

JNK signaling in cancer: in need of new, smarter therapeutic targets.

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Abbreviations

AP, activator protein; ASK, apoptosis signal-regulating kinase; ATF, activating transcription factor; ATP, adenosine triphosphate; CNS, central nervous system; DEN, diethylnitrosamine; DMBA, 7,12-dimethylbenz[α]anthracene; ERK, Extracellular signal-regulated kinases; HCC, hepatocellular carcinoma; IKK, I κ B kinase; I κ B, inhibitor of inhibitor of kappa B; IL, interleukin; JIP, c-Jun-N-terminal kinase interacting protein; JNK, c-Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; MAP2K, mitogen-activated protein kinase kinase; MAP3K, mitogen-activated protein kinase kinase kinase; MEFs, murine embryonic fibroblasts; Myt, Myelin transcription factor 1; MM, multiple myeloma; NF- κ B, nuclear factor κ B; PARP14, Poly(ADP-ribose) polymerase family member 14; PUMA, p53 up-regulated mediator of apoptosis; SCC, squamous carcinoma cells; TCF4, transcription factor 4; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; UV, ultraviolet.

ABSTRACT

The c-Jun NH2-terminal kinases (JNKs) are master protein kinases that regulate many physiological processes, including inflammatory responses, morphogenesis, cell proliferation, differentiation, survival and death. It is increasingly apparent that persistent activation of JNKs are involved in cancer development and progression. Therefore, JNKs represent attractive targets for therapeutic intervention with small molecule kinase inhibitors. However, evidence supportive of a tumor suppressor role for the JNK proteins has also been documented. Recent studies showed that the two major JNK proteins, JNK1 and JNK2, have distinct or even opposing functions in different types of cancer. As such, close consideration of which JNK proteins are beneficial targets and more importantly what effect small molecule inhibitors of JNKs have on physiological processes are an absolute must. A number of ATP-competitive and ATP-noncompetitive JNK inhibitors have been developed, but have several limitations such as a lack of specificity and cellular toxicity. In this review we summarize the accumulating evidence supporting a role for the JNK proteins in the pathogenesis of different solid and hematological malignancies and discuss many challenges and scientific opportunities in the targeting of JNKs in cancer.

INTRODUCTION

Protein kinases are the largest family of enzymes encoded by the human genome and have emerged as one of the most popular drug target class in the pharmaceutical industry (Zhang *et al.*, 2009). The role of protein kinases is to catalyze the transfer of the terminal phosphoryl group of ATP to specific amino acid residues in target proteins (substrates). This modifies the functions of the target proteins by affecting either the activities or by controlling sub-cellular localization, degradation and association with other binding partners (Manning *et al.*, 2002). Furthermore, protein kinases themselves can be activated by addition of phosphate groups to their specific amino acid residues either by an autophosphorylation event or phosphorylation by other kinases. Of these kinases, the mitogen-activated protein kinases (MAPKs) are a well-studied family of serine-threonine kinases that plays an important regulatory role in the cell (Johnson and Lapadat, 2002). MAPKs are activated via a kinase signaling cascade in which a MAP kinase kinase kinase (MAP3K) activates a MAP kinase kinase (MAP2K) that in turn activates a MAP kinase (MAPK). There are three well-characterized subfamilies of MAPKs in mammals: the extracellular signal-regulated kinases (ERKs), the p38 kinases and the c-Jun NH2-terminal kinases (JNKs), and all three MAPKs have been conserved from unicellular organisms to complex organisms including humans (Johnson and Lapadat, 2002).

The JNK kinase family includes three proteins (JNK1, JNK2 and JNK3) that are encoded by three separate genes *jnk1* (*Mapk8*), *jnk2* (*Mapk9*) and *jnk3* (*Mapk10*), and are alternatively spliced to create at least ten variants of 46 and 55 kilodaltons (Davis, 2000). Whereas JNK1 and JNK2 are expressed in most tissues, the expression of JNK3 is largely restricted to brain, heart and testes (Bogoyevitch and Kobe, 2006). The JNK proteins – also known as stress-activated protein kinases (SAPKs) – similar to other MAPKs, are activated by a series of phosphorylation events (that is the MAP kinase module) in response to multiple stimuli such as cytokines, growth factors, pathogens, stress, toxins and drugs (Bogoyevitch and Kobe, 2006; Seki *et al.*, 2012) (Figure 1). MKK4 and MKK7 are the two MAP2K protein kinases that directly phosphorylate JNKs on threonine 183 (Thr183) and tyrosine (Tyr185) residues present in a conserved tripeptide motif (Thr-Pro-Tyr) within their activation loop (Davis, 2000). In turn, MKK4 and MKK7 are activated via dual phosphorylation by MAP3Ks, such as members of MEKK family, the mixed-lineage kinase family, the ASK family, TAK1 and TPL2 (Davis, 2000). Moreover, the kinase activity of JNKs is regulated by interaction with scaffold proteins (Whitmarsh *et al.*, 2001) as well as dual-specificity phosphatases (Owens and Keyse, 2007) and NF κ B transcription factors (Papa *et al.*, 2006).

Upon activation by the upstream MAP2Ks, JNKs phosphorylate and activate a number of nuclear and nonnuclear proteins, including the transcription factor activator protein-1 (AP-1), which is formed by dimerization of the Jun proteins (c-Jun, JunB, JunD) with the Fos proteins (c-Fos, FosB, Fra-1, Fra-2), activating transcription factor 2 (ATF-2), c-Myc, p53, Elk1, NFAT as well as cell death regulators of the Bcl-2 family in the mitochondria (Bogoyevitch and Kobe, 2006). These proteins control a diversity of cellular responses, such as proliferation, differentiation, cell death and survival.

The diversity of cellular functions of JNKs underscores the diversity of disease conditions in which JNKs are implicated, including cancer (Figure 2). Indeed, aberrant expression and activation of JNKs are found in many cancer cell lines as well as in patient samples (Wagner and Nebreda, 2009). Moreover, abnormalities in JNK activity have also been associated with diabetes (Hirosumi *et al.*, 2002), inflammatory and neurodegenerative disorders (Roy *et al.*, 2004; Hunot *et al.*, 2004). Consequently, the signaling mechanisms that underline JNKs expression and activities in these diseases and the identification of JNK inhibitory molecules are the subject of intense ongoing research. JNKs are also essential regulators of normal physiological functions such as immune responses and cell and tissue morphogenesis (Waetzig *et al.*, 2005). Therefore, although targeting JNKs in the above diseases represents a worthwhile therapeutic strategy, inhibition of the JNK proteins could also be deleterious. Here, we particularly review the current knowledge on the role of JNK signaling pathways in cancer pathogenesis, with a focus on selected solid and hematological malignancies in which the progress in understanding their pathogenesis is proceeding at a rapid pace. We also offer a perspective on the principal challenges that are relevant to the development of drugs targeting JNKs.

The role of JNKs in cancer

The early findings that the transforming actions of several oncogenes such as Ras, c-fos, Met and Bcr-Abl could be JNKs dependent (Smeal *et al.*, 1991; Rodrigues *et al.*, 1997; Raitano *et al.*, 1995; Behrens *et al.*, 2000; Manning and Davis, 2003), suggested that the JNK signalling could contribute to the cellular transformation that supports cancer development. Indeed, c-Jun, the prototypical downstream target of the JNK pathway, is required for transformation induced by the oncogene Ras (Lloyd *et al.*, 1991) and for liver cancer development in mice (Eferel and Wagner, 2003; Min *et al.*, 2012). Moreover, studies in a mouse model of intestinal cancer (Apc^{min}) have shown that the oncogenic function of c-Jun is dependent on phosphorylation by JNK as mice expressing a nonphosphorylatable mutant form of c-Jun

(JunAA/JunAA/Apc^{min}) developed smaller and fewer polyps (Nateri *et al.*, 2005) thus implying a pro-oncogenic role for JNK/c-Jun axis in this context.

In support of this view, hyperactivation of the JNK proteins themselves has been reported in multiple cancer cell lines and tissue samples (Hui *et al.*, 2008; Chang *et al.*, 2009a; Chang *et al.*, 2009b; Barbarulo *et al.*, 2012), and depletion of the individual JNKs can suppress tumorigenesis depending on the tissue specificity (Wagner and Nebreda, 2009). In primary hepatocellular carcinomas (HCCs), for example, activation of JNK1 but not JNK2 is increased as compared to the non-neoplastic lesions, and absence of JNK1 impaired liver cell proliferation and tumor formation (Hui *et al.*, 2008; Chang *et al.*, 2009a). Additionally, over-activation of JNK1 in HCC tissues has been shown to associate with both a poor disease prognosis and expression of progenitor cell biomarkers (Chang *et al.*, 2009b). JNK1 has also been shown to be an important contributor to the tumor-promoting activity of tobacco smoke in lung (Takahashi *et al.*, 2010). In contrast, other studies have described an oncogenic function for JNK2 in various tumour-derived cell lines (Nitta *et al.*, 2011; Bost *et al.*, 1999; Yang *et al.*, 2003; Cui *et al.*, 2006; Yoon *et al.*, 2012) and animal cancer models (Chen *et al.*, 2001; Ke *et al.*, 2010; Chromik *et al.*, 2007). Myeloma cell lines and primary tumors have been shown to exhibit elevated JNK2 activity, which appears to be the key determinant to enhanced cell survival (Barbarulo *et al.*, 2012). Moreover, genetic deletion of JNK2 in mice reduced skin carcinogenesis induced by DMBA and TPA treatment (Chen *et al.*, 2001), further outlining the role of JNK2 in tumorigenesis. In addition, a recent study established that both JNK1 and JNK2 are required for lung tumor formation induced by inactivation of endogenous KRas gene (Cellurale *et al.*, 2011). These findings are consistent with the hypothesis that the JNK proteins may promote tumor development in a tissue- or cell-specific manner, and suggest that selective inhibition of these kinases may be effective in halting specific tumor formation.

However, despite having a role in tumor development, a substantial body of evidence implicates JNK proteins in tumor suppression (Wagner and Nebreda, 2009; Davis 2000). In the DMBA/TPA-induced skin cancer model, for example, JNK1-null mice developed a greater number of papillomas compared to the wild-type mice (She *et al.*, 2002), indicating that JNK1 negatively regulates tumorigenesis. The tumor-suppressing function for JNK1 has been further supported by experiments, which demonstrated that JNK1-null mice are highly susceptible to tumor development after inoculation of both melanoma and lymphoma cell lines (Gao *et al.*, 2005). Moreover, JNK1-deficient mice spontaneously develop intestinal tumors (Tong *et al.*, 2007). It has been proposed that the tumor suppressor activity of JNKs results from their well recognized apoptotic functions involving the mitochondrial pathway (Davis 2000). Detailed analysis demonstrated that primary murine embryonic fibroblasts (MEFs) isolated from mice

that lack expression of both JNK1 and JNK2 proteins were resistant to the induction of apoptosis and failed to release pro-apoptotic molecules from mitochondria, including cytochrome C in response to UV-irradiation (Tournier *et al.*, 2000). This indicates that stress-induced JNK signaling leads to apoptosis via activation of the mitochondrial pathway. In line with these findings, studies in multiple myeloma cell lines showed that activation of the JNK pathway by 2-Methoxyestradiol (2ME2), a potent antitumor agent is associated with apoptosis and the release of Smac/DIABLO protein from mitochondria into cytosol (Chauhan *et al.*, 2003). Moreover, the activation of the JNK pathway is required for microtubule-damaging agent-induced apoptosis of breast cancer cells through its ability to phosphorylate and inactivate the mitochondrial anti-apoptotic protein Bcl2 (Yamamoto *et al.*, 1999; Srivastava *et al.*, 1999). In addition, JNK2 has been shown to phosphorylate the tumor suppressor p53 on Serine 6, which is a key site for p53 stabilization and activation (Fuchs *et al.*, 1998; Oleinik *et al.*, 2007). Indeed, knockdown of JNK1 and JNK2 by siRNA impaired the p53-dependent apoptosis in response to anticancer drugs (Oleinik *et al.*, 2007).

In summary, the information outlined above indicate that JNK1 and JNK2 can act as either tumor promoter or as tumor suppressor kinases in different types of cancer and highlight the importance in understanding fully both the roles of JNKs and the molecular basis for these distinct functions in different tumors in order to validate the therapeutic potential of JNK inhibition. It has been proposed that differential regulation of specific cellular targets is the underlying mechanism for the distinct functions of JNK1 and JNK2 in cancer, although targets regulated by the individual JNK proteins remain largely unidentified (Figure 3). In the succeeding section, we focus on various cancers, both solid and hematological malignancies in which JNKs are reported to be involved and may be targetable.

JNKs IN SOLID CANCERS

Liver cancer

Liver cancer is a compelling example of the involvement of the JNK pathway in the initiation and progression of cancer (reviewed by Seki *et al.*, 2012). The most common type of primary liver cancer is hepatocellular carcinoma (HCC), with approximately 750,000 cases per year worldwide and few approved treatment options (Llovet *et al.*, 2003; Jemal *et al.*, 2011). HCC could arise from cirrhosis (scarring of the liver) or severe liver fibrosis caused by multiple rounds of hepatocyte death, inflammatory responses and compensatory liver regeneration, all of which involve overactivation of the JNK pathway in injured hepatocytes (Farazi and DePinho, 2006; Papa *et al.*, 2009; Seki *et al.*, 2012). Although exactly how the JNK pathway contributes to HCC development remains to be fully elucidated, evidence from studies in mice

with diethylnitrosamine (DEN)-induced HCC model suggests that the JNK pathway contributes to the development of HCC by inducing hepatocyte cell death that promotes compensatory proliferation (Seki *et al.*, 2012). For example, deletion of the NF- κ B activator IKK β in hepatocytes induced activation of JNKs, cell death that triggers compensatory proliferation of surviving hepatocytes and greatly enhanced the development of HCC in response to DEN treatment (Maeda *et al.*, 2005; reviewed by Papa *et al.*, 2009). Interestingly, deletion of *jnk1*, but not *jnk2* was able to completely abrogate the effect of deletion of IKK β in hepatocytes on cell death, compensatory proliferation and hepatocarcinogenesis in response to DEN induction, indicating overactivation of JNK1 as a key player in DEN-induced HCC (Sakurai *et al.*, 2006). This was further confirmed by studies using JNK1 deficient mice, which were much less susceptible to DEN-induced HCC development. This impaired formation of HCC was associated with decreased expression of the cell cycle regulator cyclin D and the vascular endothelial growth factor (VEGF), suggesting that JNK1 likely acts as a tumor-promoter in HCC by enhancing hepatocyte proliferation and neovascularization (Sakurai *et al.*, 2006). The critical role for JNK1 activation in HCC was further supported by experiments which demonstrated that hepatocyte-specific deletion of NEMO/IKK γ , an activator of NF- κ B, caused spontaneous formation of HCC in parallel with increased JNK1 activation, hepatocyte death and compensatory hepatocyte proliferation (Luedde *et al.*, 2007). Additionally, administration of antioxidants to these mice resulted in significantly reduced JNK activation and HCC development, further indicating a role for JNK activation in promoting HCC formation (Luedde *et al.*, 2007). JNK1 has also been shown to regulate the expression of the p53 up-regulated modulator of apoptosis (PUMA), a pro-apoptotic BH3-only protein, during fatty acid-induced hepatocyte apoptosis (reviewed by Huang and Strasser, 2000; Cazanave *et al.*, 2009). Indeed, in the DEN model, mice deficient in PUMA exhibit defects in HCC formation due to death and compensatory proliferation reduction of hepatocytes. Such effects were prevented in these mice by treatment with the JNKs inhibitor SP600125, indicating that the JNK1/PUMA axis plays a central role in DEN-induced hepatocyte apoptosis and subsequent proliferation (Qiu *et al.*, 2011).

A further proposed mechanism that accounts for the pro-tumorigenic role of JNK1 in HCC development is that JNK1 may regulate hepatocyte proliferation directly. In the DEN model, JNK1 was found to down-regulate the expression of the proliferation inhibitor p21 through transcriptional up-regulation of the growth promoter c-Myc (Hui *et al.*, 2008). Mice that lack JNK1 display decreased tumor cell proliferation following DEN administration, and genetic inactivation of p21 in JNK1-deficient mice has been shown to restore hepatocyte proliferation in these mice. Furthermore, over-expression of c-Myc has been shown to increase

proliferation of JNK1^{-/-} liver cells (Hui *et al.*, 2008). In tissue samples from patients with HCC, JNK1, but not JNK2, has been reported to be constitutively active in more than 50% of samples studied relative to adjacent normal tissues (Hui *et al.*, 2008; Chang *et al.*, 2009a). The hyper-activation of JNK1 was associated with increased tumour size and an elevated expression of genes controlling cell cycle and proliferation, indicating the relevance of JNK1 for the proliferation of liver cancer cells (Chang *et al.*, 2009a). Indeed, knockdown of JNK1 by shRNA lentiviruses impaired cell proliferation in human HCC cell line, and JNK1 has been shown to reduce expression of p21 through up-regulation of c-Myc (Hui *et al.*, 2008).

More recently, an additional aspect has emerged, with relevance to the effects of the JNK signaling on HCC development. Davis and colleagues using mice with compound deficiency of JNK1 and JNK2 in hepatocytes demonstrated that JNKs have both tumor promoting and suppressing roles in hepatocarcinogenesis that depend on the cell types of the liver. The authors generated a genetically engineered mouse model deleted of JNK1 in hepatocytes and JNK2 in the entire organism, and discovered that these mice developed more HCCs than wild-type mice following administration of DEN. The increased HCC development was associated with increased hepatocyte death, and, consequently increased compensatory proliferation as well as increased c-Myc and c-Jun expression. This indicates that JNK activity in hepatocytes functions to reduce tumor development (Das *et al.*, 2011). However, in nonparenchymal cells of liver, such as hepatic immune cells (Kupffer cells), JNK activity is reported to promote HCC development. The authors went further to demonstrate that this was mediated through creation of an inflammatory environment, including the expression of pro-tumorigenic cytokines such as IL6 and TNF α that contribute to compensatory hepatocyte proliferation (Das *et al.*, 2011).

To date, JNK1 inhibitors have not been developed to treat patients with HCC. Nevertheless, the functional roles of JNK1 in hepatocyte death and compensatory proliferation make drugs of this type promising anti-HCC therapies. Indeed, an inhibitory peptide directed against the substrate-docking domain of JNK proteins (D-JNKI1) was shown to suppress JNK activity and reduce tumor growth in the DEN-induced HCC model and in human HCC xenograft model (Hui *et al.*, 2008). In a rat DEN-induced HCC model, the administration of the JNKs inhibitor SP600125 reduced the number and size of HCCs (Nagata *et al.*, 2009). Additionally, inhibition of JNK activity has been shown to improve the efficacy of some current chemotherapeutics. For example, SP600125 in combination with the chemotherapy drug TNF-related apoptosis inducing ligand (TRAIL) was shown to increase apoptosis in human HCC cultures (Mucha *et al.*, 2009). Indeed, activated JNK1 was found in tissue samples from patients with HCC (Hui *et*

al., 2008) and correlates directly with poor therapeutic response to sorafenib, a multikinase inhibitor that is currently being tested in clinical trials for HCC (Hagiwara *et al.*, 2012). Therefore, collectively, the findings described above have illustrated the importance of JNK1 in promoting HCC and the potential of JNK1 to be a target, alone or in combination with other therapeutics, for anti-HCC therapies.

Breast cancer

In sharp contrast to tumor-promoting role of JNK1 described above, in breast cancer models the JNK pathway has been shown to suppress tumor development. One example came from the work of Cellurale and colleagues (Cellurale *et al.*, 2010). They employed mice heterozygous for the p53 tumor suppressor, which spontaneously develop breast cancer in a pattern and frequency similar to that reported for the Li-Fraumeni breast cancer in humans (Kuperwasser *et al.*, 2000). It has been shown that the crossing of p53^{-/+} mice with JNK1^{-/-} mice and JNK2^{-/-} mice causes significantly shortened tumor-free survival compared with control p53^{-/+} mice, suggesting that JNK1 and JNK2 may function to reduce breast cancer. A similar tumor-suppressing role for JNK proteins, especially JNK2, was also observed in transgenic mice that express the polyomavirus (PyV) middle T (MT) oncogene in mammary epithelial cells (Webster *et al.*, 1998). PyV MT transgenic mice develop multifocal mammary tumors, which frequently metastasize to the lung, thus emulating both early and late stages of human breast cancer. Systemic deletion of JNK2 in PyV MT transgenic mice was found to shorten latency and increase tumor multiplicity. Interestingly, PyV MT/*jnk2*^{-/-} mammary tumors showed more genomic instability, aneuploidy and impaired DNA damage response due to a reduction of DNA repair proteins and activation of the cell-cycle inhibitor p21, suggesting that in this case JNK2 expression prevents breast cancer development by regulating cell cycle progression and DNA repair mechanisms (Chen *et al.*, 2010). Further evidence for the tumor-suppressing role of JNKs in breast cancer has been provided from studies that show compound JNK deficiency in mammary epithelial cells to promote tumor formation in a KRas/p53 mouse model of breast cancer (Cellurale *et al.*, 2012). In line with these findings, inactivating mutations in the JNK activator *Mkk4* gene have been identified in human breast cancer (Su *et al.*, 1998; Su *et al.*, 2002). In addition, deletion of *Mkk7* gene (the other upstream activator of JNK) in primary murine epithelial cells facilitates oncogenic transformation in the mammary gland (Schramek *et al.*, 2011), further validating the tumor-suppressive role of the JNK cascade in breast cancer. Activation of the JNK pathway has also been shown to mediate the cytotoxic effects of the chemotherapy drug taxol in MCF7 breast cancer cells (Mamay *et al.*, 2003). Whilst these recent findings implicate the JNK cascade in mammary carcinoma suppression, they also emphasize that JNK1 and JNK2 have redundant

or at least overlapping roles. Inducing both JNK1 and JNK2 would therefore be exploited for therapeutic benefit in patients with breast cancer.

Skin cancer

Persistent ultraviolet (UV) radiation exposure is the major cause for the development of skin cancer, which comprises histologically distinct skin neoplasms. These include squamous cell carcinoma (SCC), basal cell carcinoma (BCC) and melanoma that are named after the types of skin cell from which they arise. Among these, SCC is the most common and invasive type of non-melanoma skin cancer, with limited treatment options at present, especially for the late stage (reviewed by Zhang and Selim, 2012). As JNK proteins, also termed stress-activated protein kinases (SAPKs), are highly responsive to UV radiation (Derijard *et al.*, 1994), it is not surprising that hyper-activation of JNKs is frequently observed in SCC. Early studies on this topic revealed opposite functions for JNK1 and JNK2 in regulating skin carcinogenesis (reviewed by Zhang and Selim, 2012). Using a well-characterized multi-stage model of mouse skin carcinogenesis in which tumors are initiated with DMBA (7,12-dimethylbenz(a)anthracene and promoted with TPA (12-O-tetradecanoylphorbol-13-acetate), Dong and colleagues found that in JNK2-deficient mice the multiplicity of papillomas induced by TPA was lower than that in wild-type mice, indicating that JNK2 is a crucial tumor promoter of skin cancer development (Chen *et al.*, 2001). Moreover, *Mkk4* deficiency prevented DMBA/TPA-induced skin cancer by suppressing JNK2 expression, providing the first clear evidence that the MKK4-JNK2 axis is essential for tumour formation in the skin (Finegan and Tournier, 2010). In contrast, JNK1 appears to be a crucial suppressor of skin cancer as JNK1-deficient mice exhibited a significantly higher papilloma incidence compared with the wild-type mice (She *et al.*, 2002). This was further confirmed by studies using the regenerated human SCC model, in which primary keratinocytes were transduced for expression of constitutively active JNK1 or JNK2 along with the oncogenic Ras mutant and then used for human skin regeneration on immunodeficient mice (Ke *et al.*, 2010). While tissue expressing JNK2 were hyper-proliferative and displayed clinical features of human SCC, tissue expressing JNK1 exhibited normal histological features of human skin. This indicates that JNK2, but not JNK1 cooperates with Ras to transform normal epidermal cells to malignancy with SCC features (Ke *et al.*, 2010). In line with these findings, analyses of both biopsies and keratinocytes from patients with SCC reported increased levels of JNK2 activity as compared to healthy controls (Zhang *et al.*, 2007; Ke *et al.*, 2010). The exact mechanism for the distinct functions of JNK1 and JNK2 in skin cancer development is not fully clear but has been proposed to include ERKs and Akt signalings and the AP-1 DNA binding activity (She *et al.*, 2002).

More recently, JNK1 has been shown to prevent skin cancer via a novel mechanism. After UV

irradiation, JNK1 associates with and phosphorylates the cell cycle inhibitory kinase Myt1, which induces caspase-3 cleavage and DNA fragmentation resulting in apoptosis. Knockdown of Myt1 suppresses UV-induced apoptosis in skin cancer cells, and Myt1 has been shown to phosphorylate the mitotic kinase cdc2 on tyrosine 15, which is a key site for kinase inactivation (Choi *et al.*, 2009). The opposite functions of JNK1 and JNK2 in skin cancer suggest that therapeutics discriminating between JNK1 and JNK2 proteins may offer a promising therapeutic opportunity.

Brain tumors

The most frequent primary brain tumors in adults are gliomas, which begin in the glial tissue. Indeed, gliomas account for about 70% of adult malignant primary brain tumors. According to the World Health Organization (WHO) classification system gliomas are further distinguished into three subtypes: grade 2 (low-grade astrocytoma, oligodendroglioma and oligoastrocytoma), grade 3 (anaplastic gliomas) and grade 4 (glioblastoma) (Li *et al.*, 2008). Approximately half of all gliomas are glioblastoma, by far the most severe and aggressive subtype (Ricard *et al.*, 2012). A number of cellular and xenograft studies revealed a role for JNKs in the development and progression of gliomas. For example, elevated levels of JNKs activity were observed in a panel of glioblastoma cell lines and in human glioblastoma tumor samples (Antonyak *et al.*, 2002). Similarly, phosphorylation of JNKs and c-Jun has been shown to strongly correlate with the histological grade of glioblastoma (Li *et al.*, 2008). Compared to JNK1 and JNK3, JNK2 has a dominant pro-tumorigenic role in glioblastoma. Tsuiki *et al.* (2003) reported that JNK2 possesses autophosphorylation activity and constitutive substrate kinase activity. This is in line with data showing that JNK2 is the major JNK protein that is constitutively activated in glioblastoma. JNK2 was also shown to support tumorigenesis *in vivo* by activating Akt and up-regulating the expression of eukaryotic translation initiation factor 4 (eIF4E) (Cui *et al.*, 2006), which is frequently overexpressed in many human cancers (Graff *et al.*, 2008). More recent evidences indicate a role for JNKs in maintaining glioma stem cell properties, which are responsible for the poor prognosis of glioblastoma patients. Elevated levels of the phosphorylated (active) form of JNKs were observed in sphere-cultured U87 and U373 glioma cells and in stem cells isolated from patient-derived glioma cells. Pharmacological inhibition of JNKs with SP600125 or knockdown of JNK1 or JNK2 significantly suppressed sphere formation, soft-agar colony formation, which are assay methods to evaluate stemness of stem-like cancer cells *in vitro*, and reduced the expression of stem cell markers in sphere-cultured glioma cells. Moreover, knockdown of JNK2 in glioma stem cells suppressed tumor formation *in vivo* (Yoon *et al.*, 2012). The same group reported that JNK2 promotes stemness of glioma stem-like cells through up-regulation of Notch-2

expression (Yoon *et al.*, 2012). This study reveals a new tumorigenic function for JNKs, in particular JNK2. However, there is also evidence supporting a role for JNK3, expression of which is largely restricted to the brain, as a tumor suppressor. Indeed, loss of expression of the *jnk3* gene has been identified in 10 of the 19 human brain tumor cell lines examined (Yoshida *et al.*, 2001).

JNKs IN HEMATOLOGICAL MALIGNANCIES

While the roles of JNK proteins in solid tumors have been described in recent studies, the specific functions of JNKs in hematological malignancies remain limited. This stems in part from a lack of genetic animal models of these conditions, which makes the task even more difficult.

Leukemia

Leukemia is a broad term used to describe numerous cancers of the blood and bone marrow (Murati *et al.*, 2012). These hematological malignancies are characterized by an increase in white blood cell proliferation, coupled with the failure of normal pro-apoptotic responses to neoplastic cells. Leukaemia is clinically and pathologically classified as 'acute' and 'chronic'. Additionally, these conditions are further divided according to which kind of blood cell is affected, such as lymphoblastic leukemia and myeloid leukemia. Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hemopoietic progenitor cells ("blasts"), which lose the ability to differentiate normally and to respond to normal regulators of proliferation (reviewed by Estey and Döhner, 2006). Chronic myeloid leukemia (CML) conversely involves the unregulated proliferation of more differentiated mature hematopoietic cells (reviewed by Deininger *et al.*, 2000).

One proposed therapeutic strategy for treatment of leukemia is to induce differentiation of circulating leukemia cells. This is a first step to induce apoptosis of these neoplastic cells. The precise mechanism that triggers the activation of apoptosis in mature differentiated cells has not been elucidated. JNK has been shown to enhance monocytic differentiation of human leukemia cells induced by 1,25-dihydroxyvitamin D₃, a derivative of vitamin D₃ (Wang *et al.*, 2005). Yet, the precise molecular mechanism of how JNK promotes differentiation is not completely understood. Recent studies have highlighted the distinct role of JNK1 and JNK2 in this differentiation process and described for the first time a negative interplay between JNK2 and JNK1. It has been shown that while JNK2 is a negative regulator of monocytic differentiation, JNK1 is a positive regulator. Strikingly, silencing of JNK2 increased the activity of JNK1p46, which is associated with increased cellular differentiation after treatment with

1,25D. In contrast, silencing of JNK1 reduced the intensity of 1,25D-induced differentiation, and partially abrogated activation of c-Jun/AP-1 transcription factors (Chen-Deutsch *et al.*, 2009). Accordingly, specific inhibitors of JNK2, and not JNK1, may complement the differentiation therapy induced by the vitamin D3 derivatives and, thus, may be used as an anti-leukemia drug.

Constitutive activation of JNK has been also found in T-cell acute lymphoblastic leukemia (T-ALL). Suppression of JNK activity in T-ALL cells by using general (pan)-JNK chemical inhibitors promoted cell cycle arrest and apoptosis and increased sensitivity to Fas-mediated apoptosis, whereas constitutive activation of JNK in T-ALL cells resulted in accelerated cell cycle progression and resistance to Fas-mediated apoptosis. Silencing of JNK1, but not JNK2, exhibited similar effects to JNK inhibitors, suggesting that JNK1 is the predominant form of JNK proteins and, in this case, the identification of JNK1-specific inhibitors may have some important therapeutic implications in the treatment of T-ALL (Cui *et al.*, 2009).

Other studies have demonstrated that JNK activity has been also associated with treatment failure in AML. Biochemical analyses of primary AML bone marrow samples have shown that JNK activity is associated with increased efflux activity of the multi-drug protein (MRP), whose function promotes the efflux of chemotherapeutic drugs from the treated cells, with consequential resistance to chemotherapy and hyper-proliferation of AML cells. A central cellular program involving the signaling pathway $JNK \rightarrow c-Jun/AP-1$ might coordinate the expression of MRP, thus affecting the multidrug-resistant state of AML cells (Cripe *et al.*, 2002). Notably, the involvement of JNK in multi-drug resistance has been also characterized in other human cancers (Yan *et al.*, 2010; Ronaldson *et al.*, 2010). However, there is no clear understanding of how JNK regulates the functioning of MRP, or any other member of the ATP-binding cassette family of membrane efflux pumps.

In summary, studies to precisely identify the specific function of JNK in certain type of leukemia cells may provide valuable information for the design of new inhibitors with clinical translational potential.

Multiple myeloma

Multiple myeloma (MM) is a malignant tumor of terminally differentiated B cells, characterized by accumulation of clonal, long-lived plasma cells in the bone marrow and extra-medullary sites. Notable characteristics of myeloma cells include a low proliferative capacity of plasma cells and the failure of normal apoptotic index in vivo. Accordingly, pro-survival mechanisms

play central roles in the accumulation of the malignant clones in MM. Although the role of JNK signaling in the pathogenesis of MM has been extensively addressed, the function of JNK in promoting or suppressing MM is still a debate. Initial studies from Anderson and colleagues described the biologic effect of JNK inhibition in MM cell lines. JNK-specific inhibitor SP600125 induces growth arrest of MM cell lines. Importantly, SP600125 induces activation of the transcription factor NF- κ B and its upstream molecules IKK α , suggesting that JNK may play a role in the survival of MM cells by suppressing constitutive activation of NF- κ B in MM cells (Hideshima T *et al.*, 2003a). However, these results are conflicting with the fact that NF- κ B is important for the survival of these cancer cells (reviewed by Gilmore, 2007). Constitutive activation of NF- κ B was indeed highly scored in CD138+ cells from the bone marrow of 22 MM patients. In contrast to MM patients, activated forms of NF- κ B were absent in cells from healthy individuals (Bharti *et al.*, 2004). Diverse genetic aberrations accumulated in many myeloma samples activate both the canonical and alternative pathways of NF- κ B activation (Annunziata *et al.*, 2007; Keats *et al.*, 2007; Hideshima *et al.*, 2009). A possible explanation for this apparent discrepancy is that the JNK inhibitor SP600125 might over-stimulate NF- κ B signaling independently of JNK activation. Whichever is the case, the molecular mechanisms elucidating how JNK promotes survival of MM cells are not clear (discussed below).

Although the function of JNK has been linked to MM cell survival, JNK activation is also required for apoptotic cell death of MM cells induced by chemotherapeutic drugs. The proteasome inhibitor Bortezomib (originally named PS-341) has proven successful for the treatment of relapsed and newly diagnosed MM. Notably, Bortezomib mediates anti-MM activity by inducing p53 and MDM2 protein expression; it induces the phosphorylation of p53 protein and activation of JNK activity, which is essential for the induction of caspase-3 activation and therefore apoptosis of MM cells. In addition, SP600125 blocked bortezomib-induced apoptosis in MM cells (Hideshima *et al.*, 2003b).

How can one signal pathway mediate two very different cellular responses? One plausible answer is that distinct JNK proteins, JNK1 and JNK2, may serve different functions in MM by regulating specific downstream effectors. Recently, we systematically assessed the functional roles of JNK1 and JNK2 in MM by silencing the expression of either JNK1 or JNK2 in MM cell lines and examined their activity in MM primary tumors. Interestingly, we evaluated the level of phospho-active JNK in myeloma plasma cells of BM trephine biopsies of newly diagnosed MM patients compared to normal plasma cells from tonsillar samples, and found that JNK is constitutively active in MM plasma. In addition, we observed that phosphorylation of JNK2 was significantly higher than JNK1 in a panel of MM cell lines and primary CD138+ MM cells, suggesting a pathogenic role for JNK2 in this disease. Silencing JNK2 enhanced MM

apoptotic cell death. On the other hand, ablation of JNK1 did not alter the proliferation rate of MM cells. More fascinating was the observation that silencing of JNK2 in MM cells was associated with increased JNK1 activity, and that suppression of JNK1 activity in JNK2-depleted cells restored the regular growth rate of these malignant cells, suggesting that JNK2 controls basal JNK1-mediated pro-apoptotic responses to abnormal signals (Barbarulo *et al.*, 2012). It is important to point out that the JNK2-mediated control of JNK1 activity is not a novel concept documented in tumorigenesis. In a range of human cell lines, it was shown that JNK1 is actively pro-apoptotic but is constitutively inhibited by the presence of active JNK2 (Ahmed and Milner, 2009). Moreover, JNK2 antagonizes the JNK1-mediated differentiation signal in human myeloid leukemia cells resistant to vitamin D (Chen-Deutsch *et al.*, 2009). This regulation of JNK1 by JNK2, however, was not observed in non-malignant cells (Ahmed and Milner, 2009), suggesting that the opposing roles of JNK1 and JNK2 may exist only in cancerous cells. Indeed, ablation of *Jnk2* gene in JNK1^{+/+} MEFs caused increased expression of c-Jun and cellular proliferation (Sabapathy *et al.*, 2004; Tournier *et al.*, 2000), whereas selective inhibition of JNK2 activity by a chemical genetic approach in JNK1^{+/+} MEFs caused no changes in c-Jun expression or proliferation, suggesting compensatory increases in JNK1 function (Jaeschke *et al.*, 2006). Interestingly, it has also been proposed that increased JNK1 function detected in JNK2^{-/-} MEFs are primarily due to a competition between JNK1 and JNK2 for activation by the upstream MAPKK (Jaeschke *et al.*, 2006).

How JNK2 suppresses the JNK1-mediated pro-apoptotic signal in MM? By investigating the mechanisms for the distinct role of JNK1 and JNK2 in MM, we identified and characterized a novel signaling pathway that involves JNK2-PARP14-JNK1 (Barbarulo *et al.*, 2012). Previous studies have established that PARP14 is an ADP ribosyl-transferase that transduces survival signals in murine primary B cells by regulating expression of B-cell survival factors as well as by repressing an apoptotic program involving caspases (Goenka *et al.*, 2009). In the mouse, PARP14 has been shown to facilitate B-lymphoid tumorigenesis driven by oncogenic c-Myc (Cho *et al.*, 2011). Our recent study has investigated whether expression of PARP14 is under control of JNK2 in promoting survival of malignant MM cells. Silencing of JNK2 reduced the levels of PARP14 protein and promoted apoptosis in a panel of MM cell lines. Notably, ectopic expression of PARP14 in JNK2-depleted MM cells restored the levels of PARP14 similar to those of the endogenous protein expressed in MM cells, and rescued cells from undergoing apoptosis induced by the permanent deficiency of JNK2 expression. Moreover, the rescue of JNK2-depleted cells by ectopic expression of PARP14 is associated with the suppression of JNK1 activity in MM cells. Clearly, these data indicate that JNK2 regulates expression of PARP14, which is important for the suppression of JNK1 activity. However, how this regulation

occurs is not determined yet. It is plausible that JNK2 cooperates with certain transcription factors. To date, however, the identity of the transcription factors regulating *Parp14* gene expression is not known. Moreover, regulation of PARP14 by JNK2 does not explain the constitutive inhibition of JNK1-mediated apoptosis in MM cells. In an attempt to evaluate how JNK2-PARP14 suppresses JNK1 activity, we have examined whether PARP14 protein is able to interact with either JNK1 or JNK2 in cells. Remarkably, we showed that PARP14 specifically interacts with JNK1, through its C-terminal portion, thus inhibiting JNK1 kinase activity and ultimately apoptosis. Although at the present it is not directly proved whether JNK1 kinase inactivation by PARP14 involves the enzymatic activity of PARP14, our data suggest that this might be the case. The JNK1 kinase activity is, indeed, enhanced after treatment of MM cells with PJ-34, a pan-inhibitor of PARP enzymatic activity (reviewed by Jagtap and Szabó, 2005; Barbarulo *et al.*, 2012). However, whether this over-activation of JNK1 is due to specific inhibition of PARP14 enzymatic activity remain to be elucidated. In summary, we identify a novel pathway whereby constitutively active JNK2 promotes survival of myeloma cells via PARP14, which in turn associates with JNK1 and suppresses its kinase activity, suggesting that JNK2-PARP14 is a potential therapeutic target in myeloma.

Lymphoma

Bcr-Abl is a chimeric oncogene generated by the translocation of sequences from the c-Abl protein-tyrosine kinase gene on *chromosome 9* into the *Bcr* gene on *chromosome 22*. Bcr-Abl is an activator of several signal transduction pathways that mediates malignant transformation and entry of the cell cycle, and is also a potent inhibitor of cell death (Cilloni and Saglio, 2012). Previous studies have demonstrated that survival signaling of Bcr-Abl-transformed B lymphoblasts is mediated by JNK1, as knockdown of *Jnk1* gene in mice causes defective transformation of pre-B cells by Bcr-Abl. The JNK1-mediated survival is supported via regulation of Bcl2 expression. Notably, ectopic expression of Bcl2 rescued the defective phenotype of *Jnk1*^{-/-} B-cells (Hess *et al.*, 2002). These data are consistent with a model whereby JNK1-Bcl2 mediates the pathogenesis of transformed pre-B cells. In line with this observation, it was demonstrated that JNK signaling via Egr-1 and c-Myc activation is required for survival and proliferation of B-lymphoma cells (Gururajan *et al.*, 2005). Suppression of JNK activity by SP600125 strongly inhibited cell growth in a panel of murine and human B lymphoma cells, indicating that JNK is essential for suppression of basal apoptosis (Gururajan *et al.*, 2005). Recent observations by Sabapathy and colleagues, however, underscored the redundant function of JNK1 and JNK2 in B cell lymphomas (Anbalagan and Sabapathy, 2012). These authors used the murine E μ -Myc-induced B cell lymphoma model to induce lymphomagenesis and investigate the role of JNK1 and JNK2 in the development of B cell

lymphoma. The E μ -Myc transgenic mice model resemble the *MYC/Ig t(8:14)* translocation in human Burkitt lymphoma where c-Myc is overexpressed in B lymphocytes by the immunoglobulin heavy chain enhancer (E μ) (Adams *et al.*, 1985). In this model, Myc-induced lymphoma is preceded by a prolonged pre-cancerous phase in which the high proliferative rates of E μ -Myc B cells are counterbalanced by a high apoptotic index (Maclean *et al.*, 2003). Surprisingly, the overall survival of Myc-transgenic mice deficient for *Jnk1* is not significantly different from wild-type mice, and there is also no significant difference between the *wild-type* and *Jnk2*^{-/-} group. Moreover, loss of either *Jnk1* or *Jnk2* in Myc-transgenic mice neither revealed any statistical difference in the survival of these two groups of animals nor appreciably affected the maturation status of B220⁺ B cells. Therefore, JNK1 or JNK2 alone is not dispensable for Myc-induced lymphomagenesis (Anbalagan and Sabapathy, 2012). Although in our study we have characterized only one B-lymphoma cell line, our observations are in line with this latter findings suggesting that JNK1 and JNK2 play redundant roles in B lymphoma cells, and the absence of either JNK1 or JNK2 is compensated for by the other remaining JNK isoforms for the proliferation of B lymphoma cells (Barbarulo *et al.*, 2012). These observations, therefore, suggests that JNK plays an important role in B-cell lymphoma pathogenesis and targeting the catalytic activities of both JNK1 and JNK2 would be beneficial for the treatment of these conditions.

STRATEGIES TO TARGET THE JNK PROTEINS

Clearly the JNKs play key roles in the pathobiology of many cancer types. This information has stimulated both the academic research community and the pharmaceutical industry to design, synthesize and screen chemical libraries of compounds that can act as selective inhibitors of the JNKs. Based on their mode of action, the majority of these compounds can be classified into two categories, adenosine triphosphate (ATP)-competitive inhibitors and ATP-noncompetitive inhibitors (reviewed by Siddiqui and Reddy, 2010).

Well-recognized and thoroughly studied the ATP-competitive JNK inhibitors, include small molecules from a variety of scaffolds such as indazoles, aminopyrazoles, aminopyridines, pyridine carboxamides, benzothien-2-ylamides and benzothiazol-2-yl acetonitriles, quinoline derivatives, and aminopyrimidines (LoGrasso and Kamenecka, 2008; Gaillard *et al.*, 2005; Szczepankiewicz *et al.*, 2006; Kamenecka *et al.*, 2009; He *et al.*, 2011; Plantevin Krenitsky *et al.*, 2012). Although, to date, use of these inhibitors clinically has not been reported, efficacy of a subset of these molecules has been explored in cell-based experiments and animal models of disease. For example, the anthrapyrazolone SP600125, one of the earliest and most studied ATP-competitive JNK inhibitor (Bennett *et al.*, 2001) has been efficacious in mouse model of Parkinson disease (Wang *et al.*, 2004). Another compound, the benzothiazolone

AS601245, has been shown to provide neuroprotective effects in rats after focal cerebral ischemia (Carboni *et al.*, 2004) and be effective in ischemia reperfusion injury (Ferrandi *et al.*, 2004). These inhibitors, however, display poor kinase specificity as they target the highly conserved ATP-binding site. For example, SP600125 displays action against all three JNK proteins, and at higher concentrations, also displays ability to inhibit the closely related ERKs and p38 MAPKs (Bennett *et al.*, 2001; Bain *et al.*, 2003). Likewise, AS601245 inhibits phosphorylation of c-Jun at high doses, which is due to a moderately specificity for JNKs (Gaillard *et al.*, 2005). To overcome the specificity problem, Zhang *et al.* (2012) have very recently developed and characterized a potent and irreversible inhibitor of all three JNK proteins, JNK-IN-8, that forms a covalent bond with a conserved cysteine in the ATP site. This has been achieved through the use of structure-based drug design. The major advantage of cysteine-directed covalent inhibitors is the ability to control kinase selectivity and prolong pharmacodynamics despite competition with high endogenous levels of ATP. It will be of interest to see how this novel inhibitor of JNK activity is broadly applied in the treatment of a range of JNK-associated diseases, including cancer, and whether desired therapeutic outcomes would be achieved without unwanted side effects.

Alternative strategy for the inhibition of JNKs is the development of peptide or small molecule ATP-noncompetitive inhibitors that prevent the protein-protein interactions of JNKs with the JNK-interacting protein-1 (JIP1), or with the upper kinases or downstream substrates (Bogoyevitch *et al.*, 2010). JIP1 is a “scaffolding” protein that enhances JNK signaling specificity and efficiency by bringing JNKs into proximity with their upstream activators and substrates (Whitmarsh, 2006). The site of interaction between JNK and JIP1 contains a conserved consensus sequence, which is termed D-domain (Stebbins *et al.*, 2008). Works have demonstrated that a peptide, pepJIP1, consisting of 11 amino acids (153-163) derived from the D-domain of JIP1, inhibits JNK activity potently *in vitro* and exhibits noteworthy selectivity for JNKs with little inhibition of other MAPKs (Barr *et al.*, 2002; Heo *et al.*, 2004). The inhibitory activity of pepJIP1 relies either on the competition of this peptide with the D-domains of substrates or upstream kinases, or on allosteric effects (Ho *et al.*, 2003; Barr *et al.*, 2002; Heo *et al.*, 2004). Moreover, Ngoei *et al.* (2011) have recently reported the characterization of a novel JNK inhibitory peptide PYC71N, which prevents the interaction of JNK with c-Jun and represents a novel tool to explore the JNK-mediated substrate interactions and phosphorylation.

Studies *in vivo* using cell-permeable JIP1-based peptides have also shown effective inhibition of JNK signaling. For example, the intraperitoneal administration of pepJIP1 fused to HIV-TAT (human immuno-deficiency Virus-Trans-acting activator of transcription) sequence in diabetic

mice models restores normoglycemia without causing hypoglycemia in lean mice (Kaneto *et al.*, 2004). Other example of the use of JIP1-based peptides for JNKs inhibition includes a cell-permeable and protease-resistant peptide, D-JNKI-1, which was initially found to protect against cerebral ischemia in two models of middle cerebral artery occlusion (Borsello *et al.*, 2003). Systemic intraperitoneal administration of D-JNKI-1 in mice attenuated the growth of B16-Fluc murine melanoma xenografts and cancer pain (Gao *et al.*, 2009). The same inhibitor suppressed tumor growth of subcutaneously implanted HCC cells in nude mice and in mice with DEN-induced liver cancer. Immunohistochemical analysis of excised tumors confirmed that the anti-growth efficacy resulted from inhibiting levels of activated JNK (p-JNK) in the treatment group remained inhibited 3 months after D-JNKI-1 injection, whereas tumors from the control group continuously expressed high levels of p-JNK (Hui *et al.*, 2008). Most recently, two separate studies have shown that the inhibition of the JNK activity by D-JNKI-1 *in vivo* is an effective approach to the treatment of inflammatory bowel diseases. (Reinecke *et al.*, 2012; Kersting *et al.*, 2013).

Thus, cell-permeable JIP1-based peptides have been proven to have beneficial effects to improve a number of JNK-dependent diseases, although there are several challenges that have limited the development of peptide-based inhibitors. Other chemical entities that have the ability to disrupt the interaction between JNKs and JIP1 both *in vitro* and *in vivo* have also recently been identified (Bogoyevitch *et al.*, 2010). In screening 30,000 small molecules in a Dissociation Enhanced Lanthanide Fluoro-Immuno Assay (DELFI) platform combined with a Time-Resolved Fluorescence Energy Transfer (FRET) based kinase assay, Stebbins *et al.* (2008) have identified the inhibitor BI-78D3, which was found to compete with pepJIP1 for binding to JNKs and inhibit the JNK kinase activity. The authors have also shown that BI-78D3 *in vivo* blocks concanavalin A-induced-liver damage and restores insulin sensitivity in a mouse model of type 2 diabetes. Moreover, through an extensive structure-based virtual screening Ngoei *et al.* (2013) have recently identified a thiazole-2,2' diamine compound, JD123, which binds to both the ATP-binding site and the protein substrate site and so functions as a dual inhibitor of the JNK proteins. JD123 has also been shown to inhibit sorbitol-induced cJun phosphorylation in MEFs in a dose-dependent manner. Further structural-based studies will be necessary to improve membrane permeability for its use in cell and *in vivo* studies as well as to determine the precise docking modes and interactions with JNKs and so provide further understanding of its inhibitory mechanisms (Ngoei *et al.*, 2013).

In the cancer setting, however, the strategy of using JNK inhibitors that do not display selectivity across JNK proteins may be flawed as JNK1 and JNK2, which are more ubiquitous than JNK3 in their tissue distribution, appear to have opposite functions based on tissue

specificity (Yao *et al.*, 2009; Chen *et al.*, 2001; She *et al.*, 2002; Chen-Deutsch *et al.*, 2009; Barbarulo *et al.*, 2012). JNK2 is constitutively activated in myeloid leukemia cells, and has been shown to antagonize differentiation mediated by JNK1 (Chen-Deutsch *et al.*, 2009). Yet JNK2 appears to be the main JNK protein involved in multiple myeloma pathobiology via inhibition of the pro-apoptotic activity of JNK1 (Barbarulo *et al.*, 2012). In contrast, JNK1 activity appears to be essential for proliferation of human HCC (Hui *et al.*, 2008). Thus, taken together, the results from these studies and others (Bost *et al.*, 1999; Chen *et al.*, 2001; Yang *et al.*, 2003; Cui *et al.*, 2006; Chromik *et al.*, 2007; Ke *et al.*, 2010; Nitta *et al.*, 2011; Yooh *et al.*, 2012) suggest that highly selective JNK1 or JNK2 inhibitors are necessary for treating cancer. However, to date, selective inhibitors for a specific JNK protein are not yet available commercially. Interestingly, Kaoud *et al.* (2011) have recently reported the discovery of two potent and selective JNK2-inhibitor peptides that inhibit migration of murine mammary carcinoma cells. A selective inhibitor of JNK1, AV-7, has also been developed and tested in *in-vitro* studies (Yao *et al.*, 2009). Further and more extensive studies, including *in-vivo* pharmacokinetics of these compounds will be essential to validate them for drug development.

In addition, JNK1 and JNK2 appear to differentially influence tumorigenesis through regulating distinct cellular targets or binding partners (Figure 3). Examples include the interaction of JNK1, but not JNK2, with the membrane-associated kinase Myt1 and the observation that JNK1 phosphorylates Myt1. Phosphorylated Myt1 then inhibits UVA-induced skin cancer development (Choi HS *et al.*, 2009). In multiple myeloma, we recently showed that JNK2 inhibits the pro-apoptotic activity of JNK1 through effect on PARP14 expression (Barbarulo *et al.*, 2012). In sharp contrast, JNK1 deficiency impaired liver cancer formation via modulating expression of MYC and the CDK inhibitor p21 (Hui *et al.*, 2008). Besides these, not many studies have addressed the identification of targets regulated by the individual JNK proteins and none have yet evaluated the therapeutic potential of their inhibition in cancer. Indeed, so far, most efforts to target JNK pathways in cancer focused on direct inhibition of JNKs themselves. In this respect, it is important to note that selective inhibition of the individual JNK proteins could cause adverse cellular effects and unwanted toxicity because of their relevance to normal cellular functions such as adaptive immunity and brain development (Waetzig and Herdegen, 2005). Therefore effective treatment of JNK-associated cancers will require maintaining a delicate balance between suppressing deleterious functions of individual JNK proteins and interfering with cellular physiology. Treatments aimed at inhibiting downstream cellular targets of a specific JNK protein in a tissue-specific manner may have better therapeutic efficacy and reduce adverse side effects.

CONCLUDING REMARKS

Over the past two decades, JNKs have increasingly been recognized as an attractive molecule target for the treatment of human malignancies because they regulate a number of cellular processes whose deregulation has been associated with cancer (Wagner and Nebreda, 2009). This has triggered extensive drug discovery efforts. To date the majority of developed JNK inhibitors target the highly conserved ATP-binding site, although multiple steps in the JNK pathway could be targeted for inhibition (reviewed by Siddiqui and Reddy, 2010; Bogoyevitch *et al.*, 2010). While a number of these inhibitors have been proven to work *in vivo* in animal models of disease ranging from inflammatory to neurodegenerative disorders they have not been translated into clinical use thus far. One of the main concerns associated with ATP-competitive inhibitors is the lack of specificity as they can also target other kinases thus generating off-target effects. Moreover, the high concentrations of intracellular ATP significantly reduce the efficacy of these compounds (Siddiqui and Reddy, 2010; Bogoyevitch *et al.*, 2010). Small molecule and peptide-based inhibitors, that target the docking site of downstream substrates or the scaffold proteins, are a promising alternative to the traditional ATP-competitive inhibitors with the potential for improved efficacy and specificity towards the JNKs (Chen *et al.*, 2009). Cell-permeable versions of these inhibitors have been successfully used in studies for JNK-associated disorders including cerebral ischemia (Borsello *et al.*, 2003), stroke, Parkinson's disease (Kuan and Burke, 2005) and diabetes (Kaneto *et al.*, 2004), and are indeed of increasing interest to the research and drug discovery communities. A considerable additional work, however, will be clearly needed to validate these compounds in *in-vivo* studies.

As the diverse functions of the JNK proteins in cancer are emerging, greater efforts are now being directed towards the development of selective inhibitors (ATP competitive or otherwise) that discriminate between JNK1 and JNK2 (Figure 3). However, because JNKs play a pivotal role in normal biological functions, direct inhibition of the JNKs themselves could also have undesired side effects. Indeed, one of the major challenges facing researcher is to develop selective inhibitors of JNKs aimed at treating different cancers based on their ability to target specific JNK-mediated cellular event. As research likely will inform on new downstream substrates or binding partners, further studies should also focus on developing substrates-specific inhibitors, which could make an important contribution to the future alleviation of different types of cancer.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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FIGURE LEGENDS

Figure 1

Schematic representation of the JNK signaling cascade. Like all other MAP kinases, the JNK signaling cascade consists of a three-component module of upstream MAP3 kinases (MAP3Ks) that couples the signals from the cell surface to intracellular protein effectors. Different MAP3Ks have been implicated in the JNK cascade, depending on the type of stimulus the cell receives. Once activated, MAP3Ks phosphorylate and activate components of the MAP2K module, such as MKK4 and MKK7, which in turn phosphorylate and stimulate the activity of distinct JNK isoforms through dual phosphorylation on threonine and tyrosine residues within a conserved *Thr-X-Tyr* motif. Upon activation, each JNK protein itself can phosphorylate serine and threonine residues on specific substrates, delivering different cellular activities.

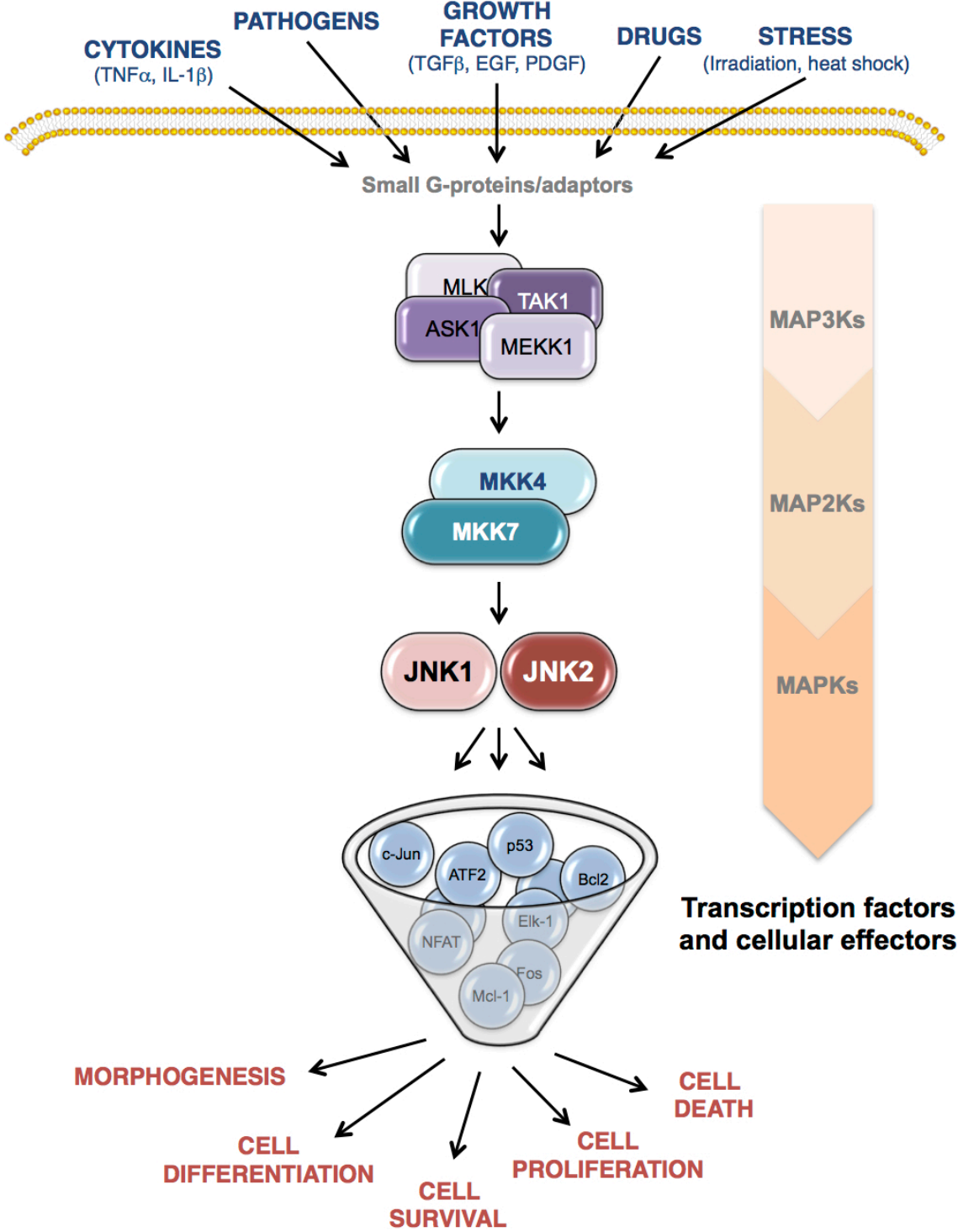
Figure 2

JNKs regulate key cellular activities involved in cancer. JNKs are activated in response to different extracellular and intracellular stimuli, such as oncogenes, dietary agents, obesity, alcohol, infectious agents and irradiation. Persistent activation of JNKs affect tumorigenesis by both transcription-dependent and transcription-independent mechanisms involved in cell proliferation, survival, transformation, inflammation, migration and suppression of cell death.

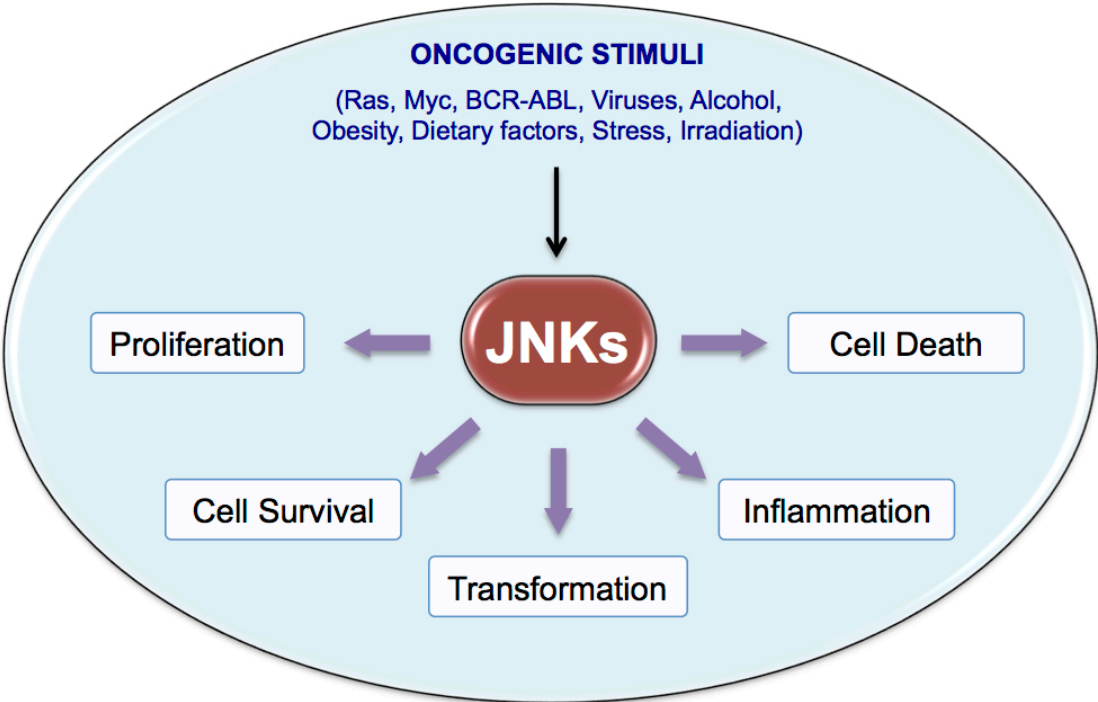
Figure 3

Specific cellular targets of JNK1 and JNK2 in different types of cancer. A graphical abstract visualizing the distinct targets regulated by the individual JNK proteins. Distinct functions of JNK1 and JNK2 in various cancers may occur through differential regulation of specific substrates and/or downstream effectors. The development of inhibitors that target specific downstream effectors of the individual JNK proteins would provide unique opportunities for therapeutic interventions.

Bubici_Figure 1



Bubici_Figure 2



Bubici_Figure 3

