

Stress kinase signaling in cancer: fact or fiction?

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Abstract

Cancer results from genetic alterations in intracellular signaling pathways, which normally orchestrate the execution of developmental programs and the organismic response to extrinsic factors. Mutations in upstream activators and components of the cytoplasmic (Ras-Raf MEK-ERK) cascade frequently occur in tumors. *In vitro* and *in vivo* studies have shown that isolated activation of this pathway is both, necessary and sufficient for transformation. During the last years two new groups of related kinases have joined the ranks of mitogen-activated protein kinases (MAPKs), stress-activated protein kinases (SAPKs)/Jun N-terminal kinases (JNKs) and p38. Their activation not only occurs during cellular responses to unphysiological stimuli but also downstream of cytokine and pathogen receptors and has been observed in tumors. In this article we will review the role of stress kinases in cancer, and discuss the mechanisms through which they regulate the transformation process.

Key words

cancer, signaling, stress kinases, JNK, p38

1. Introduction: What does it take to make a cancer gene?

The steps required for the transition from a normal to a transformed phenotype have been under close scrutiny ever since the notion arose that genetic alterations are the underlying cause for cancer. Enumerations of changes occurring on this path and the dissection of their functional consequences has painted the picture of cancer as a disease with an array of divergent genetic modification but a limited number of traits, which are required for its successful establishment [1]. The capacity to transform is not restricted to genes identified in the setting of naturally occurring or experimentally induced tumors but is also observed for many artificially mutated proteins. The use of the latter has been instrumental in the dissection of cancer pathways. Functionally, cancer genes may belong to the following groups: oncogenes, tumor suppressors, and mutator genes [1]. Oncogenes are usually derived from normal cellular counterparts, the proto-oncogenes, which in their non-mutated form function as essential components of intracellular signaling pathways. They comprise ligands, receptors, molecular switches, cytoplasmic signal transducers and transcription factors. Functional heterogeneity is also seen among proteins with tumor suppressor activities, which include ligands, phosphatases, acetylases and transcription factors. Mutations eliminate the negative impact on growth and survival and thereby contribute to tumor formation. Mutator gene products normally function in the maintenance of genetic stability by assuring genomic integrity. Functional loss allows for the accumulation of a sufficient number of mutations required for the transformation process to succeed [1].

Canonical signaling pathways involved in the transformation process include the cytoplasmic (Ras-Raf-MEK-ERK)-, the Wnt/ β -catenin-, Hedgehog-, the JAK/STAT-, PI3K/PKB- and the Notch pathway. Since all of them have been reviewed extensively in the past [2-7], only a few common principles shall be highlighted here. Activation results from qualitative or quantitative changes in individual components through mutational events and relieves the cell from the control by extrinsic factors. One further shared feature is the preponderance of transcription factors as distal effectors stressing the role of *de novo* gene transcription for the transformation process. Together these cancer pathways control a limited number of traits that are required for the establishment and maintenance of the transformed phenotype: unlimited replication potential, self-sufficiency in growth signals, protection against apoptotic cell death, *de novo* angiogenesis, and the ability to metastasize [1]. The transforming potential of proteins involved in tumor development is directly related to their ability to simultaneously control several of these features [8]. Detailed analysis of tumor cells has demonstrated a multitude of small as well as large alterations in the genome. Despite this complex picture, the number of events required for the transformation *in vivo* may be limited as exemplified by tumors induced in transgenic mice by a single oncogene (e.g. [9, 10]), the reversibility of the oncogene-induced tumor phenotype [9], and the identification of a limited number of genetic alterations required for malignant transformation of primary cells [11]. Moreover, even established tumors are still susceptible to the action of inhibitors targeting a single component of a transformation pathway (e.g. inhibition of the Bcr-Abl kinase by Glivec [12]), suggesting the presence of little redundancy in essential pathways required for the maintenance and perhaps also for the establishment of the transformed phenotype.

2. Introducing new players in the cancer game

New players are constantly added to the list of cancer genes. Within the group of the mitogen-activated protein kinases oncogenic transformation has been analyzed primarily in the context of signaling through Ras-Raf-MEK leading to the activation of the first identified MAPKs ERK1,2 [13]. This signaling pathway was shown to be both, necessary and sufficient for transformation *in vitro* as well as *in vivo* [10, 14-16]. Two recently identified MAPKs, p38 and the c-Jun N-terminal kinase (JNK), were initially described as stress-activated protein kinases (SAPKs) [17]. Mammals possess three JNK-encoding genes (*JNK1*, *2* and *3*), [18]

(also known as *SAPK γ* , *SAPK α* and *SAPK β* [19]) giving rise to at least 10 different JNK isoforms through alternative splicing [20]. JNK1 and JNK2 are ubiquitously expressed, JNK3 expression is almost exclusively restricted to the brain [21]. Phosphorylation of two amino acid residues within the activation loop by the upstream dual-specificity kinases MKK4/SEK1 and MKK7 activates JNKs [22]. Stress kinase signaling is not only involved in the cellular response to unphysiological stimuli: analysis of cytokine receptor signaling and genetic studies in mice, *Drosophila* and *C. elegans* have demonstrated an essential role for JNKs in the signal transduction downstream of cytokine and pathogen receptors [23-25]. During *Drosophila* development JNK is required for the movement and fusion of epithelial sheets in the process of dorsal closure [26] and certain forms of developmental apoptosis [27, 28]. In *C. elegans* no evidence so far surfaced, linking JNK signaling to embryonic morphogenesis, but JNK requirement has been demonstrated for the coordination of body movement, synaptic vesicle transport, and the stress response [29]. In mammals JNKs have been implicated in T-cell maturation [30] and the induction of apoptotic cell death [31].

Four *p38* genes are known, which differ in their expression pattern and affinity for upstream activators and downstream targets. Whereas the expression of the γ and δ isoforms is limited to a few tissues, the α and β isoforms are widely expressed [32]. Activation of *p38* occurs through phosphorylation by MKK3/6 following exposure to cytokines, heat shock, or high osmolarity. *p38* kinases show the same degree of evolutionary conservation, which is also observed with other MAPKs. In *Drosophila* and *C. elegans* homologues of *p38* function in neuronal development and innate immunity [33, 34]. *p38* is also involved in vertebrate adipocyte and myocyte differentiation [35]. Additional studies suggested positive as well as a negative role for *p38* in survival and proliferation, depending on the cell system and the stimulation conditions applied [35].

The study of the parallelly structured MAPK pathways revealed a high degree of crosstalk. Prolonged activation of ERK stimulates JNK activity through the induction of heparin binding epidermal growth factor (hb-EGF), which upon binding to the epidermal growth factor (EGF) receptor triggers JNK activation [36]. Activation of JNK in turn negatively regulates the signal propagation through the cytoplasmic cascade. Interruption of the signal flow from activated MEK to ERK is one underlying mechanism [37]. We have no understanding how this crosstalk affects the processes of tumor initiation and progression and whether it allows the tumor to adapt to changing requirements for signaling activities throughout the life of a tumor.

2.1. Evidence for the oncogenic potential of JNKs and p38

c-Jun was found initially in an altered form as oncoprotein of the avian sarcoma virus 17 (ASV17) and later shown to be part of the AP-1 transcription factor complex, which regulates the expression of many gene products that are required for the establishment of the transformed phenotype [38]. Presence of a bZIP domain in all members of the Jun family, which in addition to c-Jun also includes JunD and JunB, allows them to form homodimers and heterodimers to generate transcription-competent AP-1 complexes. Further complexity is achieved through Fos and ATF proteins, which also participate in the formation of AP-1 complexes. The composition of AP-1 determines the target gene spectrum and the physiological outcome [23, 39]. Retroviruses expressing v-Jun but not c-Jun induce fibrosarcomas in chicken and transform CEFs. Transformation of established mammalian cell lines occurs with much lower efficiency and requires the presence of additional events [38], which include tissue wounding as demonstrated in the formation of sarcoma in H2K-v-jun transgenic mice [40]. c-Jun expression is elevated in many oncogene-transformed cells, and, as recently demonstrated, also in primary tumors [41]. Moreover, expression of a dominant interfering mutant of c-Jun (TAM67) blocks transformation by various classes of oncogenes [42, 43]. Requirement for c-Jun in tumor formation *in vivo* has been shown directly by

demonstrating that the liver-specific inactivation of the *c-jun* gene did not interfere with normal hepatocyte function but prevented the development of chemically induced hepatocellular carcinomas (HCCs) [44]. Impaired tumor growth in *c-jun* deficient hepatocytes correlated with upregulation of p53, its target gene *noxa*, coding for a pro-apoptotic member of the Bcl-2 family, and increased apoptosis [44].

c-Jun is subject to regulation by MAPKs of the ERK and JNK subfamilies. Activation of the cytoplasmic signaling cascade is observed in many tumors as the result of mutations in Raf itself or in the activation of upstream signaling components and may be responsible for the upregulation of c-Jun expression in these cells [45]. However, transcriptional competence also requires phosphorylation modification of c-Jun by JNKs. Activation of JNK was consistently observed in oncogene expressing cells [46-50], and constitutively active forms of JNK have been detected in primary glial tumors [51, 52]. Hyperactivation of JNK in tumors is the result of the presence of deregulated upstream components (e.g. Ras, Her2) or the overexpression of JNK itself [52]. Genetic proof for the critical role of c-Jun-directed JNK kinase activity in transformation has been provided by the demonstration that c-Jun N-terminal phosphorylation is required for transformation by certain oncogenes [53]. Isozyme-specific differences were observed in a mouse model of multi-stage skin tumorigenesis, where JNK2 deficiency inhibited tumorigenesis [54], while JNK1 functioned as tumor suppressor [55]. JNK requirement for transformation of primary fibroblasts by oncogenic Ras was also disputed based on results obtained in *Jnk-null* cells [56]. However, when we probed into the transforming activity of JNK, using a constitutively active mutant of the kinase, we obtained evidence for its oncogenic potential *in vivo* and *in vitro*. Activated JNK transformed NIH 3T3 fibroblasts [57] and was sufficient for fibrosarcoma development in nude mice [58].

p38 MAPK has been discussed a potential tumor suppressor based on reports implicating p38 in the negative regulation of cell survival and growth, a process, which implies the phosphorylation and activation of p53 and p73 [59-61] and the regulation of survival and death signaling [62]. In addition, p38 activity represses cyclin D1, stabilizes p21 as well as HBP1, thus leading to cell cycle arrest [63-65]. A variety of anti-cancer drugs (e.g. cisplatin) require functional p38 [66, 67]. In contrast, only a few publications have provided evidence for an oncogenic potential of p38. Appearance of phosphorylated p38 correlated with the progression of follicular lymphoma to diffuse large B-cell lymphoma [68] and the use of a p38 inhibitor demonstrated p38 requirement for chondrosarcoma proliferation [69]. The reasons for this discrepancy are currently unclear and apart from cell line differences, the loss of negative growth control through the inactivation of tumor suppressors as frequently seen in tumor cells may unmask the transforming potential of p38. Tumor suppressor function has also been discussed in the context of the JNK signaling. One of the first reports addressing the role of the newly discovered stress kinases in tumor formation suggested a tumor suppressor function based on the demonstration that in rare cases of tumors a deletion of MKK4 was observed [70]. An anti-oncogenic function of MKK4 could not be further substantiated in recent studies [71, 72].

2.2. Effector pathways in cellular transformation by stress kinase signaling

Insight into the role of stress kinase signaling under physiological and patho-physiological conditions has been gained through the use of genetically modified mice, *in vitro* studies using dominant negative and constitutively active mutants and more recently the application of inhibitors, whose selectivity is still a matter of debate. Fibroblasts deficient in c-Jun show severely reduced proliferation caused by p53-dependent upregulation of the CDK inhibitor p21, suggesting that c-Jun induces growth by antagonizing p53 [73]. These cells are resistant to transformation by certain oncogenes, e.g. Ras and Src [74]. Moreover, mice deficient in c-Jun die during mid- to late-gestation due to massive apoptosis of hepatoblasts, erythroblasts and other cell types [75]. These findings suggest a requirement for c-Jun in the proliferation

and survival of cells. The role of JNK in cell survival is controversial. In many instances, in particular in the neuronal system, JNK activation correlates with cell death [31]. However, JNK requirement in pro-survival signaling has also been reported [76] and may result in the phosphorylation inactivation of the pro-apoptotic Bcl-2 family member BAD [77] as previously described for the survival kinases, C-Raf and PKB [78, 79]. No effect on survival but instead growth induction was observed in our own *in vivo* and *in vitro* studies using a constitutively active mutant of JNK [58]. These directly opposing functions of JNK could result from the utilization of differing cellular models, or from the application of activators and activating conditions, whose effect is not limited to JNKs. Furthermore, the strength and the duration of the JNK signal can be important, as exemplified by the observation that only sustained activation of JNK is associated with apoptotic death [80]. Persistent JNK activation has been shown in tumors, suggesting that either the pro-apoptotic effects of JNKs in tumors are actively suppressed or that JNKs do not affect the cell survival process in this setting.

Similar to data obtained in c-Jun deficient mice, genetic inactivation of MKK7, a direct activator of JNKs, affected cell proliferation and survival [81]. Early passage MEFs showed reduced growth, premature senescence and G2/M cell-cycle arrest. The G2/M cell-cycle kinase CDC2 was identified as MKK7-JNK-c-Jun target [81]. No upregulation of CDC2 but instead induction of the G1 phase cyclin D1 was observed in nude mouse tumors induced by activated JNK suggesting the existence of various mechanism for stress kinase-dependent growth regulation [58].

Growth of tumors beyond a certain size results in hypoxia and requires the formation of new blood vessels which is controlled through the production and secretion of angiogenic factors. JNK and p38 are activated by hypoxic conditions in tumor cells [82, 83]. JNK in turn phosphorylates c-Jun and stimulates AP-1-dependent transcription [82]. Another transcription factor, whose expression and activation is induced under hypoxic conditions is HIF-1 α (hypoxia-inducible factor). Over 60 HIF-1 α target genes have been identified, which regulate survival, proliferation, cytoskeleton, energy metabolism and also angiogenesis [84]. c-Jun/AP-1 functionally cooperates with HIF-1 in different cell types [85]. Hypoxia induces the phosphorylation of c-Jun at Ser63 by JNK, which is necessary for this cooperation [85]. Pro-angiogenic factors like vascular endothelial growth factor (VEGF), the VEGF-related protein VRP, oncostatin and basic fibroblast growth factor all stimulate JNKs in endothelial cells [86]. VEGF also activates p38, which is required for actin reorganization and endothelial cell migration [87]. JNK as well as p38 are both able to increase the stability of the VEGF mRNA [88]. Most convincingly the function of c-Jun in angiogenesis was shown after conditional ablation of c-Jun, which blocked the ability of human endothelial cells to form new blood vessels *in vitro* and *in vivo* [89]. Proliferation, migration, invasiveness, tubule formation and the production of metalloproteinase-2 were all substantially inhibited in these cells [89]. Endostatin, an endogenous angiogenesis inhibitor, decreases the expression of c-Jun in human microvascular endothelial cells and activity suppresses JNK and p38 [90]. Matrix metalloproteinase-2 (MMP-2) is constitutively produced by endothelial cells and is required for the switch to the angiogenic phenotype in models of tumor formation [91]. MMP-2 is necessary for the remodeling of the extracellular matrix that takes part during angiogenesis. Increased blood vessel formation was also a characteristic of JNK-induced fibrosarcomas in nude mouse [58]. These findings suggest that JNK activation plays an important role for tumor growth, both through direct effects on cell-cycle progression and indirectly through allowing the tumor to respond to limitations in oxygen supply.

2.3. What can JNKs do others can't?

Much of the evidence in support for a role for stress kinase signaling in cancer stems from the analysis of transformed cells, knockout animals and human tumors. No transgenic mice expressing activated JNK or other mouse tumor models with constitutive activation of

JNK have been reported. The recent generation of gain of function mutants of JNK allowed us for the first time to directly probe into transforming potential of JNKs *in vitro* and in a nude mouse system [58]. This work did not identify unique JNK-regulated processes but instead came up with growth induction and angiogenesis, traits, which are also controlled by other transforming pathways. The possibility remains that stress kinases are merely the effectors of other signaling pathways rather than independent players in the cancer game. To answer this question it will be important to understand the mechanisms, which lead to the activation of stress kinases. Her2 as well as JNK overexpression itself have been implicated [52]. Small-G proteins of the Rho family may comprise other potential activators operating in tumors [92]. *In vitro*, we were able to demonstrate transforming potential for the serine threonine kinases Cot/Tpl-2 [93] and MLK-3 [94], both of them potent JNK activators. However, the involvement of these kinases in human tumors has not been reported. Based on *in vitro* studies the participation of autocrine factors in the process of JNK activation presents a very likely scenario. This may in particular be true for tumors with activating mutations in the Ras-Raf-MEK-ERK pathway [36]. Analysis of Raf signaling has demonstrated the presence of multiple effectors targeted by a single oncogenic kinase. The include NF- κ B [95, 96], PI3K-PKB [97] and JNK [36]. For all of them autocrine modes of activation have been suggested. In addition, blocking PKB or NF- κ B severely impaired Raf-induced transformation [96, 97]. This example illustrates that expression of one cancer gene leads to the recruitment of several effector pathways, for which overlapping functions have been suggested (e.g. apoptosis suppression for PKB and NF- κ B), but which nevertheless are not redundant for tumor growth. Future work will help to answer the question whether stress kinase activity in tumors results from the preceding activation of other cancer pathways or is the consequence of activating mutations in this pathway itself. The further development of more specific inhibitors and the conditional inactivation of stress kinases will then allow to probe into unique roles of stress kinase signaling in cancer. Irrespective of the outcome of these experiments, the functions already assigned to JNKs make them interesting targets for future therapeutic intervention.

Concluding remarks

The situation we face currently in the assessment of the transforming potential of stress kinases is not unlike the situation previously seen with many *bona fide* oncogenes, which were recognized early on for their tumorigenic potential in *in vitro* studies and animal experiments, but formal proof of their involvement in human tumors often followed years later, as exemplified by the Raf kinases [98]. Also the characterization of these proteins showed that their function is often context dependent, as demonstrated for Ras, which has pro- and anti-apoptotic functions [99], as reported again for stress kinases (see above). Our *in vitro* experiments have suggested that activated JNK is a weak oncogene compared to v-Raf. The reason for this may simply lie in the nature of the mutation used for the *in vitro* activation of JNK. Moreover, as work on the cytoplasmic cascade demonstrated, average rather than maximum signal strength is compatible with growth [100]. So far we cannot assign any singular cancer related activities to stress kinases. But as shown for other canonical cancer pathways stress kinases target traits, which are essential for tumor growth. Understanding the complexity of signaling events occurring in tumors will allow for the simultaneous targeting of several critical players during cancer therapy thus minimizing the risk that tumor cells escape the treatment. The true realization of transformation potential of activated JNKs will require the understanding of the mechanisms leading to their activation in tumors and the availability of genetic models and highly specific inhibitors for the further analysis of their contribution to the cancer process.

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