively regulates

cell invasion (140). Indeed, VEGF increases recruitment of PTP1B to a MET/VEGFR2 heterodimer, reducing MET phosphorylation and tumor cell migration. Consequently, VEGF blockade restores and increases MET activity both in mouse models of glioblastoma and in a subset of glioblastoma patients (140). Also, preliminary results attained in phase I clinical trials on HCC patients showed that association of Sorafenib (VEGFR inhibitor) and Tivantinib resulted in a clinical benefit for patients that acquire resistance to anti-VEGFR drugs (138).

MET is also thought to play an important role in resistance to radiotherapy (141). Several publications, as discussed earlier, showed that IRs induce up-regulation of MET transcription, both in cancer-derived cell lines and primary tumors, which results in receptor over-activation and subsequent increased invasiveness and radio-resistance. Accordingly, MET blockade (via kinase inhibitors) restores sensitivity to radiation (68), strongly suggesting the potency of MET inhibition with radiotherapy.

Indirect results were obtained on the role of MET in resistance to systemic chemotherapy. It was first suggested that cisplatin treatment increased MET expression in head and neck squamous-cell carcinoma (HNSCC) and over-expressing cells acquired increased metastatic potential (142). However, more recently, the efficacy of anti-MET targeted therapy (attained either with DN30 Fab or with PHA-665752) in combination with two different chemotherapeutic regimens – cisplatin and 5-fluorouracil – was evaluated in a 'in cell' preclinical format. The authors demonstrated that tumors displaying increased MET gene copy number achieved a plateau response by MET monotherapy and do not receive further benefit by addition of either cytotoxic treatment. From an opposite but complementary perspective, widespread cytotoxic activity exerted by conventional chemotherapy in multiple cell lines not harboring MET amplification was not further enhanced by MET inhibition (163).

In summary, it is likely that HGF/MET pathway inhibitors will have a greater effect when used in combination with other targeted agents or conventional treatments, such as ionizing radiations.

1.3 RON Tyrosine Kinase Receptor and its Ligand MSP

1.3.1 Discovery and Structural Biology

MET is the prototype of a family of tyrosine kinase receptors that share structural homology and includes RON and SEA. SEA is a RON homologue expressed in chicken tissues. In 1993, RON was discovered two years after MET by Ronsin *et al.* at the Institut de Biologie (Nantes, France). They isolated a cDNA encoding a novel protein that successive sequence analysis revealed to be similar to MET proto-oncogene and named it RON (Recepteur d'Origine Nantais) (144). RON is also known as macrophage-stimulating receptor-1 (MSTR1) or stem cell derived tyrosine kinase (STK) in mice (145). RON is synthetized as a 185 kDa single chain inactive precursor that is exposed on the cellular surface after proteolytic cleavage within the endoplasmic reticulum. The mature receptor is a disulphide-linked heterodimer formed by a 35 kDa α chain and a 150 kDa α chain (146). As for MET, the α chain is completely extracellular and contains a SEMA domain, which retains the ligand-binding activity (147). The β chain is transmembrane and comprises a

juxtamembrane region, a tyrosine kinase domain and a C-terminal regulatory tail (Figure 1.4). RON displays a 25% homology with its sibling MET within the extracellular region, and 63% homology in the tyrosine kinase domain, with an overall 34% identity (46).

RON activation occurs upon binding by its ligand macrophage stimulating protein (MSP). This induces receptor dimerization followed by auto-phosphorylation of the residues Tyr1238 and Tyr1239 located within the kinase activation loop ('catalytic' tyrosines) and sequential trans-phosphorylation of Tyr1353 and Tyr1360 within the C-terminal regulatory tail ('docking' tyrosines). Upon phosphorylation, the docking tyrosines recruit several intracellular adaptors or molecules responsible of transducing the downstream signaling cascade. Similar to the MET docking tyrosines, Tyr1353 and Tyr1360 are essential in triggering signal transduction, which has been confirmed through site directed mutagenesis. These residues completely abolish any MSP-mediated biological effects, despite persistent RON kinase signaling activity (148).

The RON specific ligand is the hepatocyte growth factor-like protein/macrophage stimulating protein (HGFL/MSP) (149,150). The ligand was first described in the late 1970s as a serum protein capable of inducing spreading, migration and phagocytosis of mouse macrophages and, a decade later, HGFL/MSP was purified from human blood plasma (151–153). In 1994, two laboratories independently elucidated that MSP is the RON ligand (154,155), unveiling the MSP/RON signaling axis. MSP is an 80 kDa heterodimer and belongs to the plasminogen gene family with which it shares the main structural features. The inactive zymogen form of MSP is expressed in lungs, adrenal glands, placenta and kidney. The primary source of MSP is in the liver, where it is constantly produced by hepatocytes and Kupffer cells (the resident macrophage population of the liver) and, subsequently, released into the blood stream as a single chain inactive precursor (pro-MSP) at a concentration of approximately 400 ng/ml (Figure 1.4). Different from the HGF/MET pathway that works in a paracrine fashion, MSP works in an endocrine fashion and activates RON at distant sites. Pro-MSP is converted by proteolytic cleavage into a mature disulfide-linked heterodimer made of a 50 kDa α chain and a 35 kDa β chain. The α chain contains four Kringle domains and regulates RON functional activities, while the β chain encompasses a serine protease-like domain devoid of enzymatic activity and necessary for receptor binding (156,157). In contrast to MET, the RON high affinity binding site for MSP lies within the β chain exclusively. Pro-MSP cleavage, and resulting activation, is performed by various plasma and membrane bound proteases with different cell and tissue localization including HGFA (158), matriptase (159), hepsin (160) and various coagulation cascade proteases, such as kallikrein, factor XIIa and factor XIa (155,161). The susceptibility of pro-MSP to different proteases suggests that its conversion into the mature form could occur in various tissues, depending on different physiological and pathological conditions (162). It is important to stress once more that the same proteases that activate MSP, also activate HGF. This could suggest a physiological and/or pathophysiological role for combined HGF/MET and MSP/RON signaling, which will be discussed later.

Although RON signals through mechanisms analogous to MET and generates similar biological responses, it has been shown that RON has a weaker kinase activity compared to MET and other tyrosine kinase receptors. Therefore, it can be surmised that RON is probably less efficient in activating the downstream signaling and, thus, the resulting biological responses are generally weaker (163). RON is normally expressed at low levels in epithelial cells and macrophage population, where it regulates cellular

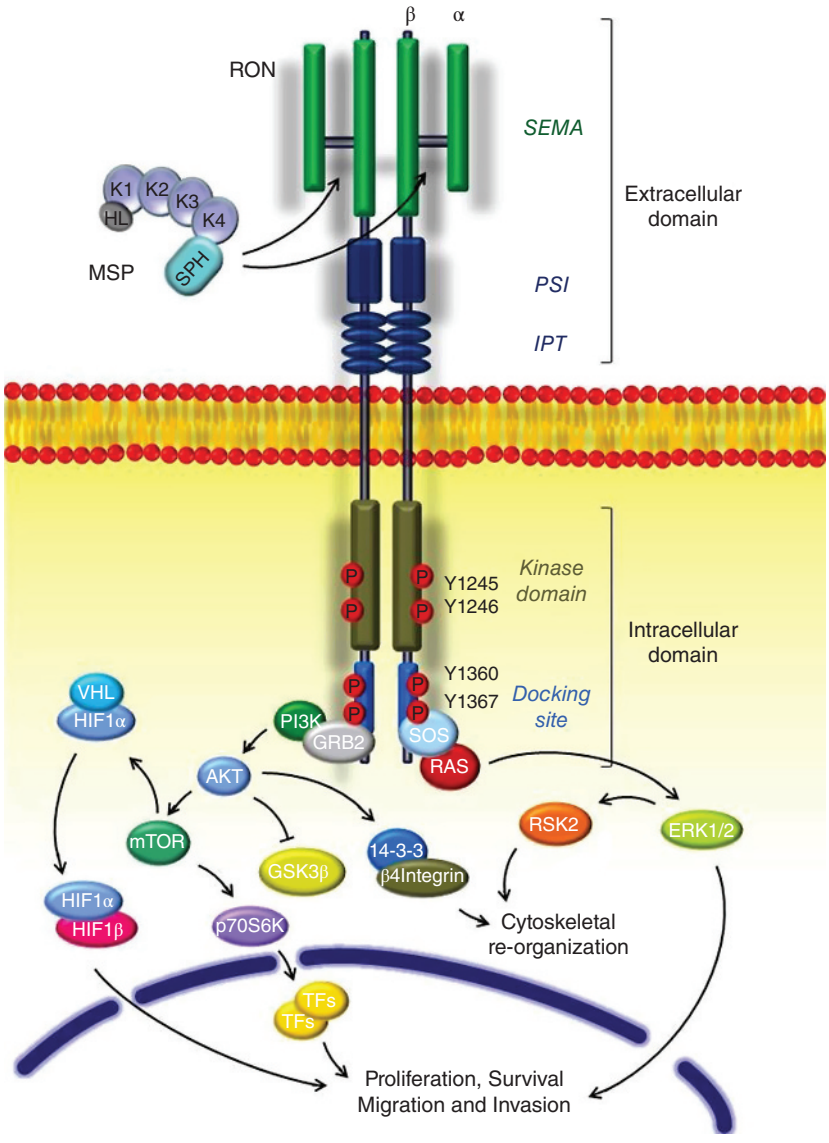

Figure 1.4 RON tyrosine kinase receptor and its ligand MSP: structure, signaling and biological activities.

MSP-induced RON dimerization is followed by receptors activation. Upon phosphorylation, the tyrosines of the docking site recruit a number of cytoplasmic molecules responsible of regulating RON-mediated biological activities such as cytoskeletal reorganization, invasion, migration, proliferation and survival.

AKT: AKT8 virus oncogene cellular homolog; ERK: extracellular signal-regulated kinase; GRB2: growth factor receptor-bound protein 2; GSK3β: glycogen synthase kinase 3β; mTOR: mammalian target of rapamycin; HF1α: hypoxia inducible factor 1α; HL: hairpin loop; IPT: immunoglobulin-plexin-transcription structures domain; MSP: macrophage stimulating protein; K: Kringle; PI3K: phosphatidyl inositol 3-kinase; PSI: plexin, semaphorin and integrin cysteine-rich domain; RAS: rat sarcoma small GTPase; RSK2: p90 ribosomal S6 kinase 2; SEMA: semaphorin domain; SOS: son of sevenless; SPH: serine-protease domain; VHL: von Hippel-Lindau protein.

proliferation, adhesion, motility and apoptosis, all events resulting in the invasive growth genetic program (45). The invasive growth genetic program occurs under specific physiological conditions (i.e. embryonic development) and, when aberrantly regulated, contributes to tumorigenesis and metastasis dissemination. The requirement for RON during embryo formation has been demonstrated in mice. Homozygous knock-out mice die very early *in utero* during the peri-implantation stage (164), whereas heterozygous mice are vital and develop to adulthood but display an altered inflammatory response, are more prone to endotoxic shock, and are unable to regulate nitric oxide (165). Another RON knock-out mouse model was generated by deleting the intracellular portion of the receptor, except for the first eight amino acids (RON TK-/-). In this situation, mice were viable and fertile but, again, showed an enhanced response to inflammatory stimuli (165), which confirmed the role of RON in the negative regulation of inflammatory response. No gross abnormalities were detected in a mouse knock-out model for the ligand (166).

The oncogenic potential of RON has been extensively investigated. First, MET mutations found in hereditary papillary renal carcinomas were reproduced in corresponding RON residues and then ectopically expressed into NIH3T3 cells. Over-expression of either wild-type RON or active mutants resulted in acquired transforming potential *in vitro* and was tumorigenic in nude mice (167). Along the same line, the introduction in RON sequence of the two mutations, Asp1232Val and Met1254Thr, found to affect the tyrosine kinase domains of KIT and RET receptors, in human mastocytosis and multiple endocrine neoplasia type 2B, respectively, generated constitutively active receptors when transfected into NIH3T3 fibroblasts. Also, it induced transforming activity as measured from focus formation and anchorage-independent growth (188). A chimeric TPR-RON, mimicking the oncogenic TPR-MET, was generated to investigate its transforming potential. The TPR fusion generated a constitutively active receptor that was inducing a pro-invasive phenotype, causing cell dissociation, motility and invasion of extracellular matrices, but unable to transform NIH3T3 cells. The deficit in transforming activity was likely the result of less potent catalytic activity of TPR-RON compared to that of TPR-MET, which results in approximately three times lower activation of the MAPK signaling cascade (163).

The role of RON in tumorigenesis was also investigated by crossing RON TK-/- mice (described earlier in the manuscript) with mice predisposed to develop mammary cancers. As a consequence of polyoma virus large T antigen under the control of the mouse mammary tumor virus promoter (MMTV-pMT), the mice developed mammary tumors from the age of three months. In this model, loss of RON signaling significantly delayed tumor initiation and growth, and reduced the number of metastasis (169). Similarly, in the context of skin carcinogenesis, RON TK-/- mice were crossed with v-Ha-RAS (Tg.AC) transgenic mice carrying a v-Ha-RAS gene fused to the promoter of the zeta globin gene. When these mice are treated with the chemical 2-O-tetradecanoylphorbol-13-acetate (TPA), they develop a vast number of papillomas, some of which undergo malignant transformation. Loss of RON resulted in increased number of papillomas, but decreased the number of papillomas that underwent malignant conversion (170). Finally, mice over-expressing RON in the lung under the lung specific surfactant C promoter (SPC) develop multiple adenomas at an early age (171), although the adenomas do not progress to malignant tumors. The animals over-expressing the receptor

under the MMTV promoter develop hyperplastic mammary glands, 100% of female animals develop mammary tumors and 90% of the mammary tumors metastasise to liver and lungs (172).

1.3.2 RON Mediated Signaling

Interaction between MSP and RON triggers RON kinase dimerization and transphosphorylation like MET, followed by downstream intracellular signaling pathways responsible for mediating a variety of cellular responses. The signaling activation cascade is extremely similar to that activated by MET, yet comprises distinct intracellular molecules including, among others, β-catenin (173) and 14-3-3 (174). Ultimately, the interaction with different intracellular mediators and the extent of such contacts result in specific RON-mediated biological responses.

As for MET, the cytoplasmic transducers can be recruited either directly, through interaction of their SH2 domains with the phospho-tyrosines located within the docking site (175), or indirectly, via scaffolding proteins such as GRB2 (22).

Activated RON receptors coordinate cellular proliferation, protection from apoptosis and invasive growth mainly through RAS-ERK and PI3K-AKT pathways. On the one hand, activation of RAS, recruited and phosphorylated by SOS, results in ERK1 and 2 activation which, in turn, stimulates p90 ribosomal S6 kinase 2 (RSK2). RSK2, by regulating gene transcription and inducing cytoskeletal re-organization, acts as a molecular switch for migration and invasion. On the other hand, activated AKT inhibits, by phosphorylation of a serine residue, GSK3β connecting RON with the β catenin pathway (173). Xu *et al*. showed that activated β-catenin cascade is one of the pathways involved in tumorigenic activities mediated by the oncogenic RON variants. Certainly, they showed that NIH3T3 cells, expressing mutated RON receptors, display increased tyrosine phosphorylation and, thus, increased stability. Also, cytoplasmic accumulation of β-catenin translocates into the nucleus and up-regulates expression of two target genes, MYC (myelocytomatosis oncogene cellular homolog) and cyclin D1. Consequently, β-catenin silencing (via siRNA) reduced significantly cellular proliferation, focus-forming activities, and anchorage-independent growth (173). In addition, AKT links RON to mTOR and 14-3-3. RON-induced mTOR phosphorylation results in the release of HIF1α from the von Hippel-Lindau (VHL) protein. In turn, HIF1α factors are free to bind hypoxia response elements (HRE), thereby inducing RON transcription (174,176). At the same time, active mTOR stimulates p70S6 kinase (p70S6K), which phosphorylates a plethora of transcription factors (TFs), leading to increased gene expression (162). Finally, AKT phosphorylates 14-3-3, which displaces α6β4 integrin from its location at hemidesmosomes (structures supporting cell adhesion) and relocates it to lamellipodia, resulting in increased cell motility (194,197) (Figure 1.4).

Similar to MET, RON activation in normal cells is a transient event; activated receptors are usually down-regulated by tyrosine specific phosphatases (PTP). Although RON-specific phosphatases have not yet been identified, the homology between MET and RON suggests that the same or structurally related enzymes may dephosphorylate both receptors and, as in MET, that the juxtamembrane region in RON may serve as negative regulatory domain. It has been shown that stimulation by MSP recruits CBL ubiquitin ligase to the multifunctional docking site, as well as to a juxtamembrane

tyrosine auto-phosphorylation site; CBL, in turn, polyubiquitylates RON molecules and induces receptors endocytosis and degradation (178).

1.3.3 Cross-talk between RON and other Receptors

Interaction between receptors of different types (cross-talk) has been shown to play a major role in receptor activation regardless of ligand stimulation, although connected receptors still retain some degree of responsiveness to ligands. Indeed, several works have shown that RON interacts with other receptor types including EGFR (179), interleukin-3 receptor (180), and plexins (43). However, RON's primary partner remains its sibling receptor MET, and the interactions between RON and MET has been extensively investigated. Follenzi *et al*. showed that:

1) trans-phosphorylation of MET and RON receptors occurs directly;
2) MET/RON complexes exist on the plasma membrane prior to ligand-induced dimerization; and
3) kinase deficient RON is sufficient to inhibit mutant MET-induced transforming activity in NIH3T3 cells, suggesting that RON supports MET transforming potential (45).

More recently, a study conducted in four different cancer-derived cell lines displaying MET amplification demonstrated that oncogene addiction to MET requires RON that is constitutively trans-phosphorylated by MET (46). Moreover, RON and MET have been co-expressed in a number of human cancers including ovarian (181), breast (182) and bladder (183) cancers, and their cross-talk confers a selective advantage to cancers growth and progression.

1.3.4 RON Activation in Human Cancers

Aberrant RON activation in cancer cells is mainly due to receptor over-expression or increased gene copy number, alternative splicing and protein truncation. Point mutations and ligand activation loops, although rare, might also occur.

As in the case for MET, much evidence advocates that RON may play a key role in cancer formation and progression. It has been found that RON is over-expressed in several human cancer cell lines where it controls migration and invasion (184,185). Moreover, the receptor has been found over-expressed and constitutively activated in several different human cancers including liver (186), lung (87), colon (187), stomach (188), ovary (181), kidney (189), pancreas (184) and bladder carcinomas (183). Furthermore, receptor over-expression has been reported in over 50% of breast neoplastic lesions, whereas it is barely detectable in normal mammary epithelial cells and benign adenocarcinomas (190). Likewise, RON over-expression usually correlates with a poor clinical outcome, and, in breast and bladder tumors, it is associated with decreased disease-free survival.

RON oncogenic potential has been shown in cancer cell lines through over expression of the receptor inducing an increase in proliferation rates (167,190), motility and invasion upon MSP treatment (187). This is also seen in transgenic mice where wild type RON expression in the distant lung epithelial cells induces tumorigenesis (171,191). On the contrary, down-regulation of RON expression (by shRNA) impairs cellular proliferation and motility while enhancing apoptosis (192). It has been also described that RON can synergize with other oncogenes, such as polyoma virus middle T antigen and

RAS, augmenting their oncogenic potential (170). The understanding of RON's contribution in tumor formation and progression continues to expand. Eyob *et al.* recently reported that MSP/RON signaling axis, by suppressing host antitumor immunity, is a key mediator in the conversion of micrometastases into bona fide metastatic lesions. They showed that blocking RON using a RON-selective kinase inhibitor resulted in a strong antitumor response mediated by CD8+ T-cells, which prevented outgrowth of lung metastasis, even when administered after micrometastatic colonies had already been established (193).

Akin to MET, several molecular mechanisms have been proposed to be responsible for RON constitutive activation:

1) autocrine and paracrine loops (87);
2) receptor over-expression, which can be achieved via gene amplification, enhanced transcription or post-transcriptional modifications; and
3) through interaction with other cellular surface receptors.

Data on genetic lesions of RON in human cancers are very scarce, since somatic mutations and copy number variations have been rarely observed. Therefore, RON oncogenic potential, as a consequence of activating point mutations described earlier, was initially shown with the experimental reproduction of mutations found in MET, KIT and RET, in RON homologous residues (168). Only relatively recently, Catenacci *et al.* reported a somatic point mutation Arg1018Gly within the juxtamembrane domain in 11% of gastro-oesophageal adenocarcinoma (194).

The RON receptor has also been found to be constitutively activated in both cancer-derived cell lines and human cancers as a consequence of a novel post-transcriptional molecular mechanism. The truncated receptors are produced via alternative splicing or alternative initiation sites. The first RON splice variant was described in human gastric cell line KATO-III. This variant lacks 49 amino acids corresponding to exon 11, coding for 3 cysteines located within the extracellular domain of the β chain and responsible for the establishment of intramolecular disulphide bridges. The resulting uneven cysteine number causes receptor oligomerization and consequent constitutive activation (195). The above described variant is known as RONΔ165. Afterwards, three other RON splicing variants have been described: RONΔ160, RONΔ155 and RONΔ55; all retain a strong oncogenic potential (196). Another RON variant, known as short-form RON (SF RON), originates from an alternative start site located within intron 10. Initially described in mice, the short form was found to be expressed in human breast and ovarian cancers as well as in many different cancer-derived cell lines. SF RON's expression results in enhanced proliferation and motility together with acquired anchorage independence growth capability's more aggressive behaviour that contributes to tumor progression (197).

1.4 Targeting MSP/RON as a Therapeutic Approach in Human Cancer

As detailed above, RON is over-expressed in many human cancer types, where increased expression leads to aberrant activation and contributes to tumor onset and progression. These findings identify RON as a clinically relevant therapeutic target in several human cancers and led to the development of agents able to inhibit its function and activity. As

for MET, various strategies are being investigated, the most well-studied being small molecular kinase inhibitors and neutralizing antibodies to the receptors able to block the downstream signaling cascade. As mentioned earlier, the small molecule protease inhibitors of HGF activation (97), also inhibit the activation of MSP, since pro-HGF and pro-MSP are both substrates for these enzymes in the pericellular space. Yet, strategies to target RON lag behind those for MET and, subsequently, drugs designed to specifically target RON are still in early stages of development (198).

Most of the small molecule kinase inhibitors developed against RON also inhibit the closely related kinase MET. The high sequence homology (63%) within their kinase domains makes it challenging to design inhibitors with high specificity (199). Some compounds (multi-target) have an even greater spectrum of activity and block several tyrosine kinase receptors including VEGFR and AXL. Nevertheless, various attempts have been performed to produce molecules that selectively block RON, with little or no residual activity against other receptors (200–202). Furthermore, it should be noted that when either MET or RON are individually inhibited, compensatory mechanisms occur and result in increased activity of the other receptor. Therefore, it is likely that simultaneously targeting RON and MET will be preferable to standalone inhibition of either target (47).

In parallel, the importance of the RON extracellular domain in ligand binding to MSP, prompted the development of anti-RON agents directed against this region; they include monoclonal antibodies, drug-conjugated antibodies and peptides. ID-1 and ID-2 were the first two antibodies reported to target the RON receptor. They were generated by immunizing Balb/C mice using MDCK (Madin-Darby Canine Kidney Epithelial Cells) cell line transfected to stably express RON (Re7). ID1 and 2 displayed no cross-reactivity with MET, were able to inhibit RON phosphorylation, and prevented MSP-induced migration in a human colon cancer derived cell line (203).

Following this original work, a number of murine antibodies targeting RON have been developed (204–206). Although *in vitro* and *in vivo* studies showed that some murine antibodies had valuable antitumor activity, none of them were humanized or entered clinical trials, thus far. The first fully human monoclonal antibody against RON, developed from ImClone Systems, was IMC-41A10. The antibody is able to inhibit MSP/RON binding, reduce ligand-induced receptor phosphorylation, and down modulate the downstream signaling. Moreover, IMC-41A10 reduces tumor growth of mouse xenografts obtained by injecting lung, colon and pancreatic cancer cell lines subcutaneously in immunocompromised mice (207). Nevertheless, no clinical trials were pursued using this antibody. Another human antibody against RON developed by ImClone Systems is IC-RON8 or Narnatumab (208). Preclinical studies showed that Narnatumab could down-regulate RON expression, inhibit MSP-mediated RON phosphorylation (without showing any agonistic activity) and, subsequently, prevent in pancreatic cancer cells MSP-driven cellular migration and tumorigenicity. Narnatumab entered a clinical trial, but was discontinued soon after without explanation (209).

1.5 Concluding Remarks

MET and RON are structurally related tyrosine kinase receptors. Either through ligand-dependent or constitutive activation, they generate a complex intracellular signaling

cascade that plays a pivotal role in controlling tissues homeostasis under physiological conditions (i.e. embryonic development and organ regeneration). Their deregulation contributes to tumor onset, progression and, above all, metastatic spread. The two sibling receptors have also been linked to anti-cancer agent resistance in patients, and MET has been associated with maintenance of cancer stem cells. Indeed, exerting a dual role as necessary oncogenes for some tumor types and adjuvant, pro-metastatic genes for others, MET, and to a lesser extent, RON, become very attractive candidates for targeted therapeutic intervention, as a reasonable number of patients would benefit from their inactivation. The accumulation of work in this area has spawned several clinical trials using MET and RON inhibitors, either alone or in combination. Inhibitor combinations where multiple genes/pathways are down-regulated simultaneously, show promising results in terms of anti-tumor efficacy and improvement of clinical outcomes. However, the clinical results obtained using anti-MET agents emphasize the need to stratify patients to select those more likely to respond. Continuous efforts are also needed to identify the molecular basis of acquired resistance and to develop therapeutic strategies able to prevent it. Novel approaches to targeting ligand-mediated MET and RON cancer cell signaling, such as inhibition of HGF and MSP ligand activation with small molecule protease inhibitors, are promising but still in the early stages of preclinical development.

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