3. PATHOGENESIS OF CANCER

3.1. Cancer onset [21,22,23]

Cancer malignancy is represented by the acquired ability of cancer cells to invade into neighbouring tissues and survive in these ectopic sites. In the process of invasion, cancer cells enter the circulation from where they can reach distant organs and eventually form secondary tumors, called metastases.

Although perceived as a subversion of tissue structure and function, the process of invasion and metastasis probably originates from a physiological property of undifferentiated stem and/or progenitor cells. During embryonic development, ectodermal cells invade through the primitive streak and occupy the subectodermal space where they form the mesoderm; neural crest cells emerge from the dorsal aspect of the neural tube, migrate to different sites in the body, and survive there to grow and differentiate under the influence of specific local factors. This transformation of the flat, two-layer germinal disc into a three-dimensional organism depends on the transient conversion of some cells from an epithelial to a mesenchymal phenotype, *i.e.* cells with a spindle-shaped morphology and motile behaviour. This epithelial-to-mesenchymal transition (EMT) is then followed by ordered cell migration and the morphogenesis of new structures.

The same process can be reactivated in post-natal life during tissue repair and organ regeneration. Although there is still some controversy, it is now widely accepted that cancer cells hijack the strategies by which the embryo grows and develops. Morphogenesis and metastasis seem to arise from the same genetic programme that instructs cells to detach from a primary colony, cross tissue boundaries, adhere to and migrate through extracellular matrices, and escape to death caused by an unfamiliar tissue context (a process known as anoikia).

For some kind of tumors, such as astrocytomas, cancers of head and neck and at least in one half of colorectal cancers, lethality is due almost uniquely to local invasion. For other kind of cancers, such as for breast cancers and melanomas, death is usually the consequence of distant metastases. Invasion and metastasis are the hallmarks of cancer malignancy. Consequently, invasion and metastasis are

major prognostic markers. For epithelium-derived cancers in particular, the rate of survival dramatically drop down if invasion occurs.

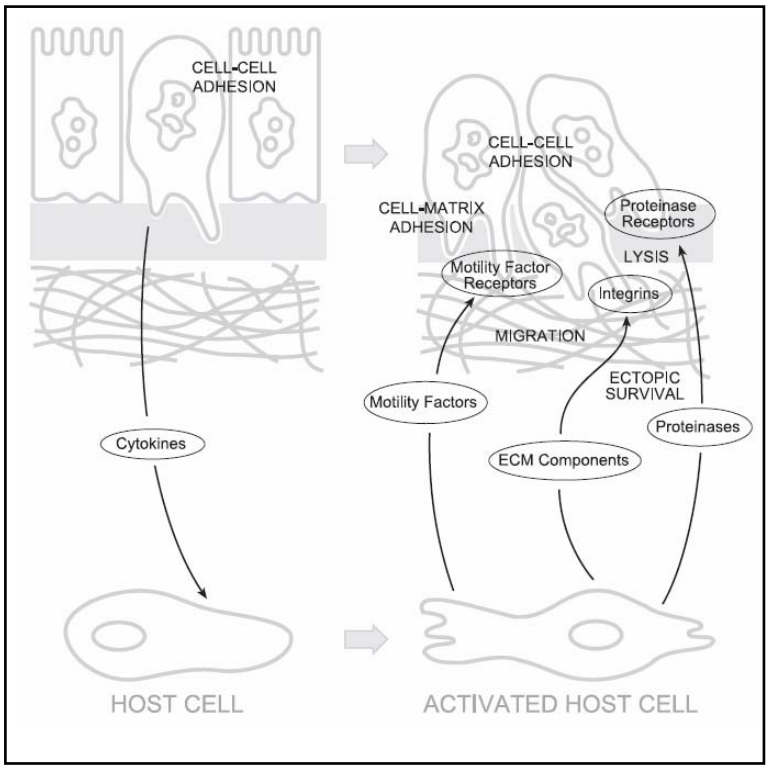
Invasive growth is regulated by specific extracellular signals. Among these, scatter factor/hepatocyte growth factor (HGF), the ligand for the receptor encoded by the *MET* proto-oncogene, is an example of a morphogenic cue that can turn into a trigger for cancer onset and progression. HGF/Met signaling has an important role in the development and malignant progression of tumors, particularly in tumor invasiveness and metastatic potential. Met is involved in epithelial cancer genesis driving to scatter and providing resistance to anoikia. In the first step of this process, cells acquire the ability to dissociate from their neighbours by breaking intercellular adherent junctions (scattering), leave their original environment and reach the circulation. Cell survival in the bloodstream is facilitated by Met-induced protection from apoptosis and the ability to transiently grow in an anchorage-independent manner. In transformed tissues, deregulation of the invasive growth program is responsible for cancer progression and metastasis. Constitutive Met activation forces neoplastic cells to disaggregate from the tumor mass, erode basement membranes, infiltrate stromal matrices and eventually colonize new territories to form metastases.

3.2. Oncogenes and oncosuppressors [21]

A series of alterations in the genome of the cell population of origin forms the basis for tumor development. The genes of interest are classified as oncogenes (or tumor-promoter genes), one allele of which is activated leading to gain-of-function events, and tumor-suppressor genes (or antioncogenes), both alleles of which are inactivated leading to loss-of-function events. The products of these genes belong to various classes of protein families, such as cytokines, cell surface receptors, signal transducers, and transcription factors. The list of oncogenes encoding cell surface receptors of the protein-tyrosine kinase family alone counts more than forty members. Mechanisms of activation of oncogenes implicate mutation, gene amplification and promoter activation. Mechanisms of tumor-suppressor inactivation are exemplified by loss of heterozygosity plus silencing of the second allele genetically, through mutation, or epigenetically, through methylation. In familial cancers, one mutation is carried with the germline.

3.3. Dissecting the pathways toward invasion [21]

Cellular activities positively or negatively associated with the invasive phenotype comprise proliferation, cell-cell adhesion, cell-matrix adhesion, ectopic survival, migration, proteolysis and neo-angiogenesis.



Cell-cell and cell-substratum adhesion modification, proliferation, migration, proteolysis, ectopic survival are necessary steps for tumor onset and progression. From Mareel and Leroy, Physiology Review 2002 (Ref. 21).

In cells that have progressed toward malignancy through activation of promoter genes and inactivation of suppressor genes, these cellular activities are regulated by autocrine and paracrine ligands, resulting in modulation of the invasive phenotype. The challenge is to trace the pathways from the ligand to the receptor to the signal transduction and finally to the cellular response that is crucial for the alteration of the invasive phenotype.

3.3.1. Proliferation [24]

Proliferation of cells is balanced by the phenomenon of programmed cell death, or apoptosis, during which cells undergo a highly regulated death that induces little or no inflammation. Tumors arise when normal regulatory mechanisms fail to maintain a balance between proliferation and apoptosis such that cells accumulate in excess numbers.

HGF/Met signaling can affect both proliferation and apoptosis depending on the cell context.

3.3.2. Players in cell-cell adhesion [21]

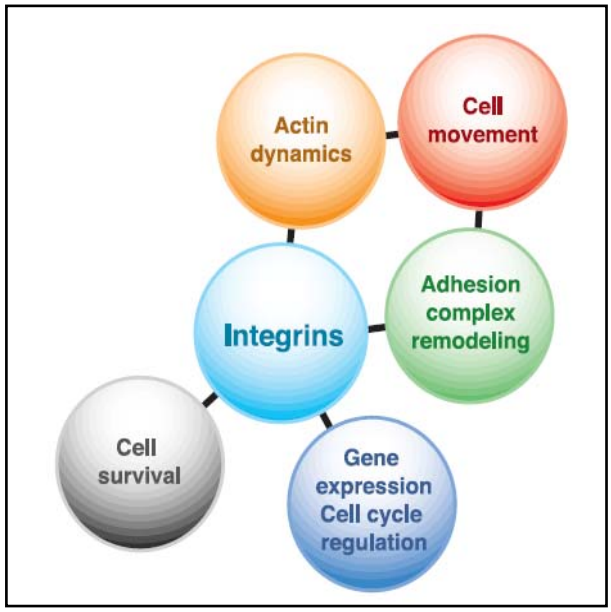
Homotypic (between cells of the same type) epithelial cell-cell adhesion counteracts the escape of cells into neighboring tissues. Heterotypic cell-cell adhesion, *e.g.* between circulating cancer cells and the vascular endothelium, promotes invasion through the vascular wall and the initiation of metastases. The prototype of homotypic epithelial cell-cell adhesion molecule operating through homophylic interaction is E-cadherin. Adherents junctions are the structure in which E-cadherin operates, but other intercellular junctions, such as tight junctions, desmosomes, and gap junctions, exist and putatively play a role in invasion. E-cadherin dimers on one cell form stable molecular bonds with E-cadherin dimers on another cell. Such extracellular interaction is sufficient for a relatively weak adhesion, whereas stronger adhesion necessitates also intracytoplasmic interactions of E-cadherin and an intact transmembrane domain. Loss of E-cadherin, eventually associated with upregulation of N-cadherin, may stimulate migration and so promote invasion. The molecular organization and the links of the E-cadherin/catenin complex with multiple other receptor and non-receptor signal-tranducing systems make it very likely that alterations of an element of the complex affects multiple cellular activities. Indeed, tyrosine phosphorylation of β-catenin by non-receptor kinases, like Src, and receptor tyrosine kinases, like EGFR and c-Met, perturbs the E-cadherin/β-catenin complex in various manners: alterations of the binding of the complex to actin, disturbance of the signaling function through conformational changes that hamper the cross-talk with other proteins, and recruitment of new partner proteins that possess phosphotyrosine-specific binding domains.

3.3.3. Cell-substratum adhesion: receptors for the extracellular matrix [21]

For cancer cell migration, a dynamic formation and dissolution of cell-substrate contacts is needed, and ECM receptors are expected to have a dual function, as they serve the adhesion to the matrix necessary for migration as well as arrest of cells inside the matrix. To coordinate all these functions, multiple extracellular and intracellular networks as well as fine tuning signaling are necessary. Structurally, cell-substrate adhesion is manifested by small focal complexes at the leading edge of migratory cells, larger focal adhesions at the end of actin stress fibres all over the surface of static cells, and hemidesmosomes between epithelial

cells and the basement membrane. Cellular receptors for ECM molecules belong mainly to the integrin family. They recognize unique short amino acid sequences, such as RGD, in the ECM molecules. Integrins are integral membrane cell surface glycoproteins composed of two subunits α and β linked by disulfide bonds; the combination of different subunits determines ligand specificity.

Epithelial cancers tend to express fewer integrins, such as $\alpha 6\beta 4$, and they do so in a disorganized pattern. ECM/integrin interactions are implicated not only in invasion but also in differentiation, proliferation and regulation of apoptosis. Invasive cells leave their natural ECM context above the basement membrane and arrive in a completely different ECM in the stroma. When such cells fail to switch on survival pathways, they go into a form of apoptosis called anoikis. Integrins transduce signals that regulate anchorage dependence of growth and share with growth factor receptors very similar signaling pathways. Activation of tumor promoter genes encoding elements of the integrin signaling pathways may overcome the necessity of integrin-mediated cell-substrate contacts for growth and survival.



Signal transduction pathways emanating from integrins regulate numerous cellular processes.
From Martin, Science 2002 (Ref. 33)

Focal adhesion kinase (FAK) is a 125-kDa non-receptor protein tyrosine kinase that is essential for signaling from the extracellular matrix to the actin cytoskeleton. Cell-substrate attachment, mediated by the direct interaction of integrin extracellular domains with the extracellular matrix, leads to clustering of integrins, recruitment to the focal adhesion complexes and tyrosine phosphorylation of FAK. Focal complexes

at the leading edge thus provide the cell with “directionality” for cell movement. The formation and remodelling of such focal contacts is a dynamic process regulated by protein tyrosine kinases and small GTPases of the Rho family.

3.3.4. Migration [21]

Cell motility plays a key role in both normal physiology and various disease processes. There are no arguments to believe that the locomotory apparatus of cancer cells is different from that of their normal counterparts. Our actual molecular concept of migration is that motility factors find their receptor on the cell surface and stimulate the cell to migrate and find its way toward the source of the motility factor. Such factors may stimulate or inhibit migration. Most motility factors are motogens as well as mitogens: they act on cell motility as well as on growth. The initial protrusive structure, filopodia and lamellipodia, contains dense arrays of actin filaments with their barbed ends (fast growing, or plus ends) oriented in the direction of protrusion. The key events regulating cell motility are polymerization of actin, formation of actin stress fibres, and focal adhesion formation.

Many forms of invasion are dependent on the small GTPases Rho, Rac, Cdc42 and Ras, which are essential for the control of the actin assembly/disassembly regulating cell movement. Through their multiple target proteins, the function of these GTPases is not restricted to migration but also involves adhesion and proliferation. Whereas Rac controls protrusion of lamellipodia and forward movement, Cdc42 maintains cell polarity and Rho mediates the cell-substrate adhesion needed for migration and stabilizes microtubules that are oriented toward the leading edge. An extensive treatment of Rho-family small GTPases-driven cellular locomotion will be presented further.

Many studies showed that HGF–Met signaling increases the motility of epithelial cells [24].

3.3.5. Proteolisis: an essential step for cancer cell homing in new sites [24]

Tumor cell metastasis requires neoplastic cells to interact with the extracellular matrix and break the barrier to the circulation. The critical event of tumor invasion is the interaction of the neoplastic cells with the basement membrane (BM). The basement membrane is composed of type IV collagen, laminin, and heparan sulfate proteoglycan as its major components. The ECM is composed of the basement

membrane along with other large molecules such as fibronectin and vitronectin. ECM degradation is a highly dynamic event requiring the production of proteinases, namely plasminogen activator and matrix metalloproteinase, which act in cascades, where proenzymes are proteolytically cleaved before they act on their own substrate.

An HGF-stimulated pathway involving MAPK1/2 signaling is important in the up-regulation of expression of the serine protease urokinase (uPA) and its receptor (uPAR), resulting in an increase of uPA at the cell surface. Certain components of the ECM can be directly degraded by uPA, and more importantly, uPA cleaves plasminogen into the broader-specificity protease plasmin, which is able to efficiently degrade several ECM and basement membrane components. Plasmin also activates metalloproteinases which have potent ECM/BM degrading abilities.

3.3.6. Angiogenesis [24]

The induction of angiogenesis (formation of new blood vessels) may be an important mechanism that permits tumor cell proliferation and eventually metastasis. Tumor-derived angiogenic factors lead to ingrowth of new capillaries with fragmented basement membranes, allowing tumor cells to more rapidly enter the circulation. The regulation of angiogenesis depends upon a balance between the activity of local factors that promote or inhibit neovascularization.

HGF acts as a potent angiogenic molecule by directly acting on vascular endothelial cells. HGF stimulation of vascular endothelial cells promotes migration, proliferation, protease production, invasion and organization into capillary-like tubes. HGF can also promote the expression of angiogenesis factors by tumor cells, namely IL-8 and VEGF.

3.4. Src and Met: prototypes of tyrosine kinases implicated in cancer development [21,22,23]

Phosphorylation and dephosphorylation are key phenomena in intracellular signaling, and genes encoding kinases and phosphatases are on the list of oncogenes and tumor-suppressor genes.

SRC is the first oncogene detected and a prototype showing many characteristics of the other oncogenes. The viral oncogene v-*SRC* has a cellular counterpart c-*SRC* that is activated by mutation to become an oncogene. The Src protein is anchored to the plasma membrane through myristoylation, receives various

signals, signals in its turn to many substrates directly and indirectly, and is implicated in numerous cellular functions, including proliferation, motility as well as cell-cell and cell-substrate adhesion.

At an experimental level, temperature-sensitive mutants of *SRC* were used to transform the MDCK epithelial cell line. Their invasion into embryonic chick heart or into collagen gels could be switched on by changing the incubation temperature from 39.5 to 35°C . In these MDCK*ts-src* cells, activation of Src leads to loss of E-cadherin functions, as evidenced by deficient cellular aggregation and gain of invasion. One of the molecular changes of interest in the E-cadherin/catenin complex is tyrosine phosphorylation of β-catenin, weakening its binding with E-cadherin.

As well as for non-receptor tyrosine kinases such as Src, receptor tyrosine kinases such as c-*MET* and *FGFR* were discovered as oncogenes. The c-Met receptor for HGF consists of a 50-kDa extracellular α-subunit that is disulfide-linked to a 145-kDa β-subunit having cytoplasmic tyrosine kinase domains and sites of tyrosine phosphorylation. The activity of tyrosine kinase catalytic domain is induced in an autocatalytic fashion by receptor transphosphorylation. The C-terminal tail constitutes the docking site that is responsible for the recruitment of a wide spectrum of downstream signaling molecules, including PI 3-kinase, the GRB2/SOS complex, the non-receptor tyrosine kinase Src, the transcription factor signal transducer and activator of transcription 3 (STAT3) and the adaptors Shc and Gab-1, which provide additional docking sites for many other signaling molecules. Moreover, recent data showed that Met functionally interacts with receptors of different families, all individually believed to be involved in cancer progression, such as B plexins, members of the EGF-receptor family, Fas, integrin α6β4 and CD44.

The role of Met in human tumors emerged from several experimental approaches and was unequivocally proved by the discovery of Met-activating mutations in inherited forms of human renal papillary carcinomas. Mutant Met induces motility in MDCK cells and metastatic potential in NIH-3T3 fibroblasts; transgenic mice develop metastatic mammary cancer.

Interestingly, the expression pattern of the c-*MET*, and of other protooncogenic receptor tyrosine kinases, with their respective ligands during embryonic development suggests that they are involved in normal epithelial morphogenesis as well. It has been shown that the scattered phenotype induced by

HGF is equivalent to an EMT, at least in defined cellular models. Scattering or EMT is a necessary event that occurs before several morphogenic processes. During organogenesis, cell stimulation by autocrine HGF stops. Instead Met, which is expressed by epithelial and myoblast progenitors, is stimulated by paracrine HGF expressed by the mesenchymal cells. The most frequent occurrence in human tumours is the increased expression of c-*MET* in the absence of mutation or the production of autocrine HGF. This type of *MET* deregulation is associated with a metastatic phenotype and a poor prognosis. *MET* overexpression is evident in colorectal carcinomas, hepatocarcinomas, gastrinomas, and carcinomas of the pancreas, stomach, prostate, ovary and breast. Met can function as an activated transforming oncogene and the driving force for clonal selection in tumour onset, but this is likely to be a rare occurrence. Alternatively and more frequently, wild-type Met can promote motility, invasion and metastasis of cancer cells, independently of the oncogenic events that cause tumour onset and progression. The two roles (transformation and invasiveness) can be executed concomitantly in cases where *MET* genetic alterations have occurred. Furthermore, the motile and invasive phenotype can be sustained when Met is overexpressed and activated by ligand binding. Conceivably, Met overexpression sensitizes cells to HGF and invasive-growth signaling, supporting the role of the tumour microenvironment in promoting metastasis. As activated oncogenes, such as *Ras*, *RET*, or *ETS*, or other mitogenic signals, induce *MET* transcription, Met overexpression is often a consequence rather than the cause of cell transformation, but it definitely has a key role in cellular metastasis.

3.5. Overview on HGF/Met signalling [21,24,25]

Signal transduction pathways have many potential branch points that provide opportunities for signaling cross-talks, and most invasion pathways are quite complex though often presented as linear. Methods used to study the invasive signal transduction implicate the genetic manipulation of cells to change their sensitivity toward putative invasion modulators and the use of pharmacological inhibitors targeting specific elements of the signaling pathways.

A large signaling complex is recruited to activated c-Met: it includes the adaptor proteins Grb2, SHC, Gab1, Crk/CRKL and the signal transducers PI 3-K, Stat3,

PLC-γ, the Ras guanine nucleotide exchange factor son-of-sevenless (SOS), the Src kinase, and the SHP2 phosphatase. Interaction of c-Met with adaptor proteins and signal transducers can occur directly via the multisubstrate docking site at the C-terminus of Met or indirectly via other adaptor proteins and signal transducers.

Activation of the receptor by HGF recruits a group of downstream molecules and/or adaptor proteins to its multidocking sites (Y^{1349}VHV and Y^{1356}VNV). The list of downstream molecules and adaptor proteins continue to grow and utilize the tyrosine residues in the docking sites differentially. Grb2 prefers to bind Y^{1356}, while other factors including Gab1, p85 PI 3-K, PLC-γ, Src and Shc associate with both Y^{1349} and Y^{1356}. Mutation of Y^{1356} uncouples Met from Grb2, the mediator for Ras/MAPK pathway through association with SOS; this mutation also impairs the interaction of Met with Gab1, a large scaffold adaptor protein which recruits several important substrates to activated Met.

PI 3-K pathway is coupled to Met through the interaction of its p85 subunit with the multi-docking sites of Met. The p85 subunit contains two SH2 domains (N- and C-terminal) and an SH3 domain and acts as an adaptor protein, allowing the p110 subunit to interact with protein tyrosine kinases and tyrosine-phosphorylated proteins, resulting in the activation of PI 3-K enzymatic activity. Ras/MAPK pathway is bridged to activated Met by the adaptor protein Grb2 which links Met to SOS, a Ras GEF. HGF, through activated ERK, also induces the phosphorylation and activation of paxillin and focal adhesion kinase (FAK). It has been shown that Ras is required for epithelial adhesion junction disassembly induced by HGF through activation of both PI 3-K and MAPK. Collectively, these studies provide strong evidence that both PI 3-K and Ras/MAPK pathways are required or HGF-induced invasion and branching morphogenesis.

In addition to PI 3-K and Ras/MAPK pathways, activated Met also recruits Gab1, Src and Stat3 to its multi-docking sites. Gab1, the large scaffolding molecule that is phosphorylated in association with activated Met, is also responsible for HGF/Met-induced scattering and branching morphogenesis of epithelial cells. Gab1 brings a number of substrates to the multi-docking sites of Met for phosphorylation and activation, including PLC-γ, Shc, Shp2, and CRKL as well as Grb2 and PI 3-K. Uncoupling of PI 3-K, PLC-γ or Shp2 from Gab1 impairs Met-mediated branching morphogenesis.

SH2 domain-containing inositol 5-phosphastase 1 (SHIP-1) was identified to be a new binding partner for Met via Y^{1356} at the multi-docking sites. Overexpression of SHIP-1 enhances HGF-induced branching tubulogenesis, while mutant SHIP-1 impairs this process, suggesting that SHIP-1 is another critical factor for HGF/Met-mediated branching morphogenesis.

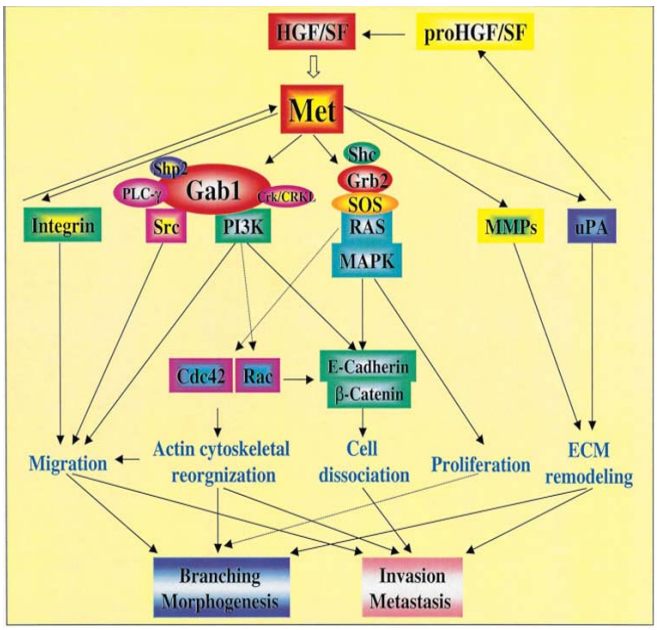
During invasion or branching morphogenesis, the driving forces for cell motility are derived from the actin cytoskeletal reorganization, which is controlled by Cdc42, Rac and Rho small GTPases. Cdc42 promotes filipodia and microspikes formation, while Rac induces lamellipodia and membrane ruffling. HGF can induce the activation of Cdc42, Rac and Rho, concomitantly with the formation of filipodia, lamellipodia and membrane ruffling. Several effectors for Cdc42 and/or Rac have also been found to be involved in HGF-induced cell-cell dissociation and migration, such as Cdc42/Rac-regulated p21-activated kinase (PAK) and neural Wiskott-Aldrich syndrome protein (N-WASP). Dominant negative Cdc42 or Rac inhibit HGF-induced lamellipodia formation and cell spreading.

Members of the Crk family of adapter proteins (c-Crk, v-Crk, and CRKL) are comprised primarily of SH2 and SH3 domains. Both c-CrK and CRKL have been implicated in signaling via HGF/Met. Crk and CRKL bind to phosphorylated Gab1. Sustained tyrosine phosphorylation of Gab1 is required for branching morphogenesis to occur. Crk appears to bind Gab1 in response to Met but not other receptor tyrosine kinases. The SH3 domain of Crk family members binds to proline-rich domains found in C3G, a guanine nucleotide exchange factor that activates the GTPase Rap1, a member of the Ras superfamily. Crk family members also bind Dock180, which binds to and activates Rac. Both Rap1 and Rac are activated by HGF/Met signaling presumably via the Crk-mediated mechanisms just described.

Integrins play a major role in facilitating communication between the ECM and the actin cytoskeleton. Integrins are cell surface, heterodimeric receptors (α, β) that bind to most proteins of the ECM and induce intracellular signal transduction (outside-in signaling). HGF/Met signaling promotes cell adhesion and invasion by increasing the avidity of integrins for their specific ligands. There is a “cross-talk” between HGF/Met and integrins and as well as with cadherins, with considerable activation of downstream focal adhesion, Rho GTPases and catenin proteins.

The focal adhesion site connects a cell with its underlying substratum via integrins, which associate with the actin cytoskeleton. In addition to this structural function, the focal adhesion also plays an important role in cell signaling. Multiple non-enzymatic proteins comprise the focal adhesion including vinculin, α-actinin, and paxillin. Other proteins localized in the focal adhesion include kinases (such as p125FAK), phosphatases (such as LAR), and proteases (such as calpain II). HGF signaling has been shown to induce the formation of focal adhesions. Paxillin is a 68 kDa protein with four tandem LIM domains at the C-terminus, a proline-rich region at the N-terminus, and several tyrosine motifs that form optimal binding sites for Crk/CRKL, Src, and other proteins. Other binding sites exist for focal adhesion proteins such as vinculin and FAK. Paxillin is tyrosine phosphorylated in response to integrin cross-linking, growth factor stimulation, and neuropeptide stimulation. HGF/Met signaling induces the phosphorylation of paxillin.

E-cadherin mediates cell–cell adhesion in epithelial tissues. E-cadherin connects epithelial cells through homotypic interactions via its association with catenins (α, β, γ). HGF stimulation promotes the tyrosine phosphorylation of β-catenin, interfering with the ability of β-catenin to bind the intracellular portion of E-cadherin. The result is a dismantling of cell-cell adhesion complexes.



Met signaling.
From Zhang and Wande Woude, J. Cell. Biochem. 2003 (Ref. 25).

The non-receptor tyrosine kinase Src is a major mediator for Met signaling and will be extensively treated further.