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The MET Receptor Tyrosine Kinase in Invasion and Metastasis

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Various cytokines and soluble growth factors upon interaction with their membrane receptors are responsible for inducing cellular proliferation, differentiation, movement, and protection from *anoikis* (a planned suicide activated by normal cells in absence of attachment to neighboring cells or extracellular matrix (EMC)). Among those soluble factors a major position is exerted by hepatocyte growth factor (HGF) together with its receptor MET and macrophage-stimulating protein (MSP) in cooperation with its receptor RON.
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Both MET and RON are tyrosine kinases crucially involved in the control of the "invasive growth" (Giordano et al., 2002). Under physiological conditions such as embryonic development and organ regeneration, they contribute to establishing the normal tissue patterning by orchestrating cellular proliferation, disruption of intercellular junctions, migration through the EMC and protection from apoptosis. In transformed tissues, receptor deregulation is responsible for cancer progression and metastasis formation and dissemination. Either upon ligand stimulation or receptor constitutive activation, cancerous cells are induced to leave the primary tumor, degrade the basal membrane, move towards different organs and there give rise to metastasis (Trusolino et al., 2001; Giordano et al., 2002).

MET and Its Ligand HGF: The Structure

MET tyrosine kinase is a disulphide-linked heterodimer originated from the proteolytic cleavage of a single chain precursor. The heterodimer is formed by a single-pass transmembrane beta chain (145 kDa) and a completely extracellular alpha chain (50 kDa). The extracellular segment contains a Sema domain, an atypical motif made by over 500 amino acids, which has a low affinity binding activity for the ligand. The extracellular portion comprises also a cysteine-rich domain (Cys domain) known as Met-related sequence (MRS), and four immunoglobulin-like structures (IPT domain), a typical protein–protein interaction region. The intracellular portion of the receptor is made of a juxtamembrane section followed by a catalytic site and a C-terminal regulatory tail. The juxtamembrane segment is vital for receptor downregulation (Trusolino and Comoglio, 2002). It contains a serine residue (Ser 985) that upon phosphorylation is responsible for inhibiting the receptor kinase activity, and a tyrosine (Tyr 1003) capable of binding CBL. CBL proto-oncogene is a ubiquitin ligase that promotes receptor polyubiquitination resulting in MET degradation (Peschard et al., 2001; Abella et al., 2005). The juxtamembrane portion is flanked by the catalytic site, which contains two tyrosines (Tyr 1234 and 1235) responsible for regulating the enzyme activity. Lastly the C-terminal tail contains two tyrosines (Tyr 1349 and 1356) that when phosphorylated create a multifunctional docking site capable of recruiting a vast cohort of intracellular adaptors in charge of transducing inside the cell the signaling triggered by the ligand–receptor interaction (Ponzetto et al., 1994) (Fig. 1). These two tyrosines have been demonstrated to be both essential and sufficient to execute MET physiological functions (Maina et al., 1996) and to elicit MET oncogenic potential (Bardelli et al., 1998).

MET high affinity ligand is known as scatter factor (SF) or HGF. SF and HGF were identified independently as a factor capable of inducing scatter (a complex mechanism 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) of epithelial cells (Stoker et al., 1987; Gherardi et al., 1989) and as a potent growth stimulator for primary hepatocytes kept in culture (Nakamura et al., 1986), respectively. The two molecules were subsequently proved to be identical (Naldini et al., 1991). SF/HGF belongs to the plasminogen family. It contains a hairpin loop, followed by four kringle domains (80 amino acid, double looped structures stabilized by intramolecular disulphide bridges), flanked by an activation portion and a serine protease domain devoid of proteolytic activity. SF is generated as a single chain inactive precursor (pro-SF) present in the ECM of nearly all tissues. Its activation occurs locally upon proteolytic cleavage operated by enzymes such as: urokinase-type plasminogen activator (uPA), coagulation factor XII, trombin, or XII-like factor (Zanetti et al., 1998) (Fig. 1).

The Invasive Growth Program

Cancer is a multistep process that results from the accumulation of mutations which either inactivate tumor-suppressor genes (such as p53, pRB, or adenomatosis poliposis coli (APC)) or activate dominant proto-oncogenes (for instance RAS or PI3K) (Hanahan and Weinberg, 2000; Vogelstein and Kinzler, 2004). These aberrant events free cells from proliferative control and allow primary tumor formation. The initial tumor growth is followed by metastatic spread and ultimately metastasis, that are resistant to conventional therapies, are the major cause of death from cancer. Over the past decades much has been learned about the genetic and biochemical basis of the earlier stages of tumorigenesis and the subsequent metastatic colonization. The ability of neoplastic cells to invade the adjacent tissues, survive in foreign compartments, and proliferate to settle at distant sites defines a biological program known as "invasive growth" (Fig. 2).

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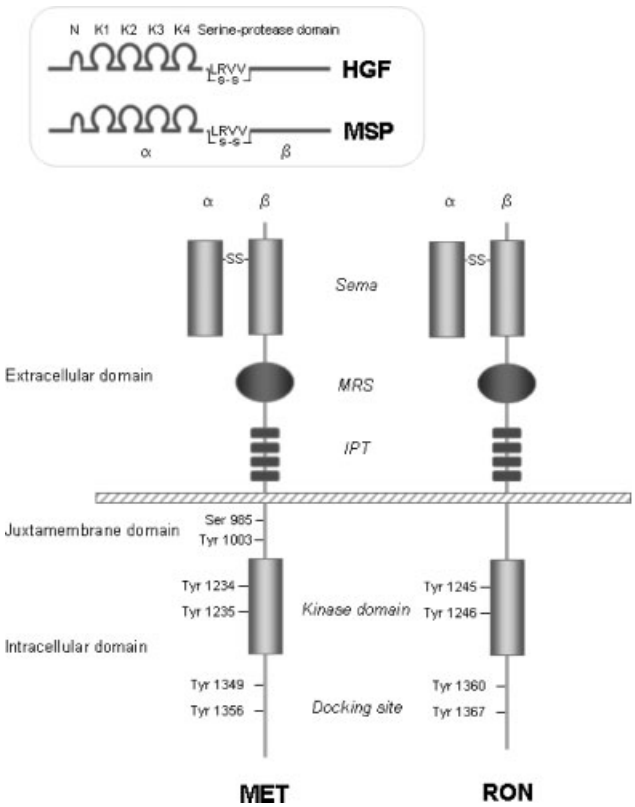

Fig. 1. Structural features of MET and RON receptors and their ligands. MET and RON tyrosine kinases are disulphide-linked heterodimers made of a single-pass transmembrane beta chain and a completely extracellular alpha chain. The extracellular region contains a Sema domain, a cysteine-rich domain called Met-related sequence (MRS), and four immunoglobulin-like structures (IPT domain). The intracellular portion of the receptor is made of a juxtamembrane section followed by a catalytic site and a C-terminal regulatory portion. MET and RON ligands, HGF and MSP respectively (also known as scatter factors) contain a hairpin loop (N), followed by four kringle domains (K1–K4), flanked by an activation portion and a serine protease domain devoid of proteolytic activity.

The invasive growth program does not occur only in cancer cells, but happens also in normal physiological conditions throughout embryonic development and organ formation and in adult life where it regulates inflammatory responses and wound healing processes as part of the acute injury repair. It consists of several stages, each of them occurring at a particular time and place, harmonically orchestrated to allow germ layers, in the embryo, and tissues, in the adult, reorganization. During embryo patterning the invasive growth program orchestrates complex events such as gastrulation, in which the embryonic epithelium originates the mesoderm; morphogenesis of epithelia; angiogenesis; nervous system formation, and myoblasts migration. Indeed all these events require cells to proliferate, migrate, overcome cell death, invade the surrounding tissues and reorganize 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 through the extracellular environment. Ultimately cells loose their epithelial phenotype to acquire a mesenchymal one. EMT is followed by cell migration and generation of new structures. All these events can be recapitulated in vitro culturing Madin–Darby canine kidney (MDCK) epithelial cells. Epithelial cells grown in suspension in a collagen matrix upon SF stimulation generate ramified tubules made of polarized epithelial cells (instead of the spherical cysts obtained under control conditions) that invade the three-dimensional region; this process is known as "branching morphogenesis" (Montesano et al., 1991).

During embryonic formation MET and its ligand HGF play an essential role coordinating muscle development, nervous system organization, bone remodeling and angiogenesis (Birchmeier and Gherardi, 1998). The formal prove that expression of either HGF or MET is mandatory during embryo development has been attained by the generation of knockout mice for either the ligand or its receptor. The null mutant mouse embryos are not vital and die in utero because of severe placental development (Schmidt et al., 1997b) and liver, muscle (Maina et al., 1996) and nerve defects. The mice show smaller livers due to loss of parenchymal cells, defects in the directional migration of myoblasts from the somites to the limbs (Birchmeier and Gherardi, 1998) and reduced and compromised nerve outgrowth and branching (Maina et al., 2001). As mentioned, in adult life MET has also an active role in orchestrating cell proliferation and migration during acute injury repair (Nakamura et al., 2000; Huh et al., 2004) when cells at the wound edge reprogram themselves and restart dividing prior to migrate towards the cut to regenerate the lacking tissue.

In other words invasive growth is a program of epithelial motility and morphogenesis that comprises several steps that occur at a particular time and place. All these events are required during embryogenesis for correct embryo development and in adult tissues to overcome injuries, but contribute to tumorigenesis and metastasis when aberrantly regulated.

The Invasive Growth Signaling

As previously mentioned MET triggers a broad spectrum of biological responses through its multifunctional docking site. When phosphorylated the two tyrosines in the receptor tail recruit numerous intracellular adaptors interacting with their SH2 domains (Ponzetto et al., 1994). The binding with the receptor can be either direct, such as for Shc (Pelicci et al., 1995), Src, Grb2, and the p85 regulatory subunit of PI3K (Ponzetto et al., 1994), or indirect through the scaffolding protein Gab1 (Weidner et al., 1996). Gab1 lacks an intrinsic enzymatic activity but upon interaction with the receptor becomes phosphorylated and provides binding sites for several proteins involved in MET signaling cascade (Trusolino and Comoglio, 2002). Gab1 is the major director of MET signaling and several biochemical studies have demonstrated that it is necessary to induce MET cellular responses. A further prove of its importance has been provided by genetic experiments: knockout mice for Gab1 phenocopies MET knockout animals (Sachs et al., 2000). The different adaptors are responsible for generating MET specific biological activities, and their harmonic coordination results in a unique response. In order to occur the invasive growth program necessitates the integrity of the entire transduction machinery.

Many works have dissected MET induced signaling either using site direct mutagenesis (Ponzetto et al., 1994) or inhibitors acting on selected pathways (Birchmeier et al., 2003). Shc and Grb2 link MET with the RAS-mitogen-activated protein kinase (MAPK) pathway, essential to induce cellular proliferation and transformation (Ponzetto et al., 1996); in addition Grb2 recruits CBL, responsible for receptor

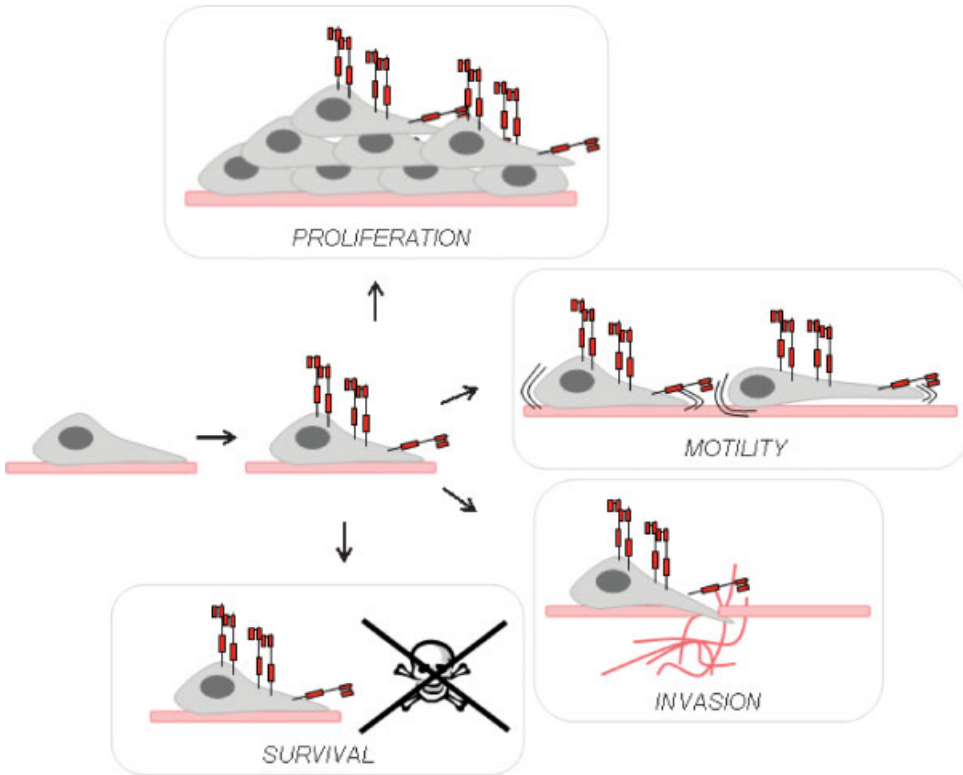

Fig. 2. The invasive growth program. Following events that ultimately result in MET over-expression and over-activation, a normal epithelial cell becomes tumorigenic and activates the invasive growth program. Invasive growth is a complex phenomenon which results from the combination of many different biological events, such as proliferation, motility, invasion, and survival, as summarized in the cartoon.

downregulation; Gab1 engages: PI3K, indispensable for cell motility (Royal et al., 1997) and protection from apoptosis (via AKT activation) (Xiao et al., 2001); PLCγ (Gual et al., 2000) and Shp2 (Maroun et al., 2000), necessary to induce branching morphogenesis through a sustained phosphorylation of MAPKs. Epithelial tubulogenesis requires also STAT3, a transcription factor directly recruited by the receptor (Boccaccio et al., 1998).

The Role of MET During Tumor Formation and Progression

It has been extensively demonstrated that when used in a deviant cellular environment and without spatial and temporal regulation, MET exerts a major role in tumor formation and progression. Cells which over-express either MET or HGF are tumorigenic when implanted into nude mice and become extremely metastatic (Rong et al., 1994), moreover transgenic mice for either MET or HGF develop metastatic tumors (Takayama et al., 1997) while, on the contrary, endogenously expressing cancer cells become less aggressive when MET is switched off. Accordingly, it was demonstrated that short hairpin RNA (shRNA) mediated MET knockdown in rabdomyosarcomas (RMS)-derived cell lines greatly affects cell proliferation, survival and invasion (Taulli et al., 2006). Furthermore in xenograft models of RMS MET silencing produced a dramatic reduction of tumor mass (Taulli et al., 2006). Similar results were obtained silencing MET in lung cancer cell lines harboring MET amplification. In those cell lines receptor silencing (once more achieved by shRNA technology) induced a significant growth inhibition (Lutterbach et al., 2007); notably the silencing sorted no effects on cell lines that did not display receptor gene amplification.

It has been extensively described, both in animal models and in normally occurring human cancers, that constitutive activation of MET can be achieved in three different ways: (i) with establishment of ligand-receptor autocrine loops; (ii) via receptor over-expression, and (iii) in presence of activating point mutations in the receptor coding sequence. Ligand-receptor autocrine circuits make cells independent from the need of growth factors (Ferracini et al., 1995); receptor over-expression triggers receptor oligomerization and reciprocal activation even in absence of ligands; point mutations generate constitutively active receptors. This last event is extremely uncommon; however, some missense point mutations have been described in MET coding sequence in certain human cancers. Particularly missense mutations located in the tyrosine kinase domain of MET were described in patients who suffer from hereditary and sporadic papillary renal-cell carcinomas (Schmidt et al., 1997a) and head and neck squamous-cell carcinomas (Cortesina et al., 2000), whereas alterations in the juxtamembrane region were mainly found in human gastric and lung cancers (Lee et al., 2000; Ma et al., 2005).

In a small number of tumors subtypes, it has been shown that the genetic lesion driving the receptor over-expression was increased gene copy number. MET amplification was initially described in gastric cancers (Kuniyasu et al., 1992; Kijima et al., 2002), tumors of the upper digestive tract such as esophageal (Miller et al., 2006) and biliary tract carcinomas (Nakazawa et al., 2005) and more recently in lung cancers (Engelman et al., 2007; Lutterbach et al., 2007).

Enhanced MET expression has been described in various solid tumors such as osteosarcomas (Ferracini et al., 1995),

renal (Natali et al., 1996), ovarian (Di Renzo et al., 1994), hepatocellular (Takeo et al., 2001), and non-small cell lung carcinomas (Olivero et al., 1996); liver metastasis of colon cancers (Di Renzo et al., 1995a); tumors of the upper gastrointestinal tract such as oral squamous cell (Morello et al., 2001), esophageal (Porte et al., 1998; Taniguchi et al., 1998), and gastric carcinomas (Taniguchi et al., 1998); pancreatic (Di Renzo et al., 1995b) and prostatic cancers (Humphrey et al., 1995). Receptor enhanced expression always correlates with poor prognosis. Yet in the majority of the tumors, MET over-expression occurs at a transcriptional level. In the last years, the transcriptional mechanisms responsible for increased MET expression and activity have been extensively investigated and some of them have been unveiled; one of them is hypoxia, a condition of oxygen deficiency that can be found in the inner tumor part (discussed later on).

In tumors in which MET is over-expressed in the absence of any alteration in the coding sequence, it was proposed that the augmented receptor transcription results from mutations of upstream genes. It was demonstrated that various oncogenes (such as RAS, RET or EST), when activated, are able to induce MET over-expression (Ivan et al., 1997). In addition, MET promoter analysis revealed the presence of several (four) putative binding sites for members of the ETS transcription factor family, notorious to be involved in invasive growth. Indeed, in vitro experiments with cell lines stably transfected with ETS1 indicated that both MET mRNA and protein production is increased. Interestingly, it was further proved that the receptor in turn can induce ETS1 mRNA, showing that ETS proteins act both upstream and downstream MET. These data suggest that ETSs contribute to the invasive growth program through MET over-expression (Gambarotta et al., 1996).

In human colorectal cancers (CRC) it has been observed that MET expression is a component of the genetic program controlled by the WNT/β catenin pathway. In CRCs the loss of APC tumor-suppressor gene results in β catenin constitutive activation and MET over-expression. MET over-expression is an early event throughout the colorectal adenoma–carcinoma sequence, as MET can be detected in the earliest neoplastic lesions of CRC (Boon et al., 2002).

Additionally other pathways have been proposed to interplay with MET signaling, among them the ones triggered by Notch and Hedgehog, however up to now the underlying mechanisms remain partly unexplored.

MET and the Microenvironment

It is now widely accepted that the development of human cancers is not only due to the sequential accumulation of somatic mutations but results from the cross-talk between cancer cells and the tumor microenvironment, which consists of ECM, blood vessels, inflammatory cells, and fibroblasts (Bottaro and Liotta, 2003; Bhowmick et al., 2004).

It was initially assumed that MET promoter was able to respond to many different stimuli coming from the extracellular environment. Indeed, quite recently, it was unequivocally demonstrated that MET transcription could be modulated by oxygen tension in tissues. Michieli and co-workers (Pennacchietti et al., 2003) demonstrated that: (i) hypoxia stimulates transcription of MET, resulting in higher levels of protein expression; (ii) hypoxic regions of human tumors show increased expression of MET while MET expression becomes very low in proximity of blood vessels; (iii) hypoxia enhances HGF signaling; (iv) hypoxia cooperates with HGF in promoting branching morphogenesis and invasion. Equally MET over-expression by itself can recapitulate hypoxia-driven invasion whilst inhibition of MET over-expression is enough to block hypoxia-induced invasive growth. These data show that hypoxia promotes tumor invasion by sensitizing cells to HGF stimulation, providing a molecular basis to explain MET over-expression in human cancers (Pennacchietti et al., 2003).

Already in 1865 a close relationship between blood coagulation and cancer was postulated (Baron et al., 1998; Rickles and Levine, 2001). However, until very recently the mechanism underlying this association remained a mystery. Only lately, Boccaccio et al. (2005) demonstrated that MET is responsible for inducing a thrombohemorrhagic syndrome, providing for the first time a strong indication that cancer and hemostasis are connected. In their work the authors showed, using a transgenic mouse model of somatic oncogene delivery, that MET activation in liver causes both tumor formation and hemostatic unbalance. The slow developing hepatocarcinogenesis was anticipated by a first stage of blood hypercoagulation (venous thromboses) and followed by a second phase culminating in internal hemorrhages. The thrombohemorrhagic syndrome induced by MET is achieved through the upregulation of two genes: plasminogen activator inhibitor type 1 (PAI-1) and cycloxygenase 2 (COX-2) (Boccaccio et al., 2005). PAI-1, by blocking plasmin maturation, prevents fibrin degradation, while COX-2 regulates platelet functions. Ultimately, the two proteins are responsible for maintaining a provisional scaffold of fibrin polymerized around the cells, and guarantee a nest-like structure, which offers the support for neo-angiogenesis and provides solid trucks for migrating cells.

MET and Other Receptor Families

As previously described, MET is a transmembrane receptor exposed on the phospholipidic cellular membrane. It has been shown that it interacts in a dynamic way with other cellular surface receptors, and the output signal triggered by the receptor derives from the combination and integration of this complex network. Many different molecules have been proved to be MET partners, among them: integrin α6β4, the adhesive molecules CD44, the group of B plexins, Fas, and other tyrosine kinase receptors such as RON, EGFR, and ErbB2.

MET is constitutively associated with integrin α6β4. Upon ligand binding and receptor activation, the integrin becomes phosphorylated, recruits intracellular signal transducers (Shc, Shp2 and PI3K), and generates a supplementary platform necessary to promote the receptor invasive growth program (Trusolino et al., 2001).

MET is also associated with CD44, the transmembrane receptors for hyaluronic acid, one of the most abundant components of the EMC. It has been described that some CD44 isoforms, generated by alternative splicing, are able to trigger or enhance MET activation. Firstly, CD44v3, containing the alternatively spliced exon 3, strongly binds to HGF being responsible for concentrating the ligand at the cellular surface and presenting the ligand in multimerized complexes that lead to receptor over-activation (van der Voort et al., 1999). Secondly, CD44v6 isoform is required for ligand dependent MET activation as it promotes HGF–MET interaction through its extracellular domain. Moreover, CD44v6 cytoplasmatic portion is necessary to activate a number of MET intracellular transducers (Orian-Rousseau et al., 2002). Indeed the interaction between MET and CD44 results in an efficient functional cooperation that drives tumor growth and metastasis.

MET interacts constitutively also with members of class B of plexins. Plexins are transmembrane receptors for semaphorins, a large family of both soluble and membrane-bound ligands, which were originally identified as axon guidance molecules in the nervous system (Tamagnone et al., 1999). It was shown that stimulation of plexinB1 with its natural ligand Sema4D induces plexin clustering and consequent HGF-independent MET activation which results in the invasive growth response (Conrotto et al., 2004).

MET can also associate with Fas, the death receptor. The interaction with MET prevents Fas homo-oligomerization and clustering and ultimately results in protection for apoptosis (Wang et al., 2002).

Finally, other tyrosine kinase receptors can be MET partners. It was firstly shown in our laboratory that MET interacts with RON, member of the same family of tyrosine kinase receptors. 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 receptors and a kinase dead RON is sufficient to block MET transforming activity (Follenzi et al., 2000). These data show that, while specific for their ligands, SF receptors cross-talk and combine forces to provoke specific intracellular signaling pathways.

MET has been shown to interact with EGFR via mechanisms that occur at different levels. Firstly, in human hepatoma and epidermoid carcinoma tumor cell lines, it was demonstrated that EGFR activation, through its ligand TGFα, leads to phosphorylation and activation of MET even in absence of HGF (Jo et al., 2000). Accordingly, constitutive MET phosphorylation in the same cell lines was inhibited by neutralizing antibodies against TGFα and/or EGFR (Jo et al., 2000). Secondly, in thyroid carcinoma cell lines aberrant EGFR activation leads to MET over-expression and ligand-independent constitutive activation (Bergstrom et al., 2000). Finally, in human hepatocellular and pancreatic carcinoma cells, the MET receptor becomes tyrosine phosphorylated not only upon EGF stimulation but also in response to G protein-coupled receptor (GPCR) agonists. Both GPCR ligands and EGF are shown to increase the intracellular level of reactive oxygen species (ROS). ROS inhibit tyrosine phosphatases, thus resulting in receptor activation (Fischer et al., 2004).

Lastly, it is well established that MET cooperates with ErbB2 to support the invasive growth, although a directed physical interaction between the two tyrosine kinase receptors has never been observed. The two receptors synergize to enhance the malignant phenotype, promoting the break-down of cell–cell junctions and enhancing cell invasion, particularly in cancers where ErbB2 is over-expressed and HGF is a growth factor physiologically present in the stroma (Khoury et al., 2005).

Concept of Oncogene Addiction and Tailored Cancer Therapy

As previously said human cancer is a complex multistep process that arises throughout the acquisition of several different genetic alterations, which ultimately are responsible for activating oncogenes and inactivating tumor-suppressor genes. Unexpectedly, however, not all the mutations exhibit the same significance in the tumor context. Certain mutations turn out to be more important and the tumors become dependent on the activity of a solo-mutated gene for tumor maintenance. This concept was formulated in late nineties and is known as "oncogene addiction" (Weinstein, 2002). Oncogene addiction indicates the dependence of cancer cells on an overactive gene or pathway for cell survival and proliferation, and consequently the disruption of that gene/event leads to apoptosis and growth arrest. The oncogene addiction theory represents a milestone in cancer therapy. Virtually, to destroy any cancer it is enough to identify and turn off "the" driving gene.

It has been recently shown that a number of cancer cell lines displaying an increased gene copy number of some tyrosine kinase receptors, such as EGFR (Moroni et al., 2005) or HerB2 (Tagliabue et al., 1991; Konecny et al., 2006), in vitro are dependent on that gene for growth and survival and the addiction shown in culture is reflected in vivo by tumor behavior. Indeed tumors treated with anti-EGFR (Cetuximab or Panitumumab) or anti-HerbB2 (Trastuzumab) monoclonal antibodies display a remarkable "shrinkage" response when they bear genomic amplification of the EGFR or HerbB2 loci.

Recently, it has been proved that certain human cancers such as gastric cancers, RMS and lung cancers are addicted to MET, as MET is an absolute requirement for tumor proliferation and maintenance. To begin with, it was shown that gastric cancer cell lines with high levels of MET amplification, which results in constitutive receptor activation, are exquisitely sensitive to MET inhibitor PHA-665752. The compound induces massive apoptosis in all cell lines harboring MET amplification without affecting the ones lacking receptor amplification (Smolen et al., 2006). It was therefore demonstrated that gastric cancer cell lines in which MET is amplified are dependent on the kinase receptor for growth in vitro. Equivalent results were obtained in the two major histological subtypes of RMS tumors: embryonal RMS (ERMS), and alveolar RMS (ARMS) (Taulli et al., 2006).

Evaluating a panel of lung cancer cell lines for MET amplification, expression, and activation, it was shown that shRNA mediated MET knockdown resulted in growth arrest only in cell lines displaying increased MET copy number, while little or no effects were seen in cell lines with no amplification (Taulli et al., 2006; Lutterbach et al., 2007). These works unequivocally prove that cancer cell lines can be dependent on a single amplified tyrosine kinase for growth and survival, and MET is one of them.

Targeting MET as a Therapeutic Approach in Human Cancer

In the last few years, compelling evidence regarding the tyrosine kinase receptor MET and its family have proposed MET as an optimal pharmacological target in anti-cancer therapy: (i) the receptor central role throughout cancer formation and metastatic spread; (ii) the presence of somatic mutations in MET coding sequence or increased gene copy number in certain human cancers.

As noted earlier, invasion and metastasis are the main cause of dead for all cancer patients. Therefore, therapeutic approaches directed at impairing the metastatic spread are required. Thus, MET tyrosine kinase and its ligand HGF, responsible for triggering the metastatic process in a wide variety of human cancers, become very attractive therapeutic targets as a vast cohort of cancer patients would benefit from MET pathway inactivation. Moreover, some patients (the ones harboring MET mutated or amplified) would exclusively benefit from anti-MET targeted therapy.

Based on the above considerations, in the last decade, several attempts to interfere and abrogate MET signaling have been made. The strategies adopted to neutralize the receptor can be divided into three groups: (i) ligand antagonists, molecules created to specifically prevent the ligand binding to the receptor but unable to activate the downstream signaling; (ii) kinase inhibitors; and (iii) receptor competitors.

One of the earliest ligand antagonists to be developed was NK4. NK4 is a molecule bearing the N-terminal region and the four kringle domains of HGF. It competes with the ligand for the receptor binding but fails to activate the receptor, thereby blocking the downstream pathways and the biological outcomes. Moreover, the molecule was shown to strongly inhibit angiogenesis. As expected the compound, when used in experimental mouse tumor models (either administered in a conventional manner or delivered by gene transfer) (Heideman et al., 2004), effectively impaired tumor growth, invasion, metastasis, and angiogenesis (Matsumoto and Nakamura, 2003) (Fig. 3).

Uncleavable HGF, an unprocessable form of HGF can be classified as ligand antagonist. Michieli and colleagues

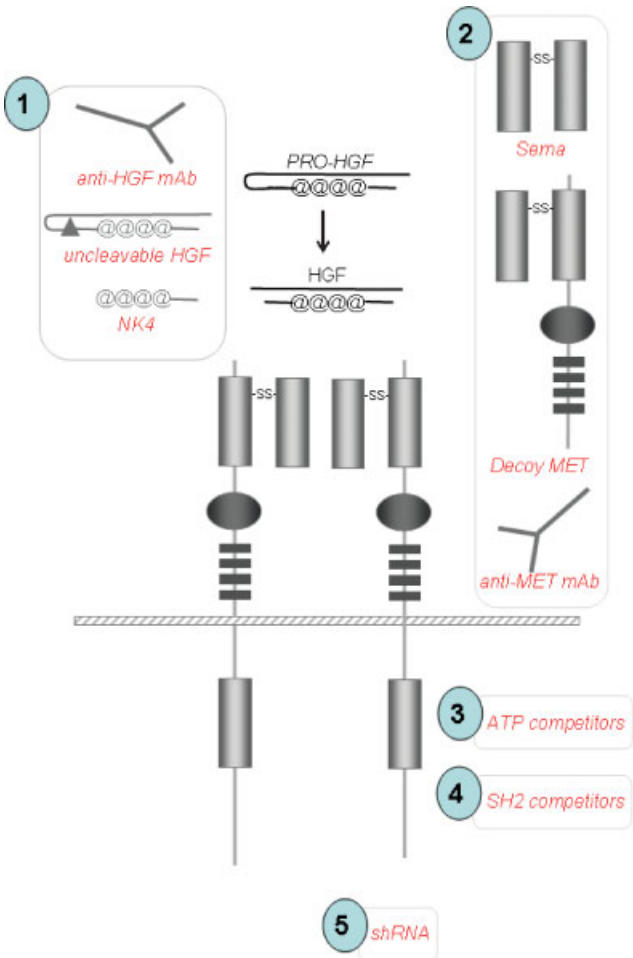

Fig. 3. Strategies to block MET signaling. Several strategies have been persued to block MET activity. They can be divided into different categories: (1) ligand antagonists, molecules created to specifically prevent the ligand binding to the receptor but unable to activate the downstream signaling (such as anti-HGF monoclonal antibodies, uncleavable HGF and NK4); (2) receptor competitors (among them Sema recombinant proteins, Decoy MET and anti-MET mAbs); (3) kinase inhibitors; (4) competitor peptides; (5) shRNA.

engineered the ligand introducing a single amino-acid substitution in the proteolytic site. The mutation 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: it competes with endogenous pro-HGF for the catalytic domain of the enzymes in charge of processing the precursors, thus inhibiting endogenous pro-HGF processing and maturation; and it binds to the receptor with high affinity displacing the mature ligand, thus impairing HGF induced activation. This latter mechanism is possible in a scenario in which the ligand precursors bind the receptors forming quiescent complexes that become active only upon pro-HGF cleavage and activation. In their work, the authors provide evidence that both local and system expression of uncleavable HGF inhibits tumor growth, impairs tumor angiogenesis and notably prevents metastatic colonization (Mazzone et al., 2004) (Fig. 3).

Neutralizing anti-HGF antibodies are also classified as ligand competitors. After a first pioneering work in which it was demonstrated that a minimum of three antibodies, each one acting on different HGF epitopes, was required to prevent MET tyrosine kinase activation (Cao et al., 2001), other works demonstrating that fully human monoclonal antibodies can individually bind and neutralize human HGF have been published. These antibodies are capable of binding HGF at subnanomolar concentrations, inhibiting HGF-mediated receptor phosphorylation, cellular proliferation, survival, and invasion. Importantly, in vivo these antibodies impair HGF-dependent tumor growth and cause significant regression of tumor xenografts (Burgess et al., 2006). Similarly, Kim et al. (2006) showed that blocking HGF–MET interaction with systemically administered anti-HGF mAb, it is possible to obtain a striking anti-tumor effect even within the central nervous system. The major limit regarding this first class of compounds is that they block only HGF-dependent receptor activation.

An alternative strategy to block tyrosine kinase receptors utilizes kinase inhibitors. The inhibitors are low molecular weight molecules able to compete for the ATP binding site of the receptor and prevent receptor transactivation and recruitment of the downstream effectors. These compounds comprise K252a, PHA-665752, and SU11274. K252a is a potent inhibitor of all the receptor tyrosine kinase family. It hampers MET-induced biological activities if used at nanomolar concentration. Interestingly, the inhibitor seems to be more effective when MET is mutated and specifically displays the mutation M1268T, found in papillary carcinoma of the kidney (Morotti et al., 2002). PHA-665752 is able to inhibit the catalytic activity of MET kinase with a relatively high specificity 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 abrogation of the main biological phenotypes elicited by the receptor (Christensen et al., 2003). More recently, it was shown that treatment with PHA-665752 triggers massive apoptosis in gastric cell lines harboring MET amplification and not in the ones with normal diploid gene copy number (Smolen et al., 2006). Finally, SU11274 is able to effectively block, affecting the kinase activity and subsequent signaling, two mutant forms of MET receptor: M1268T and H1112Y, while it does not hit two other variants, L1213V and Y1248H (Berthou et al., 2004). The main concern regarding the use of those inhibitors is their selectivity: none of them is fully specific for MET and possible side effects may occur (Fig. 3).

Another approach to block MET is the generation of receptor competitors. To this end, a whole bunch of compounds have been designed to bind the receptor extracellular domain, thus impairing receptor dimerization. Among these compounds there are monoclonal antibodies directed against the receptor. Following the binding these antibodies block receptor activation either by preventing the ligand binding or promoting receptor downregulation. Recently, Petrelli and co-workers showed that a monoclonal antibody directed against the extracellular portion of MET (DN30) induces receptor shedding, prevents MET activation and abrogates its biological activity. The mechanism through which DN30 efficiently downregulates MET is via the proteolytic cleavage of the extracellular portion, which results in "shedding" of the ectodomain, and cleavage of the intracellular domain, which is successively degraded by the proteasome machinery (Petrelli et al., 2006) (Fig. 3).

As it has been extensively demonstrated that the extracellular Sema domain is the one involved in ligand binding and receptor dimerization (Kong-Beltran et al., 2004; Wickramasinghe and Kong-Beltran, 2005), another way to neutralize the receptor activity is to develop soluble recombinant Sema proteins or anti-Sema antibodies. As expected these molecules produce a downregulation of the

downstream signaling triggered by the receptor, either in presence or absence of the ligand (Kong-Beltran et al., 2004) (Fig. 3). Analogous results were obtained engineering a soluble MET receptor (decoy MET) capable of preventing both ligand binding and receptor homodimerization (Michieli et al., 2004).

Alternatively at least two other strategies could be pursued to specifically block the receptor: (i) peptides competing with the intracellular transducers for the receptor's docking site (Fig. 3), and therefore blocking the downstream pathways (Birchmeier et al., 2003) and (ii) reduction of the number of receptor molecules exposed on the cellular surface. Suppression of MET signaling pathway has been achieved by targeting the tyrosine kinase receptor using shRNA technology and adenovirus vectors carrying small-interfering RNA constructs (Shinomiya et al., 2004). Firstly, Shinomiya et al. (2004) were able to drastically reduce MET expression in a subset of mouse, canine, and human tumors cell lines (among them human prostate cancer, sarcoma, glioblastoma, and gastric cancer cells lines), which results in impaired cell proliferation and viability, inhibition of scattering and invasion in vivo, and a substantial reduction of tumor growth in vivo. More recently, MET was silenced in ERMS and ARMS cell lines using shRNAs expressed in lentiviral vectors under an inducible promoter. MET downregulation significantly affected cell growth, survival, and invasion in vitro and promoted a considerable decrease in tumor growth in xenograft models (Taulli et al., 2006).

RON: MET Little Brother

Recepteur d'origine nantais (RON), also known as stem cell-derived tyrosine kinase (STK) in mice, belongs to the tyrosine kinase receptors family of which MET is the prototype (Comoglio and Boccaccio, 1996).

RON tyrosine kinase is a 185 kDa heterodimer formed by a 35 kDa alpha chain and a 150 kDa beta chain. The mature receptor presented on the cell surface originates from the proteolytic cleavage of a single chain precursor (Wang et al., 2003). As for MET, the alpha chain is completely extracellular and contains a Sema domain which retains a ligand-binding activity (Angeloni et al., 2004); the beta chain traverses the cellular membrane and comprises a juxtamembrane domain, a tyrosine kinase domain, and a C-terminal regulatory tail. RON displays with its sibling MET a 25% homology in the extracellular region and 63% in the tyrosine kinase domain (Fig. 1).

The ligand that binds to RON is the HGF-like protein/MSP (HGFL/MSP) (Han et al., 1991; Bezerra et al., 1993). MSP is an 80 kDa heterodimer and belongs to the plasminogen gene family with which it shares the main structural features. MSP is primarily produced in hepatocytes and released into the blood stream as a single chain inactive precursor at a concentration of approximately 400 ng/ml (Fig. 1). The precursor is activated by proteolytic cleavage operated by membrane bound proteases such as matriptases or members of the coagulation cascade (Wang et al., 1994).

RON signals through mechanisms analogous to the ones used by MET and generates similar biological responses. As for MET, also RON has been shown to be required during embryo formation: RON homozygous knockout mice die very early in utero during the peri-implantation stage (Muraoka et al., 1999); heterozygous mice are vital and mature to adulthood but show altered inflammatory response (Waltz et al., 2001).

RON is preferentially expressed in epithelial cell types and macrophage populations where it regulates cellular proliferation, adhesion, motility, and apoptosis, all events resulting in the complex invasive growth program (Follenzi et al., 2000).

As in the case of the more famous family member MET, several evidences suggest that RON may play a 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 (Tamagnone and Comoglio, 1997; Camp et al., 2005). Moreover, the receptor has been found over-expressed and over-activated in several different human cancers, such as colon (Chen et al., 2000), lung (Willett et al., 1998), breast (Maggiora et al., 1998), stomach (Okino et al., 2001), ovary (Maggiora et al., 2003), pancreas (Camp et al., 2005), bladder (Cheng et al., 2005), liver (Chen et al., 1997), and kidney (Rampino et al., 2003). Besides RON over-expression correlates with a bad clinical outcome in some human cancers, and in breast and bladder tumors it is associated with decreased disease free survival (Wang et al., 1994).

In addition, RON oncogenic potential has been shown in cells manipulated to over-express the receptor and in transgenic mice where the receptor induces an increase in cell proliferation (Maggiora et al., 1998; Peace et al., 2001), motility and invasion upon MSP treatment (Chen et al., 2000) and drives tumorigenesis (Chen et al., 2002a,b), respectively. On the contrary, downregulation of RON expression in RON expressing cancer cell lines achieved via the introduction of RON specific shRNA impairs cell proliferation and motility and enhances apoptosis (Xu et al., 2004). It has been also described that RON is able to synergize with other oncogenes such as polyoma virus middle T antigen (pMT) and RAS, augmenting their oncogenic potential (Chan et al., 2005).

Nevertheless, although more information has been recently gained on RON contribution in tumor formation and progression, little is known about RON biology mainly because of the lack of valid investigation tools. Only very recently an antagonist of RON activity has been tested in vivo to validate RON as a potential cancer target (O'Toole et al., 2006). O'Toole and colleagues developed a fully human anti-RON monoclonal antibody that binds the receptor with high affinity and blocks the binding with its natural ligand MSP. In their work, the authors show that the mAb inhibits the downstream pathways and ultimately blocks tumorigenesis.

Similarly to MET, several molecular mechanisms have been proposed to be responsible for RON constitutive activation. Autocrine and paracrine loops (Willett et al., 1998); receptor over-expression, which can be achieved via gene amplification, enhanced transcription or post-transcriptional modifications; and finally interaction with other cellular surface receptors. Up to now no somatic mutations in RON coding sequence have been identified in human cancers although the experimental reproduction of mutations found in MET, KIT, and RET, in RON homologous residues results in an oncogenic phenotype. A novel mechanism to activate RON receptor both in cancer-derived cell lines and in several human cancers types has been described: the generation of truncated receptors 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 three cysteines located in the extracellular domain of the beta chain and responsible for the establishment of intramolecular disulphide bridges. The resulting uneven cysteine number causes receptor oligomerization and consequent constitutive activation (Collesi et al., 1996). The above-described variant is known as RONΔ165. Afterward, other three RON splicing variants have been described, RONΔ160, RONΔ155, and RONΔ55 all shown to have a strong oncogenic potential (Zhou et al., 2003). Another RON variant known as short form RON (SF RON) originates from an alternative start site in intron 10. Initially described in mice, the short form was shown to be expressed also in human breast and ovarian cancers as well as in many different cancer-derived cell lines where its expression results in enhanced proliferation and motility together with anchorage independence (Bardella et al., 2004).

Future Perspectives

MET and RON tyrosine kinase receptors have been shown to be over-expressed and over-activated in a large cohort of human cancers and their role in human oncogenesis has been extensively proved. These facts together with the gigantic progresses made in cancer therapy with the use of tyrosine kinase inhibitors, make MET and RON superlative targets for cancer therapy. Unquestionably, a strong case can be made to optimize and develop new drugs aimed at blocking effectively either MET or RON activity.

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