CHAPTER

2

Targeting the Hepatocyte Growth Factor Receptor to Overcome Resistance to Targeted Therapies

Nele Van Der Steen[*,**], *Ingrid Garajova*[†,‡], *Christian Rolfo*[**], *Andrea Cavazzoni*[§], *Elisa Giovannetti*[*,¶]

[*]VU University Medical Center, Amsterdam, The Netherlands
[**]Center for Oncological Research, University of Antwerp, Antwerp, Belgium
[†]Sant'Orsola-Malpighi Hospital, University of Bologna, Bologna, Italy
[‡]Interdepartmental Centre of Cancer Research "Giorgio Prodi", University of Bologna, Bologna, Italy
[§]University of Parma, Parma, Italy
[¶]University of Pisa, Pisa, Italy

Abstract

The hepatocyte growth factor (HGF) and its tyrosine kinase receptor cMET (MET proto-oncogene) entered into the spotlight mainly as a bypassing pathway for other targeted therapies (e.g., therapies targeting epidermal growth factor receptor). However, the HGF–cMET signaling axis can be oncogenic by itself. Aberrations in HGF–cMET occur in many cancer types, such as non-small-cell lung cancer, pancreatic cancer, and renal carcinoma. This resulted in the development of several inhibitors targeting this signaling axis. Biomarkers for these targeted therapies include cMET amplification and cMET exon 14 skipping. Activation of cMET by HGF results in the activation of several downstream pathways, among which are the mitogen-activated protein kinase cascade and phosphatidylinositol-3-kinase-Akt signaling. During embryogenesis, they control the development of tubules and are involved in the migration and invasion of several cell types. These functions are mirrored in cancer growth, whereby cMET is a known activator of cell migration and metastasis. Therefore, in this chapter, we start by describing the road to discovery, the functions in development, and explain the HGF–cMET signaling in detail. Next we focus on HGF–cMET in cancer, describing the possible aberrations and providing an overview of inhibitors (under development). Finally, we pay attention to the role of HGF–cMET as a resistance mechanism against other (targeted) therapies.

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ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
BMP-2	Bone morphogenetic protein-2
ccRCC	clear cell renal cell cancer
CDA	Cytidine deaminase
CRC	Colorectal cancer
CSC	Cancer stem cell
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
ERM	Ezrin, radixin, moesin protein family
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
Gab1	Grb2-associated binding protein 1
GGA3	Golgi-localized gamma-ear containing Arf-binding protein 3
Grb2	Growth factor receptor bound protein 2
GSK3ß	Glycogen synthase kinase 3 beta
HGF	Hepatocyte growth factor
iNOS	inducible NOS
JNK	C-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MET	Tyrosine-protein kinase Met
MKK1	Mitogen-activated protein kinase kinase 1
MSP	Macrophage-stimulating protein
mTOR	mammalian target of rapamycin
NF-kB	Nuclear factor-kB
NK	N-terminal and kringle
NSCLC	Non-small cell lung cancer
PDAC	Pancreatic ductal adenocarcinoma
PI3K	Phosphoinositide 3 kinase
PKC	Protein kinase C
pRCC	papillary renal cell cancer
PSC	Pancreatic stellate cells
RCC	Renal cell carcinoma
SF	Scatter factor
SFR	Scatter factor receptor
SHC	Src homology 2 domain containing
Shp2	Protein-tyrosine phosphatase 2C
STAT3	Signal transducer and activator of transcription 3
TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor

2.1 DISCOVERY AND STRUCTURE OF HEPATOCYTE GROWTH FACTOR

The first report of hepatocyte growth factor (HGF) dated back to 1984. Almost simultaneously, two articles described the isolation of a new growth factor in rats, named HGF or hepatotropin [1,2]. This growth factor was isolated from serum and enhanced DNA synthesis and mitogenesis in hepatocytes [1,2]. Platelet-derived serum showed only 50% activity when compared to serum from whole blood. The addition of platelet lysates restored this decreased

activity, suggesting an interaction with platelets [2]. The other article showed that HGF displayed high affinity for heparin, suggesting association with platelets, but they were not able to confirm this [1]. Reduction of the disulfide bonds inactivated HGF, as well as heat and increased acidity. In 1988, human HGF was isolated for the first time. Purification from plasma of a patient with fulminant hepatic failure resulted in a protein of 76–91 kDa, which consisted of two chains of 31–34 and 54–65 kDa, respectively [3]. HGF was shown to activate the cMET receptor [4].

In 1973, a scatter factor (SF) that promotes cell migration and overgrowth in SV40-transformed cells was described in Balb/c3T3 cells [5]. This factor was later shown to be produced by embryonic fibroblasts and stimulated migration in epithelial cells only, suggesting a paracrine effect. This SF affected locomotion in cell colonies, whereas in single cells it only leads to membrane ruffling [6]. Further research into these effects showed that cancer cell lines neither produce nor respond to this factor [7]. Purification of the SF succeeded in 1989. The purified factor retained all the previously described features of the fraction, isolated from a conditioned medium. This SF increased motility and modulated morphology. It inhibits junction formation of single epithelial cells and promotes cell scattering in epithelial cell sheets, without influencing cell growth [8]. The SF receptor was identified as the product of the cMET proto-oncogene [9].

Further study of both factors led to the conclusion that HGF and SF are one and the same growth factor [10]. Both factors were mapped onto the same chromosomal region (7q11.2-21), and their protein sequence was indistinguishable. Moreover, SF was able to promote hepatocyte growth, whereas HGF showed SF activity [10,11]. Both SF and HGF were shown to bind the cMET β-chain with high affinity and heparansulfate proteoglycans with low affinity. Cell responses to HGF were also shown to be mediated by cMET activation [12].

The HGF gene was mapped to chromosome 7 at the 7q21.1 position [13]. The gene contains 18 exons and spans over 70 kbp [14]. Exon 1 encodes a signal sequence and the 5′-UTR. The α-chain is encoded by exons 2–11, exon 12 results in a spacer sequence, and the β-chain is encoded by exons 13–18. HGF is translated as a single-chain 83 kDa protein: pre-pro-HGF. In the first step, the signal sequence is removed, resulting in pro-HGF. In the second step, pro-HGF is cleaved between Arg494 and Val495, resulting in a 69 kDa α- and 34 kDa β-chain that are connected by disulfide bonds [15].

HGF is part of the plasminogen-related growth factor family, together with HGF-like/macrophage-stimulating protein (HGF1/MSP). HGF and HGF1 share a common ancestor protein with plasminogen and apo(A) and evolved most likely through gene duplication [16]. This evolution is visible when comparing the very similar exon–intron structure coding for the first three kringle domains of plasminogen and the α-chain of HGF and HGF1. In the case of HGF, the fourth kringle domain most likely resulted from internal duplication, since its organization is very different from the fourth kringle domain in plasminogen. The serine protease domain of the β-chain of HGF is not active. This inactivity is caused by point mutations resulting in replacement of two out of three amino acids of the catalytic triad (His534Gln and Ser673Tyr) [17]. In plasminogen, this catalytic triad is formed by His–Asp–Ser, whereas the triad consists of Gln–Asp–Tyr in HGF and Gln–Gln–Tyr in HGF1. This suggests that mutation of the serine protease domain happened before the duplication of HGF and HGF1, but after the duplication of plasminogen, which retained its activity.

In contrast to plasminogen, the N-terminal domain of HGF is not cleaved [16] but is necessary for receptor binding. The N-terminal and kringle 1 domain of HGF (NK1) is able to

associate with the receptor, but with reduced affinity. Adding the second kringle domain (NK2) doubles this binding affinity. The binding affinity of NK2 when compared to complete HGF is still fivefold lower. The addition of kringle domains 3 and 4 did not lead to further improvement of binding affinity. This points toward a 2:2 complex of HGF and cMET [18]. The cleavage of pro-HGF results in an open form of HGF [19]. The 7-bladed propeller sema-domain of cMET contains the binding site for HGF [20]. A first high-affinity binding site is occupied by the N-terminal domain and the first kringle domain of HGF [19]. A second low-affinity binding site of cMET is bound by the serine protease domain on the β-chain of HGF [21]. Hereby, the mutations of the catalytic triad play a role in the association of HGF to cMET [21]. Complete HGF, consisting of both the α- and β-chains, is needed to activate cMET signaling [19].

Two naturally occurring splice variants of HGF have been described: NK1 [22] and NK2 [23,24]. The NK1 variant consists of the N-terminal region and the first kringle domain. This variant is capable of binding to both heparin and cMET. This splice variant is able to induce phosphorylation of cMET, but a 16× higher concentration is needed to induce the same effect as full-length HGF [22]. In a concentration ranging from 5 to 10 nM, NK1 is capable of inducing scattering, however, it is about 50 times less potent in comparison to HGF. When adding NK1 in 40-times molar excess to HGF, it reduces activity of HGF by 70% and is able to inhibit DNA synthesis [22]. The second splice variant consists of the N-terminal domain and the first two kringle domains (NK2) [24]. This variant competes with HGF for cMET binding and inhibits HGF-dependent mitogenesis [24].

2.2 ACTIVATION OF THE HGF-MET SIGNALING PATHWAY

HGF is the only known ligand of cMET. Mature HGF, consisting of an alpha and beta chains, binds cMET and causes receptor dimerization (Fig. 2.1). This is a paracrine process, since HGF is mainly secreted by mesenchymal cells, and cMET is mainly expressed on epithelial cells [6,7,11,25]. Binding of HGF leads to cross-phosphorylation of cMET at positions Tyr1003, Tyr1234, Tyr1235, Tyr1349, and Tyr1356. Once Tyr1349 and Tyr1356 are phosphorylated, they serve as docking sites for the adaptor proteins Grb2 (Growth Factor Receptor Bound protein 2), Gab1 (Grb2-associated binding protein 1), and SHC (Src homology 2 domain containing) [26]. These adaptor proteins link the receptor to its downstream signaling effectors [26–28]. In this process, several coreceptors are involved. The most important coreceptor of cMET is CD44v6, a splice variant of CD44 containing exon 6. A complex is formed between cMET-HGF and CD44v6 [29]. Orian-Rousseau et al. [30] revealed a two-step process. In the first step, this complex is formed and HGF-induced phosphorylation of cMET and its adaptor proteins takes place. In the second step, the cytoplasmic domain of CD44v6 is necessary to induce phosphorylation of MEK and ERK through HGF–cMET signaling [30]. The cytoplasmic domain of CD44v6 is also necessary to induce internalization of the HGF–cMET signaling complex. Phosphorylated Tyr1003 is the binding site for the E3-ligase c-Cbl [31,32]. Internalization of HGF–cMET is dependent on the association of the cytoplasmic domain of CD44v6 with ERM proteins (Ezrin, Radixin, Moesin) and their connection to the cytoskeleton [33]. A second coreceptor/enhancer is α6β4 integrin [34]. This integrin forms a complex with cMET that functions as a signal amplifier for HGF–cMET

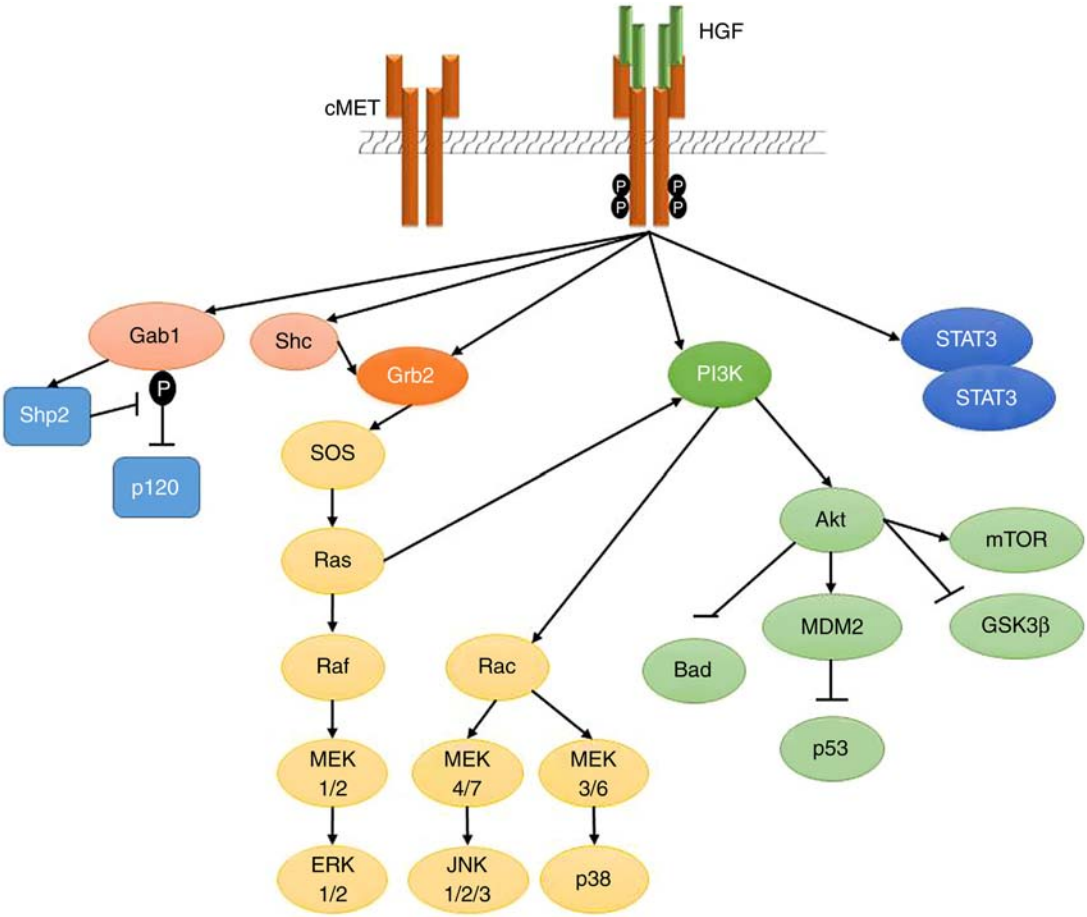

FIG. 2.1 Schematic of HGF–cMET signaling. Abbreviations: HGF, hepatocyte growth factor; Gab1, Grb2-associated binding protein 1; Grb2, growth factor receptor bound protein 2; Shp2/PTPN11, tyrosine protein phosphatase nonreceptor type 11; p120, p120 catenin; Shc, Src homology 2 domain containing; SOS, Son of Sevenless; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p38, p38 mitogen-activated protein kinase; PI3K, phosphoinositide 3 kinase; Akt, protein kinase B; MDM, mouse double minute 2; GSK3β, glycogen synthase kinase 3 beta; mTOR, mammalian target of rapamycin: STAT3, signal transducer and activator of transcription 3.

signaling. Active cMET phosphorylates the β4 subunit, which results in the recruitment of SHC and activation of phosphatidylinositol-3-kinase (PI3K) [34]. A third coreceptor/amplifier is the Semaphorin 4D ligand and its receptor Plexin B1 [35,36]. Association of this complex with cMET is involved in cMET-induced angiogenesis [37]. Semaphorin 4D association with cMET is able to induce phosphorylation without the binding of HGF. However, this stimulation is weaker and delayed compared to HGF activation of cMET. Moreover, Semaphorin 4D–cMET is able to induce cell motility and morphogenesis, but is not able to stimulate cell proliferation. It also has no mitogenic effect on endothelial cells and is not

capable of inducing VEGF expression [37]. This association with cMET has been shown for all class B plexins [36], but more studies are warranted to elucidate downstream effects of these interactions. However, this interaction is reported to be antimigratory in the case of breast carcinoma [38] and melanocytes [39].

2.2.1 Mitogen-Activated Protein Kinase Cascades

HGF activation of cMET leads to the induction of several mitogen-activated protein kinase (MAPK) cascades. Signaling from the Grb2 adaptor protein leads to activation of Ras, either through direct association of Grb2 to cMET or indirectly by SHC binding to cMET [40]. In the meantime, activation of Ras is stimulated indirectly. The adaptor protein Gab1 recruits Shp2, which in turn dephosphorylates a phosphorylation site on Gab1 involved in the activation of p120-Ras-GAP. Inhibition of the p120 Ras GTPase activating protein leads to increased levels of active Ras coupled to GTP [41]. Activation of Ras continues through several signaling pathways. First, Ras activates the Raf–MEK1/2–ERK1/2 cascade. This results in cell proliferation and transformation. Second, Ras activates PI3K signaling. This stimulates Rac that phosphorylates MEKK1/2/3/4. On the one hand, MEK4/7 are phosphorylated, leading to activation of JNK1/2/3 [42] and, on the other hand, MEK3/6 activation leads to activation of p38 $\alpha/\beta/\gamma/\delta$ [43,44]. The activation of both JNK and p38 signaling is associated with cell proliferation, transformation, differentiation, and apoptosis [42].

2.2.2 PI3K-Akt

Binding of HGF to cMET also results in the recruitment of the p85 subunit of PI3K to the receptor [45]. This leads to the activation of PI3K-Akt signaling. PI3K has several functions. First, it has an antiapoptotic effect [46] by inhibiting Bad, an effector of apoptosis [47], and at the same time activating MDM2, an inhibitor of p53 [48]. Second, it stimulates cell growth and proliferation through activation of mTOR [49], resulting in protein translation and cell growth, and by inhibiting GSK3β that activates cell-cycle regulators [50], leading to cell proliferation.

2.2.3 STAT3

STAT3 is another downstream effector of cMET. STAT3 is recruited directly to the cytoplasmic tail of cMET and upon phosphorylation forms homodimers that translocate to the nucleus [51]. In the nucleus, STAT3 binds to the promoters of several genes. STAT3-regulated genes are reported to play a role in tubulogenesis, leading to cell proliferation and differentiation [51]. Furthermore, STAT3 also plays a role in pro-cancer immunity. IL6 is upregulated, activating a feed-forward loop by increasing STAT3 activation. Through the sequestering of RelA in the nucleus, NF-κB is activated. Moreover, STAT3 signaling is necessary for regulatory T-cell expansion, T_h17 cells development, and the immunosuppressive effects of myeloid-derived suppressor cells [52].

2.2.4 HGF-Independent Activation and cMET Internalization

Besides activation by HGF, cMET can also be activated through cell adhesion. Hereby, α5β1 integrin plays a role. Once this integrin associates with type IV collagen, it forms a

complex with cMET, thus activating the receptor. Next, cMET phosphorylates Src, which in turn leads to the activation of FAK [53–56].

As already briefly mentioned, Tyr1003 in the juxtamembrane domain of cMET is phosphorylated upon activation. Once phosphorylated, this Tyr is part of a docking site for the E3-ligase c-Cbl [31]. This starts the breakdown process for cMET. Hereby, c-Cbl has two tasks. First, it recruits an E2-ligase that in turn ubiquinates cMET, marking cMET for internalization through clathrin-coated pits in a dynamin-dependent manner [57,58]. Second, c-Cbl attracts the CIN85–endophilin complex. This complex is involved in the invagination and scission of these clathrin-coated pits [59]. From here, intracellular cMET trafficking goes over the microtubules from the early endosomal complex to the late perinuclear complex and finally cMET is broken down by the 26S proteasome [60].

Recycling of cMET from endosomes has also been reported. Hereby, GGA3 (Golgi-localized gamma-ear containing Arf-binding protein 3) plays an important role. After internalization, cMET is contained in Rab4/Rab5-positive endosomes. GGA3 colocalizes with these endosomes and stimulates recycling to the plasma membrane [61]. GGA3 has been shown to bind to Crk, which in turn binds the Gab1 adaptor protein that is associated to the cMET receptor [62]. Loss of GGA3 results in increased breakdown of HGF-bound cMET, a decrease in ERK signaling, and a decrease in cell migration [61]. This decrease in ERK signaling can be explained by the continuous signaling of the cMET receptor once it is internalized [63]. PKCα is involved in the microtubule-dependent trafficking of cMET to the late endosome, whereas PKCε, on the other hand, controls sustained phosphorylation of ERK1/2 by cMET from the early endosome. Once internalized, cMET continues to signal and phosphorylate ERK1/2. PKCε is involved in the translocation of ERK1/2 from the cytoplasm to the focal complexes at the plasma membrane [58]. Here, ERK1/2 phosphorylates paxillin, thus contributing to cell migration [64].

Signaling from HGF–cMET continues from the perinuclear endosome. Rac1 associates with cMET from the moment of internalization, however signaling from the perinuclear endosome is necessary for sustained activation of Rac1. This is caused by the cMET-dependent activation of Vav2, a Rho GEF able to activate Rac1. Next to Vav2, activation of PI3K is also necessary for sustained Rac1 activation [65]. The p85 subunit associates with cMET in the perinuclear endosome, and the PI3K product PIP3 regulates Vav2 activity [66]. Active Rac1 on the edges of the cell leads to membrane ruffling, cell migration, and invasion [65].

Activation of STAT3 also requires HGF–cMET signaling from the perinuclear endosome. Although phosphorylated STAT3 is assumed to diffuse freely to the nucleus, there is a long period of exposure to phosphatases between the cell membrane and the nucleus. By activating STAT3 in proximity to the nucleus, this exposure is considerably more limited, resulting in a strong STAT3 signal and translocation to the nucleus [67].

2.2.5 Receptor Shedding

The first report of cMET shedding dates back to 1995. Herein, Galvani et al. [68] described a reduction of cMET expression of 50% in reaction to the growth factor inhibitor suramin. Shedding of cMET can be caused by stress signals like TNF-α-actinomycin D and camptothecin [69], by activation of another receptor tyrosine kinase, e.g., epidermal growth factor

receptor (EGFR) [70] or by antibody treatment, e.g., DN30 [71]. This shedding has an extra advantage for treatment with antibodies, as it also functions as a decoy receptor for HGF, thus preventing cMET activation by two distinct mechanisms. However, not all antibodies cause cMET shedding, as is illustrated by onartuzumab that does not induce shedding or internalization of the receptor and prevents clearance of s-cMET [72]. ADAM10 or ADAM17 recognize the 998-SVD-1000 motif in cMET, which is mainly dependent on D1000 [69]. Next, a second cleavage is performed in the membrane by γ-secretase [73], resulting in two intracellular parts of cMET that are degraded by the proteasome [74]. This process is independent of the kinase activity of cMET. The two remaining intracellular parts are pro-apoptotic in a kinase-dependent manner [69]. Soluble-cMET has been reported to be a biomarker for pre-eclampsia [75] and hepatocellular damage [76] and points toward a poorer outcome in non-small cell lung cancer (NSCLC), where it correlates with cMET expression [77].

2.3 HGF FUNCTIONS

HGF has a critical role in the development and organogenesis of several tissues, as described in the following sections.

2.3.1 Placenta

The importance of HGF in placenta formation is illustrated by $HGF^{-/-}$ or $cMET^{-/-}$ mice. Homozygous mice die *in utero* around E13.5–E15.5 and show deformation of the placenta, among other defects [78]. These homozygous-deficient embryos show reduced numbers of labyrinthine trophoblast cells, along with a decrease in labyrinth area size and poorly developed embryonic vessels and maternal sinuses. These defects can be rescued by stimulation with HGF *in vitro*. During embryogenesis, HGF is produced by the allantoic mesenchyme, whereas the cMET receptor is expressed on the extraembryonic ectoderm. Upon contact between these tissues, mitotic activity and scattering of trophoblasts are induced [78]. HGF is also needed in an earlier stage, the early postimplantation growth. Here, HGF does not stimulate the scattering of trophoblasts, but stimulates cell division of trophoblasts [79]. Pre-eclampsia is associated with impaired trophoblast invasion [80]. A study using human placenta explants showed that in the case of pre-eclampsia, the production of HGF was 25% less over 24 h [80]. This is confirmed by the results of a second study, where the level of soluble cMET (s-cMET) in serum of pregnant women was determined, in both healthy subjects and patients with pre-eclampsia [81]. Here a significant lower concentration of s-cMET was measured in women suffering from pre-eclampsia when compared to healthy subjects, both in the first and second trimester of the pregnancy. Even more, a significantly lower s-cMET concentration was detected in the serum of women with early and serious pre-eclampsia when compared to women with later and milder pre-eclampsia [81].

HGF induces trophoblast motility by activation of the PI3K and MAPK pathways. Activation of p42/44 MAPK is necessary for motility and for induction of inducible NOS (iNOS) [82]. This activation of iNOS can be inhibited by transforming growth factor-β (TGF-β) [83]. Downstream of PI3K, activation of p70S6 [84] and Akt [85] are needed to induce

trophoblast motility. PI3K signaling also causes phosphorylation of GSK3β, a member of the Wnt signaling cascade [86]. In the case of GSK3β, phosphorylation causes inhibition and not activation. Inhibition of GSK3β leads to the activation of NF-κB and β-catenin. In turn, β-catenin activates the Lef/Tcf transcription factor [87], leading to cell survival.

There are four different isoforms of HGF identified that play a role during growth of the placenta [88]: the NK1 and NK2 isoforms and the dNK1 and dNK2 isoforms. The last two contain a deletion of five amino acids in the first kringle domain of HGF. All of these isoforms are expressed in the endometrium, but dNK2 is expressed at higher levels than NK2. Presumably, expression of the HGF antagonists NK1 and dNK1 moderates the activity of HGF in the endometrium. In contrast, NK1 and dNK1 are not expressed in the placenta, whereas NK2 and dNK2 are expressed in equal amounts [88].

2.3.2 Muscle

The development of muscles can be divided into several steps. First, the precursor cell pool in the dermomyotome needs to be established. Second, these precursors need to be delaminated and migrate to their final position. Third, the precursor cells proliferate and finally differentiate to form skeletal muscles [89]. HGF is one of the three important players in this process.

Pax3 is the first gene to become active. It is responsible for the establishment of the precursor pool and the delamination and migration of the cells [90]. This is visible in the *Splotch* mutant. This mutant contains a deletion in Pax3 [91] and lacks the limb muscles, whereas development of the axial and body wall musculature is not affected [92]. Pax3 induces cMET expression. This is shown in the *Splotch* embryos wherein cMET expression is lacking [93,94]. Activation of the cMET–HGF axis is important to induce the delamination and migration of the myogenic precursor cells from the somites into the limb buds [95]. In mice embryos that are deficient in either cMET or HGF, these precursor cells develop normally in the dermomyotome, but fail to start the migration to the limb buds, resulting in the lack of hypaxial muscle groups. On the other hand, skeletal muscles outside the limb develop normally [95], showing that cMET signaling is not necessary in further differentiation of muscle cells. In normal embryos, cMET expression is detected in the migrating myogenic precursor cells [96]. HGF, on the other hand, is expressed in the mesenchyme [97]. It has been shown that HGF expression is stimulated by fibroblast growth factor (FGF) in the apical ectodermal ridge. When this zone is removed, HGF expression was detected until the 18/19th stage, when the apical ectodermal ridge is formed. This shows that FGF is necessary for the continuation of HGF production [96]. The zone of polarizing activity, on the other hand, controls HGF expression by expression of BMP2. The removal of this zone leads to ectopic and enhanced expression of HGF [96]. The homeobox gene Lbx1 is induced before delamination, expressed during migration and downregulated during differentiation. This gene is responsible for the correct guiding of the precursor cells during migration [89]. Before the final differentiation of the muscle progenitor cells, a proliferation step takes place. Here, cMET also plays a role, since a mutation of the Grb2 binding site in cMET leads to a decrease in the size of skeletal limb muscles [98]. In these homozygotic $cMET^{Grb2/Grb2}$ mice, the placenta and liver are developed normally. In contrast, muscles are reduced in size, although no effect on migration or differentiation of myoblasts are observed during

embryogenesis. Even more, defects in the appendicular and axial muscles are observed. These muscle groups do not derive from migratory precursor cells. A reduction in the secondary muscle fibers is observed in all muscle groups, pointing toward a role for cMET during proliferation of myoblasts forming secondary muscle fibers [98].

2.3.3 Liver

During embryogenesis, the liver develops from the ventral endoderm under the influence of the HNF3β and GATA-4 transcription factors [99]. Interaction of the liver bud with the cardiac mesoderm leads to hepatic induction. The cardiac mesoderm produces FGF-8 before hepatic induction [100] and FGF-1 and FGF-2 at the time of hepatic induction [101]. These FGFs have been shown to be necessary and sufficient for hepatic induction, but not sufficient for further liver development [101]. Next to the influence of the cardiac mesoderm, the septum-derived mesenchyme also plays a role in liver development. Here, bone morphogenetic protein (BMP) is produced that, in parallel to FGF, induces liver development [102]. BMP4 is necessary during hepatic specification and to inhibit the expression of pancreatic genes. This is shown in BMP4 knockout mice that present with a delay in liver bud formation. Also the use of Noggin, a BMP inhibitor, inhibits the outgrowth and migration of hepatic cells [102]. HGF is also expressed by the septum transversum mesenchyme, whereas its receptor cMET is expressed on hepatocytes [103]. A knockout of either HGF or cMET leads to death *in utero*, due to strongly reduced liver growth and disturbed hematopoiesis [103,104]. When culturing hepatocytes from $HGF^{-/-}$ embryos *in vitro* in medium supplemented with growth factors supporting epithelial growth, they showed no difference on the survival or growth of these remaining hepatic cells [103], confirming the mitogenic influence of HGF on hepatocytes. Activation of cMET leads to the expression of the c-Jun transcription factor. Knockout c-Jun embryos display the same phenotype as HGF or cMET knockout embryos, due to reduced hematopoiesis that is not capable of sustaining the growing embryo [104,105]. These matching phenotypes point toward the involvement of the HGF/cMET/c-Jun axis in liver development.

2.3.4 Kidney

During the development of the renal system, contacts between the ureteric bud and the metanephrogenic mesenchyme starts the branching process [106]. During branching and invasion into the mesenchyme, several growth factors work together to coordinate this process [107]. In the first step, GDNF**31** and formin IV [108,109] are important to induce ureteric bud formation. Disruption of the formin IV isoform has been shown to lead to renal agenesis/dysgenesis due to failure in ureteric bud outgrowth and branching [108]. Formin IV, a known actin nucleator, colocalizes with the actin cytoskeleton and in the perinuclear zone in quiescent epithelial cells. After stimulation with HGF, formin IV transiently translocated in these cells to the nucleus in a MAPK-dependent manner [110]. However, its exact role in the nucleus is not known to date.

Embryos from $HGF^{-/-}$ mice are reported to have normal renal development. However, homozygous deletion of HGF or cMET leads to death *in utero*, with 12% of embryos reaching the E14.5 stage and 2% the E16.5 stage [103], whereas the induction of renal branching

is situated around stage E13.5 [111]. This urges for caution on drawing conclusions on the necessity of HGF for renal development based on an unconditional HGF knockout model. Several findings point toward an important role for HGF during renal development. First, glycosaminoglycans are necessary for ureteric bud development. When these glycosaminoglycans are removed, the development of the ureteric bud stops, and this effect is rescued by addition of HGF only [112]. Second, contact with the mesenchyme is necessary to start the development of the kidney. When the mesenchyme and epithelium are separated, development stops, whereas restoration *in vitro* rescued this effect [106]. With the mesenchyme being the main producer of HGF and the epithelium mainly expressing cMET [113], this also points toward an important role for the HGF–cMET axis in kidney development. Third, when growing developing murine kidneys (d11.5–12.5) were cultured *in vitro*, addition of anti-HGF serum resulted in significant growth inhibition, a poorly defined ureteric duct and poorly developed branches [111,113].

To control the branching of the nephrotic duct, BMPs serve as negative signals. They prevent the branching into already colonized regions of the mesenchyme and stimulate branching into new regions [114]. BMP4 has been shown to inhibit branching, but stimulates tubule elongation [115]. In high concentrations, BMP7 inhibits branching, whereas in low concentration it stimulates branching [116]. Finally, MMP9 is needed to facilitate the growth of the branches into the mesenchyme [117].

The first critical pathway for HGF-induced branching is the MAPK pathway [118]. HGF induces phosphorylation of MKK1 through activation of cMET, whereas EGF induces phosphorylation of both MKK1 and ERK5. Inhibition of MKK1 by PD98059 abolishes HGF-induced cell migration and branching completely, whereas it only partially inhibited EGF-induced motility and branching. Phosphorylation studies showed that this inhibitor is able to inhibit MKK1 completely, whereas ERK5 is still partially active. Using the U0126 inhibitor, which inhibits both MKK1 and ERK5, the effects of both HGF and EGF were abolished completely. Expression of a dominant-negative ERK5 only influenced cell motility and branching after activation of EGF and did not affect the HGF-induced effects [118]. The manner of signaling, either transiently or endured, is important for the correct induction of branching. The cell-surface heparan sulfate Gpc4 is needed for sustained signaling through MAPK by HGF [119]. Without Gpc4, HGF is still able to induce cell migration and proliferation, but fails to induce branching. Branching can be rescued by addition of EGF [119]. Although the exact role of Gpc4 in HGF signaling has not been revealed yet, possibly this role is similar to that of Gpc1. Gpc1 is able to induce lipid rafts formation, thus stabilizing the HGF–cMET signaling complex, which leads to sustained downstream signaling [120].

Next to the MAPK pathway, activation of the PI3K pathway is also involved in branching [119,121,122]. Addition of PD98059 (MAPK inhibitor) or LY294002 (PI3K inhibitor) leads to inhibition of scattering, but morphologically different outcomes. A constitutively active form of PI3K was able to induce scattering, but active MAPK was needed during this process. Activation of Akt of Rac did not lead to scattering of the cells, whereas induction of scattering by active Ras or PI3K was sensitive to both the LY294002 and PD98059 inhibitors. These findings point toward a role for PI3K act signaling in branching, but this signaling does not include downstream activation of Akt or Rac, suggesting a different downstream signalization path [122].

2.3.5 Nervous System

Nervous tissue arises from the node of Hensen, which gives rise to the nervous streak during early stages of embryogenesis. HGF is expressed in this node of Hensen [123] and plays a role in retaining the neural competence of this tissue [124]. A marker of this competence is $L5^{220}$. When the expression of $L5^{220}$ decreases, the level of competence of the node decreases. Addition of HGF after its normal expression window leads to a longer expression of $L5^{220}$ and thus a prolonged time of complete competence. Ectopic addition of HGF leads to the formation of a neural streak/neural plate-like structure, which does not possess the completely undetermined character of the normal neural plate [124]. However, HGF or cMET knockout embryos do not show large defects in the neural system up to their death *in utero* at E13.5–E15.5, which suggests that HGF is involved but not necessary in these early stages of neuronal development [103].

In later stages, HGF is involved in neuronal survival, proliferation, and outgrowth. First, HGF enhances the uptake of dopamine by the dopaminergic neurons in the brain [125]. In the brain, HGF is mainly produced by microglia [126], where it functions as a neurotrophic factor (together with bFGF) through the induction of Ras signaling [126]. In contrast, cMET is expressed in microglia cells, astrocytes, and neurons [126]. Second, in the case of sympathetic neurons, HGF plays a role in survival and axonal outgrowth [127]. Both HGF and cMET are expressed during the development of the sympathetic ganglia [127]. When anti-HGF antibodies are added, a reduced number of sympathetic neuroblasts is observed, along with reduced survival [127]. Expression of a mutated form of cMET also leads to increased sympathetic neuroblast apoptosis *in vivo* [127]. Third, signaling mutants of cMET lead to a reduction in sensory nerves in the skin of limbs and the thorax [128]. Together with NGF, HGF is responsible for the axonal outgrowth from the dorsal root ganglion (DRG) neurons and sustains a part of these DRG neurons. No synergy with other neurotrophic factors as BDNF and NT3 is detected. Disruption of the Grb2 docking site of cMET does not lead to defects in the development of these sensory neurons, pointing toward downstream signaling that is independent of Grb2 [128]. Finally, HGF acts as a mitogen for Schwann cells [129]. However, anti-HGF antibodies do not lead to impaired development of Schwann cells. This suggests that HGF is not necessary for Schwann cell development [129].

2.3.6 Testis

During the development of the testis, one of the first steps is the migration of cells from the mesonephric mesenchyme toward the celomic epithelium [130]. During this stage, HGF is expressed by the celomic epithelium, whereas cMET expression is detected in the mesonephric mesenchyme [131]. HGF functions as a chemoattractant for the migrating cells from the mesonephric mesenchyme [132]. These migrating cells are necessary for the testicular cord morphogenesis [130]. At stage E12.5, cMET is expressed in the developing testicular cords, whereas HGF production is situated in the stroma and myoid cells [131,132]. Organ culture *ex vivo* demonstrated that HGF is sufficient for testis differentiation and testicular cord formation from undifferentiated male urogenital ridges [132]. Around E17.5, cMET expression in the testicular cord is downregulated, cMET expression increased in interstitial fetal Leydig cells, and HGF is now being produced by the

interstitial compartment [133]. During this stage, HGF promotes testosterone production [133] and Leydig cell survival and differentiation [134].

2.4 DYSREGULATION IN CANCER

2.4.1 cMET Mutations

The cMET receptor has several domains that are crucial for its function. A mutation in each one of these domains can deregulate its activity and have oncogenic potential. Mutations in the ligand-binding domain can affect HGF affinity, whereas mutations in the kinase domain might lead to a constitutively active receptor. Mutations in the juxtamembrane domain, containing the E3-ligase c-Cbl docking site, can lead to impaired cMET breakdown. Many mutations are known in cMET, but only a few of them are carcinogenic. Therefore, we will limit our description to mutations that influence treatment and resistance; for the complete list of mutations, we refer the reader to the COSMIC and ClinVar databases.

Several splice site mutations are known that cause exon 14 skipping, which occur in around 3% of adenocarcinomas [135]. Exon 14 forms part of the juxtamembrane domain and contains the c-Cbl docking area. Therefore, a deletion of this exon leads to impaired breakdown of cMET and prolongs signaling. These mutations can be present in the 5′ or 3′ splice sites, the branching A or the polypyrimidine tract that are all necessary for the splicing process. Known types of mutations that cause exon 14 skipping are missense mutations and deletions [136,137]. Patients with detected exon 14 skipping have been shown to respond to several cMET small-molecule inhibitors like crizotinib, capmatinib, and cabozantinib [137].

Hereditary papillary renal cell carcinoma is the only cancer type with known germline mutations in cMET to date. These mutations are mostly situated in the kinase domain of cMET and lead to an autosomal-dominant syndrome. Known mutated codons include V1110L/I, H1112R/L/Y, M1149T, V1238I, D1246N/H, V1238I, and M1268T [138–140]. Some of these mutations show homology to previously known mutations in oncogenes. The D1246N/H missense mutations are compatible with the D816V mutation in cKit [141,142], whereas the Y1248C/H/D mutations are related to the M918T mutation in RET [143]. All of these above-mentioned mutations have transforming capabilities and lead to constitutive, activated cMET.

Mutations causing resistance to cMET-targeted therapies have been reported: Y1230C [144] and D1228N/V [145,146]. The activation loop of cMET controls the activity of the receptor. This loop closes the ATP binding pocket of the receptor by interacting through salt bridges with amino acids at positions K1110 and D1228. Y1230 is located on the activation loop and is turned inwards through interaction with A1226 [147]. Type I small-molecule cMET inhibitors (crizotinib, capmatinib, tepotinib) interact with the receptor through the formation of π-stacks with the Y1230 residue. These π-stacks are abolished by the substitution of Tyr to Cys at this position [144]. Type II cMET inhibitors (cabozantinib, glesatinib) have an extra interaction with cMET through the association with a hydrophobic pocket on the receptor, thus being less dependent on these π-stacks [148]. A missense mutation at D1228, on the other hand, leads to a destabilization of the activation loop. These mutations have been reported in

both patients presenting with cMET exon 14 skipping and cMET amplification after EGFR-TKI treatment [144–146] and lead to resistance against type I cMET small-molecule inhibitors, but can be overcome by type II inhibitors.

2.4.2 Amplification and Fusion Protein of cMET

Amplification of cMET has been described to occur in approximately 1.5% of NSCLC adenocarcinomas [135] and in around 5% of gastric carcinoma [149,150] and glioblastomas [151]. A consensus of scoring criteria is lacking to determine the exact cut-off for cMET amplification, leading to the reporting of varying percentages. However, this cut-off might prove to be important for treatment outcomes as has been reported in the first results of the crizotinib trial in NSCLC [152]. Herein, patients with a cMET/CEN7 ratio >5 showed a partial response to crizotinib in 50% of cases, when compared to 20% in the intermediate amplified population (>2.2 to <5) and 0% in the low amplified population (>1.8 to ≤2.2) [152]. Moreover, Noonan et al. [153] showed that patients with a low and intermediate ratio showed concomitant driver mutations in other genes in 52% and 50% of cases, respectively, whereas for the high amplification this was 0%. It is important to make a distinction between cMET amplification and polysomy, given that besides cMET, the genes for EGFR and HGF are also located on chromosome 7. In NSCLC patients that originally presented with a sensitizing mutation in EGFR and that received an EGFR-TKI, the percentage of cMET amplification rises to approximately 20% [154,155]. We will discuss this in more detail in the next section. In addition to amplification, a fusion protein of cMET is also known: TPR-MET [156,157]. This fusion protein was originally identified in the MNNG-HOS cell line and is the result of a translocation between the TPR gene on chromosome 1 and the cMET gene [158]. The fusion protein is located intracellularly, and through the bindings of the coiled coils of TPR the kinase domain of cMET autophosphorylates and is constitutively active [159]. Moreover, the juxtamembrane domain of cMET is missing in the fusion protein, resulting in impaired breakdown of the fusion protein, thus contributing to its oncogenicity [160]. Because the kinase domain of cMET is retained, TPR-MET is inhibited by small-molecule inhibitors, like SU11274 [161]. In addition to NSCLC, TPR-MET has also been reported in gastric carcinoma [157].

2.4.3 cMET Overexpression

Overexpression can result from several dysregulations. First, epigenetic regulation through the loss of DNA methylation might result in higher promotor activity, causing cMET overexpression [162]. Second, miRNA regulation of the promotor can also result in increased expression [163]. Finally, the transcription of cMET can be upregulated, leading to higher translational activity and expression [164]. In the case of NSCLC, cMET overexpression has not proved to be a good biomarker for response to cMET small-molecule inhibitors, as was shown in the onartuzumab phase III trials [165]. The percent of cMET-overexpressing tumors varied substantially between the different studies, ranging from 14% to over 50% of cases in NSCLC in various patient populations [166–169]. The same variation is seen in gastric carcinoma [150,170], glioma [151,171], and pancreatic cancer [172,173].

2.4.4 HGF Overexpression

Like cMET overexpression, HGF overexpression can be caused by transcriptional [174] or epigenetic changes [162]. However, autocrine or paracrine loops can also cause overexpression of HGF and thus increase cMET activity [175]. Therefore, a distinction between HGF expression between the stroma and the tumor cells themselves is important. The reported percentages of cells with HGF overexpression range from 25% to 82% in tumor cells [176,177]. These loops have been described in multiple cancer types: hepatoma [178], lung carcinoma [179], and multiple myeloma [180], among others.

2.5 HGF INHIBITORS FOR CANCER THERAPY

The HGF–cMET signaling pathway represents a promising drug target for novel anticancer strategies. Targeting the HGF ligand or the cMET receptor are two possible ways to influence the activation of this pathway. In general, agents against the HGF ligand can be further divided into HGF activation inhibitors or HGF inhibitors [181]. HGF activation inhibitors prevent the cleavage of pro-HGF into the active form and HGF inhibitors block the direct binding of HGF to the cMET receptor. These inhibitors exist as monoclonal antibodies or small molecules. Moreover, blocking the cMET receptor by cMET antagonists or cMET tyrosine kinase inhibitors (TKIs) represents alternative therapeutic strategies for modifying HGF–cMET signaling pathway [181,182]. HGF inhibitors can be used as single agents or in combination with other anticancer drugs such as cytostatic drugs or targeted compounds [182,183]. Most of the HGF–cMET inhibitors have shown encouraging results when used in drug combination studies.

2.5.1 HGF Activation Inhibitors

The inactive precursor of HGF (pro-HGF) transforms to its active form, thanks to the balance between the activators (HGFAs) and the inhibitors (HAIs) [184,185]. Several authors investigated the role of HAIs in solid tumors. HAI-1 was identified as a prognostic marker for prostate cancer and might be a novel target for the treatment of this malignancy. The authors described that the level of HAI-1 changes in prostate cancer compared to benign prostate hyperplasia. Further, a low level of HAI-1 correlated with a high Gleason score, a more advanced pathological stage as well as worse clinical outcome [186]. The same trend was observed by Tsai et al. [187] for HAI-2 in human prostate cancer progression. The expression of HAI-2 decreased throughout progression in cell invasion capability, contributing to tumor genesis and metastasis. Therefore, HAI-1 and HAI-2 might represent novel prognostic markers as well as therapeutic targets. To date, however, no clinical tests for HAIs are available.

2.5.2 HGF Inhibitors

HGF inhibitors bind to HGF, blocking binding to the cMET receptor and resulting in subsequent effects on downstream activation of the pathway. Several monoclonal antibodies against HGF have been tested in preclinical studies, such as rilotumumab, ficlatuzumab, and TAK701. Rilotumumab is a fully humanized IgG2 monoclonal antibody [188]. Early preclinical

data showed synergistic cytotoxic effects with temozolomide and docetaxel [189,190]. In a phase II study evaluating the efficacy of rilotumumab in combination with capecitabine vs placebo plus capecitabine in patients affected by gastric or esophagogastric junction tumors, the patients with cMET overexpression experienced better progression-free survival (PFS) and overall survival (OS) with rilotumumab and capecitabine combination [191]. Ficlatuzumab is a humanized anti-HGF IgG1 monoclonal antibody. Mok et al. [192] evaluated its efficacy in combination with gefitinib. In the overall Asian population, no significant benefit from the addition of ficlatuzumab to gefitinib in patients with advanced stage lung adenocarcinoma was reported, though patients classified as "VeriStrat poor" may benefit from ficlatuzumab combination therapy [192]. TAK701 is a humanized monoclonal antibody directed against HGF [193]. Addition of TAK-701 to gefitinib is a promising strategy to overcome EGFR-TKI resistance induced by HGF in NSCLC with activating EGFR mutation [194].

2.5.3 MET Antagonists

cMET antagonists compete with HGF for binding to cMET which causes cMET degradation and inactivation of the pathway [195]. Several cMET antagonists have been investigated in preclinical studies. However, disappointing results from recent clinical studies have been obtained with onartuzumab, in particular, for colorectal cancer (CRC), gastric cancer, glioblastoma, and NSCLC, such as in the phase III study NCT01456325 METlung.

This study analyzed the efficacy and safety of combination therapy with onartuzumab and erlotinib in patients with locally advanced or metastatic NSCLC, after progression on first-line with a platinum-based chemotherapy regimen. Moreover, patient selection according to MET expression by immunohistochemistry was provided. The authors concluded that the combination of onartuzumab and erlotinib did not improve clinical outcomes and, on the contrary, shorter OS was observed in the onartuzumab arm, compared with erlotinib in patients with cMET-positive NSCLC [196]. Further, Wakelee and coworkers [197] observed that onartuzumab does not provide any clinical benefit when given in combination with current first-line standard-of-care chemotherapy regimen for nonsquamous NSCLC. Recently, the results of a randomized phase II trial investigating the role of onartuzumab in metastatic CRC were published. Onartuzumab combined with FOLFOX (5-FU and oxaliplatin regimen) and bevacizumab did not significantly improve efficacy outcomes in this setting and, moreover, MET expression was not a predictive biomarker [198]. The same disappointing results were obtained in the METGastric Randomized Clinical Trial where the addition of onartuzumab to FOLFOX did not significantly improve OS, PFS, or ORR vs FOLFOX and placebo. Moreover, as in previous trials, cMET expression did not predict the clinical response to onartuzumab treatment in patients affected by metastatic gastric cancer [199]. Finally, in another phase II study, there was no evidence of further clinical benefit with the addition of onartuzumab to bevacizumab compared with bevacizumab plus placebo in unselected patients with recurrent glioblastoma [200].

2.5.4 MET Kinase Inhibitors

Targeting intracellular cMET with cMET kinase inhibitors represents another approach for HGF–cMET signaling pathway deactivation. These kinase inhibitors can be divided

into two groups: selective cMET kinase inhibitors (tivantinib, savolitinib) and nonselective cMET kinase inhibitors (crizotinib, cabozantinib, foretinib). A phase II study evaluated the efficacy of the tivantinib with erlotinib in patients with locally advanced or metastatic EGFR mutation-positive NSCLC after progression on EGFR-TKI (gefitinib or erlotinib) [201]; this study enrolled 45 NSCLC patients with acquired resistance to EGFR-TKIs and who continued treatment with the combination of tivantinib and erlotinib. The median PFS and median OS were 2.7 months and 18.0 months, respectively. Interestingly, better clinical outcomes were obtained in patients whose tumors overexpressed cMET [201]. In another study, the combination of tivantinib and erlotinib was compared to erlotinib monotherapy in nonsquamous NSCLC patients with one or two lines of prior chemotherapy. No statistically significant difference in OS between the arms was observed, although a significant improvement in PFS was observed in the group of patients with KRAS-mutant tumors ($P = 0.006$) [202]. Eng et al. [203] evaluated the role of tivantinib in combination with CETIRI (irinotecan and cetuximab) in patients with metastatic KRAS wild-type CRC, as a second-line treatment. The combination of tivantinib and CETIRI was well tolerated but did not significantly improve PFS. Subgroup analyses trended in favor of tivantinib in patients with overexpression of cMET, though further studies are needed to confirm these results [203]. In HCC patients, a significant improvement in OS was observed in patients with cMET-overexpressed tumors in a phase II study; the results of a phase III study in the subgroup of cMET-overexpressed HCC tumors after sorafenib failure will be soon to come [204].

Crizotinib is a small-molecule inhibitor of the anaplastic lymphoma kinase (ALK) with additional activity against the cMET, ROS, and RON receptors. Crizotinib was approved for use in ALK-rearranged advanced NSCLC [205–207]. In a case report recently published by Pietrantonio et al. [208], the authors described a patient affected by metastatic CRC, BRAF-mutated. This patient initially responded to combination treatment with anti-EGFR (panitumumab) and anti-BRAF (vemurafenib). At progression, rebiopsy was provided and cMET amplification was determined (not found in the pretreatment specimen). Probably, ectopic cMET overexpression led to resistance to panitumumab and vemurafenib. Based on these data, the patient was treated with crizotinib and vemurafenib, with rapid and marked effectiveness [208].

Cabozantinib is a small-molecule inhibitor of cMET, VEGFR2, KIT, and RET followed by AXL and FLT3 [209] and is approved for the treatment of metastatic medullary thyroid cancer and for the second-line therapy for metastatic renal cell carcinoma (RCC) [210,211]. A phase II trial compared cabozantinib with sunitinib as the first-line therapy in patients with metastatic RCC. Cabozantinib demonstrated a significant clinical benefit in PFS and ORR over standard-of-care sunitinib as the first-line therapy in patients with intermediate- or poor-risk metastatic RCC [212]. Daud et al. [213] evaluated the role of cabozantinib in patients affected by malignant melanoma. Median PFS was 4.1 months with cabozantinib and 2.8 months with placebo (hazard ratio of 0.59; $P = 0.284$). The authors concluded that cabozantinib has clinical activity in patients with metastatic melanoma, including uveal melanoma, although further clinical investigation is warranted [213]. Interestingly, a small, randomized phase II trial evaluated the efficacy of erlotinib or cabozantinib monotherapy, or combination treatment with erlotinib and cabozantinib as second-line or third-line treatment of patients with wild-type EGFR and advanced NSCLC. This trial showed that

cabozantinib alone or combined with erlotinib had superior efficacy to that of erlotinib alone [214]. The efficacy of cabozantinib was also evaluated in a cohort of metastatic breast cancer patients in a phase II study and demonstrated clinical activity including objective response and disease control [215].

2.6 TARGETING HGF–CMET PATHWAY IN SOLID TUMORS TO OVERCOME DRUG RESISTANCE

HGF–cMET signaling pathway is implicated in mechanisms of acquired resistance to inhibitors against EGFR (gefitinib), VEGF (sunitinib), HER-2 (trastuzumab and lapatinib), and BRAF (vemurafenib) [216–220]. In the following paragraphs, we describe how targeting of the HGF–cMET signaling pathway can overcome the drug resistance in NSCLC, RCC, pancreatic cancer (PDAC), and CRC.

2.6.1 Lung Cancer

Lung cancer is the leading cancer worldwide, both in incidence and in mortality [221]. Eighty percent of lung cancers are NSCLC. In turn, NSCLC can be divided into two main histological subtypes: adenocarcinoma and squamous cell carcinoma. Adenocarcinomas exhibit glandular differentiation and mostly arise in the lung periphery, whereas squamous cell carcinomas often present with keratinization and intercellular bridges. Squamous cell carcinomas occur mostly in the main and lobular bronchi [222]. The 5-year survival rate for NSCLC is very low: around 15%. The current treatment regime [223] is mainly dependent on the stage. For early stages of NSCLC (stage I/II/IIIa), local surgery is the preferred treatment option, with adjuvant chemotherapy for stage II patients. This chemotherapy mostly consists of a two-drug combination with cisplatin. If surgery is not an option, radiotherapy is a suited treatment option. In locally advanced NSCLC, chemotherapy is given to patients that can tolerate it. This regimen consists mostly of a cisplatin–vinorelbine or cisplatin–etoposide doublet; combinations with carboplatin are used to a lesser extent. Resectable tumor surgery is an option after induction therapy [223]. Recently, immunotherapy has also become available for treatment of NSCLC [224].

Several oncogenic drivers have been identified in NSCLC and the list is continuously expanding: EGFR, ALK, cMET, ROS1, RET, FGFR, and NTRK [225]. These drivers are mainly expressed in the adenocarcinoma subtype. In the case of EGFR and ALK, multiple generations of inhibitors have been approved for therapy, whereas for the other drivers, clinical trials are mostly ongoing [226].

Focusing on the HGF–cMET axis, cMET amplification and cMET exon 14 skipping are known as oncogenic drivers in NSCLC. Both occur in around 2% of NSCLC and in both the adenocarcinoma and squamous cell carcinoma subtypes. They are not mutually exclusive and both are responsive to cMET small-molecule inhibitors [136,137]. Several TKIs are currently in clinical trials for the treatment of cMET aberrations, with the dual ALK-cMET inhibitor crizotinib being the most advanced [227].

In the context of resistance, cMET in NSCLC is mostly known to cause resistance against EGFR-TKIs (e.g., erlotinib, gefitinib, afatinib, osimertinib). Primarily cMET amplification is

reported in patients with resistance [216,228]. The percentage of cMET amplification increases in this population of EGFR-TKI-treated patients to approximately 20% [154,155]. The resistance mechanism of cMET activation can be explained by the extensive cross-talk between EGFR and cMET signaling [229], with largely overlapping downstream signaling effectors like MAPK and PI3K-Akt. In total, a core network of around 50 proteins can be identified [229]. Moreover, activated EGFR is able to stimulate cMET activation and vice versa [230]. Active EGFR influences cMET in several ways. It increases cMET phosphorylation and expression and decreases ubiquitination of cMET. This effect is enhanced by Her3. Active cMET, in turn, activates EGFR indirectly through MAPK activation [230]. This extensive cross-talk also explains the many clinical trials that immediately focus on combinations of cMET- and EGFR-targeted therapies [227]. However, not all of these combinations have proved to be successful, as was illustrated in the onartuzumab + erlotinib trial that was stopped due to futility [165,231] and the tivantinib + erlotinib trial that did not reach its primary endpoint [232].

Activation of cMET also plays a role in combination with conventional therapies (chemotherapy, radiation therapy). First, radiation therapy is known to upregulate cMET expression [233]. This effect can be explained by the involvement of cMET in tissue repair [234] and homologous recombination [235] that are both activated in a reaction to restore damage by radiation therapy. However, the combination of cMET inhibition and radiotherapy still needs to be tested in patients. Second, cMET inhibition also has an effect in combination with chemotherapy. This interplay is more complex and seems to be dependent on the type of chemotherapy and the form of cancer. cMET is reported to play a role in chemoresistance in both pancreatic cancer (gemcitabine) [236] and ovarian cancer (cisplatin and paclitaxel) [237]. Inhibition of cMET is able to overcome this resistance in ovarian cancer (carboplatin, paclitaxel) [238] and gastric carcinoma (irinotecan) [239]. *In vitro* results show that combining cMET and cisplatin did not lead to synergism [240], whereas functional studies show that addition of HGF increases chemoresistance toward cisplatin in NSCLC cells through downregulation of AIF [241]. Reports on cMET and chemotherapy are often contradicting, and clinical trials are necessary to elucidate the profit of combining cMET inhibitors and chemotherapy in patients.

2.6.2 Renal Cancer

Renal cancer (RCC) is the third most frequent cancer originating from the genitourinary organs [242]. Based on the place of origin of cancer (proximal tubule of the kidney or collecting duct), four main histological types can be recognized: clear cell (ccRCC), papillary (pRCC, type 1 which consists of predominantly basophilic cells and type 2 which consists of mostly eosinophilic cells), chromophobe, and collecting duct tumor. The most frequent type is ccRCC. Division into histological types influences the patient outcome: in particular, metastatic pRCC has a worse prognosis than ccRCC [243]. Moreover, type 1 and type 2 pRCC have different clinical features. Type 1 pRCC is characterized by an indolent clinical course, and type 2 pRCC by more aggressive clinical behavior [244]. The HGF–cMET signaling pathway plays an important role in RCC pathogenesis. In particular, mutation or functional inactivation of the von Hippel–Lindau (VHL) gene is present in the majority of ccRCC cases. Absence or decreased levels of the VHL protein leads to transcriptional activation of HIF-targeted genes, including VEGF, PDGF, TGF-α, HGF, and cMET [245–247]. The expression of cMET and HGF in RCC is 70% and 60%, respectively [248]. Moreover, some authors reported that cMET expression

is higher in pRCC than in ccRCC, and higher in type 1 pRCC compared to type 2 pRCC [249]. pRCC often shows trisomy of chromosome 7, where the cMET gene is located [250]. Further, both sporadic and hereditary forms show cMET gene mutations [251]. Interestingly, some RCC cancer stem cells (CSCs) overexpress the cMET receptor which might play a role in the pathogenesis of bone metastases induced by RCC CSCs in mice and humans [252].

Nowadays, first-line treatment for patients affected by metastatic RCC includes several anti-VEGF inhibitors (sunitinib, pazopanib, and bevacizumab) and the mTOR inhibitor (temsirolimus) [211]. VEGF-targeted therapies have demonstrated a survival benefit in RCC patients with PFS of about 9–11 months [211]. Cross-talk between the VEGFR and HGF–cMET pathways is implicated in the resistance to anti-VEGFR therapies and therefore the clinical testing of this combination is very promising [253,254]. In fact, Ciamporcero et al. [247] evaluated the effects of either monotherapy or a combination treatment strategy targeting the VEGF (axitinib) and cMET (crizotinib) pathways in animal models affected by ccRCC. As expected, the combination treatment was more effective than the monotherapy with crizotinib. In another study, increased expression of HGF was observed in mouse models resistant to sunitinib. Combination with a cMET inhibitor was able to overcome sunitinib resistance [217]. Interestingly, foretinib—a dual inhibitor of cMET and VEGFR showed efficacy in pRCC with germline cMET mutations [255]. Another cMET inhibitor, savolitinib, is undergoing clinical development for various cancer types, including pRCC [256]. This agent can suppress the cMET signaling pathway and the duration of target inhibition is dose related. The authors have suggested that savolitinib could have therapeutic potential in sunitinib-resistant pRCC patients [257]. Temsirolimus is an mTOR inhibitor and is used in RCC patients with poor prognosis [211]. mTOR inhibitors such as temsirolimus and everolimus might overcome HGF-dependent resistance to EGFR-TKIs in preclinical studies [258]. Microphthalmia transcription factor (MITF)-associated (MiT) tumors are a family of rare malignancies, including translocation-associated renal cell carcinoma (tRCC) that show dysregulation in MITF family proteins. The cMET gene is transcriptionally activated by MITF family proteins which provides a rationale for the use of cMET inhibitors in this setting. A small study investigated the activity of tivantinib in patients with MiT-associated RCC patients ($n = 6$) with modest antitumor activity [259].

Simultaneous blocking of cMET and VEGFR pathways represents a promising treatment approach for RCC treatment since this will target multiple pathways involved in angiogenesis, tumor survival, and metastasis.

2.6.3 Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy and the fourth leading cause of cancer-related death in developed countries [260]. In the metastatic setting, cytostatic drugs in different combinations are the gold standard, while no targeted therapy is being used in clinical practice. The chemotherapy choices include gemcitabine as monotherapy, gemcitabine in combinations with nab-paclitaxel or the combination of 5-FU, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) or recently nanoliposomal irinotecan [261,262]. The expression of cMET and HGF is increased in >70% and >35% of PDAC patients, respectively [248]. cMET overexpression correlates with worse clinical outcome of PDAC patients (worse TNM stage, presence of lymph node invasion, poor tumor differentiation) [172,263,264]. Zhang et al. [265] developed a novel c-MET CTC assay for detecting cMET

CTCs in patients with cMET amplifications. HGF–cMET overexpression causes chemoresistance by different mechanisms of action. PDAC stroma is the predominant source of HGF with a subsequent increase in VEGF production by stromal cells [266,267]. Interestingly, some preclinical studies have demonstrated that overexpression of cMET is correlated with EMT-like changes in PDAC cells with acquired resistance to gemcitabine [236]. Pancreatic stellate cells (PSCs) are cells that produce the PDAC stroma. They secrete HGF and promote cancer cell growth but do not express the cMET receptor which is present in cancer cells. Pothula et al. [268] evaluated the effect of HGF inhibition, using a neutralizing antibody AMG102, in monotherapy or in combination with gemcitabine *in vitro* (PDAC cells) and *in vivo* (orthotopic model). The authors concluded that HGF inhibition was as effective as standard chemotherapy with gemcitabine at inhibiting local tumor growth. Moreover, HGF inhibition reduced metastasis, although this antimetastatic effect was lost when combined with gemcitabine. Therefore, carefully combining HGF inhibitors with existing treatment modalities is warranted [268]. Additionally, cMET as a marker of pancreatic CSCs was associated with PDAC aggressiveness, metastatic behavior, and intrinsic resistance to chemotherapy [269]. Avan et al. [270] evaluated the synergism of gemcitabine and crizotinib in an orthotopic mouse model of primary PDAC. Crizotinib prolonged survival and promoted gemcitabine uptake, accompanied by an increased activity of the human equilibrative and concentrative nucleoside transporters (hENT1 and hCNT1), and a decreased cytidine deaminase (CDA). Interestingly, crizotinib targets CSC-like subpopulations, interferes with cell proliferation and cell survival, decreases cell migration, and synergistically interacts with gemcitabine [271]. Similar results were obtained with cabozantinib which targets pancreatic CSCs and increases the cytotoxic effect of gemcitabine in a human pancreatic cancer model grown orthotopically in NOD SCID mice [264]. Hage et al. [272] demonstrated that cabozantinib interferes with PDAC cell survival and increased the efficacy of gemcitabine in high-gemcitabine-resistant PDAC, suggesting a capacity to overcome gemcitabine resistance. Brandes et al. [273] evaluated the efficacy of INC280 (cMET inhibitor) *in vitro* and *in vivo*. The combination of gemcitabine with INC280 significantly prolonged survival in an orthotopic syngeneic tumor model and, therefore, this combination warrants further clinical evaluation.

Previous studies have shown that HGF must dimerize to activate cMET. Small-molecule antagonists with homology to a "hinge" region within the putative dimerization domain of HGF have been developed that bind to HGF and block dimerization with subsequent inhibition of cMET signaling. Apart from cMET signaling, which leads to cancer progression, the MSP/Ron (MSP receptor) systems characterized by structural and sequence homology have the same role in PDAC cells. Church et al. [274] hypothesized that the inhibition of HGF by the hinge analogs may be extended to MSP, resulting in more efficient blocking of tumor progression. As expected, hinge analog compounds inhibited HGF and MSP activity and resulted in decreased cMET and Ron activation which may represent a new therapeutic approach for the treatment of metastatic PDAC [274].

2.6.4 Colorectal Cancer

Only about 2% of metastatic CRC tumors show cMET amplification [275,276]. cMET amplification in CRC is correlated with increased aggressiveness and worse clinical prognosis. Moreover, Bardelli et al. [277] showed that cMET amplification is associated with

acquired resistance in CRC patients that do not develop KRAS mutations during anti-EGFR therapy. His functional studies demonstrated that cMET activation confers resistance to anti-EGFR therapy both *in vitro* and *in vivo*. Interestingly, cMET amplifications were present in circulating tumor DNA before clinically evident radiologic relapse was observed [277]. The efficacies of several agents targeting the HGF–cMET signaling pathway have been investigated in preclinical studies or clinical trials in metastatic CRC patients, as, e.g., NK4. NK4 is an HGF antagonist that binds to cMET and inhibits HGF-induced tyrosine phosphorylation of the receptor [278]. Wen et al. [278] demonstrated that NK4 has inhibitory effects on angiogenesis in CRC cells. In a randomized phase II clinical trial, the combination of rilotumumab with panitumumab in previously pretreated patients with wild-type KRAS metastatic CRC was evaluated. This combination led to a higher response rate and showed a trend for a better outcome in the population with cMET overexpression [279]. Recently, Bendell et al. [198] published the results of a phase II randomized trial exploring the efficacy of cMET inhibitor onartuzumab in combination with FOLFOX and bevacizumab. The addition of onartuzumab to the standard first-line treatment did not significantly improve efficacy outcomes. Further, cMET expression by immunohistochemistry was not predictive in this setting [198]. Lastly, a phase II randomized study of biweekly CETIRI (cetuximab and irinotecan) plus tivantinib or placebo was restricted to patients who had received only one prior line of chemotherapy and were characterized by wild-type KRAS. Subgroup analyses showed a trend in favor of tivantinib in patients with cMET overexpressing tumors, with low expression of PTEN, or with those pretreated with oxaliplatin, though the subgroups were too small [203].

2.7 BIOMARKERS FOR HGF/MET INHIBITORS

Nowadays, personalized cancer treatment for every patient is an important aim of clinical oncologists. As the efficacy of anticancer treatment is always reserved to a certain group of patients, it is important to identify biomarkers that could predict response or resistance to a specific class of agents, including HGF/cMET inhibitors. Several predictive biomarkers of HGF/cMET inhibitors have been investigated.

Circulating HGF and cMET were evaluated as pharmacodynamic biomarkers of cMET inhibition in different clinical trials, although the results have been inconclusive and it seems that their potential as predictive biomarkers of response depends on the type of cMET inhibition [280–286]. cMET protein expression might be prognostic of clinical outcome in selected cancer types with specific molecular aberrations. Some authors have demonstrated that in certain cancer types, overexpression of cMET protein, as determined by immunohistochemistry, may be associated with poor prognosis. Iveson et al. evaluated the subgroup of cMET-overexpressing gastric tumors treated with a chemotherapy regimen containing epirubicin, cisplatin, and capecitabine with or without rilotumumab. In this phase II study, cMET overexpression was correlated with better clinical outcome in the patients treated with rilotumumab [287]. These results were not confirmed in the phase III RILOMET-1 study [288]. In another study exploring the role of tivantinib in NSCLC, it was shown that overexpression of cMET might have predictive potential only in cMET-positive patients with nonsquamous histology [289]. Amplification of the cMET gene locus with overexpression of the receptor on the cell surface or cMET gene mutations might activate the HGF–cMET signaling pathway. cMET gene amplification, copy

number, and mutations appear to be relatively conservative biomarkers, though they are rare. High levels of cMET amplification correlated with responsiveness to crizotinib in esophagogastric adenocarcinoma patients [290]. Germline cMET mutations were predictive of clinical response in pRCC [255]. Interestingly, some authors analyzed changes in cMET phosphorylation before and after treatment with several anti-cMET drugs, although it remains unclear whether changes in these markers are predictive of clinical responses [291–293].

2.8 CONCLUSIONS AND FUTURE DIRECTIONS

With over 25 years since its first discovery, the cMET receptor is emerging as an important target for personalized cancer therapy. cMET is a unique RTK, expressed in the epithelial cells of many organs during embryogenesis and in adulthood, with a versatile role in control and regulation of several biological functions in response to HGF. In addition to its importance in normal physiology, a wide variety of human malignancies have sustained cMET stimulation, overexpression, or genetic aberrations, including lung cancer, as well as upper gastrointestinal cancers such as pancreatic, gastric, and hepatocellular cancers. This aberrant activation of the cMET/HGF signaling pathway is associated with tumor development, progression and aggressive phenotype, and poor clinical outcome.

As summarized in this review, a variety of different strategies to inhibit this signaling pathway have been developed, and several cMET inhibitors are now under clinical investigation in different tumors, with encouraging results. Crizotinib has been approved for ALK-rearranged NSCLC, while cabozantinib is approved for the treatment of metastatic medullary thyroid cancer and for second-line therapy for metastatic RCC. However, the clinical efficacy of several other cMET inhibitors needs to be ultimately validated in ongoing phase II and III randomized trials. Moreover, recent studies suggested the potential role of crizotinib and other cMET TKIs for the treatment of selected upper gastrointestinal cancers, including gastric cancers with cMET amplification and pancreatic tumors, where the HGF–cMET axis plays a pivotal role in progression and invasive growth.

Several important questions remain to be answered. Most clinical studies testing anti-cMET agents have been conducted in combination with other anticancer drugs, such as erlotinib in the context of mutated or wild-type EGFR NSCLC patients, as a means of overcoming acquired EGFR-TKI resistance. Further preclinical and clinical studies to evaluate a possible role for cMET monotherapy and for combination with other agents are warranted. Knowledge gained from these studies should be complemented with molecular and biochemical studies on the function of cMET and related pathways in response to cMET inhibition, as well as studies on the acquired resistance to this treatment. Finally, identification and selection of the optimal patient populations that will benefit from treatment would provide valuable direction and innovative strategies for the clinical development of cMET targeted therapies.

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