

## Review

# Molecular mediators of cell death in multistep carcinogenesis: a path to targeted therapy

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## Abstract

**A consistent, if not invariant, feature of cancer cells is the acquired ability to evade apoptosis. The pioneering work of Dr. Stan Korsmeyer was invaluable in characterizing the molecular foundations of cell death signaling mechanisms during normal development and during multistep carcinogenesis. This foundation now forms the basis for the rational design of therapeutic strategies to selectively activate cell death in cancer cell populations. These strategies are currently being evaluated in an increasing number of clinical trials targeting diverse tumor types.**

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**Keywords:** apoptosis; bcl-2; p53; cancer models; myc; cancer**Abbreviations:** IR, ionizing radiation; shh, sonic hedgehog; ptc, patched; smo, smoothened; NMSC, nonmelanoma skin cancer; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; Tag, T antigen; ECM, extracellular matrix; FAK, focal adhesion kinase; ASO, antisense oligonucleotide; IMD, integrin-mediated cell death

## Introduction

Cancer is a complex disease that arises from accumulated genetic, and sometimes epigenetic, alterations of critical cell regulatory genes. These genetic alterations confer upon tumor cells qualities that define the malignant phenotype. Cell death resistance is a common feature of most, if not all, tumors.<sup>1</sup> It is now over 30 years since it was proposed that hyperplasia could result from decreased apoptosis rather than increased mitosis.<sup>2</sup> The detection of apoptosis in normal, adult tissues confirmed the long held assumption that cells must be continuously lost from tissues to balance the proliferation that is comparatively easy to observe. It is now also widely

appreciated that apoptosis can be initiated in response to conditions that are likely to be encountered during tumor development including DNA damage, oncogene activation, hypoxia and loss of appropriate growth signals. Apoptosis, thereby, serves as an inherent mechanism to reduce the likelihood that abnormal cells with the potential to form tumors will remain viable.

Oncogenes and tumor suppressor genes were first recognized by virtue of their abilities to regulate cell cycle progression and to undergo either gain-of-function or loss-of-function mutations, respectively. Similarly, cell death regulators can undergo either gain-of-function or loss-of-function genetic, or epigenetic, alterations including mutation, amplification, translocation, deletion or CpG island methylation. Gain-of-function alterations typically affect cell death suppressors, for example, bcl-2, whereas cell death sensitizers, for example, bax, typically experience loss of function during the course of tumorigenesis.<sup>3–5</sup> These genetic or epigenetic changes can occur as primary events initiating the neoplastic cascade or secondary events that contribute to tumor progression and metastatic dissemination.

Recognizing that disordered cell death contributes to multistep carcinogenesis has significant conceptual implications regarding the biology and therapy of cancer. It is, simply put, no longer adequate to consider that cancer is a disorder of 'uncontrolled' proliferation. More appropriately, it should be considered that many malignancies are better characterized by a gradual accumulation, rather than a proliferation, of neoplastic cells. As such, traditional chemotherapeutic agents that affect only proliferating cells would be expected to be of limited utility in the clinical management of most cancers. Further, the efficacy of such agents in the management of low growth fraction malignancies is widely understood to be a function of cell death induction and not cytostasis.<sup>6</sup>

This review will focus on the identification and molecular characterization of several well-known regulators of cell death in the context of multistep carcinogenesis. The findings will be discussed from a biologic perspective as well as forming a basis for a consideration of the targeted manipulation of cell death for therapeutic effect.

## Primary Cell Death Suppression is an Oncogenic Genetic Event

Although the field of cell death research is among the most active and rapidly advancing areas in the biological sciences, this was not always the case. Recognition of the morphologic features of apoptosis enabled an appreciation of the significance and ubiquitous nature of this process during normal development and in the maintenance of tissue homeostasis.<sup>2</sup> Further, it was speculated that disordered cell death could be anticipated to contribute to many disease

states including autoimmunity and cancer. However, understanding the mechanistic basis of cell death awaited the availability of the molecular tools to experimentally and specifically manipulate this process.

The paradigm that proto-oncogenes could be deregulated by chromosomal translocations developed from the recognition that the normal cellular homologues of viral-transforming genes, the proto-oncogenes, could be located at the sites of recurrent chromosomal breakpoints found in leukemias and lymphomas. Two notable examples were the deregulation of *c-myc* associated with the t(8;14) of Burkitt's lymphoma and *c-abl* associated with the t(9;22) of chronic myelogenous leukemia. Based on these observations, it was reasoned that it should be possible to discover genes that are important in neoplastic transformation by identifying transcriptionally active rearrangements present in specific malignancies. Indeed, much of our understanding of the biology of follicular lymphoma has resulted from the molecular characterization of the t(14;18)(q32;q21) found in approximately 85% of these tumors. Cloning of the derivative chromosome 14 breakpoint revealed a new transcriptional element that was termed *bcl-2*.<sup>7-10</sup> The marked deregulation of the *bcl-2*-Ig fusion gene suggested that it was the pathologically important consequence of the t(14;18). However, unlike previously characterized mammalian proto-oncogenes, there was no recognized viral-transforming gene that was homologous to *bcl-2*.

The contribution of the t(14;18) translocation and deregulated *bcl-2* expression to the development of lymphoma was studied prospectively using a transgenic mouse model.<sup>11</sup> The transgenic constructs recreated the molecular features of the t(14;18). The overexpression of *bcl-2* resulted in hyperplasia of the splenic white pulp secondary to an increase in B-lymphocytes. Cell cycle analysis, surprisingly, indicated that there were no substantial differences in the fraction of cycling cells isolated from the transgenics and controls. Approximately 95% of the cells resided in G<sub>0</sub>/G<sub>1</sub>.

The observations regarding the consequences of overexpressing *bcl-2* at first seemed paradoxical. Vaux *et al.*<sup>12</sup> provided insight into how overexpression of *bcl-2*, in the apparent absence of enhanced proliferation, could result in lymphoid hyperplasia. They observed that IL-3-dependent cell lines transfected with *bcl-2* expression vectors could remain viable in the G<sub>0</sub> state in the absence of IL-3, suggesting that *bcl-2* imparted a survival signal to the cell that could substitute for the requirement for exogenous IL-3. Nunez *et al.*<sup>13</sup> also demonstrated that *bcl-2* contributes to the survival of IL-3-dependent cell lines after growth factor withdrawal. These results were confirmed and extended when *bcl-2*-Ig transgenic splenocytes were placed into culture media containing only 5% fetal calf serum. Approximately 10% of the *bcl-2* transgenic lymphocytes that were initially plated displayed a 500-fold enhancement in survival compared to control littermates.

The enhancement of viability by *bcl-2* was shown to be mediated by the inhibition of programmed cell death, or apoptosis.<sup>14</sup> The disease process in the *bcl-2*-Ig transgenic mice, although initially indolent, progressed to malignant lymphoma after an average latency period of 15 months.<sup>15</sup> The lymphomas arising in the *bcl-2*-Ig transgenic mice were monoclonal B-cell malignancies. Results obtained from the

*bcl-2*-Ig transgenic mouse provide direct evidence that enhanced viability, resulting from a molecular lesion such as the deregulation of *bcl-2*, renders affected cells susceptible to malignant transformation and tumor progression. Secondary changes that contribute to the process of multistep lymphomagenesis, once identified, would be expected to genetically complement with *bcl-2* and accelerate the rate of tumorigenesis.

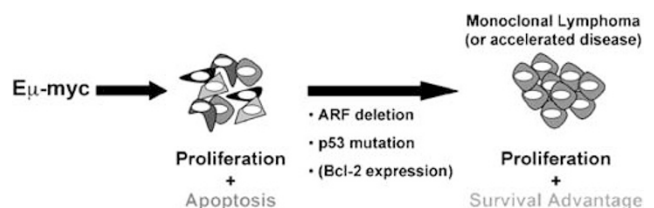
The *bcl-2*-Ig model provided convincing evidence that the deregulated expression of *bcl-2* resulting from the t(14;18) translocation is causative in lymphomagenesis. This was the first appreciation that inappropriate cell survival is important for the acquisition of further genetic changes that can produce a malignant phenotype. This pioneering work led by Stan Korsmeyer, as well as other groups, placed *bcl-2*, cell death regulation and tumorigenesis firmly on the map, together.

## Compensating for 'Oncogenic Stress' During Multistep Carcinogenesis

The long latency period and conversion from polyclonal B-cell hyperplasia to monoclonal B-cell lymphoma suggested that tumor progression in the *bcl-2*-Ig model was a multistep process. Approximately half of the lymphomas arising in *bcl-2*-Ig transgenic mice also displayed rearrangements of the *c-myc* oncogene. This provided *in vivo* perspective on previous observations that *bcl-2* can synergistically cooperate with *c-myc* in cellular transformation.<sup>12,16,17</sup>

Translocations involving *c-myc* and Ig gene enhancers are frequently observed in Burkitt's lymphomas. These high-grade malignancies are characterized by high rates of proliferation and apoptosis. E $\mu$ -*myc* transgenic mice were used to assess the contribution of *c-myc* deregulation in lymphomagenesis.<sup>18</sup> E $\mu$ -*myc* mice develop clonal B-cell lymphoma with a latency of 3–5 months. Interestingly, in contrast to *bcl-2*-Ig transgenic mice, which exhibit a polyclonal hyperplasia prior to tumor development, no hyperplasia was observed in E $\mu$ -*myc* mice despite significantly elevated rates of proliferation. This observation suggested that a compensating deletion of cells was able to balance the increased rate at which they were being generated (Figure 1).

It is now understood that inappropriate, or deregulated, *c-myc* expression results in the induction of apoptosis as well as



**Figure 1** Tumor progression in the E $\mu$ -*myc* lymphoma model. Myc expression can drive proliferation as well as apoptosis. Genetic alterations that disrupt myc-induced apoptosis, without affecting proliferation, contribute to disease acceleration or progression to malignant lymphoma. E $\mu$ -*myc*/*bcl-2* double transgenic mice develop lymphomas much more rapidly than E $\mu$ -*myc* mice. Advanced, monoclonal malignant lymphomas arising in E $\mu$ -*myc* mice often display deletion of ARF or mutation of p53, both of which disrupt myc-induced, p53-dependent apoptosis

mediating cell cycle progression.<sup>19</sup> Myc-induced apoptosis is especially robust when cells are placed under growth-limiting conditions. For example, in the presence of myc, following IL-3 withdrawal, IL-3-dependent cells die more rapidly than cells without myc expression. Similar observations were made in myc expressing Rat1 cells when shifted to low serum.<sup>20</sup> Bcl-2 can efficiently block myc-dependent apoptosis without affecting the rate of cell proliferation.<sup>20,21</sup> Genetic cooperation between deregulated c-myc and bcl-2 was established by generating myc/bcl-2 double transgenic mice.<sup>22,23</sup> These mice develop high-grade lymphomas after a latency of only 3 weeks. The functional basis of this cooperation is the ability of bcl-2 to inhibit myc-associated apoptosis without altering myc-induced proliferation (Figure 1).<sup>23</sup>

It is clear that bcl-2 expression is just one way that cancers acquire a survival advantage to enable myc-driven oncogenesis. Somatic mutations of myc can also circumvent apoptosis and enhance tumorigenesis.<sup>24</sup> Several myc mutations, occurring in hotspot regions, have been identified in human tumors. Importantly, some myc mutants display increased transformation potential *in vitro* that is associated with a corresponding reduction in apoptosis. The impact that specific myc mutations can have on tumorigenesis *in vivo* was demonstrated using hematopoietic progenitor cells expressing wild-type or mutant myc (T58A).<sup>24</sup> When injected into irradiated mice, the T58A myc cells developed malignancies more rapidly than mice injected with wild-type myc-expressing cells. Cells overexpressing mutant myc exhibited a reduction in apoptosis but equivalent proliferation rates compared to wild-type myc-expressing cells. The mechanistic basis of these observations was shown to be an inability of the T58A mutant to induce the BH3-only protein Bim. Bim is expressed at high levels in B cells in which myc is deregulated and can suppress myc-driven lymphomagenesis. In the presence of such mutations, the inactivation of p53 or ARF often seen in E $\mu$ -myc lymphomas, does not occur. Inappropriate expression of myc can activate the p53 axis via ARF and induce p53-dependent apoptosis.<sup>25</sup> Direct evidence that disruption of p53 and ARF can accelerate lymphoma development in E $\mu$ -myc mice has been provided (Figure 1).<sup>26</sup>

## Apoptosis Induction is an Important Component of Tumor Suppression

DNA damage, resulting in gene mutation or deletion, is a principal contributor to neoplastic transformation. DNA damage is a consequence of a variety of endogenous and exogenous sources of cellular stress. The major sources of DNA damage are endogenous reactive oxygen species, which can be byproducts of normal cellular respiration.<sup>27</sup> Accordingly, cells possess sophisticated mechanisms to detect and respond to DNA damage. One such mechanism is the activation of the p53 tumor suppressor protein. Other stimuli commonly encountered by tumor cells, including oncogene activation and hypoxia, can also activate p53. In response to these forms of cell stress, high levels of activated and stabilized p53 protein accumulate in the nucleus.<sup>28,29</sup> Activated p53 can induce cell cycle arrest, DNA repair processes and apoptosis. These cellular outcomes minimize

the accumulation of deleterious mutations that can promote transformation or eventually contribute to a fully malignant phenotype.<sup>30</sup> The importance of this pathway is highlighted by the observation that over 50% of cancers have acquired p53 gene mutations, ostensibly allowing the tumors to bypass this p53 checkpoint.<sup>31,32</sup>

Numerous *in vitro* and *in vivo* studies have established p53 as a potent tumor suppressor gene. p53-null (p53 $-/-$ ) and heterozygous (p53 $+/-$ ) mice have been generated independently by several groups, each with similar results.<sup>33–35</sup> Homozygous and heterozygous p53 knockout mice are viable and susceptible to tumor formation. The majority of p53 knockout mice develop lymphomas by 6 months of age.<sup>33</sup> p53 $+/-$  mice develop a spectrum of tumor types after a prolonged latency. Importantly, tumors arising in p53 $+/-$  mice display deletion or somatic inactivation of the remaining wild-type p53 allele.<sup>33,34,36</sup>

It is generally accepted that the ability of p53 to induce apoptosis is important for its tumor suppressor function. Thymocytes from p53 $-/-$  mice are resistant to apoptosis induction in response to treatments causing DNA damage (ionizing radiation (IR) and etoposide).<sup>37</sup> Transgenic expression of an SV40T antigen mutant protein (T<sub>M</sub>), which does not bind p53, induces tumor formation after a longer latency period compared to tumors arising in wild-type T antigen (Tag) mice. Tumors arising in the T<sub>M</sub> mice also exhibited higher levels of apoptosis compared to tumors arising in the Tag mice. Tumors arising in p53-null/T<sub>M</sub> mice exhibited tumor latencies and rates of apoptosis comparable to those arising in Tag mice, confirming the significance of p53 inactivation in tumor formation.<sup>38</sup>

Most p53 alterations in human tumors are missense point mutations, not gross deletions. Therefore, mouse models have been created to address the effect of various point mutations on tumorigenesis. A knock-in mouse model of human R175H (R172H in mice) has been developed, referred to as 515A.<sup>39</sup> Mice heterozygous for the knock-in allele develop tumors with the same kinetics as p53 $+/-$  mice. However, in contrast to p53 $+/-$  mice, tumors arising in p53 $+/-$ 515A animals exhibit the ability to metastasize. This suggests a gain-of-function activity for the R175H mutation, facilitating tumor metastasis. Upon exposure to IR, p53 $+/-$  embryos exhibit high levels of apoptosis. Under the same conditions, very little apoptosis is observed in p53 $+/-$ 515A embryos. This raises the possibility that inhibition of p53-induced apoptosis, through a mutant p53 gain-of-function mechanism, contributes to tumor metastasis. Similar results were obtained with an R175H knock-in model using a different inbred strain of mouse.<sup>40</sup>

There is also evidence indicating that the tumor suppressor function of p53 in the context of primary c-myc gene deregulation is not strictly limited to apoptosis induction.<sup>41</sup> The p53 mutant R175P can induce cell cycle arrest but is defective for induction of apoptosis.<sup>42</sup> A knock-in model with this mutation (designated 515C) was used to investigate the role of cell cycle arrest in p53-mediated tumor suppression. p53<sup>515C/515C</sup> cells do not undergo apoptosis in response to adriamycin treatment, serum withdrawal or IR. p53<sup>515C/515C</sup> behave as p53 $-/-$  cells in these apoptosis assays. However, they retain partial ability to transactivate p21 and induce cell

cycle arrest. Importantly, p53<sup>515C/515C</sup> mice showed a dramatic delay in tumorigenesis compared to p53<sup>-/-</sup> animals, suggesting that p53-induced cell cycle arrest has a direct role in tumor suppression.

## Disruption of Apoptosis During Tumor Progression and Therapeutic Resistance

Compared to hematopoietic neoplasms, less is understood about the genetic complexities that contribute to the development of epithelial malignancies. Even so, it is evident that cell death deregulation as a primary or secondary event can facilitate tumor formation, progression and therapeutic resistance. For example, in advanced-staged prostate cancer, the therapy of choice is hormone ablation by surgical or chemical castration. This treatment is effective because androgens are necessary for the survival and proliferation of androgen-dependent prostate carcinoma cells. While 80% of patients will exhibit a favorable response to androgen ablation, the treatment is not curative and androgen-independent tumor relapse is a predictable outcome. Androgen-independent cells are typically resistant to further hormone therapy, radiation or chemotherapy. As such, there are no effective therapies to fight androgen-independent prostate cancer.

Progression to androgen independence is thought to involve the acquisition of an apoptosis-resistant phenotype.<sup>43</sup> Several molecular alterations of genes associated with apoptosis resistance and disease progression have been identified in prostate cancer. These include loss of PTEN, loss of p53 and upregulation of bcl-2 expression. p53 alterations are not commonly found in primary tumor sites. They are more commonly found in advanced stage and metastatic disease.<sup>44–46</sup> This can explain, in part, the resistance of advanced stage prostate cancer therapeutic interventions predicated on inducing apoptosis by DNA-damaging agents.<sup>47</sup>

Bcl-2 expression is associated with androgen independence, disease progression and resistance to therapy. Several investigators have observed that bcl-2 is expressed at higher levels in relapsed androgen-independent prostate cancers compared to androgen-dependent tumors.<sup>43,48–50</sup> Bcl-2 can confer resistance to androgen ablation in xenografts of the androgen-sensitive prostate cancer cell line, LNCaP.<sup>51</sup> Additionally, a transgenic mouse model of bcl-2 gene deregulation was generated using the probasin promoter to target bcl-2 expression to the prostatic glandular epithelium in transgenic mice.<sup>52</sup> Compared to control prostates, bcl-2 expression did not alter steady-state levels of proliferation or apoptosis. However, bcl-2 significantly reduced apoptosis induction following castration in the prostatic glandular epithelial cells. Further, bcl-2 could cooperate with the genetic events and accelerate prostate tumor formation in this transgenic mouse model.<sup>53</sup>

Together, these observations suggest that bcl-2 can provide a survival advantage following androgen ablation and, thereby, contribute to androgen-independent tumor progression. Recent studies also indicate that androgens act to suppress bcl-2 expression in prostate glandular epithelial cells that possess an intact androgen-signaling

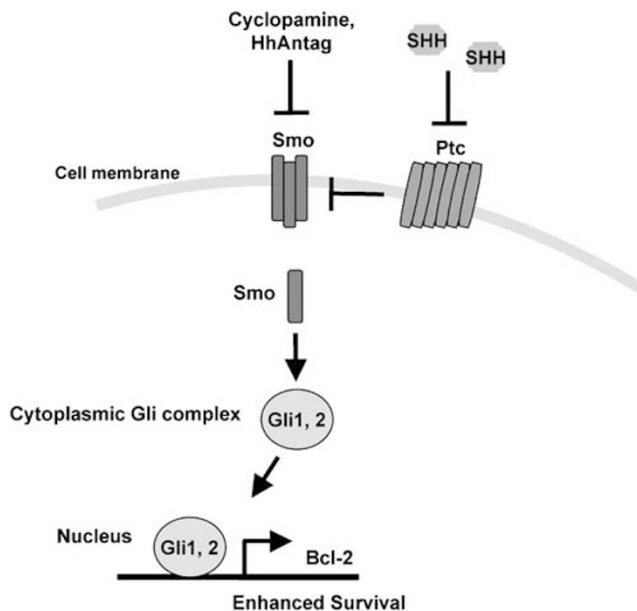
axis.<sup>54,55</sup> These findings imply that, while initially effective, hormonal ablation itself may have the unintended consequence of enabling tumor cells to adapt to androgen deprivation by derepressing a compensatory survival pathway. Similar to lymphoid neoplasms, this viability advantage would facilitate the acquisition of complementing molecular events ultimately resulting in tumor relapse.

Skin cancers are among the most common epithelial-derived malignancies worldwide. The neoplasms are comprised primarily of nonmelanoma skin cancers (NMSC), including basal cell (BCC) and squamous cell (SCC) carcinomas. These tumors exhibit locally invasive growth but rarely metastasize. It has also been shown that bcl-2 family member proteins are differentially expressed in skin and in NMSC.<sup>56,57</sup> The potential impact of bcl-2 deregulation during multistep skin carcinogenesis was assessed in a transgenic mouse model in which bcl-2 was targeted to all keratinocyte cell layers comprising the epidermis using a modified keratin 1 promoter (HK1-bcl-2 mice).<sup>56</sup> Keratinocytes from HK1-bcl-2 transgenic mice were resistant to apoptosis induced by ultraviolet (UV)-irradiation and mutagens compared to those from control littermates. The HK1-bcl-2 mice also developed papillomas at a significantly greater rate and shorter latency than the control mice following initiation with DMBA (2,4-dimethylbenzoic acid) and promotion with TPA (12,0-tetradecanoylphorbol-13-acetate). These findings suggest that bcl-2 deregulation, and subsequent inhibition of apoptosis, is one of several genetic steps that lead to the development of skin cancer.<sup>58</sup>

Similar findings have been obtained in skin carcinogenesis studies using bax-deficient mice. Interestingly, in addition to exhibiting an attenuation in apoptosis following UV-irradiation,<sup>59</sup> bax-deficient keratinocytes also exhibited a delay in the repair of cyclobutane pyrimidine dimers.<sup>60</sup> Thus, bax, in addition to its ability to mediate cell death, signaling events also facilitates repair of UV-induced DNA damage by nucleotide excision repair mechanisms.

One method to assess genetic cooperation during multistep skin carcinogenesis utilizes organotypic 'raft' cultures of keratinocytes. In raft cultures, keratinocytes are grown on a layer of fibroblasts in a collagen-derived matrix. Upon exposure to the air-liquid barrier, the keratinocytes stratify and differentiate in patterns representative of the different layers of the epidermis.<sup>61</sup> Somatic Ha-ras activation is a common event in NMSC. To study potential functional interactions involving activated ras and bcl-2, corresponding clones were generated in HaCaT keratinocytes using standard gene transfer techniques.<sup>62</sup> Ras cultures were associated with higher rates of proliferation and lower rates of spontaneous apoptosis. However, these cultures displayed far higher rates of apoptosis following UV-irradiation compared to control or bcl-2 expressing cultures. In contrast, in ras + bcl-2 rafts relative to ras cultures, apoptosis was largely abrogated following UV-irradiation.<sup>62</sup> These findings demonstrate a functional cooperation between ras and bcl-2 in response to a biologically relevant genotoxic stress.

Although high levels of bcl-2 protein have been observed in a variety of epithelial malignancies, the mechanistic basis for this remains largely unknown. However, in BCCs, and possibly other tumor types, the sonic hedgehog (shh)



**Figure 2** The sonic hedgehog (shh) signaling pathway. The transmembrane shh receptor ptc functions to inhibit smo. Binding of shh to ptc relieves this inhibition and allows smo to internalize and promote Gli transcription factor activation. Active Gli proteins translocate to the nucleus and activate or repress downstream target genes. Inappropriate activation of this pathway may contribute to apoptosis resistance through Gli-dependent transactivation of bcl-2

signaling pathway appears to modulate the expression of bcl-2 (Figure 2). Hedgehog proteins, desert hedgehog (dhh), India hedgehog (lhh) and shh, are small secreted molecules crucial to normal development. Shh, the most widely expressed family member, is a lipid-modified protein that binds the transmembrane repressor, patched (ptc). Shh binding to ptc relieves repression of the G-protein-coupled receptor smoothened (smo). Activated smo internalizes to the cytoplasm where it causes the activator gli transcription factors (gli1 and gli2) to translocate to the nucleus.<sup>63</sup> In humans, a third gli transcription factor, gli3, acts mainly as a transcriptional repressor.

Genetic analysis of patients with nevoid BCC syndrome (NBCC or Gorlin's syndrome) revealed mutations in the ptc gene and predisposes individuals to early-onset BCCs and, less frequently, medulloblastomas.<sup>64</sup> Inappropriate activation of the shh pathway is also observed in almost all spontaneous BCCs. The deregulation of the hedgehog signaling pathway has subsequently been demonstrated in tumors of the brain, gastrointestinal tract, prostate, lung, skin, ovary and esophagus. Transgenic mice engineered to overexpress gli2 in keratinocytes develop spontaneous BCCs.<sup>65</sup> Similar to BCCs arising in humans, these tumors tend to express bcl-2. Gli1 transgenic mice, utilizing the keratin 5 promoter, which targets gli1 expression to the epidermis, develop tumors closely resembling BCCs.<sup>66</sup>

Recent evidence suggests that one consequence of deregulated hedgehog signaling is enhancement of cell survival by upregulation of bcl-2.<sup>67,68</sup> The bcl-2 promoter could be directly transactivated by gli1. Further, gli1 transgenic mice generated with an inducible keratin 14 promoter

exhibited upregulation of endogenous bcl-2 following induction compared to uninduced mice.<sup>67</sup>

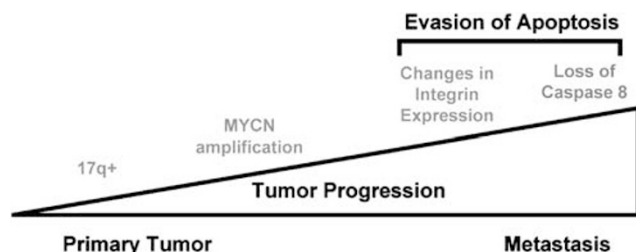
## Cell Death Mechanisms Implicated in Metastatic Tumor Dissemination

Metastatic tumor dissemination is a common feature of cancer lethality. The process of metastasis is complex and involves many steps including invasion of surrounding and distant tissues, cell migration and survival in a foreign tissue environment. These processes are intimately linked to the physical interaction between a cell and the extracellular matrix (ECM). Perhaps not surprisingly, cell death occurs in response to disrupting cell–matrix interactions.

Integrins are heterodimeric cell surface receptors made of combinations of different  $\alpha$  and  $\beta$  subunits with varying specificities for ligands within the ECM.<sup>69</sup> Integrin-mediated cell–ECM interactions transmit signals that play a role in cell shape, adhesion, gene transcription, migration and cell survival. Several mechanisms by which integrins signal for cell survival have been proposed including activation of focal adhesion kinase (FAK) and regulation of bcl-2 family members. Ligated integrins cluster and activate FAK. Inhibition of integrin-mediated FAK signaling in some systems triggers apoptosis.<sup>70</sup> Notably, constitutively active FAK is sufficient for cells to survive anchorage-independent growth.<sup>71,72</sup> Integrin signaling can also inhibit bcl-2 degradation, increase transcription of bcl-x<sub>L</sub>, and, through AKT, inactivate BAD.

Anoikis occurs when anchorage-dependent cells detach from the ECM.<sup>72</sup> Integrin-mediated death, IMD, is a term coined for apoptosis that occurs when adherent cells with specific integrins fail to find their appropriate ligand in the surrounding ECM. For successful metastasis, cells must evade anoikis (acquire anchorage-independent growth) and evade IMD (overcome death signals from unligated integrins in a foreign ECM). Both anoikis and IMD appear to serve as potent barriers to tumor metastasis.<sup>73</sup> While ligated integrins can signal for survival, the presence of specific unligated integrin complexes on the cell surface of adherent cells can signal for apoptosis. For example,  $\beta 3$  integrin-expressing T24E cells undergo apoptosis when cultured in a collagen matrix. Collagen provides a ligand for  $\beta 1$  but not  $\beta 3$  integrins. T24E cells without  $\beta 3$  integrin expression are resistant to apoptosis in this matrix.<sup>73</sup> These findings demonstrate that expression of an integrin in the absence of appropriate ligand can induce cell death. Conversely, reduction of unligated integrins can reduce apoptosis in ECM-attached cells. IMD results in the activation of caspase-8. Inhibition of death receptor-activated caspases prevents IMD. Additionally, fluorescently labeled, active caspases colocalize with unligated integrin in cells undergoing IMD. More persuasively, active caspase-8, but not other caspases, can be immunoprecipitated with integrin complexes in these cells.

Given the above observations, alteration of integrin expression or reduction of caspase-8 could provide a survival advantage, through evasion of IMD, and enhance the process of metastasis (Figure 3). Interestingly, loss of caspase-8 is commonly observed in disseminated neuroblastoma.<sup>74,75</sup>



**Figure 3** Evasion of IMD can contribute to tumor metastasis. Various genetic alterations and changes in gene expression can be observed in the course of neuroblastoma progression.<sup>129</sup> In some cases, increases in 17q copy number and NMYC amplification are observed in primary tumors. Other changes can occur later that specifically inhibit IMD (alteration of integrin expression, and loss of caspase-8) and promote metastasis

Recent work in a chick embryo cancer model has investigated the role of caspase-8 and IMD in metastasis.<sup>76</sup> Clinically derived neuroblastoma cell lines with or without endogenous caspase-8 expression were tested for the ability to metastasize in chick embryos. The presence or absence of caspase-8 did not affect the growth or apoptotic index of primary tumors. However, more apoptosis was observed in locally invasive cells with caspase-8 expression compared to caspase-8-negative cells. Importantly, bone marrow and lung metastases occurred more readily in embryos seeded with caspase-8-negative cells. Reconstitution of caspase-8 suppressed these metastases, but had no effect on primary tumor growth. Metastatic murine neuroblastoma sublines displayed reduced caspase-8 expression compared to nonmetastatic parental cells.

Altered integrin expression was also identified as a route to increased metastasis of neuroblastoma cells in this model. Subpopulations of NB7 cells with and without  $\alpha 3 \beta 1$  integrin expression were isolated. Type I collagen does not supply a ligand for  $\alpha 3 \beta 1$  integrin complexes, but is the major ECM component of the chorioallantois, through which cells must pass to metastasize in the chick embryo model.  $\alpha 3 \beta 1$ -deficient cells demonstrated increased survival within a type I collagen matrix and increased metastasis *in vivo*.<sup>76</sup> These data suggest that evasion of IMD via reduced caspase-8 expression or altered integrin expression can enhance the survival of invasive cells and facilitate metastasis. In addition to integrin expression pattern changes, some SCC tumors harbor rare integrin gain-of-function mutations that promote tumorigenesis, in part, by inhibiting apoptosis.<sup>77</sup>

## Targeting Cell Death in Cancer Therapy

It is widely appreciated that the deregulation of cell death, through activation of a survival pathway and/or inactivation of a death signaling pathway, is critical for the expression of the malignant phenotype. This realization has had profound implications in the design and development of cancer therapy. It is noteworthy that the idea of the cell death 'rheostat', first proposed by Stan Korsmeyer as an important determinant of cell death sensitivity,<sup>78</sup> remains a central concept in how these therapies are conceived. In general, therapies are being considered which either restore cell death sensitivity or

selectively disrupt signaling pathways on which the tumor depends for its survival. In this regard, the p53 tumor suppressor gene and the bcl-2 proto-oncogene are popular targets for anticancer therapeutics. These, along with new therapies influencing the shh pathway, are discussed below and represent a small sampling of apoptosis-modulating therapies currently being evaluated. Other active areas of translational and clinical investigation include apoptosis induction through TRAIL receptors and inhibition of XIAP. The use of recombinant TRAIL and TRAIL receptor agonistic antibodies are being evaluated.<sup>79</sup> Similarly, antisense oligos, small-molecule inhibitors, peptides and SMAC mimetics are being used to inhibit XIAP.<sup>80,81</sup>

p53 represents an attractive therapeutic target for compelling reasons. It is among the most common genes to undergo loss of function mutations in a variety of human malignancies. Further, the presence of p53 mutations is frequently correlated with poor prognosis in many, but not all, cancers.<sup>82–84</sup> Preclinical observations indicated that wild-type p53 gene transfer could inhibit tumor growth and increase the sensitivity to apoptosis by chemotherapeutic agents and radiation.<sup>85–88</sup> p53 gene transfer using replication-defective adenoviral vectors (Ad-p53) was shown to induce apoptosis in tumor cells independent of endogenous p53 gene status.<sup>89–94</sup>

The feasibility of restoring cell death signaling selectively in human cancer was established in non-small-cell lung cancer (NSCLC) patients enrolled on clinical trials evaluating viral-mediated p53 gene transfer.<sup>95</sup> Nine patients, all with tumors harboring a mutated p53 gene, were given intratumoral injections of p53. Six patients displayed tumor regression or stabilized disease. p53 transgene expression was detected along with the induction of apoptosis. More recently, a randomized clinical trial of radiation treatment, with or without Ad-p53, in 90 head and neck cancer patients was reported.<sup>96</sup> Of the patients receiving combination treatment, 64.7% achieved a complete remission compared to 20% in patients receiving radiation alone. Importantly, the results of this study provided sufficient evidence for the approval of Ad-p53 in the treatment of head and neck cancer by the China State Food and Drug Administration. The results of these and ongoing clinical trials will ultimately determine the optimal clinical context of p53 replacement gene therapy.<sup>97</sup>

One of the limitations in the use of replication-defective viral delivery systems is that the virus must infect individual tumor cells to induce an effect. The use of replication-competent viral vectors can solve this problem, but, to spare normal cells, the importance of tumor selectivity becomes even more critical. With these issues in mind, 'oncolytic' viral vectors have been designed that replicate only in p53-deficient cells. In this case, viral infection, and oncolysis, may spread among p53-deficient tumor cells while leaving normal cells unaffected. With this approach, an anticancer therapeutic can selectively target cells that have acquired the ability to evade p53-induced apoptosis. The ONYX-015 agent is a type 2/5 chimeric adenovirus in which the E1B-55K gene has been modified so that the protein product cannot inactivate p53 and allow for viral replication. Therefore, this virus cannot replicate in cells harboring wild-type p53 protein.<sup>98,99</sup> ONYX-015 has been evaluated in a variety of phase I/II clinical trials as a single agent and in combination with chemotherapeutic

agents. These trials have demonstrated the feasibility and safety of ONYX-015 delivered intratumorally, and in some tumor types, there is evidence of antitumor activity.<sup>100,101</sup> Based on these studies, ONYX-015 is now undergoing phase III trials of head and neck cancer. Despite this initial success, there have been some disappointing clinical data in ovarian cancer. In a phase I trial of ONYX-015 in patients with recurrent ovarian cancer, there was no compelling evidence of clinical response in any patient.<sup>102</sup>

An understanding of the molecular regulation of steady-state p53 protein levels in cells has also led to strategies predicated on augmenting endogenous levels of p53 for therapeutic effect. These strategies are based on disrupting the interaction of p53 with the MDM2 protein. The p53 protein physically associates with MDM2, a ubiquitin ligase, in unstressed cells and undergoes ubiquitination and subsequent proteasomal degradation.<sup>103,104</sup> MDM2 also maintains p53 in an inactive state by binding directly to the transactivation domain of p53 and shuttling p53 to the cytoplasm.<sup>105–107</sup> In this way, p53 protein levels remain low in unstressed cells. Conditions that result in the activation of the p53 pathway, such as genotoxic stress, disrupt the interaction of p53 with MDM2, resulting in the stabilization and rapid accumulation of p53 protein.

A group of small-molecule MDM2 antagonists, consisting of *cis*-imidazoline analogs, have recently been identified by screening a synthetic chemical library.<sup>108</sup> These molecules, termed Nutlins, displace p53 from its complex with MDM2 at nanomolar concentrations. These compounds bind MDM2 at the p53 binding site and disrupt the interaction, resulting in p53 stabilization and activation. Nutlins were shown to induce growth arrest and apoptosis in tumor cells possessing wild-type p53 as well as inhibit the growth of tumor xenografts. These molecules, alone and in combination with radiation or chemotherapeutic agents, exhibit significant antitumor activity in preclinical models.<sup>109,110</sup> All the findings from the preclinical studies appear promising, however, the safety and ultimate utility of Nutlins has yet to be evaluated or established in clinical trials.

There is compelling evidence that the inappropriate expression of bcl-2 as a primary or secondary event can contribute to tumor development, progression and therapeutic resistance. From this perspective, bcl-2 represents an attractive target for therapeutic manipulation. However, enthusiasm for targeting bcl-2 in the clinical setting was initially hindered by consideration of the normal distribution of bcl-2 protein, such as in neurons of the central nervous system, and the potential redundancy of this family of proteins. Genasense (oblimersen sodium) is a phosphorothioate antisense oligonucleotide (ASO) that targets human bcl-2 mRNA. In preclinical studies using bcl-2 antisense oligos in the androgen-dependent mouse Shionogi tumor model, bcl-2 ASO was administered following castration. Bcl-2 ASO accelerated tumor regression and delayed emergence of androgen-independent disease.<sup>111</sup>

In a phase I/II trial, Genasense in combination with decarbazine reduced bcl-2 protein levels in melanoma biopsies and correlated with significant clinical response.<sup>112</sup> In a randomized phase III trial, patients with advanced melanoma were treated with decarbazine with or without

Genasense. Addition of Genasense resulted in an increase in progression-free survival but not overall survival.<sup>113</sup> The US Food and Drug Administration, however, did not consider the findings sufficient to merit approval for this application. In general, as a single agent in pretreated patient populations, oblimersen has demonstrated modest effects, however, evaluating its activity in combination with other agents is warranted.<sup>114,115</sup> Currently under consideration is the use of Genasense plus fludarabine and cyclophosphamide for patients with relapsed or refractory chronic lymphocytic leukemia (CLL).

An alternative strategy targeting the bcl-2 survival pathway involves BH3 domain peptidomimetics and is based on disrupting protein–protein interactions between members of the bcl-2 family, thereby facilitating apoptosis. A hydrophobic pocket on the surface of the bcl-2 protein is responsible for protein–protein interactions with the BH3 domain of proapoptotic family members.<sup>116</sup> Peptide and nonpeptide organic compounds have been identified that bind this hydrophobic pocket and disrupt the inhibitory effect of bcl-2 on proapoptotic protein partners, thus inducing apoptosis. Cell-permeable bcl-2-binding peptides can induce apoptosis *in vitro* and inhibit the growth of leukemia cells in immunocompromised mice.<sup>117</sup> HA14-1 is a nonpeptide ligand of the bcl-2 hydrophobic pocket discovered by computer screening of virtually designed compounds.<sup>116</sup> HA14-1 efficiently inhibits bcl-2 binding to bak and causes apoptosis in primary leukemia cells and cancer cell lines.<sup>118</sup> Recently, a high-affinity BH3 small-molecule mimetic, ABT-737, has been identified that binds to multiple antiapoptotic bcl-2 family members at nanomolar concentrations.<sup>119</sup> In preclinical studies, ABT-737 was able to induce significant, or complete, tumor regression in established tumor xenografts. Already, a phase I/II clinical trial has evaluated a small-molecule bcl-2-antagonist, GX15-070 (obatoclax), in patients with CLL.<sup>120</sup> If effective, these agents will represent a significant advance in mechanism-based cancer therapies.

The activation of the shh signaling pathway is observed in many human cancers and may contribute to tumorigenesis by enhancing cell proliferation and/or cell survival (Figure 2). Inhibitors of the pathway have been characterized in preclinical studies. Cyclopamine is a naturally occurring shh pathway inhibitor isolated from the lily *Veratrum californicum*. Cyclopamine inhibits shh signaling by binding to and inhibiting smo.<sup>121</sup> In a mouse model of prostate cancer, noninvasive prostate cancer cell lines were made invasive by overexpression of gli1. The addition of cyclopamine was shown to inhibit tumor growth and induce apoptosis.<sup>122</sup> Similarly, treating colorectal or hepatocellular cancer cell lines with cyclopamine induces apoptosis.<sup>123,124</sup> It has also been shown in gastric carcinoma xenograft models that cyclopamine effectively induced apoptosis specifically in tumor cells, resulting in near-complete tumor regression in 12 days.<sup>125,126</sup> Synthetic inhibitors of the pathway have recently been identified. High-throughput screening methods have identified small molecule smo inhibitors.<sup>127</sup> One compound, referred to as HhAntag, has a higher affinity for smo than cyclopamine and has been tested in a *ptc* +/– *p53* –/– medulloblastoma model. This compound can inhibit gli1 expression *in vivo*. Furthermore, HhAntag administration caused complete tumor

regression, at the highest dose, and prolonged survival.<sup>128</sup> Phase I clinical trials evaluating a topical hedgehog antagonist are currently accruing patients with single or multiple BCCs.

Collectively, these studies reflect a high level of interest in mechanism-based strategies to manipulate cell death resistance mechanisms in human cancer. These strategies are based on our understanding of the molecular regulation of cell death in normal cells and how these processes are subverted during multistep carcinogenesis.

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