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Targeting Myc function in cancer therapy

Review Article

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Summary

The development of novel therapeutic strategies to improve the survival rate of patients with cancer requires a better understanding of the critical events that underlie the origins and progression of tumors. The Myc family of transcription factors play important normal roles in regulating cell proliferation and their deregulated or elevated expression is one of the most common features of cancer cells. Here, we review mechanisms thought to underlie Myc-dependent tumor formation and discuss possible strategies for disrupting the oncogenic activity of Myc family proteins.

I. Introduction

Deregulated expression of members of the Myc family of genes is a common feature of diverse malignancies. Myc gene amplification and gene translocation are often responsible, but abnormally high Myc levels are also observed in numerous tumors that show no such defects. Although it is not possible to discriminate between cause and effect when evaluating the role of Myc in human tumors, a large collection of experimental results from cell culture and animal models clearly demonstrate that deregulated Myc expression can function as a root cause of cancer.

How do Myc proteins contribute to the tumor phenotype? The use of transgenic mice containing inducible Myc genes or activatable forms of Myc, together with more traditional types of transgenic models, have led to, or confirmed, the identification of several Myc activities that can be a factor in tumor formation. These activities include stimulating cell proliferation, promoting vasculogenesis and, paradoxically, promoting or sensitizing cells to apoptosis. Although Myc driven apoptosis can be regarded as a safeguard or tumor suppressor mechanism (Huebner and Evan, 1998; Pelengaris et al, 2002a), when combined with its affects on cell proliferation and vasculogenesis, this activity has the potential to ultimately have the reverse effect. Because Myc deregulation/overexpression can stimulate both proliferation and apoptosis, it has the capability of applying strong selection pressure for the development of cells that escape cell death. This type of scenario was shown to play out in cultured primary cells exposed to high c-Myc levels (Zindy et al, 1998). Typically, cells that escaped Myc-driven apoptosis in culture, harbored defects in the p53 tumor suppressor pathway (Zindy et al, 1998), which serves as an important mediator apoptosis in general and of Myc-driven apoptosis specifically (Sherr, 2001). Mutations in the p53 pathway, in theory, help clear the path for Myc-driven tumorigenesis by not only preventing apoptosis (**Figure 1**), but by also disabling important checkpoints governed by p53 that prevent excessive cell proliferation (Sherr, 2001). Proof of this principal has been obtained in the results of crosses between transgenic mice that overexpress c-Myc and ones that have abrogated p53 pathway function. In this environment, tumorigenesis is typically accelerated, often dramatically (Nilsson and Cleveland, 2003). Taken together, these results demonstrate that Myc deregulation has the potential to function as an early, initiating event in the evolution of tumor cells and, at least theoretically, may be partially responsible for the high proportion of human tumors that exhibit mutations in genes encoding p53 or its positive regulator p19ARF.

In addition to mutations that disrupt the p53 pathway, Myc-dependent apoptosis can be disarmed through a variety of other mechanisms (Nilsson and Cleveland, 2003). Most notably, this can be accomplished by overexpression of anti-apoptotic proteins such as Bcl2 and $BclX_L$ (Strasser et al, 1990; Pelengaris et al, 2002b) and

loss of proapoptotic proteins such as Bax (Eischen et al, 1991). Thus, events that cripple Myc-dependent apoptosis, but leave its other proliferation-promoting activities intact, cooperate to drive tumor formation (**Figure 1**).

Based on the model presented above, tumors that exhibit excessively high and/or deregulated Myc expression, must either have lost their apoptotic response to Myc or are not programmed to respond in this manner. The latter situation appears to exist in certain cell types, such as skin keratinocytes (Gandarillas and Watt, 1997; Pelengaris et al, 1999;Waikel et al; 1999, 2001). When c-Myc expression was induced in suprabasal mouse keratinocytes, cells committed to terminal differentiation, they reinitiated cell proliferation and formed highly

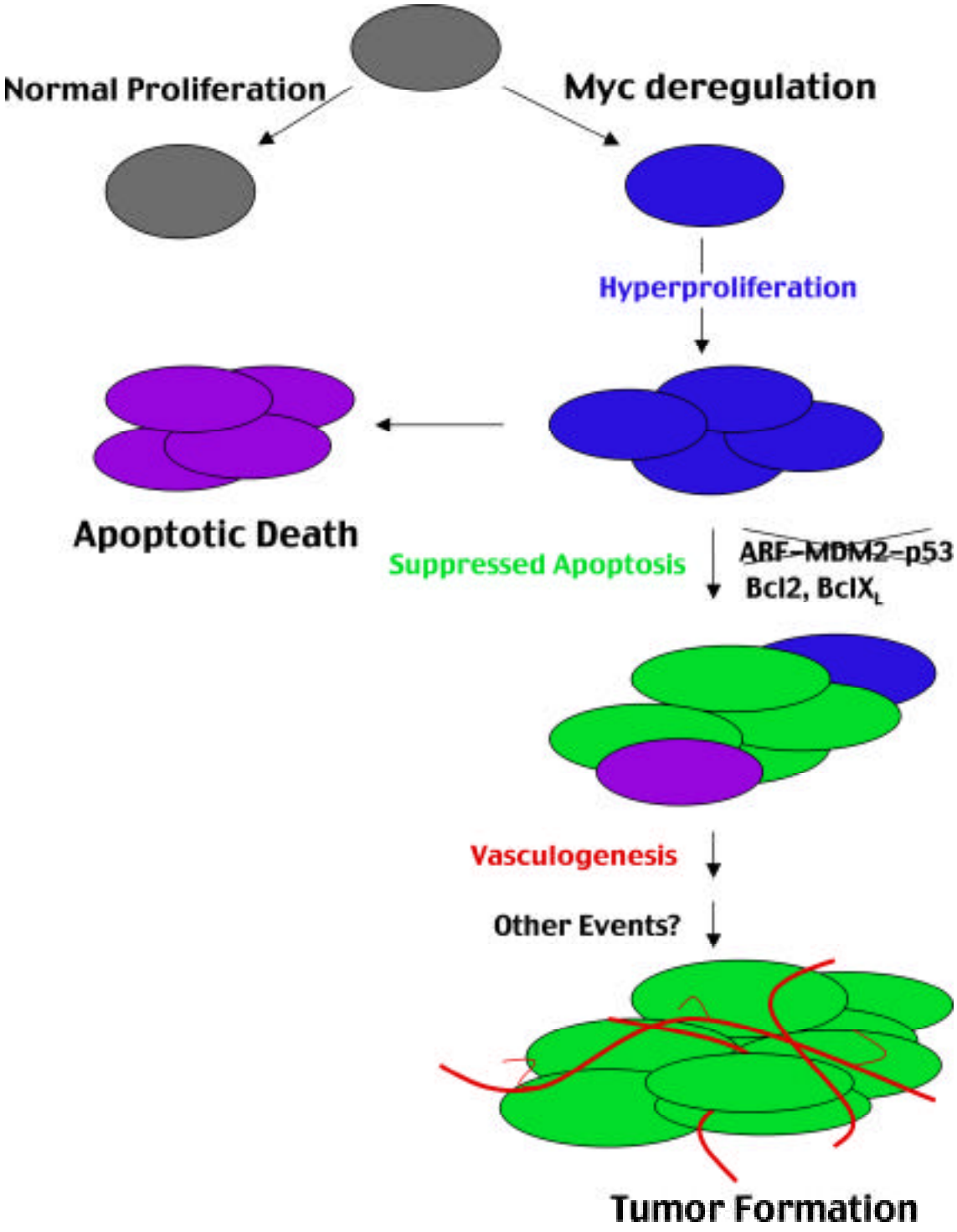

Figure 1. Model outlining activities and events associated with Myc-dependent tumor formation. When normal cells (gray) are subjected to Myc deregulation (blue), they become hyperproliferative. In the absence of sufficient growth/survival factors and nutrients to support hyperproliferation, cells are stressed to the point that they undergo apoptosis (purple). Myc overexpressing cells that sustain secondary events allowing escape from an apoptotic fate, such as mutational disruption of p53 pathway function or upregulation of anti-apoptotic proteins such as Bcl2 and $BclX_L$, continue to proliferate (green), In addition to promoting cell proliferation, Myc stimulates vasculogenesis and angiogenesis, activities that ultimately drive tumor formation.

vascularized papillomas (Pelengaris et al, 1999). Although apoptosis appears to be minimal in this setting, the formation of tumors was limited due to the retention and advance of the keratinocyte terminal differentiation program. In other words, Myc seemed to cause suprabasal keratinocytes to revert to a basal-like phenotype that ultimately produced differentiated "squames" that slough off the skin surface (Pelengaris et al, 1999; Waikel et al, 2001). This is a surprising result since Myc has the demonstrated activity of suppressing the differentiation programs of many other cell types while promoting their proliferation (Grandori et al, 2000). Moreover, in terms of the response to deregulated/elevated Myc expression, the lack of increased apoptosis in keratinocytes appears to be the exception rather than the rule.

A potential explanation for these results is that skin keratinocytes have a higher threshold for induction of apoptosis. Because of their location at the body surface and therefore frequent exposure to stresses capable of inducing apoptosis (e.g. UV light), a higher apoptosis threshold may have evolved specifically in keratinocytes to help insure the integrity of our skin. For example, keratinocytes may have naturally high levels of certain anti-apoptotic proteins or low levels of proapoptotic proteins compared to other cell types. Clearly, there is still much to be learned about the conditions that determine the response primary cells in vivo have to deregulated and/or overexpressed Myc and mechanisms that ultimately lead to tumorigenesis. Moreover, understanding the detailed molecular mechanisms that underlie Myc-dependent tumorigenesis in different cancers will ultimately provide specific, efficatious targets for the development of therapeutic drugs.

II. Potential therapeutic strategies that target Myc expression and activity

A. Turning Myc off

The most obvious way to prevent Myc-dependent tumorigenesis is to target its downregulation or inactivation in tumors. Transgenic mice expressing Myc under the control of an inducible promoter or expressing an activatible form of Myc (Myc-estrogen receptor fusions), have clearly demonstrated that tumors induced by ectopic Myc expression typically remain dependent on the artificially deregulated and typically elevated Myc levels (Felsher and Bishop, 1999; Pelengaris et al, 1999, 2002b; D'Cruz et al, 2001). Thus, "turning off" Myc subsequent to tumor formation has been found to lead to rapid tumor regression. Although in some settings a subpopulation of cells ultimately become resistant to Myc downregulation, these results clearly indicate that therapies targeting inactivation of Myc in tumors would at least temporarily slow tumor growth. Indeed, this would most likely be true whether or not a tumor exhibited Myc deregulation/overexpression, as targeted deletion of c-Myc in both primary and "immortal" cells has been demonstrated to cause a dramatic reduction in their ability to proliferate (Mateyak et al, 1997; Trumpp et al, 2001; de Alboran et al, 2001).

These latter results and the finding that homozygous deletion of c-Myc and N-myc cause mid-gestation lethality, also illustrate the seemingly obvious point that, even if Myc genes could be targeted for downregulation in vivo, this would probably have to be largely confined to the tumor cell population. However, the great majority of tumors occur in adults, which of course contain a much smaller pool of proliferating cells than a fetus or prepubescent individual. Thus, assuming that the only effect of targeting Myc downregulation is decreased proliferation, this strategy may actually be less destructive to normal proliferating cell populations than many standard chemotherapeutic agents that may also negatively impact non-proliferating cells. Moreover, because of the overlapping tissue expression patterns of the three well-characterized Myc family genes, c-Myc, L-Myc and N-Myc, systemic downregulation of any one of the Myc genes – in an attempt to target its overexpression (or normal expression) in a specific tumor – may have a quite limited deleterious effect overall. This would probably be most true for L-Myc and N-Myc, which exhibit a more limited expression range than c-Myc (Mugrauer et al, 1988; Downs et al, 1989; Hatton et al, 1995). Thus, for example, targeting L-Myc downregulation to treat small cell lung carcinoma, which frequently exhibit L-Myc amplification, by systemic application of L-Myc anti-sense oligos, morpholinos, or siRNA, may have a minimal organism-wide deleterious effect. Further, unlike the lethality caused by N-Myc and c-Myc deletion in mice, mice lacking L-Myc appear normal, supporting the hypothesis that there would be minimal impact outside of a L-Myc-dependent tumor.

It has been demonstrated that antisense oligonucleotides targeting c-Myc, L-Myc and N-Myc can be effective at slowing the proliferation of particular tumor cell types in culture and in partially ameliorating tumor-associated phenotypes (Wickstrom et al, 1988; Schmidt et al, 1994; Dosaka-Akita et al, 1995; Smith et al, 1998; Waelti and Gluck, 1998; Iversen et al, 2003; Pastorino et al, 2003). Further, it has been observed that systemic introduction of Myc antisense agents can lead to significant tumor regression in mouse tumor xenografts (Schmidt et al, 1994; Iversen et al, 2003; Pastorino et al, 2003). However, these studies have been largely preliminary in nature and, to date, there has been no follow-up evidence supporting the notion that this type of approach works on human tumors. It seems that the greatest limitation to this approach is instability of antisense agents and consequently an inability for them to effectively reach and enter enough tumor cells to have a significant impact. Perhaps the development of next generation antisense Myc agents that may have a longer half-life in vivo (Iversen et al, 2003) or adjuvant vehicles to better deliver the agents to tumors will aid their effectiveness.

B. Restoring Myc-dependent apoptosis in tumors

As discussed above, transgenic models of Myc-driven tumor formation using inducible and/or activatible systems have demonstrated that most tumors regress

following "inactivation" of Myc. In this setting, a basic assumption had been that reactivating or turning Myc back on would reinitiate tumorigenesis. Surprisingly, this was found not to be the case in osteosarcomas produced in transgenic mice using an inducible c-Myc expression system (Jain et al, 2002). Termination of ectopic c-Myc expression caused restoration of osteocyte differentation and tumor regression and subsequent restoration of ectopic c-Myc expression led to apoptosis and a failure to promote tumor formation (Jain et al, 2002). Mechanisms underlying this unexpected phenomenon have yet to be defined and it is not known whether this is a general response of cells to temporary downregulation of oncogenic levels of Myc. Although many questions remain, reactivation of Myc-driven apoptosis has obvious implications for tumor therapy (Felsher and Bradon, 2003). For example, some tumors might be especially vulnerable to *transient* downregulation of Myc protein levels using existing antisense and siRNA technologies as discussed above. Such a protocol would ameliorate the potential side effects of sustained systemic delivery of such agents. Further, there transient use, combined with chemotherapeutic drugs known to exacerbate Myc-driven apoptosis, might offer even more promise.

Because defective apoptosis appears to be a common mechanism underlying Myc-dependent progression to tumor formation, as well as tumor progression in general, restoring apoptosis in tumors offers great promise as a cancer therapy. The prevalence of p53 pathway defects in tumor cells, has made restoring p53 pathway function the primary focus in this area. Indeed, considerable progress has been made in this effort and drugs with the potential of restoring wildtype p53 function to mutated and defective p53 proteins have been identified and are currently being tested in clinical trials (Wang and El-Diery, 2004).

The anti-apoptotic BCL-2 family proteins are also attractive targets for drug design, as they are known to cooperate with ectopic Myc expression in tumorigenesis and are expressed at elevated levels in a wide variety of tumor types (Nilsson and Cleveland, 2003). BCL2-specific antisense oligonucleotides have been developed that show broad anti-cancer activities in pre-clinical models and are currently being tested in several late-stage clinical trials (Hu and Kavanagh, 2003; Manion and Hokenbery, 2004). While drugs that target restoration of apoptotic pathways appear to have general anti-tumor activity, tumors that exhibit deregulation and/or overexpressed of Myc family proteins may be particularly vulnerable to this type of therapy.

C. Targeting disruption of functional Myc complexes

The biological function of Myc family proteins is highly dependent on the integrity of its basic-helix-loop-helix leucine zipper motif (bHLHZip – Grandori et al, 2000). The HLHZip motif mediates interaction with another bHLHZip protein, Max, which facilitates binding of the basic regions of the Myc:Max heterodimer to the DNA sequence CACGTG and related "E-box" sites . The Myc:Max heterodimer can activate transcription in reporter assays, an activity mediated primarily through a conserved tripartite activation domain in the N-terminal half of Myc family proteins (Grandori et al, 2000). Many different proteins have been found that interact within this region and mediate or modulate Myc-dependent transcription. As if this were not complicated enough, Myc proteins can also repress transcription, an activity that involves protein-protein interactions in regions that sometimes overlap with their activation domains (Grandori et al, 2000).

Because of the obligate role Max plays in Myc function, interaction between Myc and Max and between Myc:Max heterodimers and DNA offer attractive targets for drug design. The same is true for protein – protein interactions that mediate the transcriptional properties of Myc. Drugs that interfere with either the Myc:Max interaction or with Myc:Max DNA binding would be expected to abolish Myc activity, whereas drugs that interfere with interactions between Myc and coactivator or corepressor proteins may have a more limited or selective affect on Myc function. Because Max interacts with a number of other proteins that contain Myc-like HLHZip regions (Grandori et al, 2000), there is the real problem of specificity in targeting the Myc:Max interaction, as drugs that interfere with Myc:Max interactions may also interfere with other Max - HLHZip interactions, with unknown consequences for the cell. Nonetheless, small molecules have been identified that disrupt Myc:Max heterodimerization using a yeast two-hybrid approach, and they seem to have specific effects in suppressing Myc activities (Yin et al, 2003). Potential problems of specificity may also arise in drugs that target Myc:Max DNA binding, as they may affect DNA binding by members of a large number of additional proteins that contain a "basic" region DNA binding motif.

Finally, because the molecular mechanisms that mediate the transcriptional activities of Myc family proteins are still confusing, it is not clear whether targeting any of the many interactions thought to control Myc transcription would cripple its functions in tumorigenesis. However, one potential target is the interaction between Myc and the coactivator TRRAP (McMahon et al, 1998 –**Figure 2**). Interaction with TRRAP was found to be required for Myc-dependent transformation (McMahon et al, 1998; Park et al, 2001) and regions within these proteins that mediate the interaction have been mapped. Thus small molecules that disrupt this interaction might be effective in blocking tumor-promoting activities of Myc.

A second potential target is the interaction between Myc and Miz1 (Wanzel et al, 2003). Miz1 is a transcriptional activator whose activities are repressed by interaction with Myc which causes displacement of the Miz1 coactivator protein CBP (Staller et al, 2001, Herold et al, 2002). Through this mechanism, Myc was found to disrupt Miz1-dependent transcriptional activation of the genes encoding cyclin-dependent kinase inhibitors p15INK4D and p21CIP1 (Herold et al, 2002; Seoane et al, 2002). The p21CIP1 gene is a key transcriptional target of p53, and by suppressing its transcription, Myc appears to suppress the cell cycle arrest functions of p53, but not its pro-apoptotic function. Therefore, in cells that have an intact p53 pathway, the development of drugs that disrupt.

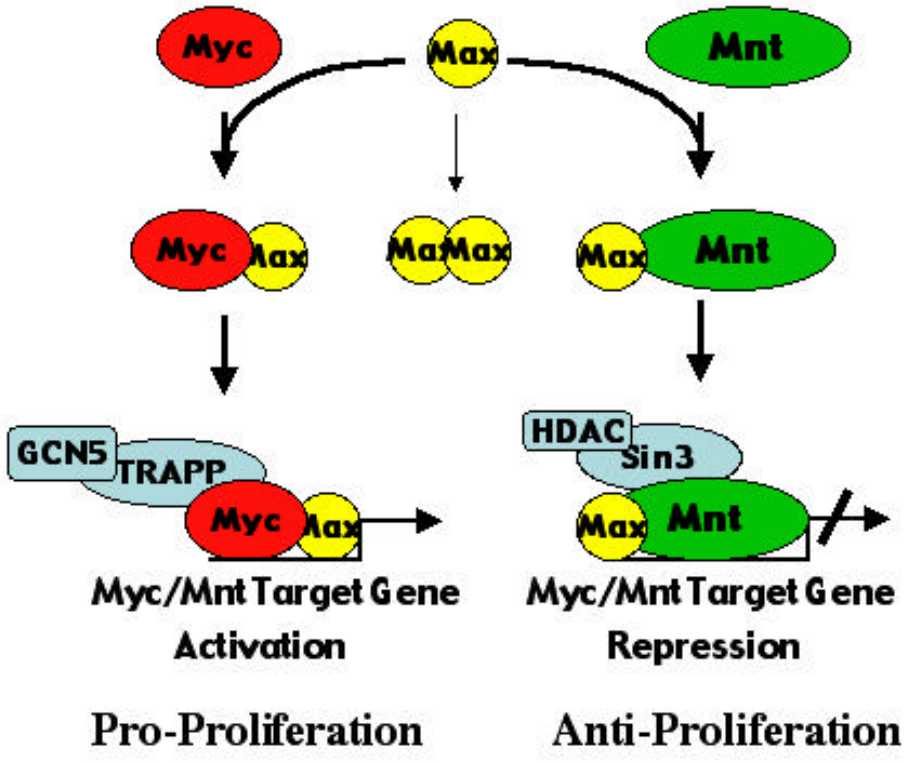

Figure 2. Speculative Myc-Mnt antagonism model. Myc (c-Myc, N-Myc and L-Myc) and Mnt compete for interaction with their obligate heterodimerization partner Max and for binding and regulation of shared transcriptional target genes. Myc:Max complexes activate transcription through recruitment of coactivator proteins such as TRRAP and TRRAP-associated GCN5, a histone acetyltransferase. Of note, TRRAP is one of many proteins found to interact with Myc and affect its ability to activate transcription. In contrast to Myc, Mnt represses transcription through its interaction with Sin3 corepressor proteins, which tethers histone deacetylating (HDAC) enzymes. Ubiquitous Mnt:Max complexes are postulated to create a threshold of transcriptional repression at shared Myc/Mnt target genes that is overcome, and proliferation promoted, when Myc levels are expressed at sufficiently high levels.

intact p53 pathway, the development of drugs that disrupt interaction between Myc and Miz1 would theoretically cause increased susceptibility to Myc-dependent apoptosis.

D. Interfering with downstream pathways regulated by Myc

Because Myc family proteins are transcriptional regulators, it would seem that disrupting the function of proteins encoded by its transcriptional target genes would offer an effective way at disarming Myc function. However, the identification of bona fide Myc target genes has been at best difficult and at worse, misleading (Eisenman, 2001). Moreover, recent findings support the view that Myc functions are not mediated by it's regulation of a small number of key transcriptional targets, but instead through it's binding and regulation of perhaps thousands of different genes (see http//www.myc-cancer-gene.org for an updated list). Although it is not clear how many different genes Myc actually regulates, it is clear that it broadly affects the gene expression profile of cells. This is also reflected at the protein level, where changes, up and down, of broad categories of proteins have been observed following ectopic Myc expression (Ishii, et al, 2002). Therefore, instead of - or in addition to - trying to zero in on specific Myc transcriptional targets as candidate drug targets, it may be fruitful to focus on disrupting more downstream events that ultimately contribute to the oncogenic activity of Myc. In many, and perhaps most cases, such events are probably not unique to Myc-driven oncogenesis, but represent general attributes of tumor cells that Myc can provoke or enhance.

One example of this is vasculogenesis - the production of new blood vessel networks, and angiogenesis - the remodeling and expansion of this blood vessel networks. Vasculogenesis and angiogenesis provides for the increased blood supply required to support the ever-growing nutritional needs of neoplastic tissues during tumorigenesis. Ectopic expression/activation of Myc in transgenic mice has been found to stimulate angiogenesis and vasculogenesis (Pelengaris et al, 1999, 2002b). Further, the vasculature network formed in neoplastic tissues was dependent on continued ectopic Myc expression. In addition, it was recently revealed that angiogenesis and vasculogenesis is defective in c-Myc null embryos and this deficiency was linked to the inability of c-Myc null embryonic stem cells to form tumors in *Skid* mice (Baudino et al, 2003). These studies, together with data indicating that Myc can regulate, either directly or indirectly, the expression of a number of important factors involved in angiogenesis and

vasculogenesis (Baudino et al, 2003 and references therein), support the idea that drugs that disrupt neovascularization will be effective in disrupting Myc-dependent tumorigenesis. Because neovascularization is a common and necessary feature of tumor growth in general, the development and testing of such drugs has been the focus of intense study for several years. However, the drugs that have been developed have, so far, yet to prove effective against human tumors (Siemann et al, 2004). Thus, perhaps models of Myc-driven tumorigenesis may provide a useful setting to more precisely define the critical mechanisms responsible for neo-vasculogenesis and a useful system to test novel drugs designed to disrupt new blood vessel formation.

Another pathway modulated by Myc family proteins that is likely generally important in tumorigenesis is cell growth. Cell growth refers to the increased cell size associated with progression through specific phases of the cell cycle. Before cells divide, they increase their cell mass and volume in order to maintain a consistent size of daughter cells (Saucedo et al, 2002). It is hypothesized that Myc regulates cell size by stimulating the expression, directly or indirectly, of genes encoding proteins required for protein synthesis (Jones et al, 1996; Greasly et al, 2000) and by assisting RNA polymerase III in the transcription activation of transfer and ribosomal (5S) RNAs (Gomez-Roman et al, 2003). Although these Myc activities might be considered potential targets for therapeutic intervention in tumors, disrupting fundamental components of the protein synthesis machinery, that are not necessarily coupled to cell proliferation, might be expected to have strong adverse effects on non-tumor tissues as well. However, the activity of mTOR, a central regulator of cell growth, survival and protein translational control is a key target of the drug rapamycin and related compounds that show promise as anticancer agents (Bjornsti and Houghton, 2004). Indeed, rapamycin has been shown to be effective at reversing chemotherapeutic resistance of Myc-dependent mouse lymphomas that express Akt, an important regulator of mTOR activity and cell survival (Wendel et al. 2004). In addition, inhibition of mTOR activity by rapamycin can lead to c-Myc downregulation in some cell types, (Gera et al, 2004), and has been shown to inhibit transcription of the telomerase catalytic subunit hTERT gene (Zhou et al, 2003), a direct target of c-Myc transcriptional activation (Grandori et al, 2000) and putative oncogene. Thus, inhibitors of mTOR activity may ultimately prove efficacious on human tumor subsets that can be defined as exhibiting Myc deregulation, particularly ones showing activation of Akt/mTOR signalling.

III. Stimulating endogenous Myc antagonists

Besides Myc family proteins, Max interacts with another set of bHLHZip proteins that include the four Mad family proteins (Mad1, Mxi1-Mad2, Mad3 and Mad4), Mnt and Mga (Grandori et al, 2000). Like Myc:Max, these alternative Max complexes bind to E-box sequences, but appear to function as dedicated repressors. Furthermore, each of these proteins can suppress the ability of Myc family proteins to transform normal cells in culture to tumor-like cells (Grandori et al, 2000). From these results it has been speculated that this group of proteins normally function as Myc antagonists in cells and would therefore act as tumor suppressors in vivo. Although there is no definitive evidence for a role as tumor suppressors in human cancers for any of these proteins, disruption of mouse Mxi1 (a.k.a. Mad2) and Mnt genes was shown to predispose certain cell types to tumorigenesis (Schreiber-Agus et al, 1998; Hurlin et al, 2003). In the case of Mnt, conditional deletion in mammary epithelium led to the formation of breast tumors. A conditional deletion approach was required in these experiments because homozygous germline deletion of Mnt is early postnatal lethal (Hurlin et al, 2003; Toyo-oka et al, 2004) and studies are underway by our group to test whether loss of Mnt leads to tumors in other tissues.

Further support for the idea that Mnt functions as a Myc antagonist comes from cell culture experiments. MEFs lacking Mnt were found to exhibit several of the hallmark attributes of cells caused by ectopic Myc expression, including being sensitized to apoptosis, having cell cycle entry defects and showing an enhanced rate of senescence escape (Hurlin et al, 2003). Suppression of Mnt by siRNA also caused increased apoptosis, even in an immortal cell line lacking c-Myc (Nilsson et al, 2004). Although these data generally support the notion that Mnt is a Myc antagonist, because of the complicated transcriptional activities of Myc family proteins, this is somewhat difficult to unequivocally prove and requires much more work. Nonetheless, these data, particularly the finding of increasing sensitivity to apoptosis by Mnt deficiency, raise the possibility that Mnt and possibly other Max-interacting repressor proteins may serve as future cancer therapeutic targets.

IV. Conclusion

Myc family proteins serve as essential regulators of cell proliferation and events that uncouple Myc transcriptional gene expression from growth factor signaling, push cells into a proliferative mode and makes them prone to malignant conversion. If the local growth/survival factor and nutrient environment is sufficient, cell proliferation will occur, but when the environment is, or becomes unfavorable to cell proliferation, apoptotic cell death typically ensues. Thus, sustained Myc-driven proliferation, and ultimately tumor formation, is thought to require cooperation with secondary events that either provide a favorable growth factor/nutritional environment or that suppress apoptosis (or both). This understanding of Myc-dependent tumorigenesis has led to efforts to directly suppress Myc expression in tumors and initiatives to restore defective pro-apoptotic pathways in tumors. While these approaches may ultimately be successful, the identification and development of new therapeutic strategies and eventually drugs targeting Myc functions in tumorigenesis will require a more precise understanding of the complicated molecular mechanisms underlying the normal and oncogenic activities of Myc family proteins.

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