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IGF-I Influence on Breast Cancer Cell Survival

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Abstract. The insulin-like growth factor system has been implicated in the proliferative control of breast cancer cells. In addition to this function, IGF action can also protect cells from programmed cell death. Substantial knowledge has been gained about death effector molecules and their regulation in breast cancer. IGF receptor can influence several key cell death pathways. In this review, we will focus on the intracellular mechanisms activated by IGF-I that influence cell survival.

INTRODUCTION

For many years investigators attributed the protumorigenic properties of insulin-like growth factor-I (IGF-I) in breast cancer to its ability to stimulate breast cancer cell proliferation [1–3]. Indeed IGF-I does serve an important role in both normal and breast cancer biology as a mitogen; however, in the past decade researchers have realized that IGF-I's role as a survival factor is equally if not more important to tumor growth and treatment resistance [4–7]. This review will discuss current knowledge regarding intracellular targets for IGF-I mediated survival effects with an emphasis on breast cancer models. Given the recent expansion of knowledge regarding both survival and apoptosis mediated signaling molecules, it is clear that much work is yet to be accomplished in this area.

Overview of cell death pathways

Several families of proteins have been identified which contribute to the process of apoptosis. Apoptosis differs from necrosis in that cells undergoing apoptosis (or programmed cell death, PCD) do so in a step-wise fashion that requires the function of proteins with specific tasks. PCD is an energy-requiring process that leads to the dismanteling of the cell with hallmark morphological changes including blebbing of the plasma membrane, nuclear condensation, and DNA cleavage. Apoptosis is a necessary response of normal cells; its role in normal development and immune response is readily appreciated. In the normal mammary gland apoptosis serves an imperative role in involution; upon weaning, involution of the mammary gland is highly dependent on a dramatic increase in apoptosis.

Important to cancer research are questions regarding the identity of the proteins that regulate PCD and how the functions of these proteins have gone awry in cancer. Answering these questions may help offset the development of tumors and improve tumor response to therapeutic interventions. The field of apoptosis is rapidly expanding and for the purposes of this review we will focus on the families of proteins that are best characterized and clearly important to IGF-I survival responses in breast cancer.

The primary executioners of apoptosis are the caspase proteins. Caspases are cysteine proteases which are further defined as either initiator or effector (executioner) caspases. After a death stimulus, initiator caspases (caspases-2, -8, -9, and -10) are induced to undergo oligomerization via CARD or DED domains. Oligomerization permits autocatalytic activity and subsequent cleavage of the effector caspases (see refer-

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ences [8,9] for review). The two primary pathways for the execution of apoptosis are defined as either the extrinsic (receptor mediated) or intrinsic (mitochondria-mediated) pathway (Fig. 1). The extrinsic pathway is initiated by caspases-8, whereas; the intrinsic pathway is initiated by caspase-9. Each pathway appears to respond to different types of stress stimuli [10]. For example, caspase-8 mediated apoptosis is regulated via activation of the TNF (tumor necrosis factor) superfamily of receptors induced by cytokines including TNF-α, TRAIL or Fas. [11]. Proteolytically cleaved (activated) caspase-8 then cleaves effector caspases-3 and -7 [12, 13]. Caspase-8 can also cleave Bid (a BCL-2 related protein) to its truncated form (tBid) [14]. tBid translocation to mitochondria induces mitochondrial release of cytochrome c and subsequent activation of caspase-9, thus amplifying the death signal by recruitment of the intrinsic pathway.

Non-cytokine receptor mediated stress, such as UV (ultraviolet) irradiation, gamma irradiation and chemical treatment, typically initiate apoptosis through mitochondrial release of cytochrome c [15] resulting from the production of ROS (reactive oxygen species) and the loss of mitochondrial membrane potential. In the caspase-9 initiated pathway, cytochrome c release into the cytoplasm triggers the formation of the apoptosome, a multi-protein complex containing cytochrome c, dATP, Apaf-1, and caspase-9 [16,17]. In addition, the presence of caspase-3 and/or -7 within the apoptosome may result in more optimal cleavage of caspase-9. Ultimately, caspase-9 serves as the initiator caspase which further amplifies an apoptotic signal by activating caspase-8 and -2 [18]. Other downstream responses to caspase-9 include cleavage of pro-caspase-3, -6 and/or -7. Since both the intrinsic and extrinsic pathways utilize the same effector caspases, the morphological effects of these pathways are similar and involve proteolytic degradation of substrates like PARP (poly(ADP) ribose polymerase), fodrin, and Lamin B, among others [19,20].

As with most biological systems, mechanisms also exist that temper the activation of caspase mediated death pathways. The BCL-2 family of proteins primarily functions upstream of the mitochondria to either enhance or inhibit cytochrome c release. BCL-2 proteins function as dimmers. The anti-apoptotic members BCL-2 and BCL-XL can homodimerize to inhibit apoptosis by maintaining mitochondrial membrane potential in the presence of an apoptotic stimuli (i.e. cellular stress) [21,22]. In contrast, other BCL-2 family members possess pro-apoptotic functions. For example, Bid, Bak, Bad, Bax, and Bim, as well as many other BH3-only containing BCL-2 proteins (reviewed [23, 24]), are important for inducing mitochondrial release of cytochrome c in response to various types of cellular stress. Cellular local of BCL-2 related proteins is also highly regulated; when cells are stressed the pro-survival proteins like BCL-2 and BCL-XL move from the cytoplasm to the mitochondrial membrane. Pro-apoptotic BCL-2 members may either bind the pro-survival members or oligomerize and move to the mitochondrial membrane to form pores in the outer membrane. With cellular death, heterodimers of the pro- and anti-apoptotic BCL-2 proteins are thought to overcome the survival effects of the BCl-2 or BCL-XL homodimers. Thus, cellular fate depends largely upon the intracellular predominance of anti- versus pro-apoptotic BCL-2 related protein dimers. Beyond dimer formation, the function of BCL-2 related proteins can also be altered post-translationally by kinases which are sensitive to either growth factors or stress. IGF-I mediated kinases often regulate proteins via post-translational modification to alter cellular responses to stress.

Apoptosis models for breast cancer

The induction and execution of apoptotic pathways are extremely dynamic; the timeframe for induction of caspase pathways may be initiated and completed within hours, in a similar fashion to growth factor mediated pathways. In order to study the dynamics of programmed cell death, in vitro assays using cell lines have been the mainstay. In understanding the role of IGF-I in breast cancer mediated apoptosis the tools become more restrictive since only a handful of breast cancer cell lines are IGF-I responsive. Further, in vitro culture of cell lines can also lead to clonal variations. These factors should be considered before extending findings toward clinical implications. Despite such limitations, cell lines have been invaluable for identifying proteins important for apoptosis and understanding their functional interactions.

Another valuable tool for apoptosis research is the mouse model where a particular protein can be over-expressed or knocked out in a mammary specific manner. Although mouse models have been invaluable for breast cancer research in general, they have not been used extensively in a mammary specific fashion for apoptosis. Furthermore, it currently appears that in the absence of certain caspases, as with knockout mice, other caspases may compensate in PCD, providing little to no phenotypic changes (reviewed by [25]). An im-

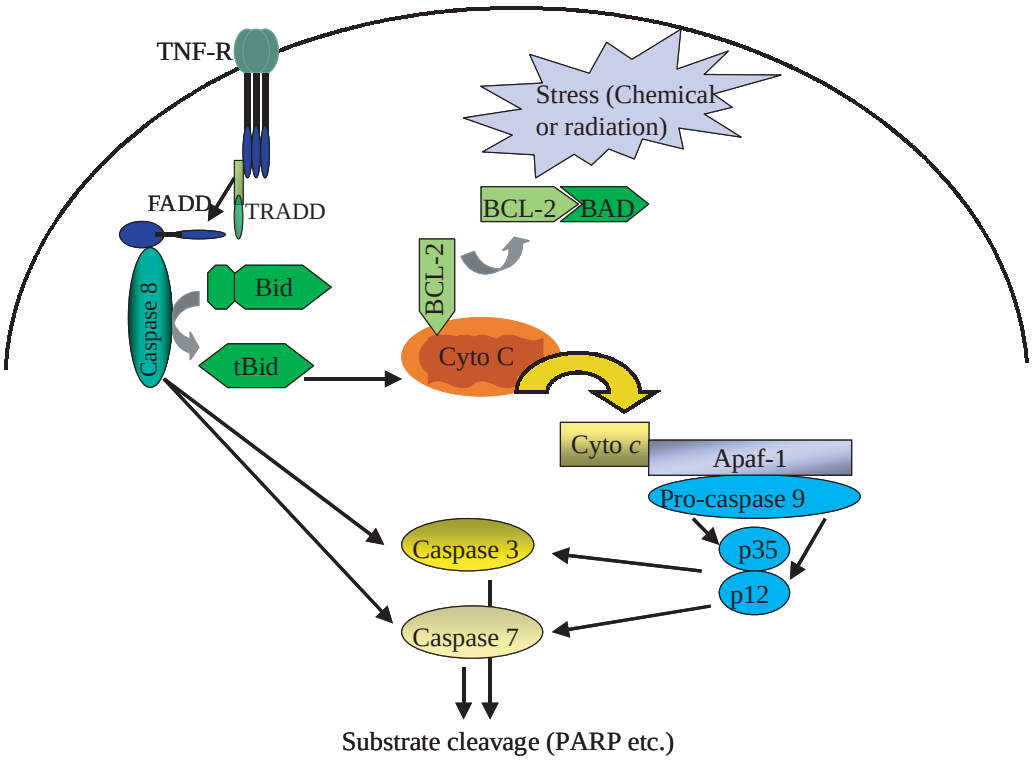

Fig. 1. Schematic model of cellular response to various forms of stress. Activation of cytokine receptors like TNF-R (tumor necrosis factor-receptor) induces the extrinsic pathway by inducing caspase-8 oligomerization and autocatalytic activation. Activated caspase-8 then cleaves effector caspase-3 and -7. Bid can also be cleaved by caspase-8, truncated Bid (tBid) then translocates to the mitochondria to induce cytochrome *c* release. In contrast, chemical treatment or UV and gamma irradiation of cells leads to ROS (reactive oxygen species) production and loss of mitochondrial membrane potential resulting in mitochondrial release of cytochrome *c*. Increased cytoplasmic cytochrome *c* levels induce the formation of the apoptosome (a protein complex containing Apaf-1, caspase-9, cytochrome *c*, and dATP) which initiates the intrinsic caspase pathway with subsequent activation of effector caspase-3 and -7 leading to morphological changes associated with the cleavage of caspase substrates.

portant in vitro example of such redundancy also exists in the IGF-I responsive, human MCF-7 breast cancer cell line. Since the discovery that MCF-7 human breast cancer cells do not express full length caspase-3, due to a 47-base pair deletion within exon 3 of the caspase-3 gene, debate ensued regarding the ability of MCF-7 cells to undergo programmed cell death [26,27]. Such an argument is no longer made. In fact, recent evidence indicates that caspase-3 expression may be low in human breast tumors [28], indicating that the expression status of caspase-3 in the MCF-7 cell line may be reflective of the disease. Moreover, use of this cell line is an important tool for the study of caspase-3 dependent and independent effects. Though less studied, caspase-7 (also an effector caspase) conveys many of the same effects as caspase-3 since it can cleave many of the same substrates. This again supports the presence of redundancy amongst caspases. With respect to animal models, however; lack of phenotypic changes in knock-out models, especially caspase knockout models, may limit our ability to make useful observations without further addition of pharmacologic caspase inhibitors or the use of compound knockout mice. These limitations should also be kept in mind as we develop therapeutic agents that target specific pathways [29].

Ultimately in order to best understand mechanisms for breast tumor growth and treatment resistance, human tumor samples must be evaluated. Some of the BCL-2 protein family members have been evaluated as both prognostic and predictive indicators and these studies indicate that tumors frequently overexpress survival proteins such as BCL-2 and BCL-XL [30–35]. As with most biological markers, evaluating a single component of a complex pathway may not allow us to appreciate the multiple mechanisms available for cancer cells to advert death. For these reasons, this review provides many observations that are directly attributed to certain models or cell lines but the generalizability is yet to be determined.

IGF-IR (Insulin-like Growth Factor-I Receptor) survival responses through Akt activation

IGF-IR is frequently overexpressed in breast tumors [36]. Moreover, its autophosphorylation and kinase activity is enhanced in breast tumors [37]. IGF ligands (including IGF-I and -II) convey biological responses by transactivating the IGF-IR, leading to the subsequent binding of adaptor proteins including the IRS (insulin receptor substrate) proteins. Of the four IRS proteins identified so far, IRS-1 is best characterized and probably mediates the majority of IGF-IR mediated survival responses in breast cancer cells [38]. IRS-1 undergoes receptor mediated tyrosine phosphorylation and then recruits SH2 containing proteins like the p85 subunit of phosphatidylinositol (PI) 3-kinase, Ras, and Grb2, among others. Activation of extracellular regulated kinases (ERK) 1 and 2 also occurs, and may mediate IGF-I mitogenic effects and possibly some anti-apoptotic responses. However, the majority of these responses are attributed to activation of PI 3-kinase in breast cancer cells [37,39–41]. Growth factor stimulated PI 3-kinase then regulates these effects through 3-phosphoinositide dependent protein kinase 1 (PDK1) and its downstream protein kinases including Akt (also known as PKB (protein kinase B)), p70 S6 kinase, JNK, and mTOR [42] (reviewed by [43,44]).

Akt is the kinase best characterized for its anti-apoptotic function in a vast range of cell types [45–47]). Akt is highly expressed in breast cancer cell lines and its activity is frequently induced by the presence of IGF-I. Further, estradiol co-treatment of MCF-7 breast cancer cells results in enhanced IGF-I activation of Akt [48]. Akt conveys survival responses primarily through phosphorylation of substrates which then inhibits their pro-apoptotic functions (see Fig. 2). As mentioned previously, BCL-2 related proteins function upstream of the mitochondria to mediate cellular response to stress. In many non-breast cancer cell lines, IGF-I induced Akt and p70 S6 kinase phosphorylate Bad [49–51] along with PKA (protein kinase A). Serine phosphorylation of Bad inhibits its pro-apoptotic responses by sequestering phosphorylated Bad to cytoplasmic 14-3-3 proteins [52,53]. Heterozygote mice expressing a triple Bad mutant gene, where alanines replace the growth factor targeted serine residues, show a defect in survival factor mediated inhibition of PCD in lymphocytes. However, IGF-I stimulation of isolated neurons is still able to convey survival responses, suggesting that Bad may not be the only substrate for IGF-I mediated effects [54].

Another substrate for Akt is IκB kinase α (IKKα) which when phosphorylated by Akt increases IκB degradation and results in NF-κB translocation to the nucleus where NF-κB enhances transcription of survival genes [55]. In multiple myeloma cell lines IGF-I treatment leads to enhanced resistance to Apo/TRAIL (a member of the TNF receptor superfamily) via increased transcription of FLICE-like inhibitory protein (FLIP), survivin, cellular inhibitor of apoptosis (cIAP-2), and X chromosome-linked inhibitor of apoptosis (XIAP) in an Akt dependent fashion [56,57]. Each of these proteins inhibits caspase mediated apoptosis. Although these initial works have documented a role of BAD and IKKα in Akt mediated survival, their role in IGF-I mediated survival effects in breast cancer cells still remains to be clearly identified.

Akt also phosphorylates caspase-9 on Ser-196. By phosphorylating caspase-9, Akt inhibits its proteolytic activity [58]. Members of the apoptosome including caspase-9 and Apaf-1 also appear to function downstream of p53 mediated apoptosis. Given both the frequency of IGF-IR overexpression and p53 mutations in human breast tumors, caspase-9 is an attractive therapeutic target for breast cancer. For this reason, determining if IGF-I mediates its survival response through inhibition of caspase-9 has recently become a focus of our research efforts. Like many other investigators, we have been unable document Akt mediated phosphorylation of caspase-9 in MCF-7 cells but while investigating caspase-9 mediated apoptosis we have made other significant observations regarding breast cancer models. As expected, IGF-I treatment inhibits PCD of MCF-7 breast cancer cells after exposure to many types of stress including paclitaxel, UV, TNF-α, and gamma irradiation. UV irradiation is a classical activator of mitochondrial cytochrome c release (i.e. the intrinsic pathway) in most cell types. Despite the ability of MCF-7 cells to undergo apoptosis, as marked by both caspase-7 and PARP cleavage, no changes in caspase-9 cleavage are observed after UV treatment. Both data from the transfection of wildtype caspase-9 in MCF-7 cells and *in vitro* caspase-9 cleavage assays suggest there are no aberrations in either endogenous caspase 9 or cytochrome c expressed in MCF-7 cells. In fact, the only stimulus that is able to induce caspase-9 cleavage in MCF-7 cells lysates is exogenous cytochrome c [41]. Since no aberrations are observed in stress induced cytochrome c release from the mitochondria, these data may indicate that MCF-7 cells express a cytoplasmic inhibitor of cytochrome c which inhibits the formation of the apoptosome and subsequent caspase-9

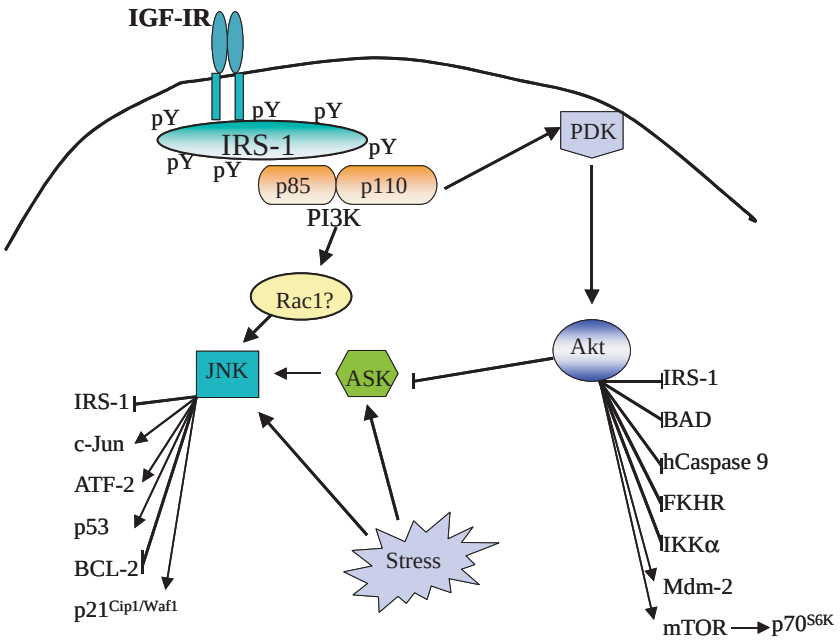

Fig. 2. IGF-I and stress activated pathways which mediate cellular outcome. Activation of the IGF-IR results in tyrosine phosphorylation of IRS-1 and recruitment of downstream kinases including PI3K. Activation of PI3K induces Akt activity, which in turn phosphorylates several substrates involved in the regulation of apoptosis (namely IRS-1, BAD, caspase-9, ASK1, FKHR, IKKα, Mdm-2 and p70^{S6} kinase). Alternatively, IGF-I treatment can also activate JNK (independent or dependent of PI3K). Thus, both IGF-I and stress treatment can activate JNK to down-regulate IGF-I signaling. IGF-IR (insulin-like growth factor-I receptor); PI3K (phosphatidylinositol 3-kinase); IRS-1 (insulin receptor substrate); ASK1 (apoptosis signal-regulating kinase 1); IKK (IκB kinase), Mdm-2 (murine double minute 2); JNK (c-Jun N-terminal kinase).

cleavage [41]. Other reports have described a modest amount of caspase-9 cleavage in MCF-7 cells [59] or have described MCF-7 cells as cytochrome c insensitive [60]. Overall it appears that MCF-7 cells do not depend heavily on the intrinsic pathway for apoptosis. Expression of high amounts of an inhibitor of cytochrome c may provide yet another mechanism for cancer treatment resistance.

Despite the unresponsiveness of caspase-9, MCF-7 cells are still sensitive to apoptosis using various types of treatment (UV, paclitaxel, and doxorubicin). Our current data indicate that UV treatment enhances caspase-8 cleavage which then leads to cleavage of caspase-7 and PARP, independent of mitochondrial release of cytochrome c [41]. Inhibition of caspase-8 significantly reduces both caspase-7 and PARP cleavage. Further, IGF-I inhibits stress induced caspase-8 cleavage in a PI 3-kinase sensitive fashion and its effect is unaltered by inhibition of both transcription and translation. This perhaps indicates that Akt has a yet-unidentified target involved in the caspase-8 mediated cell death pathway.

Another potentially useful cell line for the study of caspase-9 mediated function is the MDA MB-231 human breast cancer cell line. Even though MDA MB-231 cells express mutated p53, they are extraordinarily sensitive to UV irradiation making them a promising tool to evaluate the ability of IGF-I treatment to inhibit apoptosis via Akt mediated phosphorylation of caspase-9. Generally the MDA MB-231 cell line is not thought to respond to IGF-I as a mitogen, however, we have observed significant IGF-I mediated survival in these cells. IGF-I co-treatment of UV treated MDA MB-231 cells inhibits caspase-9 cleavage and this effect is lost with inhibition of PI 3-kinase. To date, we have been unable to observe evidence of Akt mediated serine phosphorylation of caspase-9 even in these cells. Alternatively, the survival effect of IGF-I in MDA MB-231 cells may also be mediated by upstream inhibition of Bad (leading to inhibition of cytochrome c release and subsequent cleavage of caspase-9) [54] or by caspase-8 activation of tBid and subsequent release of mitochondrial cytochrome c.

Bad and caspase-9 are both examples of apoptotic targets where Akt inhibits the function of a pro-apoptotic protein, post-translationally. Akt can also phosphorylate forkhead transcription factors to inhibit the synthesis of pro-apoptotic proteins. The three fork-

head proteins FKHR, AFX, and FKHRL1 all possess three Akt phosphorylation sites [61,62]. When located in the nucleus, unphosphorylated forkhead transcription factors induce the expression of FasL, IGFBP-1, and $p27^{Kip1}$ [63–65]. The protein products of these genes then either enhance apoptosis or induce cell cycle arrest. Phosphorylation of forkhead proteins by Akt increases their cytoplasmic localization where they bind to 14-3-3 proteins. Such a change in cellular localization inhibits the transcriptional activation of forkhead gene targets [63]. In this fashion, Akt is able to regulate G1 transit and cell survival.

A few studies have provided some insight into the importance of forkhead transcription factors in breast cancer models. Jackson et al. have demonstrated that endogenous FKHR cytoplasmic versus nuclear distribution is regulated in a growth factor mediated fashion using epidermal growth factor (EGF) responsive MDA MB-231 breast cancer cells [66], while Schurr and colleagues have reported that estrogen receptor α can bind FKHRL1 to regulate the cell cycle distribution of estrogen in MCF-7 breast cancer cells [67]. Furthermore, Hutchinson and colleagues have reported that mammary expression of both the Middle T Antigen and constitutively active Akt in bigenic mice enhances phosphorylation of FKHR in the mammary glands. Mammary tumors developing in these bigenic mice also display significantly smaller apoptotic indices. Given the short latency and high tumorigenicity of this mouse model, FKHR is a likely substrate for Akt mediated survival effects [68]. Outside of these reports, direct evidence showing that IGF-I stimulated Akt targets forkhead transcription factors to convey its survival responses is not yet forthcoming.

IGF-I regulation of stress mediated pathways

As discussed so far, IGF-I activates anti-apoptotic pathways like Akt to convey a survival response. Alternatively, IGF-I may also inhibit stress mediated pathways to circumvent apoptosis. Many protein functions are altered in response to stress and IGF-I would most likely interfere with stress signaling pathways to ultimately influence cellular fate. Two such signaling proteins include p38 kinase and JNK (c-Jun N-terminal kinase). Some types of stress such as TNF-α and paclitaxel can induce both p38 kinase and JNK [40,69], while other types of stress induce the activity of one or the other. In general, p38 kinase is induced in response to osmotic stress, whereas; JNK is activated by genotoxic stress, oxidative stress, and microtubulin interfering agents [70–72]. In fact, JNK has been shown to mediate the efficacy of several types of chemotherapy drugs, namely, doxorubicin, paclitaxel, and cisplatin [51,72,73].

Like p53, JNK activity is induced by DNA damage and JNK frequently enhances cell death when activated. p53 and JNK responses seem to be closely coupled; stress-activated JNK phosphorylates p53 and $p21^{Cip1/Waf1}$, enhancing their stability and transcriptional activity and resulting in p53 downstream effects, including cell cycle arrest or PCD. In cells lacking p53 function, investigators have recently shown that activated JNK serves a very different role by *augmenting* survival. Antisense mediated inhibition of JNK2 in MCF-7 breast cancer cells results in a significant reduction in cell viability when wildtype p53 is inactivated by HPV E6 oncoprotein expression. Thus, JNK activity may contribute to cancer treatment resistance in tumors lacking wildtype p53 [74]. In reference to breast cancer and clinical correlates of response, enhanced JNK activity has been observed both in patient breast tumors that have *acquired resistance* to tamoxifen and in mice bearing tamoxifen resistant MCF-7 xenografts [75,76]. Unfortunately, these studies, by nature of their design, were unable to determine if increased JNK activity is a consequence or the cause of tamoxifen resistance.

These results strongly indicate that JNK activity is an important predictor of cancer sensitivity to current treatment modalities. If stress treatments require activation of JNK to induce cell death then one mechanism for IGF-I may be its ability to inhibit JNK activation. Indeed some models have shown such an effect. LeRoith's group initially described the ability of IGF-I pretreatment to inhibit stress mediated activation of JNK in human embryonic kidney cells (the HEK 293 cell line) [77]. Other investigators later showed that ASK1 (apoptosis signal-regulating kinase 1, a stress-activated kinase) is upstream of stress activated JNK. Interestingly, ASK1 is phosphorylated on Ser-83 by Akt. IGF-IR also binds and phosphorylates ASK1 on a tyrosine residue in the N-terminal domain [78,79]. Both the phosphorylation of ASK1 by Akt and IGF-IR inhibit the ability of ASK1 to induce JNK in non-breast cancer models. Thus, ASK1 may be a point of convergence between stress and survival signaling pathways. However, the regulation of JNK's function(s) appears to be much more complicated than this simple model.

In addition to JNK's role in the cellular stress, we and others have previously shown that JNK activity is strongly increased in response to IGF-I treatment of MCF-7 and T47D breast cancer lines [40,80]. In a

similar fashion as Akt, IGF-I activation of JNK is PI 3-kinase dependent in MCF-7 cells [40]. Although other investigators have reported JNK activation by growth factors in a PI 3-kinase dependent fashion [81,82], little information is available about the effects of JNK activation by growth factors on cellular fate, especially with cancer models. Therefore, we decided to ascertain the biological consequences of growth factor activated JNK. Surprisingly, when MCF-7 cells are exposed to both stress and IGF-I treatment, we observe significantly more JNK activity than with either treatment alone. Inhibition of Akt, but not PI 3-kinase, further increases JNK activity by IGF-I (unpublished results). The biological effect of increased JNK signaling through overexpression of JNK in MCF-7 cells shows that even though JNK is induced by diverse agents like paclitaxel and IGF-I, its primary function in either case appears to be pro-apoptotic. In fact, IGF-I activation of JNK appears to result in negative feedback of IGF-I signaling [40].

One mechanism whereby JNK may downregulate IGF-I signaling is via serine phosphorylation of IRS-1. When IRS-1 is tyrosine phosphorylated after IGF-I ligand binding to the IGF-IR, downstream kinases such as PI 3-kinase, Akt, and ERK 1/2 are induced, conveying IGF-I pro-survival and proliferative responses. The Ser-312 residue of human IRS-1 has gained particular interest since it lies adjacent to the PTB (protein tyrosine binding) domain, the region required for receptor binding [83]. JNK binding to IRS-1 and its phosphorylation of this site inhibits ligand-induced insulin receptor binding to IRS-1. Activated JNK (activated by TNFα, anisomysin, and PI 3-kinase sensitive kinases) phosphorylates this site to inhibit subsequent IRS downstream signaling [84]. These data suggest that stress activated kinases may downregulate IGF-I signaling in a similar fashion to Akt's inhibition of stress signaling pathways. Such cross-talk amongst pathways may ultimately dictate cellular outcome.

Although transfection studies provide important information regarding the function of a protein and its downstream targets, it is increasingly important to study the regulation of endogenous proteins to provide better insight to apoptotic regulation in cancer cells. Until recently, the lack of a specific pharmacologic inhibitor of JNK has made interpretation of data difficult in this field. Moreover, the complexity of JNK's functions can be realized when considering the multiple JNK substrates discovered thus far. JNK substrates like ATF-2, c-Jun, Bcl-2, Bcl-XL, p21$^{\mathrm{Cip1/Waf1}}$, and p53 have been identified and have provided insight about the ultimate effect(s) of activated JNK within varying cellular contexts. c-Jun is JNK's most-studied substrate, and it alone leads to complex responses. Phosphorylated c-Jun homo- or heterodimerizes with Fos or ATF-2 to form AP-1 complexes, that serve as transcription factors to alter gene expression. Thus, AP-1 is a downstream effector of JNK activity, and it regulates genes such as cyclin D1, p53, p21$^{\mathrm{cip1/waf1}}$, p19$^{\mathrm{ARF}}$, and p16 that then affect cell proliferation and apoptosis (reviewed by references [85–87]).

Non-transcriptional responses of stress-activated JNK include its localization to the mitochondria and its phosphorylation BCL-2 and BCL-XL. JNK phosphorylation of BCL-2 inhibits BCL-2 survival effects in stress-induced cells [88]. In addition, binding of JNK to mitochondrial localized proteins may also alter JNK mediated effects in the cytoplasm or nucleus, ultimately inhibiting JNK's apoptotic functions [89]. The literature is currently filled with conflicting results about the biological responses of JNK which vary from apoptosis, to survival, and to cell cycle regulation. Such discrepancies may be introduced when direct JNK functions are inhibited (such as JNK phosphorylation of proteins like p53 and p21$^{\mathrm{Cip1/Waf1}}$) versus secondary responses associated with activity of its substrates like loss of AP-1 transcriptional effects. Also, given the multiple pathways that JNK can influence, cellular context must certainly influences cellular fate.

In contrast to JNK, the clinical importance of p53 is better established in cancer models. Loss of p53 function plays an important role in breast cancer development, as is indicated by the frequency of p53 mutations in breast tumor specimens. Normally, wild-type p53 expression and stability are enhanced in response to DNA damage [90], resulting in cell cycle arrest for either DNA repair or induction of programmed cell death. Most p53 mutants fail to bind DNA [91] and do not regulate p53-inducible genes [92]. Mutant forms of p53 are expressed in 30–48% of human breast tumors ([Bhargava, 1994, ref. 386], and references therein), suggesting that these tumors have lost the ability to undergo p53 dependent cell cycle arrest in response to DNA damage. p53 mutation is only one mechanism by which p53 function can be abrogated. Amplification or overexpression of mdm-2 (murine double minute-2) also results in inactivation of p53. Binding of mdm-2 to p53 inhibits p53-induced transactivation [94] and targets p53 for proteosome-dependent degradation [95]. Overexpression of mdm-2 mRNA or protein occurs in 71–73% of human breast tumors [96]. Thus, p53 function is altered in a high percentage of breast tumors by either mutant or increased expression of mdm-2 [94].

One of the predicted outcomes of the loss of p53 function is a resistance to apoptotic stimuli. Two components of the apoptosome, caspase 9 and Apaf-1, are downstream targets for p53 [97,98]. Inactivation of Apaf-1 or caspase 9 substitute for p53 loss in promoting soft agar growth and tumor formation of Myc-expressing cells [99]. When $p53-/-$ mouse embryo fibroblasts cells are transfected with a temperature sensitive p53 ($p53^{val138}$) and cultured at the permissive temperature (resulting in a functional p53), the cells undergo massive apoptosis. No apoptotic effect is observed in Apaf-$1-/-$ or caspase $9-/-$ cells transfected with the same p53 mutant treated with the same conditions. Therefore, Apaf-1 and caspase-9 represent two potentially important p53 targets in regulating apoptosis. Thus, the ability of IGF-I treatment to inhibit caspase 9 cleavage downstream of p53 remains a very interesting question in regards to treatment resistance of breast tumors.

With regard to p53 mediated apoptosis, another mechanism has recently been shown to exist whereby Akt can inhibit p53 mediated functions. Mdm-2, the protein that binds to p53 and targets it for ubiquitination, is another substrate for Akt. When mdm-2 is phosphorylated by Akt, it localizes to the nucleus where p53 binding and degradation are enhanced [100,101]. Akt-dependent regulation of mdm-2 localization and function results in decreased p53 binding to $p19^{ARF}$ and resistance to chemotherapy-induced apoptosis [100,102, 103]. Thus, it would be expected that there are intersections between p53 and Akt in the regulation of apoptosis, as well as pathways that may be unique to each of these regulatory molecules.

With so many potential targets for IGF-I mediated survival effects in breast cancer, it is unlikely that only one Akt substrate would mediate all of IGF-I effects. The survival responses conveyed by Akt action are strong and undisputed using various models. With these biologic responses in mind, the recent emphasis to target Akt for cancer treatment is well-justified. However, further research to study cross-talk between stress and survival mediated pathways may prove most fruitful therapeutically since such a strategy may allow us to enhance response to therapeutic modalities while also inhibiting the proteins that convey treatment resistance.

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