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Review

Initiation of epithelial-mesenchymal transition by c-met receptor tyrosine kinase signaling

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ABSTRACT

Epithelial-mesenchymal transition (EMT) occurs when individual epithelial cells detach from the epithelial tissue and then migrate to distant sites. This process occurs throughout development, where cells from primordial epithelial tissues are triggered to detach, migrate, and colonize distant locations to establish new tissues. This process is also a fundamental part of cancer metastasis of epithelial tumors. In development, EMT events are triggered by specific and carefully controlled signal transduction pathways, including signaling initiated by the c-met receptor tyrosine kinase. In addition to driving EMT, c-met signaling also activates cell proliferation and increased cell survival. Despite a potentially central role in cancer progression and other diseases, signaling downstream of c-met receptor is not well characterized and thus efforts to perturb c-met signaling during disease progression are hindered. Here we discuss recent advances in our molecular understanding of how c-met signaling is transduced, with an emphasis placed on reconstructing the architecture of the c-met signaling pathway at the molecular level.

KEYWORDS: c-met, HGF, signaling, receptor tyrosine kinase, epithelial-mesenchymal transition (EMT), adhesion, migration

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INTRODUCTION

Scatter factor or hepatocyte growth factor (SF/HGF) triggers scattering of epithelial cells in culture. Cell treated with SF/HGF undergo dramatic changes in cell morphology, including cell spreading, increased migration, and detachment of cell-cell adhesions. Cells that were tightly integrated into epithelial tissues instead become solitary, migratory, and invasive. This process mimics the early stages of epithelial-mesenchymal transition, a developmental program in which individual epithelial cells detach from tissues and migrate to distant sites as individual cells or groups of cells. In addition to triggering cellular events that strikingly resemble epithelial-mesenchymal transition, SF/HGF also triggers increased cell proliferation and survival. In development, SF/HGF triggers EMT in several instances, most notably the scattering cells of the dermamyotome [1]. SF/HGF signaling is also linked with cancer progression, driving changes in cell proliferation, survival, and the cellular events that drive metastasis. Cellular events associated with metastasis include breakdown of cell-cell adhesions, initiation of migration, invasion of surrounding tissues, and colonization of distant tissues with tumor cells, a strikingly similar series of events to developmental EMT programs.

SF/HGF is the growth factor ligand for the c-met receptor tyrosine kinase [2]. Receptor tyrosine kinases (RTKs) are typically activated through ligand-induced dimerization, and the c-met receptor is no exception. In this article we will explore the structure of the c-met signal transduction pathway

in an effort to understand how the cell uses the c-met signaling pathway to drive epithelial-mesenchymal transition. Since the structure of the c-met signaling pathway is shared with receptor tyrosine kinase signaling generally, we will also consider how network dynamics and signaling context might allow cells to differentiate c-met activation from activation of other receptor tyrosine kinases.

Overall signaling pathway architecture is constrained by the activation of both nuclear and cypolasmic end target proteins, which requires a branched pathway. The activation of distinct cellular responses following c-met activation further demonstrates that the c-met signaling pathway is branched. That initiation of signaling results in a final phenotypic outcome, namely transition of cells from epithelial to mesenchymal, suggests to biologists at the outset that the overall structure of the c-met signaling pathway is linear, rather than as an adaptive network. Activation of the receptor is transmitted through multiple nodes and ends at a large variety of downstream events, including changes in the regulation of specific genes and proteins. Given the large number of target proteins and genes, it is not surprising that the linear c-met architecture must be branched, though this does not rule out interconnections between distinct branches. While consideration of the c-met network as a branched linear network is appealing, there is also evidence that the network works as an adaptive network. C-met activation does result in a transcriptional increase in production of c-met receptors [3, 4]. This could act as a large positive feedback loop system that perpetuates initiation of c-met signaling. This subtle change in signal architecture converts the linear network to an adaptive network system (Figure 1).

C-met as receptor tyrosine kinase

C-met is one of a diverse array of receptor tyrosine kinases, which are involved both in normal homeostasis and in various disease states. There are 58 receptor tyrosine kinases belonging to 20 families of between one and six members. These receptors bear an extracellular ligand binding site and an intracellular tyrosine kinase domain. These two

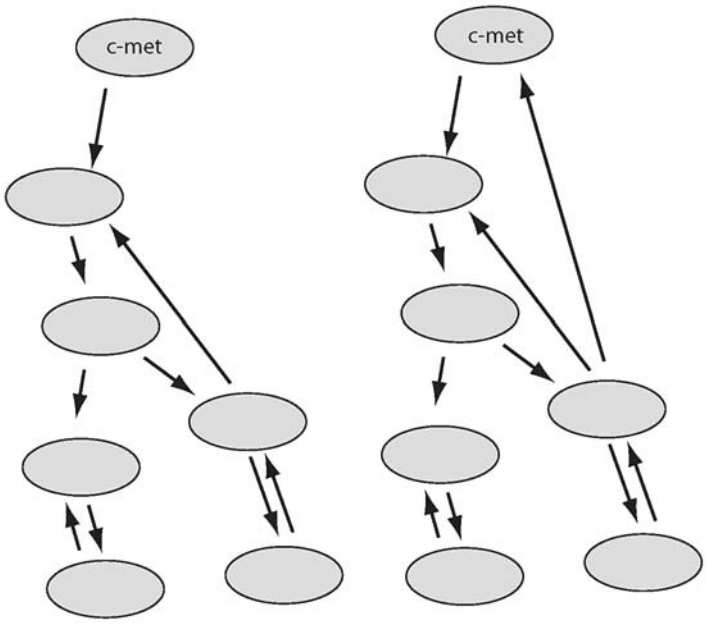

Figure 1. C-met as a linear pathway and network system.
A subtle change, namely a positive feedback loop to the c-met receptor, renders a linear pathway (left) into an adaptable network system (right) and thus complicates its experimental reconstruction.

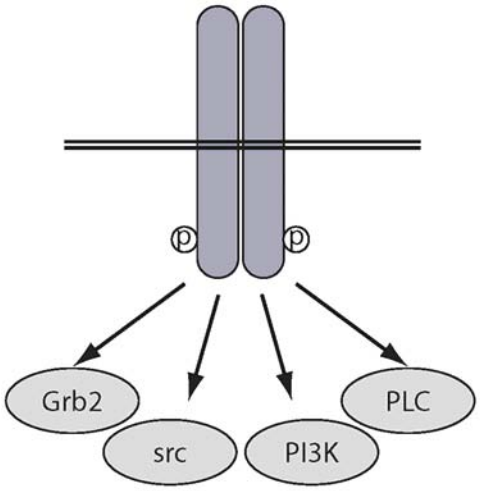

Figure 2. C-met signal branching downstream of receptor phosphorylation.

domains are separated by a single transmembrane domain. While each subfamily of receptor tyrosine kinases is activated by a different ligand, the mechanism of ligand-induced activation is largely conserved [5]. Receptor dimerization, driven by ligand binding, brings the tyrosine kinase domains of receptor tyrosine kinases into proximity to facilitate *trans* phosphorylation. Receptor phosphorylation is the initial event that then triggers downstream activation of a number of signaling modules. In fact, phosphorylated receptors act as a critical component in RTK signaling, allowing recruitment of a wide variety of SH2 domain-containing downstream effectors. Thus signal branching can occur at the level of the receptor, as multiple signaling modules headed by different SH2 domain containing proteins are activated following receptor phosphorylation (Figure 2).

Proteins recruited to phosphorylated receptor tyrosine kinases have been extensively studied. One such protein is Grb2, which, besides being used by c-met specifically, is also an important signaling node in networks downstream of most other receptor tyrosine kinases. Grb2 is a critical component of an important signaling module in RTK signaling networks. It stands at the extreme upstream of the MAP/ERK kinase cascade, acting to recruit the Ras GEF SOS1 into an RTK/Grb2/SOS1 signaling complex and thus to the membrane, thereby initiating activation Ras and of the downstream MAPK/ERK kinase cascade. In fact, SOS1 recruitment to the membrane and the subsequent activation of Ras is a major theme in RTK signaling and can occur through additional mechanisms. SOS1 can be recruited to the membrane by formation of several other signaling complexes, including c-met/RanBP9/SOS1 [6] and c-met/Grb2/Shc/SOS1 [7, 8]. In addition to direct recruitment of SOS1 by activated c-met receptors, activation of the oncogenic kinase Src by phosphorylated c-met receptors [9] can induce formation of a FAK/Grb2/SOS1 complex [10], which then stimulates Ras nucleotide exchange and activation [11]. This indirect mechanism may allow for activation of the SOS1/Ras signaling module at different times following RTK activation.

Other examples of important signaling nodes directly recruited and activated by phosphorylated receptors include phosphatidylinositol-3-kinase (PI3K), PLC, and the namesake of the SH2 domain, src. Bearing SH2 domains, these proteins are activated downstream of a wide variety of RTKs. Activation of these proteins can be accomplished by direct activation through their SH2 domain-mediated recruitment into c-met receptor tyrosine kinase complexes [12]. These proteins can also be activated downstream of RTKs indirectly, which can affect the timing of activation following receptor activation. For example, PI3K can also be activated downstream of c-met indirectly, downstream of GTP-bound Ras [13, 14]. Activation of PI3K downstream of Ras requires c-met induction of Ras nucleotide exchange by the Ras GEF SOS1, an event that follows SOS1 recruitment to the membrane by the multiple possible mechanisms mentioned in the preceding paragraph.

Activation of the above signaling components is highly conserved within diverse RTK signaling networks, as illustrated by reports where the activity of one RTK is able to compensate for the loss of function of another related RTK. For example, expression of c-met is sufficient to rescue an EMT phenotype when EGFR function is blocked [15]. Despite similarities in usage of major signaling modules, different RTKs generate different cellular responses in the same cell type. MDCK cells are stimulated to undergo EMT when the c-met RTK is activated, but not in response to

activation of EGFR or VEGFR receptor tyrosine kinase systems. This is also true in terms of molecular effects following activation of different RTK signaling networks; c-met and EGFR have opposite effects on Gab1 expression, for example, with c-met upregulating protein activity and with EGF downregulating it [16].

The striking conservation of signaling mechanisms between c-met and other RTK signaling networks begs the question of how the cell differentiates between c-met activation and activation of other RTK systems in order to generate the molecular and phenotypic responses that drive EMT. Some RTKs employ a greater number of proteins to control signal transduction through the same signaling modules (an example is FGF receptor tyrosine kinase, which has a larger number of regulatory proteins than the closely related EGF receptor tyrosine kinase [17]), which might account for differences in RTK signaling outcomes. This is important since it can affect the relative timing or intensity of shared signaling module activation between otherwise similar RTK signaling network systems, thus generating different cellular signaling outcomes. In fact the timing of signaling seems to alter the outcome of a signaling network significantly. It has been observed that alterations in the timing of MAPK/ERK signaling are associated with distinct signaling outcomes, namely proliferation versus apoptosis, in response to TNFα signaling in intestinal epithelial cells [18]. Thus, it is likely that it is more than the static architecture of a signaling network that provides information to the cell in determining cellular responses to network activation.

Feedback loops within signaling modules are also a common feature within receptor tyrosine kinase signaling networks, affecting the intensity of signaling module activation. Generally, negative feedback loops provide stability to signaling networks, while positive feedback loops provide exponential signal amplification or suppression. Both types are found in c-met signaling (Figure 3) and examples of both types of feedback loops affect the MAPK/ERK signaling module. In addition to the Ras-dependent activation discussed earlier, the MAPK/ERK signaling module can also be activated by c-met/Gab1/Shp2 [19] or c-met/Grb2/Gab1/Shp2 complexes [20].

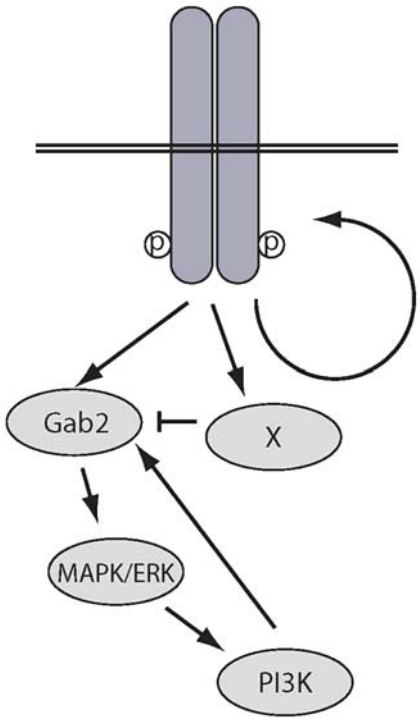

Figure 3. Three examples of feedback loops in c-met signaling.

These Shp2-containing complexes are affected by positive and negative feedback loops. First, the MAPK/ERK module increases activity of PI3K activity, which increases Gab1 activity, which in turn increases activation of the MAPK/ERK module and drives yet more PI3K activity. Thus, activation of MAPK/ERK module signaling creates a positive feedback circuit through PI3K and Gab1 to exponentially increase signal output [21-23]. In contrast, a negative feedback loop that reduces late signaling intensity occurs when prolonged activation of c-met induces degradation of Gab1 through targeted ubiquitylation, though this circuit remains more poorly defined at the molecular level [24]. Importantly, arrangement of multiple feedback loops within a single signaling module can generate complex effects on overall signaling output, particularly as a function of time (e.g. oscillatory signaling).

Perhaps a more obvious mechanism for generating distinct responses from highly similar RTK systems is to alter the context of signaling by forcing crosstalk of specific RTKs with other signaling networks. Association of RTK systems

with other signaling receptor systems at the cell surface allows for such crosstalk. C-met signaling has been found to be highly dependent on CD44. Deletion of the CD44 gene renders c-met haploinsufficiency lethal, demonstrating collaboration between CD44 and c-met [25], though other receptor systems, most notably ICAM-1, appear to be able to compensate for loss of CD44 in some instances [26]. CD44 is a receptor that drives increased migration during wound healing and, like c-met itself, is tightly associate with cancer progression [27]. CD44, and particularly splice variant 6, plays a critical role in the c-met signaling transduction network, where it functions in multiple steps [28]. Interestingly, in order for CD44 to facilitate Ras signaling downstream of c-met receptors, it must associate with actin filaments via ezrin/radixin/moesin proteins [29], suggesting that c-met, CD44, and ezrin scaffold the formation of a large signaling particle. Unlike the SH2 interaction with phosphorylated receptors, these lateral associations vary within RTK systems. Since c-met signaling is dependent on the lateral association of c-met receptor tyrosine kinases with other plasma membrane receptors, a highly conserved and otherwise undifferentiated RTK signaling network may operate distinctly from other RTK networks simply from alterations of the context in which RTK signaling occurs. In other words, activation of a generic RTK network in combination with different accessory signaling receptors could allow the cell to differentiate its cellular response.

Additional signaling nodes and modules have been implicated in modulating c-met signaling, also perhaps providing specific context to RTK signaling. Recently an increasing number of studies have recognized the importance of Ca^{2+} fluxes in RTK signaling [30, 31], particularly in the case of c-met [32-34]. In EGFR signaling, this Ca^{2+} influxes result from microtubule-dependent vesicular trafficking of Ca^{2+} channels to the plasma membrane [35]. The potential role of calcium influxes in the c-met signaling network illustrates how activation of signaling within context might also relate to timing of signaling events, as the precise pattern of calcium influx periodicity plays a critical role in cellular interpretation of calcium signaling. Perhaps different patterns of calcium influxes combined with the same RTK signaling network could elicit different cellular responses.

HGF-induced epithelial-mesenchymal transition

Epithelial-mesenchymal transition is a higher order process that involves many complicated changes in cellular behavior. Activation of EMT by a single cellular signaling network requires the coordinated control of many cell biological processes. The principal signaling modules that lead from c-met receptor activation and that initiate epithelial-mesenchymal transition are those which drive the individual cellular processes of actin rearrangement, cell spreading, detachment of cadherin-based cell-cell adhesions, accelerated cell migration, and invasion through extracellular matrices. Spatiotemporally coordinated induction of the correct signaling modules thus drives the larger EMT process. Additional signaling modules not directly tied to epithelial-mesenchymal transition are also activated by c-met and responsible for EMT-independent cellular behavior changes, including inhibition of apoptosis and increased proliferation that will be discussed in another section. The number of distinct cellular processes targeted by c-met signaling suggests that network branching is required for control of different cellular events. The branching downstream of receptor phosphorylation, described in detail in the previous section, seems well suited to this purpose, though the biological reality is much more complex. Here, we will address the individual signal transduction modules that lead from the c-met receptor to specific changes in cellular behavior. It is important to consider where cytosolic signaling alone, without changes in gene transcription, could account for cellular responses to SF/HGF stimulation. In instances where the localization and activity of proteins is altered by signaling, as might drive actin dynamics or changes in cadherin internalization, it is possible that cytosolic signaling may account for the entirety of the specific cellular response. Conversely, events that rely on changes in gene transcription and altered levels of protein production, such as in the case of expression of surface proteases required for invasion, gene transcription is clearly fundamental. There are thus undoubtedly additional

branch points in the overall c-met signaling pathway that depend on whether cellular effects are controlled at the pre- or post-transcriptional levels. Here we will examine the connection of signaling nodes with specific cellular responses to c-met activation, providing a picture of the overall c-met signaling network structure.

Induction of actin rearrangement

Morphological changes in cells are driven by actin dynamics and HGF-induced EMT is no exception. HGF stimulation results in dramatic reorganization of the actin cytoskeleton [36]. Actin rearrangements are an essential first step required for nearly every other cell biological process that underlies epithelial-mesenchymal transition [37]. Actin rearrangements are driven by altering the activity or abundance of numerous actin regulatory proteins. Given the large number of proteins that participate in actin dynamics, it is likely that multiple signaling proteins and their regulatory circuits provide the interface between the HGF signaling network and changes in actin organization. Central players in this interface appear to be small GTPases of the Rho family, which act as master regulators of actin dynamics in a number of cellular processes, as well as phosphatidylinositol-3-kinase (PI3K) [38, 39].

Induction of cell spreading

Cell spreading is an early event in HGF-induced epithelial-mesenchymal transition. Epithelial cells stimulated with SF/HGF roughly double the area of cell-substrate adhesion, an event that occurs prior to disruption of cell-cell adhesions. Cell spreading results from coordination of actin rearrangements [40] and modulation of integrin-based adhesion with the cell substratum. On certain substrates, cell spreading does not occur effectively [41], perhaps accounting for why the robustness of epithelial-mesenchymal transitions varies greatly depending on the matrix type [42]. Cell spreading, like actin rearrangements generally, relies extensively on Rho GTPases, particularly Rac1. In this pathway, Rac1 activation relies heavily on the Rac1 GEF, βPIX [43], which is in turn activated by the focal adhesion kinase (FAK)/Src-Yes-Fyn complexes [44-46]. FAK/Src-Yes-Fyn complexes assemble in response to src-dependent phosphorylation of FAK and have been shown to play a critical role in numerous events during EMT [47].

Induction of cell-cell detachment

In order for cells to complete scattering during epithelial-mesenchymal transition, epithelial cell-cell adhesions must be disassembled. Detachment of epithelial junctions occurs once cell spreading and initiation of cell migration have been completed. Disassembly of cadherin-based adhesions appears to rely on several possible mechanisms, alone or in combination.

Cadherin switching, meaning altering cadherin family member expression, is thought to occur early in epithelial-mesenchymal transition. Typically cells switch expression of the epithelial E-cadherin for that of N-cadherin. Interestingly, each cadherin is associated with different actin structures, namely E-cadherin with the actin organization that is observed in epithelial cells and N-cadherin with the actin organization of more mesenchymal cells. It remains unclear whether cadherin switching is a result of changes at the transcriptional levels, or whether this switch is a post-translational event with cells altering the preferential endocytosis of cadherin family members [48, 49].

C-met also appears to alter the function of cadherin adhesion receptors at cell-cell adhesions, primarily be changing the retention of this protein at the cell surface. E-cadherin has been observed to enter endocytic vesicles concurrently with the c-met receptor [54]. Phosphorylation of E-cadherin by src has been proposed to alter cadherin complex formation and, thus, cadherin function and distribution at the cells surface [50]. C-met signaling also can affect cadherin trafficking by Ras-dependent activation of Rin2, which stimulated Rab5-dependent vesicle trafficking to endosomes [55].

Another mechanism for abrogating cell-cell adhesion is by downregulation of E-cadherin transcription. Snail expression is increased in response to the nuclear translocation of transcriptional regulator EGR1, which is activated by the MAPK/ERK signaling module [56].

Physical rupture of cell-cell adhesion may also play a role in cell-cell detachment, especially during early EMT. This process relies on gaining a sufficiently strong grip on the cell substrate to pull apart cell-cell junctions as the cell contracts [57].

Tyrosine kinase-induced cell contractility relies on the Rho-ROCK-myosin pathway, which generates actomyosin-based contractile forces on cell-cell contacts [58]. Here c-met receptors activate p120-4A, which activates the RhoA signaling module [59].

Disruption of the tight junction system relies on increased Rac1 activity. Maintenance of the tight junction in the absence of c-met signaling is thought to rely on Par3 recruitment into aPKC/Par3/Par6 complexes, where is serves to locally depress Rac1 activity. Upon c-met activation, Src activity increases and phosphorylates E-cadherin, causing release of Numb. Numb binds phosphorylated aPKC/Par3/Par6 complexes, displacing Par3 and allowing it to translocate to the nucleus [50]. Without Par3 at the tight junction, local Rac1 activity increases and tight junctions are disassembled [51]. Interestingly, it has been shown that HGF can induce Rac1-mediated disassembly of adherens junctions in a Crk-dependent manner [52], which promotes redistribution of paxillin to focal adhesions. The result is the formation of a Crk/Paxillin/GIT2/ βPIX complex, which may then activate Rac1 and generate a potential positive feedback loop that exacerbates cell-cell junction disassembly [53].

Induction of increased cell migration

Cells responding to c-met stimulation increase cell motility, increasing their rate of migration by as much as 2 fold. Migration is driven largely by changes in actin organization and dynamics, further demonstrating the central role of actin reorganization in epithelial-mesenchymal transitions. Actin drives cellular protrusions at the leading edge that are required for cell translocation across a substrate, while remodeling of cell-substrate adhesions is also required for translocation. Rho GTPases are known to be central players in cell migration, both in regulating actin dynamics at cell protrusions and actin connections to cell-substrate adhesions. Like many essential processes in EMT, there are multiple, partially redundant circuits present in c-met signaling for inducing cell migration. Like many of the circuits discussed above, c-met-induced cell migration relies on several parallel circuits, each able to partially compensate for loss of function in its neighboring circuits.

Essential to c-met-induced cell migration is FAK, which is required to drive enhanced migration [60]. Studies have shown that focal adhesion kinase is activated by phosphorylation by several kinases, including src, c-met [61], and the MAPK/ERK module [62]. Phosphorylated FAK increases migration by altering membrane protrusion formation at the leading edge. Here FAK acts to facilitate the activation of actin regulatory proteins, including N-WASP [63]. At the trailing edge of the cells, FAK facilitates activation of the RhoA/myosin contractility pathway by forming a complex with PDZRhoGEF and cooperating in RhoA nucleotide exchange. FAK can also induce migration through activation of Arf6, which stimulates vesicle trafficking pathways that alter Rho GTPase activity, actin dynamics, and cell migration [64]. FAK/Src-Yes-Fyn complexes, described previously, also facilitate migration, acting via BMX [65], Grb7 [66], Rac1 [65-69] and focal adhesion disassembly at the trailing edge [70, 71].

Induction of cell invasion

Individual, solitary cells resulting from epithelial-mesenchymal transitions acquire the ability to penetrate through connective tissues by remodeling or degrading extracellular matrix. In the context of epithelial-mesenchymal transitions in development, this allows individual cells to transit through tissues to their final target destination. In the context of metastatic cancer cells from epithelial tumors, this allows cancer cells to invade through surrounding tissues and colonize distant sites. Surface proteases are the primary mediators of cells' ability to invade through connective tissues and cells undergoing epithelial-mesenchymal transition are no exception. Tumor invasion relies on the activity of surface matrix metalloproteinase 9 (MMP9) to degrade extracellular matrix proteins, a protein that is also expressed in response to SF/HGF signaling [72]. HGF-induced transcription of MMP proteins is mediated E1AF, an Ets family transcription factor [73]. Ets is activated through nuclear localization that results from signaling through the MAPK/ERK module [74]. In addition, Ets-1 has also been shown to participate in an important feedback loop in c-met signaling, namely driving SF/HGF-induced activation of c-met transcription [75].

C-met signaling in processes independent of EMT

Though EMT is a major cellular response to c-met signaling network activation in epithelial cells, other cellular responses to c-met signaling are also observed. These are inhibition of apoptosis and increased cell proliferation. HGF-induced inhibition of apoptosis results from inhibition of Bad, a pro-apoptotic member of the Bcl-2 family. This is accomplished by activating PI3K, which in turn generates survival signaling by acting on PDK1 and then AKT [76]. Inhibition of apoptosis can also result from upregulation of signaling through the NF-κB module and a downstream increase in Bcl-2 expression [77]. Additionally, it has also been shown that SF/HGF induces improved cell survival through phosphorylation of GATA-4 in a MAPK/ERK signaling module-dependent manner [78].

Increased cell proliferation is also mediated by NF-κB signaling module [79] or alternately, through the MAPK/ERK module and PI3K node. It is interesting to note that cell proliferation negatively correlates with EMT. It has been demonstrated that in proliferating cells, SF/HGF stimulation induces TIMP-2 to inhibit the cell surface protease MMP2, leading to increased matrix deposition and a reduction in invasion. In contrast, SF/HGF stimulation induces quiescent cells to increase matrix degradation through inhibition of TIMP-1 [80]. Clearly proliferative states play a major role in determining the outcome of c-met signaling, suggesting another instance of crosstalk between cellular processes and the c-met signaling network that provides context to signaling.

SUMMARY

Cellular signaling in response to c-met activation is highly relevant to a number of disease processes, including fibrosis, wound healing, cancer progression, and angiogenesis. A complete map of individual interactions and components in the c-met signaling network (the static network structure), however, is unlikely to provide a complete understanding of the connection between c-met signaling and normal cellular processes. Dissection of the c-met signaling network as an adaptable network system, where temporal dynamics of signaling and cellular context (crosstalk) are considered to be as important as network structure, will be critical to defining how c-met activation drives normal cellular processes and disease progression.

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