c-Myc in breast cancer

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Introduction

Ever since Bishop and his co-workers discovered the c-myc gene in the late 1970s (Bishop 1982), voluminous literature has documented its central role in proliferation and malignant transformation of human and animal cells (Amati et al. 1998, Bouchard et al. 1999, Dang et al. 1999). Most, if not all, types of human malignancy have been reported to have amplification and/or overexpression of this gene, although the frequency of these alterations varies greatly among different reports (Nesbit et al. 1999). In 1992, researchers started to realize that aberrant expression of c-myc could cause apoptosis (Evan et al. 1992, Shi et al. 1992), although the phenomenon had actually been observed much earlier (Wurm et al. 1986). Studies in recent years have further shown that the c-myc gene regulates growth, both in the sense of cell size and in the context of tissue differentiation (Gandarillas & Watt 1997, Iritani & Eisenman 1999, Johnston et al. 1999, Schmidt 1999, Schuhmacher 1999). Thus, it is now known that the c-myc gene participates in most aspects of cellular function, including replication, growth, metabolism, differentiation, and apoptosis (Packham & Cleveland 1995, Hoffman & Liebermann 1998, Dang 1999, Dang et al. 1999, Elend & Eilers 1999, Prendergast 1999).

The c-myc gene and c-Myc proteins

The c-myc gene is transcribed to three major transcripts that start from different initiating sites (Fig. 1), yielding three major proteins named c-Myc1, c-Myc2, and c-MycS (Henriksson & Luscher 1996, Facchini & Penn 1998, Xiao et al. 1998). c-Myc2 is an approximately 62-kDa protein that is the major form of the three c-Myc proteins and the one referred to as 'c-Myc' in most studies. c-Myc1 arises from an alternative initiation site at an in-frame, non-AUG codon, yielding a protein 2–4 kDa larger than c-Myc2. c-MycS arises from a leaky scanning mechanism, and initiates at two closely spaced downstream AUG codons, resulting in a protein lacking about 100 amino acids at the N-terminus of c-Myc2 (Claassen & Hann 1999). An unusual property of the c-myc gene that is often neglected by investigators is that the antisense strand of the gene also yields transcripts (Spicer & Sonenshein 1992). Therefore, one should exert caution when using antisense expression as the control for judging the level of c-myc mRNA.

The amino terminus of each full-length c-Myc protein (c-Myc1 and c-Myc2) harbors a transactivation domain (TAD), within which are two regions that are highly conserved among members of the Myc family; these regions are termed Myc homology boxes I and II (MBI and MBII) (Fig. 1). The carboxyl terminus of the c-Myc proteins contains a basic region and a helix-loop-helix/leucine zipper (HLH/LZ) domain. Through the HLH/LZ domain, a c-Myc protein heterodimerizes with another transcription factor, Max; the c-Myc/Max complex then binds to a specific DNA recognition sequence, the so-called E-box element that contains a central CAC(G/A)TG motif (Henriksson & Luscher 1996, Amati et al. 1998, Facchini & Penn 1998, Dang 1999). Genes containing this Myc E-box element in their regulatory regions may be c-Myc targets, and thus subjected to transactivation or transrepression by the c-Myc/Max complex (Cole & McMahon 1999). Within the TAD, the MBI has been shown to be required for the transactivation properties of c-Myc, whereas the MBII is needed for the trans-suppression activities (Cole & McMahon 1999, Sakamuro & Prendergast 1999). c-MycS lacks the MBI but still retains the MBII in its TAD; this may be the reason why c-MycS is deficient for transactivation but retains the activity of trans-suppression (Xiao et al. 1998, Sakamuro & Prendergast 1999). Thus, c-Myc1 and c-Myc2 can both activate and repress transcription of specific target genes, whereas c-MycS can only repress transcription and cannot function as a dominant-negative inhibitor of certain (but not all) activities of the full-length c-Myc proteins (Xiao et al. 1998, Sakamuro & Prendergast 1999).
Figure 1 Schematic diagram of the Myc family proteins. Within the TAD at the N-terminus there are two myc homology boxes (MBI and MBII) which are conserved among Myc family proteins. At the C-terminus, the HLH/LZ domain links to the basic region (BR) of the c-Myc protein. The initiation sites of the three c-Myc proteins are indicated.

Under normal growth conditions, expression of c-Myc1 and c-Myc2 proteins is differentially regulated (Batsche & Cremisi 1999). In cell culture, c-Myc2 is synthesized in growing cells, while expression of c-Myc1 increases dramatically, to a level equal to or greater than that of c-Myc2 as the cells approach high density (Ryan & Birnie 1996). These observations lead to an hypothesis that in this context c-Myc1 may act as a growth inhibitor, expression of which may be triggered by contact-inhibition (Ryan & Birnie 1996). Regulation of c-MycS is much less known, relative to c-Myc1 and c-Myc2. It has been shown that its expression is increased to the levels comparable to those of c-Myc2 during rapid cell growth, and constitutively high levels of c-MycS have been found in some tumor cell lines as well (Spotts et al. 1997).

Role of c-Myc proteins in cell proliferation

In physiological situations, the central role of c-Myc may be its promotion of cell replication in response to extracellular signals, by driving quiescent cells into the cell cycle. This function was originally thought to be elicited mainly via activation of transcription of those c-Myc target genes that are positive regulators of the cell cycle (Amati et al. 1998), such as cyclins D1, D2, E and A, cdk4, e2f1, e2f2, cdc25A and B, etc. (Barrett et al. 1995, Amati et al. 1998, Ben-Yosef et al. 1998, Dang 1999, Dang et al. 1999). However, of these putative c-Myc target genes, only cdk4 (Hermeking et al. 2000), e2f2 (Sears et al. 1997), and cyclins D1 (Daksis et al. 1994) and D2 (Perez-Roget et al. 1999) seem to encompass a Myc E-box element in their regulatory regions. More confusingly, several in vitro studies show that c-Myc actually suppresses the transcription of the cyclin D1 gene (Jansen-Durr et al. 1993, Philipp et al. 1994). One explanation for this paradox may be that activation of most of these cell cycle components by c-Myc may be by indirect mechanisms. Other possibilities include the involvement of other c-Myc binding proteins, in addition to Max (Sakamuro & Prendergast 1999). Concurrent binding of these proteins may redirect the Myc/Max dimer to promoters with non-canonical E-box elements, or may stabilize the weak binding of the Myc/Max to other promoter sites (Claassen & Hann 1999, Sakamuro & Prendergast 1999). TRRAP (TRansformation/tRanscription domain Associated Protein) (McMahon et al. 1998), BIN1 (Box-dependent myc-INteracting protein–1 or Bridging INtegrator–1) (Elliott et al. 1999), and BRCA1 (Wang et al. 1998) are examples of such c-Myc-binding proteins. TRRAP seems to be required for Myc-mediated transformation (McMahon et al. 1998), whereas BIN1 bound to c-Myc inhibits Myc-mediated transformation (Elliott et al. 1999).

In principle, promotion of cell cycle progression by c-Myc can also be achieved by suppression of transcription of growth inhibitory genes (Alexandrow & Moses 1998). Examples of these genes include gadd45 (Marhin et al. 1997), cdk (cyclin-dependent kinase) inhibitors p21cip1 (Mitchell & El-Deiry 1999, Collet et al. 2000), p19ARF (Dang 1999) and probably also p27kip1 (Amati et al. 1998, Donjerkovic et al. 1999, Wu et al. 1999). There is evidence that under certain conditions the role of c-Myc in cell cycle progression may require only its activity of trans-suppression, not that of transcription (Claassen & Hann 1999). For instance, c-MycS, which lacks the transactivation activity but retains the trans-suppression activity, can still promote proliferation of several types of
Roles of c-Myc in transformation

Transformation of a cell may not be a physiological function of c-Myc; rather, it may occur only when c-Myc is aberrantly expressed or genetically altered. Although transfection of c-Myc alone transforms Rat1 cells, as measured by an anchorage-independent growth assay, transformation of rat embryonic fibroblasts (REF) or certain primary cultured cells by c-Myc requires its co-transfection with another oncogene or growth factor gene, such as c-rasH or transforming growth factor-α (tgftα) (Amati et al. 1998). In addition, temporary overexpression of c-Myc in Rat1 cells has been shown to induce genetic instability (Felsher & Bishop 1999). Since Rat1 cells are immortalized, it is generally believed that this property of Rat1 cells contributes to their unusual susceptibility to c-Myc-induced transformation and genetic alteration. Like c-Myc2, c-MycS alone is able to transform Rat1 cells. Unlike c-Myc2, however, c-MycS cannot cooperate with c-rasH to transform REF (Xiao et al. 1998). Our laboratory has also found that c-MycS fails to induce DNA damage-induced check-point abrogation (Sheen & Dickson 2000). One explanation for these results is that transformation of REF by c-myc/c-ras cotransfection requires transactivation of certain proliferation-related genes to immortalize the cells as the priming step of transformation. c-Myc2 can induce proliferation of both REF and Rat1, whereas c-MycS can only induce proliferation of Rat1 (Xiao et al. 1998). These data further imply that some genes required for the replication of mortal cells may not be needed for the replication of immortalized cells, and that activation of these genes requires transactivation by c-Myc2. Perhaps the ability of c-Myc2 to immortalize a cell is required to make REF cells susceptible to transformation by c-rasH, as c-rasH has been shown to induce senescence (Serrano et al. 1997). Since c-rasH is a signaling molecule engaged by many growth factors, one may further surmise that the requirement of co-transfection with growth factor genes or other oncogenes may also be due to the requirement of activation of proliferation-related genes for immortalizing the cells. It remains to be determined whether the c-Myc-induced genetic instability also requires its transactivation ability and contributes to its transformation activities.

The role of c-Myc in transformation may be directly related to its regulation of expression of the human telomerase transcriptase gene (hTERT) (Greenberg et al. 1999), since telomerase functions to immortalize cells. Analysis of the 5′-flanking sequence of hTERT further reveals that transcription of this gene is dependent on a proximal 181 bp region of the promoter, which is essential for its transactivation in immortalized and cancer cells (Oh et al. 1999). This promoter region contains c-Myc E-boxes and GC-boxes (the consensus binding sequence for Sp1), and thus presumably is responsible for the observed cooperation between c-Myc and Sp1 in transcriptional activation of the hTERT gene (Oh et al. 1999, Kyo et al. 2000). In addition, estrogen has also been shown to activate hTERT, in part via its activation of the expression of c-Myc (Kyo et al. 1999). On the other hand, hTERT is also transcriptionally repressed by Mad (Gunes et al. 2000, Oh et al. 2000), a protein that can compete with c-Myc in binding to Max, via directly binding to the hTERT promoter. Since the Max/Mad complex acts in an antagonistic manner to c-Myc/Max-induced transactivation, elevation in Mad is anticipated to suppress hTERT expression also indirectly by decreasing both the abundance of the c-Myc/Max complex and the transactivation activity of c-Myc/Max.

Roles of c-Myc in apoptosis and their connection to carcinogenesis

Two sets of conflicting phenomena have frequently been reported in the literature pertaining to the role of c-Myc in apoptosis. (1) Constant overexpression of c-Myc by the approaches of transfection, viral-infection or transgenic animals may induce apoptosis (Packham & Cleveland 1995, Alarcon et al. 1996, Hagiyma et al. 1999, Prendergast 1999), usually following or associated with cell proliferation (Hoffman & Liebermann 1998), whereas cells transfected with c-myc antisense oligodeoxynucleotides to decrease the c-myc levels become resistant to apoptotic stimulus (Lee et al. 1997). (2) A decrease in c-Myc levels by techniques such as an antisense approach may also cause apoptosis of certain tumor tissues or tumor cells (Balaji et al. 1997, Citro et al. 1998, Putney et al. 1999, Wu et al. 1999), or may increase the sensitivity of the cells to apoptotic stimuli (Kang et al. 1996, Rupnow et al. 1998, Loeffler et al. 1999). In addition, constant overexpression of c-myc has also been shown to reduce sensitivity to UV-induced apoptosis (Waike et al. 1999). These conflicting observations suggest that c-Myc is capable of both inducing and suppressing apoptosis. To induce a tumor, c-Myc may need not only to promote cell proliferation but also simultaneously to inhibit its tendency for cell death, so as to increase the cell number to form a tumor mass (Cory et al. 1999, Lowe & Lin 2000). Therefore, the role of c-Myc in inhibiting apoptosis is easily connected to its tumorigenicity. Whether and how c-Myc-induced apoptosis also contributes to carcinogenesis is much less clear, although, in general, apoptosis is suggested to accelerate cell turnover and thus facilitate the progression of cells to more and more malignant phenotypes during the carcinogenic process (Vakkala et al. 1999). A puzzle is that, although c-Myc is frequently overexpressed in various tumor
tissues in human and in animals under spontaneous or experimental conditions, little, if any, evidence has shown that the apoptosis appearing in these tumors is related to the increased levels of c-Myc.

The foundation of the concept of 'c-Myc-induction of apoptosis' is built mainly on the systems where c-Myc expression is induced in a constant manner by approaches of transfection, viral-infection, or a specific promoter-driven c-myc transgene, while the expression level of the endogenous c-Myc is down-regulated (Prendergast 1999, Packham & Cleveland 1995). These artificial systems are different from the physiological situation in which c-Myc expression arises under its own promoter, specifically during G0/G1 transit of the cell cycle. Perhaps a drop in the levels of c-Myc later in the cell cycle is required for the rise of other proliferation-related genes, such as G1 cyclins. Thus, a constantly high level of c-Myc may disrupt the cyclic pattern of expression of these genes; the cell may then be signaled to die. Currently, it is still technically impossible to test this hypothesis, since it is impossible to induce c-Myc transiently and specifically at the G0/G1 transit. Moreover, in the in vivo situation, a cell that has already proliferated may remain untransformed, or it may be transformed but still retain certain critical differentiated features such as contact-inhibition. Such a cell may be terminated through an apoptotic pathway in order to maintain a particular physiological condition, such as the normal size of the organ. This may be one of the reasons why c-Myc-overexpressing cells, which usually have already undergone proliferation, may commit apoptosis.

How c-Myc induces apoptosis is still unclear, despite the fact that many apoptotic pathways, such as those that are p53-dependent and -independent, have been suggested by different experimental systems (Packham & Cleveland 1995, Hagiyama et al. 1999, Prendergast 1999, Soengas et al. 1999). The transactivation domain of the c-Myc protein has been shown recently to modulate the apoptosis directly (Chang et al. 2000). Another study shows that C-MycS retains the ability to induce apoptosis of several types of cells, and the MBII of c-MycS is needed for this function (Xiao et al. 1998), indicating that in certain situations c-Myc-induced apoptosis may require trans-suppression, but not transactivation, of c-Myc proteins.

The c-myc gene in human breast cancer

As shown in our recent meta-analysis (Deming et al. 2000) and in Table 1 that lists the most references found by search in Medline, a range of 1 to 94%, 15.5% on average, of breast cancer biopsies bear c-myc gene amplification of threefold or greater. The great variation in the frequencies may be attributed to the low sensitivity of some methods used (Soini et al. 1994), the tumor grades studied, and the small number of cases in many of the studies. Early diagnosis of many cases may be another reason, as the gene may be amplified during both early and late stages of cancer progression. The reported frequencies of overexpression of c-myc are also greatly variable. A recent report reveals that only about 22% of the tumor cases show increased c-myc mRNA expression, and the overexpression was rarely due to the gene amplification (Bieche et al. 1999). However, several other studies (Table 1) show much higher percentages of breast cancer cases with mRNA overexpression (Guerin et al. 1988, Mariani-Costantini et al. 1988, Tervahauta et al. 1992, Escot et al. 1993, Nagai et al. 1994, Le et al. 1999). Some of these studies suggest that the overexpressed mRNA might be related to gene amplification (Mariani-Costantini et al. 1988). Since most of these mRNA studies were carried out using Northern blot, dot blot, or PCR-based techniques with tissue lysates, but not using in situ hybridization, the increase in the expression may actually be assessed with great bias. This is because normal breast tissue is dominated by fat tissue; it differs greatly from tumor tissue in its epithelial cellularity, and thus is not a rigorously normal counterpart for comparisons involving mRNA extraction.

As listed in Table 1, many studies utilizing immunohistochemistry show that about 50–100% of breast cancer cases have increased levels of c-Myc proteins (Agnantis et al. 1992, Pavelic et al. 1992a,b, Saccani et al. 1992, Hehir et al. 1993, Spaventi et al. 1994, Pietilainen et al. 1995). In one of those reports, 95% of the cases show positive staining of c-Myc in the cytoplasm, and only 12% of the cases reveal either nuclear or both nuclear and cytoplasmic staining (Pietilainen et al. 1995). Other investigations also report a predominantly cytoplasmic localization of c-Myc proteins, although nuclear localization is also observed (Mizukami et al. 1991). The meaning of the cytoplasmic localization is currently unknown. Regardless of the cellular location of c-Myc, it seems that there is a higher percentage of breast cancer cases showing aberrant c-Myc protein levels than the percentage with the gene amplification. This implies that, in many cases, altered expression or altered stability of the mRNA or protein may be the mechanism for the increased levels of c-Myc, consistent with the initial report that c-Myc overexpression precedes its gene amplification and plays a role in amplification of multiple other genes and in other chromosomal instability events (Mai 1994, Mai et al. 1996).

In breast cancer, amplification of c-myc may correlate positively or negatively with alterations in other genes (Courjal et al. 1997, Cuny et al. 2000). The amplification of the chromosomal region that contains the c-myc gene has also been reported to contain, apparently through translocation events, the p40 subunit of eukaryotic translation initiation factor 3 (eIF3) and the Her2 gene (Nupponen et al. 1999), although the frequency of co-localization of these two genes in a common amplicon has not been established (Deming et al. 2000). Her2 (also termed erbB2) is a gene in
Table 1 Summary of the amplification, RNA or protein expression of c-myc in human breast cancer

<table>
<thead>
<tr>
<th>Reference</th>
<th>Methods</th>
<th>Main c-myc-related conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escot et al. (1986)</td>
<td>SN,NB</td>
<td>Not associated with ER or PR status</td>
</tr>
<tr>
<td>Whittaker et al. (1986)</td>
<td>Dot-B</td>
<td>Expressed in both benign lesions and cancer</td>
</tr>
<tr>
<td>Cline et al. (1987)</td>
<td>SB</td>
<td>More common in recurrent tumors</td>
</tr>
<tr>
<td>Spandidos et al. (1987)</td>
<td>IHC</td>
<td>Expressed in both benign lesions and cancer</td>
</tr>
<tr>
<td>Varley et al. (1987a)</td>
<td>SB</td>
<td>Correlated with poor prognosis</td>
</tr>
<tr>
<td>Varley et al. (1987b)</td>
<td>SB</td>
<td>Identified a rearrangement with deletion</td>
</tr>
<tr>
<td>Biunno et al. (1988)</td>
<td>SB,NB,Slot-B</td>
<td>No clinicopathologic parameters mentioned</td>
</tr>
<tr>
<td>Bonilla et al. (1988)</td>
<td>SB,NB</td>
<td>Not correlated with clinicopathologic parameters</td>
</tr>
<tr>
<td>Guerin et al. (1988)</td>
<td>ISH</td>
<td>RNA overexpression related to the gene amplification</td>
</tr>
<tr>
<td>Meyers et al. (1988)</td>
<td>NB</td>
<td>Gene rearrangement and mutation</td>
</tr>
<tr>
<td>Garcia et al. (1989)</td>
<td>SB</td>
<td>Associated with the inflammatory type of the cancer</td>
</tr>
<tr>
<td>Gutman et al. (1989)</td>
<td>IHC</td>
<td>Not related to any clinicopathologic parameters</td>
</tr>
<tr>
<td>Locker et al. (1989)</td>
<td>IHC</td>
<td>Not related to any clinicopathologic parameters</td>
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<tr>
<td>Machotka et al. (1989)</td>
<td>SB</td>
<td>Not correlated with survival</td>
</tr>
<tr>
<td>Spandidos et al. (1990a)</td>
<td>ELISA</td>
<td>Not correlated with survival</td>
</tr>
<tr>
<td>Spandidos et al. (1990b)</td>
<td>IHC</td>
<td>Not correlated with node metastasis</td>
</tr>
<tr>
<td>Tauchi et al. (1990)</td>
<td>IHC</td>
<td>Not correlated with ER, tumor histology or sizes</td>
</tr>
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<td>Tavassoli et al. (1989)</td>
<td>SB,Slot-B</td>
<td>Correlated with tumor grade, but not with metastasis</td>
</tr>
<tr>
<td>Tsuda et al. (1989)</td>
<td>Slot-B</td>
<td>Correlated with poor prognosis</td>
</tr>
<tr>
<td>Walker et al. (1989)</td>
<td>ISH,IHC</td>
<td>No correlation among amplification, RNA and protein</td>
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<tr>
<td>Guerin et al. (1990)</td>
<td>NB</td>
<td>Associated with poor prognosis</td>
</tr>
<tr>
<td>Meyers et al. (1990)</td>
<td>SB</td>
<td>Only 1% frequency of amplification</td>
</tr>
<tr>
<td>Tang et al. (1990)</td>
<td>SB</td>
<td>Associated with lymphocyte infiltration of the tumors</td>
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<tr>
<td>Mizukami et al. (1991)</td>
<td>IHC</td>
<td>Correlated with ER, but not with clinical parameters</td>
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<tr>
<td>Escot et al. (1991)</td>
<td>ISH</td>
<td>Methodology on RNA quantitation</td>
</tr>
<tr>
<td>Fukutomi et al. (1991)</td>
<td>IHC</td>
<td>Related to cell surface sugar chains</td>
</tr>
<tr>
<td>Le Roy et al. (1991)</td>
<td>ISH</td>
<td>The RNA expression reduced by tamoxifen</td>
</tr>
<tr>
<td>Paterson et al. (1991)</td>
<td>Slot-B</td>
<td>Existence of co-amplification with Her2</td>
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<tr>
<td>Pavelic et al. (1991)</td>
<td>IHC</td>
<td>Mainly nuclear location; related to positive nodes</td>
</tr>
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<td>Tauchi et al. (1991)</td>
<td>IHC</td>
<td>Do not regulate HSP70 expression</td>
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<tr>
<td>Tsuda et al. (1991)</td>
<td>Slot-B</td>
<td>Amplification in both primary and metastatic tumors</td>
</tr>
<tr>
<td>Agnantis et al. (1992)</td>
<td>IHC</td>
<td>Elevated in benign lesions, as a pre-cancer marker</td>
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<tr>
<td>Berns et al. (1992a)</td>
<td>SB</td>
<td>Associated with high copies of IGF1R amplification</td>
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<td>Berns et al. (1992b)</td>
<td>SB</td>
<td>Correlated with tumor size &amp; node-positivity, not ER</td>
</tr>
<tr>
<td>Berns et al. (1992c)</td>
<td>SB</td>
<td>Inversely correlated with neu amplification and PR</td>
</tr>
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<td>Berns et al. (1992d)</td>
<td>SB</td>
<td>Correlated with shorter survival</td>
</tr>
<tr>
<td>Borg et al. (1992)</td>
<td>SB,Slot-B</td>
<td>Related to early recurrence and death, not to ER</td>
</tr>
<tr>
<td>Pavelic et al. (1992a)</td>
<td>IHC</td>
<td>More frequent in invasive tumors</td>
</tr>
<tr>
<td>Pavelic et al. (1992b)</td>
<td>IHC</td>
<td>Mainly nuclear location; related to positive nodes</td>
</tr>
<tr>
<td>Roux-Dosseto et al. (1992)</td>
<td>SB</td>
<td>Correlated with early recurrence</td>
</tr>
<tr>
<td>Saccani et al. (1992)</td>
<td>IHC</td>
<td>Not related to nodal status, ER or PR</td>
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<tr>
<td>Tervahauta et al. (1992)</td>
<td>PCR,ISH,IHC</td>
<td>No clinicopathologic parameters mentioned</td>
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<td>Bootsma et al. (1993)</td>
<td>SB</td>
<td>Not associated with somatostatin receptor expression</td>
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<td>Escot et al. (1993)</td>
<td>ISH</td>
<td>Higher expression in post-ovulatory phase</td>
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<td>Gaffey et al. (1993)</td>
<td>SB</td>
<td>Co-amplified with Her2</td>
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<td>Heri et al. (1993)</td>
<td>IHC</td>
<td>Also overexpressed in benign lesions</td>
</tr>
<tr>
<td>Henry et al. (1993)</td>
<td>SB</td>
<td>Associated with poor tumor differentiation, not prognosis</td>
</tr>
<tr>
<td>Krepe et al. (1993)</td>
<td>SB</td>
<td>Related to proliferation</td>
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<tr>
<td>Nagayama &amp; Watatani (1993)</td>
<td>SB</td>
<td>Related to node metastases &amp; tumor sizes</td>
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<tr>
<td>Ottestad et al. (1993)</td>
<td>SB</td>
<td>Only 1.1% amplification frequency</td>
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<td>Pertschuk et al. (1993)</td>
<td>Slot-B,IHC</td>
<td>Correlated with recurrence</td>
</tr>
<tr>
<td>Yamashita et al. (1993)</td>
<td>SB</td>
<td>Related to tumor size, but not clinical parameters</td>
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<td>Watson et al. (1993)</td>
<td>SB,PCR</td>
<td>Related to early progression</td>
</tr>
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<td>Bleche et al. (1994)</td>
<td>SB</td>
<td>Associated with loss of heterozygosity on 1p32</td>
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<td>Bolufer et al. (1994)</td>
<td>SB</td>
<td>Associated with neu amplification and ER</td>
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<td>Breuer et al. (1994)</td>
<td>IHC,WB</td>
<td>c-Myc protein elevated in both tumors and serum</td>
</tr>
<tr>
<td>Champeme et al. (1994a)</td>
<td>SB</td>
<td>Not related to metastasis-free survival</td>
</tr>
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</table>
are negative for estrogen receptor (ER-) (Persons et al. 1994) and/or progesterone receptor (PR-) (Adnane et al. 1989, Berns et al. 1992c), although other investigations do not find such inverse correlation or even show the opposite correlation (Table 1). In our recent meta-analysis, only the correlation of c-myc amplification with PR negativity was of statistical significance (Deming et al. 2000). Amplification of the cyclin D1 gene (ccnd1) is also seen frequently in human breast cancer, which occurs preferentially in the cases without c-myc amplification (Barnes & Gillett 1998). Although this reciprocal amplification seems to be consistent with the in vitro observation that c-Myc represses the transcription of cyclin D1, direct evidence is still lacking for a reciprocal

Table 1 Continued

<table>
<thead>
<tr>
<th>Reference</th>
<th>Methods</th>
<th>Main c-myc-related conclusions</th>
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</thead>
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<tr>
<td>Champeme et al. (1994b)</td>
<td>SB</td>
<td>Not correlated with survival</td>
</tr>
<tr>
<td>Haranda et al. (1994)</td>
<td>SB</td>
<td>Not related to clinicopathologic parameters</td>
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<td>Nagai et al. (1994)</td>
<td>NB</td>
<td>Not correlated with ER RNA levels</td>
</tr>
<tr>
<td>Spaventi et al. (1994)</td>
<td>IHC</td>
<td>Not related to clinicopathologic parameters</td>
</tr>
<tr>
<td>Pechoux et al. (1994)</td>
<td>SB,ISH,ISHC</td>
<td>Also overexpressed in benign lesions</td>
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<tr>
<td>Soini et al. (1994)</td>
<td>SB,ISH</td>
<td>No clinicopathologic parameters mentioned</td>
</tr>
<tr>
<td>Berns et al. (1995a)</td>
<td>SB</td>
<td>Related to relapse</td>
</tr>
<tr>
<td>Berns et al. (1995b)</td>
<td>SB</td>
<td>Inversely related to the Rb gene alteration</td>
</tr>
<tr>
<td>Bland et al. (1995)</td>
<td>IHC</td>
<td>Not related to recurrence</td>
</tr>
<tr>
<td>Brotherick et al. (1995)</td>
<td>Cyotometry</td>
<td>Associated with c-erbB3 expression</td>
</tr>
<tr>
<td>Contegiacomo et al. (1995)</td>
<td>SB</td>
<td>Not related to clinicopathologic parameters</td>
</tr>
<tr>
<td>Correnti et al. (1995)</td>
<td>Slot-B</td>
<td>No clinicopathologic parameters mentioned</td>
</tr>
<tr>
<td>Ito et al. (1995)</td>
<td>SB</td>
<td>Not associated with ER or PR status</td>
</tr>
<tr>
<td>Janocck et al. (1995)</td>
<td>Dot-B</td>
<td>No clinicopathologic parameters mentioned</td>
</tr>
<tr>
<td>Lizard-Nacol et al. (1995)</td>
<td>SB</td>
<td>Amplification only in cancer, not in benign lesions</td>
</tr>
<tr>
<td>Lonn et al. (1995)</td>
<td>PCR</td>
<td>Correlated with survival</td>
</tr>
<tr>
<td>Pietlilainen et al. (1995)</td>
<td>IHC</td>
<td>Related to better survival; mainly in cytoplasm</td>
</tr>
<tr>
<td>Ried et al. (1995)</td>
<td>CGH</td>
<td>Amplification found; no clinicopathology mentioned</td>
</tr>
<tr>
<td>Berns et al. (1996)</td>
<td>SSCP</td>
<td>Related to poor prognosis</td>
</tr>
<tr>
<td>Courjal &amp; Theillet (1997)</td>
<td>CGH</td>
<td>CGH more sensitive than SB</td>
</tr>
<tr>
<td>Courjal et al. (1997)</td>
<td>SB</td>
<td>Correlated with ER-</td>
</tr>
<tr>
<td>Persons et al. (1997)</td>
<td>FISH</td>
<td>Related to S phase and ER-</td>
</tr>
<tr>
<td>Visscher et al. (1997)</td>
<td>FISH</td>
<td>No clinicopathologic parameters mentioned</td>
</tr>
<tr>
<td>Kononen et al. (1998)</td>
<td>Arrays</td>
<td>Amplification detected in tissue microarrays</td>
</tr>
<tr>
<td>Mimori et al. (1998)</td>
<td>RT-PCR</td>
<td>Correlated with ODC expression</td>
</tr>
<tr>
<td>Stanta et al. (1998)</td>
<td>RT-PCR</td>
<td>No clinicopathologic parameters mentioned</td>
</tr>
<tr>
<td>Bieche et al. (1999)</td>
<td>RT-PCR</td>
<td>Correlated with tumor size but inversely with survival</td>
</tr>
<tr>
<td>Le et al. (1999)</td>
<td>NB</td>
<td>Related to positive nodes</td>
</tr>
<tr>
<td>Nupponen et al. (1999)</td>
<td>SSH</td>
<td>Associated with eIF3-amplification</td>
</tr>
<tr>
<td>Schrami et al. (1999)</td>
<td>FISH</td>
<td>Amplification detected in tissue-microarray</td>
</tr>
<tr>
<td>Scorilas et al. (1999)</td>
<td>SB,NB</td>
<td>Related to survival and local recurrence</td>
</tr>
<tr>
<td>Sierra et al. (1999)</td>
<td>IHC</td>
<td>Related to metastasis when Bcl-2 also increased</td>
</tr>
<tr>
<td>Vos et al. (1999)</td>
<td>SB,CGH</td>
<td>No amplification in DCIS</td>
</tr>
<tr>
<td>Cuny et al. (2000)</td>
<td>SB</td>
<td>Correlated with ER- and PR-, but not with prognosis</td>
</tr>
<tr>
<td>Han et al. (2000)</td>
<td>IHC</td>
<td>Inversely correlated with Mad1 expression</td>
</tr>
<tr>
<td>Jonsson et al. (2000)</td>
<td>WB</td>
<td>Not related to Beta-catenin</td>
</tr>
<tr>
<td>Rao et al. (2000)</td>
<td>PCR</td>
<td>Up to 94% biopsies with amplification</td>
</tr>
<tr>
<td>Sierra et al. (2000)</td>
<td>IHC</td>
<td>Not related to clinicopathologic parameters</td>
</tr>
</tbody>
</table>

CGH, comparative genomic hybridization; Dot-B, dot blot; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; ISH, in situ hybridization; NB, northern blot; RT-PCR, reverse transcription and PCR; SB, southern blot; Slot-B, slot blot; SSCP, single-strand conformation polymorphism; SSH, suppression subtractive hybridization; WB, western blot; IGF1R, insulin-like growth factor-1; DCIS, ductal carcinoma in situ.

the epidermal growth factor receptor (EGFR) family and is amplified in about 20–30% of human breast cancer biopsies (Gaffey et al. 1993, Bolufer et al. 1994, Cuny et al. 2000). Moreover, some investigations have shown that amplification of Her2 and c-myc genes is positively correlated or simultaneously occurs in certain breast cancer biopsies (Guerin et al. 1988, Gaffey et al. 1993, Bolufer et al. 1994). However, inverse correlation between amplification of Her2 gene and c-myc has also been reported in a few other studies (Berns et al. 1992c, Sears et al. 1997).

Several studies show that amplification or overexpression of c-myc occurs more frequently in the cases that are negative for estrogen receptor (ER-) (Persons et al. 1997, Bolufer et al. 1994) and/or progesterone receptor (PR-) (Adnane et al. 1989, Berns et al. 1992c), although other investigations do not find such inverse correlation or even show the opposite correlation (Table 1). In our recent meta-analysis, only the correlation of c-myc amplification with PR negativity was of statistical significance (Deming et al. 2000). Amplification of the cyclin D1 gene (ccnd1) is also seen frequently in human breast cancer, which occurs preferentially in the cases without c-myc amplification (Barnes & Gillett 1998). Although this reciprocal amplification seems to be consistent with the in vitro observation that c-Myc represses the transcription of cyclin D1, direct evidence is still lacking for a reciprocal
relationship between the expression of c-Myc and that of cyclin D1 in any human cancer tissue.

In cultured breast cancer cells, c-Myc is able to mimic estrogen to induce cyclin E/cdk2 activity by maintaining the p27kip1 in the cyclin D1/cdk4 complex (earlier in the cell cycle), so as to keep the cyclin E2 complex free from p27kip1-binding (Prall et al. 1998). Pathological data also show that levels of p27kip1 and cyclin D1 are associated with each other in breast cancer (Gillett et al. 1999, Leong et al. 2000). Other roles of c-Myc to activate cyclin E/cdk2 activity include its direct induction of expression of cyclin E, its possible induction of an as yet unidentified factor to sequester the p27kip1 from binding to the cyclin E/cdk2, its promotion of the ubiquitin-degradation of p27kip1, and its possible repression of the expression of p27kip1 (Amati et al. 1998, Donjerkovic et al. 1999, Obaya et al. 1999, Wu et al. 1999). Based on these in vitro data, a higher level of c-Myc would be expected to be associated with lower levels of p27kip1. Since several reports have shown that a lower p27kip1 level predicts a poorer prognosis of breast cancer (Cariou et al. 1998, Gillett et al. 1999), this is surmised to be related partly to the elevated levels of c-Myc. Currently, however, no studies have been reported to prove or disprove this speculation on the relationship between c-Myc and p27kip1 and on their combined effect on the outcome of breast cancer. However, it has been observed in patients with cervical cancer that those with a low p27kip1 level survive longer than those with low levels of both p27kip1 and c-Myc (Dellas et al. 1998).

Breast cancer-associated gene 1 (BRCA1) is a putative tumor suppressor gene, loss or inactivation of which especially increases the risk of breast and ovarian cancer (Deng & Scott 2000). BRCA1 can physically bind to the c-Myc protein and repress c-Myc-mediated transcription (Wang et al. 1998). Moreover, BRCA1 can reverse the phenotype of REF transformed by activation of c-Myc and c-Ras (Wang et al. 1998). These data indicate that the mechanism for BRCA1 to function as a tumor suppressor may be related, in part, to its binding to c-Myc and its repression of the transcriptional activity of c-Myc. Loss of BRCA1 is therefore expected to result in relatively increased c-Myc activity and transforming potential. It would be interesting to test this speculation in familial breast cancers of BRCA1 carriers.

PTEN (phosphate and tensin homolog deleted on chromosome ten) is a tumor suppressor gene that is inactivated in a number of tumor types, including breast cancer (Ali et al. 1999, Di Cristofano & Pandolfi 2000). In a myc-CAT (chloramphenicol acetyl transferase) reporter gene system, ectopic expression of PTEN has been shown to repress transcription of c-myc in MCF-7 and MDA-MB 486 breast cancer cells (Ghosh et al. 1999), indicating that among its activities, PTEN may be a transcription factor and c-myc may be its target gene. The repression of c-myc by PTEN in these cells is coupled with increased apoptosis and with growth inhibition of the tumor developed from these cells in nude mice (Ghosh et al. 1999). It is thus conceivable that the tumor suppressive role of PTEN may be exerted, in part, by down-regulation of c-myc. It would be interesting to test this speculation in familial breast cancers of PTEN carriers (Cowden’s syndrome).

**The c-myc gene and mammalian gland carcinogenesis in transgenic mouse models**

Transgenic mice have been generated to target the c-myc gene to the mammary glands by placing the transgene under the control of the long terminal repeat of the mouse mammary tumor virus (MMTV) or the whey acidic protein (WAP) promoters (reviewed in Amundadottir et al. 1996a, Nass & Dickson 1997). In MMTV-c-myc transgenic mice, the transgene is expressed at high levels, specifically in the mammary and salivary glands of females (Stewart et al. 1984). Spontaneous carcinomas develop in mammary glands at a frequency of roughly 50% at about one year of age in the virgin females; distant metastasis is rare (Amundadottir et al. 1995, 1996a,b, Rose-Hellekant & Sandgren 2000). Males do not develop the tumors. Multiple pregnancies significantly increase the incidence and shorten the tumor latency (Stewart et al. 1984, Amundadottir et al. 1995, 1996a,b, Nass & Dickson 1997), indicating that certain physiological growth stimuli to the mammary gland, such as estrogen or progesterone, may serve as promoters of carcinogenesis. Female WAP-c-myc mice, on the other hand, must undergo pregnancy to develop mammary carcinomas (Sandgren et al. 1995). The incidence and tumor latency are thus likely to depend on the rounds of pregnancies. In the WAP-c-myc model, pregnancy is required for activation of the promoter, but it complicates the model as well, since pregnancy may also provide additional promotion of carcinogenesis, as seen in MMTV-c-myc mice.

Since in these c-myc transgenic models the latency periods for tumor onset are long and the tumor incidences are relatively low, the c-myc gene itself may not be sufficient for the induction of carcinogenesis; other endogenous promoting factors such as female sex hormones may still be required to complete the carcinogenic process. This hypothesis is in line with the in vitro experiments, showing that transfection of cells with c-myc alone fails to transform cells, and that co-transfection with another oncogene or growth factor gene is required. Consistent with the co-transfection experiments, double transgenic mice carrying c-myc and another gene, such as c-ras, tgfα, bcl-2, or c-neu develop mammary carcinomas at much higher frequencies and at earlier ages (reviewed in Amundadottir et al. 1996a, Nass & Dickson 1997).
Virgin female mice transgenic with MMTV-c-neu, the mouse counterpart of Her2, develop mammary carcinomas as well, with a 50% incidence at about 7 months, slightly earlier than that in MMTV-c-myc mice (Muller et al. 1988, Cardiff et al. 1991). However, about 90% of the MMTV-c-neu/MMTV-c-myc double transgenic mice, generated by mating these two strains, develop cancer at 4.6 months, a much shorter latency than the single transgene carriers (Muller et al. 1988, Cardiff et al. 1991). In human breast cancer, co-amplification of Her2 and c-myc has also been reported to be associated with a reduced survival in some studies (Gaffey et al. 1993, Bolufer et al. 1994, Cuy et al. 2000). Interestingly, in vitro c-myc has been shown to repress transcription of c-neu and reverse c-neu-induced transformed morphology of cultured NIH 3T3 cells (Suen & Hung 1991). Probably when a situation occurs to allow these two oncogenes to cooperate rather than to antagonize each other, it results in a more aggressive tumor type. Such a situation occurs in the double transgenic animals where both transgenes are driven by a transgenic MMV promoter, and may also occur when both genes are co-amplified and/or translocated during development of human breast cancer.

MMTV-v-ras<sup>h</sup> transgenic mice develop mammary adenocarcinomas at 50% frequency at an average latency period of 10 months for males and 5.6 months for virgin females (Sinn et al. 1987). The tumors from v-ras or c-neu transgenic mice bear some genetic alterations that are absent in the tumors from c-myc transgenic mice (Morrison & Leder 1994), indicating that the carcinogenesis initiated by these oncogenes may undergo different pathways. MMTV-v-ras<sup>h</sup>/MMTV-c-myc double transgenic mice develop mammary tumors at 50% incidence after an average latency of 46 and 100 days in females and males respectively (Sinn et al. 1987), again showing a synergistic effect of both oncogenes. One disadvantage of the v-ras<sup>h</sup>/c-myc and c-neu/c-myc dual transgenic models for the study of multistaged carcinogenesis is that, of the two oncogenes, it is difficult to tell which one is the major carcinogenic factor and which one is the synergist, since female carriers of either transgene develop the tumors.

Dual carriers of metallothionein–1 promoter-(MT)-tgfα and MMTV-c-myc also develop mammary gland carcinomas at virtually 100% frequency in both male and female mice (Amundadottir et al. 1995, 1996a,b). The latency for the appearance of frank tumors in both sexes is similar, about 66 days in our studies. Unlike MMTV-v-ras<sup>h</sup> and MMTV-c-neu single transgenic mice, virgin female MT-tgfα mice develop only moderate hyperplasia in their mammary epithelium (Sandgren et al. 1990, Amundadottir et al. 1995), although a low frequency of mammary tumors may occur after the mice undergo multiple pregnancies (Sandgren et al. 1995, Humphreys & Hennighausen 2000). This characteristic defines a synergistic role for the transforming growth factor-α (TGFα) in this double transgenic virgin model. The tgfα/c-myc tumors also grow faster than the c-myc tumors. In addition, the equal incidence and latency in both sexes of animals suggest that the strong synergism between the two transgenes may take place at exceedingly early ages, when sex differentiation in the endocrine environment has not been well-established nor effective. Moreover, before a macroscopic tumor appears, all cells in the hyperplastic mammary glands from MT-tgfα/MMTV-c-myc mice have already displayed displastic morphology. These properties suggest a unique feature that, although neither tgfα nor c-myc alone is a sufficient carcinogenic factor, their synergism is potently carcinogenic and can transform mammary epithelial cells as early as during the early stages of development.

Parous female mice carrying dual WAP-bcl–2/ MMTV-c-myc transgenes develop mammary cancer at an average latency period of 3.3 months, slightly, but significantly shorter than the latency (4.3 months) of tumors in parous female MMTV-c-myc carriers, whereas parous female WAP-bcl–2 mice do not develop cancers (Jager et al. 1997). There is evidence suggesting that bcl–2 and c-myc may also be synergistic in human breast cancer (Sierra et al. 1999). Interestingly, this synergism in mammary carcinogenesis is opposite to the antagonism in liver carcinogenesis observed in the bcl–2/c-myc double transgenic mice (de La et al. 1999). The reason behind this organ difference is unclear.

Loss of one p53 allele seems to have little effect on c-Myc carcinogenicity, since female MMTV-c-myc/p53<sup>+/−</sup> dual carriers develop mammary tumors at a frequency and a latency period similar to that seen in their MMTV-c-myc counterparts (McCormack et al. 1998). Study of the mammary carcinogenesis in MMTV-c-myc/p53<sup>+/−</sup> mice is unfortunately impossible, because of very early development of lymphomas (Elson et al. 1995, McCormack et al. 1998). However, these mice manifest dramatic hyperplasia in the mammary gland at an earlier stage than the MMTV-c-myc mice (McCormack et al. 1998), suggesting the possibility that lack of both p53 alleles may still have certain promoting effects on c-Myc-induced mammary carcinogenesis, although the synergy would be much weaker compared with the synergistic effects in lymphomagenesis (Blyth et al. 1995, Elson et al. 1995).

**Distinctive morphology of mammary tumors in transgenic animals**

In a series of analyses, Cardiff and coworkers (Cardiff et al. 1991, 2000, Cardiff & Munn 1995, Cardiff & Wellings 1999) and Halter et al. (1992) noticed that the mammary tumors developed in various different transgenic mouse models manifest distinctive morphology. While more than 95% of the mammary tumors occurring spontaneously in ordinary laboratory mice can be categorized using the Dunn classification, only 9% of the tumors from various transgenic...
mice could be placed into standard categories by this classification (Cardiff et al. 2000). Over 90% of the c-myc transgene-induced tumors are glandular large cell carcinomas, and the rest are adenocarcinomas (Cardiff et al. 1991, Cardiff & Munn 1995). Mammary tumors developed from parous MT-tgfnα and parous MMTV-tgfnα mice are all adenocarcinomas. While we also observed similar results, we noticed that c-myc/tgfnα dual transgenic tumors were mainly acinar carcinomas, according to the classification of Cardiff et al. (2000), which was quite different from the c-myc or tgfnα tumors. These morphological features suggest that tumor phenotype may reflect genotype, as proposed by Cardiff and coworkers. It is an intriguing question why the tgfnα/c-myc double transgenic mice have a tumor phenotype different from that in either tgfnα- or c-myc only mice. Does it mean that each of the tgfnα, c-myc, and tgfnα/c-myc genotypes selects its own favorable target cells in the mammary glands as the tumor progenitor? Clarification of this question will improve our understanding of how tgfnα and c-myc cooperate in the in vivo situation.

The mammary tumors arising in virgin female c-myc transgenic mice are characterized by the large number of apoptotic cells (Amundadottir et al. 1996b, Hundley et al. 1997, Bearn et al. 2000, Liao et al. 2000). About 15% of the tumor cells are apoptotic as identified by TUNEL staining, in contrast to the 1–2% in tgfnα/c-myc double transgenic tumors (Liao et al. 2000). In c-myc tumors, the apoptotic cells are organized in clusters, which in sections stained by the TUNEL method are manifested as many small stained cells and proliferating cells may be c-Myc-related, rather than mammary gland-specific, since similar ‘clusters’ of apoptotic cells are also discerned in the renal ducts that express a c-myc transgene (Trudel et al. 1997). One of the logical explanations may be that a paracrine or juxtacrine mechanism is involved in the c-Myc-induced formation of the apoptotic cell islands, whereas the mechanism for the c-Myc-induced cell proliferation occurs within the proliferating cells without involving their neighboring cells.

Another morphological feature of c-myc and tgfnα/c-myc tumors is that they contain little stromal tissue. Stromal cells and matrix, as well as blood capillaries are all much fewer, compared with the mammary tumors from parous MT-tgfnα transgenic mice or from other experimental mice reported in the literature. This difference in the abundance of stroma has already become prominent in the hyperplastic mammary tissue before the appearance of frank tumors. Expression of c-Myc in lung cancer cells has recently been shown to suppress the expression of vascular epithelial growth factor (VEGF) (Barr et al. 2000). It is thus conceivable that in the c-myc and tgfnα/c-myc mammary tissue and mammary tumors, the low abundance of stroma in general and of blood capillaries in particular may, in part, be related to the suppression of VEGF by c-Myc. This trait may also be partially responsible for the rareness of distant metastases of these tumors.

Possible multiple stages of c-Myc-induced mammary carcinogenesis

In principle, carcinogenesis is a multi-staged process of initiation, promotion, and progression; each of these stages also consists of multiple steps. However, none of the current animal models of mammary carcinogenesis has been observed to manifest a clear multi-step nature, except for the noticeable hyperplastic lesions prior to tumor formation. In our studies of the c-myc transgenic model, we noticed that in some of the relatively larger (>1 cm in diameter) mammary tumors, there are focal areas of tumor cells that are both hematoxylin (H)- and eosin (E)-phobic on routine H-E stained sections (Liao et al. 2000). In these focal lesions, the number of apoptotic cells are much fewer, while the number of proliferating cells are much greater compared with the surrounding tumor areas. Although these foci show a clear boundary of demarcation from surrounding tumor areas, they are not encompassed by connective tissue capsules. Usually, some portion of each focus exhibits infiltration into the adjacent tumor areas, a typical feature of invasive growth. All these morphological properties of the ‘tumor-within-a-tumor’ foci in c-myc tumors suggest that they may belong to a tumor phenotype that is more aggressive than their adjacent tumor area, and may thus represent a second step of tumor progression.

A question then raised is whether the appearance of these ‘tumor-within-a-tumor’ foci is unique for the c-myc transgenic model or, rather, is a common phenomenon among experimental models of mammary carcinogenesis. In our study of the tumors from MT-tgfnα/MMTV-c-myc double transgenic mice, we have not observed such foci. However, the rates of cell proliferation and apoptosis in the entire tumors were comparable to those in the foci of the c-myc tumors. Thus, it seems that the second stage of tumor progression may be circumvented in this double transgenic model, and the multiple steps of progression may have been completed very early, before the transformed cells develop into a frank tumor. However, it cannot be excluded that this second step of progression may still appear in other single or double transgenic models of mouse mammary carcinogenesis.

C-Myc and sex hormones

Estrogens play complex roles in mammary gland development and carcinogenesis. The roles of estrogens in
cell proliferation in their target organs are presumably exerted, in part, via a set of estrogen-responsive genes, including c-fos, c-jun, and c-myc (Schuchard et al. 1993, Hyder et al. 1994). Many in vitro studies and some in vivo experiments (mainly in uterine tissue) have shown that expression of c-myc mRNA is induced by treatment of estrogen (Schuchard et al. 1993, Shiu et al. 1993, Hyder et al. 1994). A 116 bp DNA sequence, which does not contain the canonical estrogen-responsive element (ERE), in the promoter region of the human c-myc gene is responsible for the transcriptional activation by estrogen (Dubik & Shiu 1992). It is likely that activation of the c-myc gene by estrogen requires binding of some ER-associated proteins to ER. It remains obscure if and how estrogen-ER signaling regulates c-myc expression in human breast tumors (Miller et al. 1993), as several reports (but not all) show that overexpression and/or amplification of c-myc occurs preferentially in ER-negative tumors.

ER-positive breast tumors from patients who have received tamoxifen treatment show a decreased level of c-myc mRNA, compared with their counterparts without tamoxifen treatment (Le, X et al. 1991). Similar inhibition of c-myc expression by antiestrogen has also been observed in ER-positive T–47D and MCF–7 cells (Wong & Murphy 1991, Tsai et al. 1997). These results suggest that tamoxifen antagonizes the effects of estrogens on c-myc expression both in vivo and in vitro. However, treatment with tamoxifen has also been shown to induce apoptosis of both ER-negative and ER-positive breast cancer cells, in association with an induction of c-myc expression (Kang et al. 1996). Moreover, tamoxifen also inhibits the growth of MCF–7 tumor growth in nude mice, in association with an induction of c-myc expression (Santoni-Rugiu et al. 1998). The role of increased c-Myc in this latter case was considered, without direct proof, to be related to tumor cell differentiation.

A strong, synergistic role of androgen in estrogen-induced leiomyomas and sarcomas in the uterus and scent gland in hamsters has been known for thirty years (Kirkman & Algard 1970a,b, Kirkman 1972, Dodge et al. 1976). Accumulating epidemiological data also suggest that elevated androgens, mainly testosterone secreted from ovaries, may contribute to the development of breast cancer in women (Berrino et al. 1996, Lopez-Otin & Diamandis 1998, Cauley et al. 1999, Yu et al. 2000). In an attempt to induce prostate cancer in male rats, Li et al. (1998) unexpectedly found that all male rats receiving both hormones develop invasive mammary cancer at a time point when the tumors had not yet developed in the rats receiving only estrogen. Soon afterwards Xie et al. (1999a,b) also reported similar findings in the female Noble rats. These data are the first experimental evidence demonstrating a synergistic effect of testosterone and estrogen in the induction of mammary cancer, thus raising a concern on the use of androgens in certain hormone replacement therapy in women patients (Bartlik & Kaplan 1999, Basson 1999, Hoeger & Guzik 1999).

There is currently no clue as to how testosterone plays a role in carcinogenesis of mammary gland and uterus. It is possible that testosterone may be converted to estrogen by aromatase and thus function as increased estrogen (Henderson & Feigelson 2000). However, since normal mammary glands and most breast tumors in both human and rodents express significant amounts of androgen receptor (AR) (Wilson & McPhaul 1996, Liao et al. 1998), it is also possible that testosterone may bind directly to the AR and perform an as yet undefined role in promotion of carcinogenesis. Androgen can stimulate or inhibit the transcriptional regulation of AR (termed autoregulation), depending on the cell-type (Kokontis et al. 1994, Asadi & Sharifi 1995, Umekita et al. 1996, Kokontis & Liao 1999). C-Myc/Max heterodimer has been shown to bind to a Myc E box element in the AR gene and participate in the autoregulation of AR by AR (Grad et al. 1999). On the other hand, it has been shown that androgen induces c-myc expression to promote proliferation of prostate cancer cells in culture (Kokontis et al. 1994, Umekita et al. 1996, Kokontis & Liao 1999), although it is unclear if similar effects also appear in other AR-expressing tissues. Thus, it cannot be excluded that in mammary gland and breast cancer, c-myc expression is subjected to the transcriptional regulation not only by estrogen but also by elevated androgen. The increased c-Myc may participate in autoregulation of AR expression to control the as yet undefined role of androgen in breast cancer development.

Progesterone influences differentiation, proliferation, and other functions of the mammary gland by mechanisms that are more complicated and less understood (Clarke & Sutherland 1990). Combined treatment with both progesterone and estrogen has been shown to have a stronger effect than estrogen alone on the induction of mammary tumors in male rats (Hannouche et al. 1982). The question as to whether progesterone has an impact on breast cancer development in humans is an important one, because progesterone is used widely in oral contraceptives and in hormone replacement therapy for postmenopausal women. Treatment of cultured breast cancer cells (MCF-7 and T47-D) with progestin results in transient acceleration of the G1 phase, followed by cell cycle arrest and growth inhibition (Musgrove et al. 1991, 1998). These data led to an hypothesis that the action of progesterone is to accelerate the replication of the cells already progressing through G1, which are then arrested early in G1 after completing a round of cell replication (Musgrove et al. 1991, 1998). The c-Myc protein is considered to be the mediator in this transient growth stimulation followed by growth inhibition (McMahon et al. 1998). Indeed, expression of c-myc mRNA is rapidly but transiently induced by progestin treatment, whereas
relatively long-term treatment of progesterin results in suppression of its expression (Musgrove et al. 1991, Wong & Murphy 1991). Consistent with this suppressive role, overexpression and/or amplification of the c-myc gene has been observed to occur preferentially in PR-negative breast cancer cases (Adnane et al. 1989, Berns et al. 1992c).

The promoter of the human c-myc gene contains a 15-bp sequence with homology to the progesterone response element (PRE) (Moore et al. 1997). Binding of PR to this sequence results in activation of the reporter gene, as studied in a CAT assay. However, it is still unclear whether this PRE-like sequence is involved in the transient stimulation and then inhibition of expression of the c-myc gene by progesterone in breast cancer cells. In avian oviducts, PR activates expression of the c-myc gene (Fink et al. 1988), which is mediated by the interaction of certain nuclear matrix-associated steroid receptor binding proteins (Barrett et al. 2000). It seems that the effects of progesterone on PR-responsive cells are cell-type specific, and that the specificity may be related to the mediation of some PR-associated proteins.

Prolactin is both a mitogen and a differentiating agent in the mammary gland. In several rodent models prolactin has been shown to have potent, promotive effects on mammary cancer development (Vonderhaar 1999). A role in human breast cancer has also been suggested, although unproved (Vonderhaar 1998, 1999, Hankinson et al. 1999). Prolactin receptors are present in about 70% of human breast cancer biopsies (Clevenger et al. 1995). Cultured breast cancer cells respond to prolactin as a mitogen. Prolactin is synthesized by human breast cancer cells, and inhibition of the binding of prolactin to its receptors inhibits the cell growth (Vonderhaar 1998). Prolactin can cause a dose-dependent increase in the levels of c-myc mRNA in hepatocytes both in vivo and in vitro (Crowe et al. 1991, Zabala & Garcia-Ruiz 1989). However, how prolactin affects c-myc expression in the reproductive organs and tissues is unknown. Since activation of the prolactin-prolactin receptor pathway simulates the Sos/Ras and Vav/Rac signaling cascades in several breast cancer cell lines (Vonderhaar 1998), it is conceivable that c-Myc is also mediated in certain functions of prolactin in mammary gland.

**Relevance of the c-myc gene to breast cancer prognosis and therapy**

Several reports have shown an association of c-myc gene amplification with a poor prognosis of breast cancer (Berns et al. 1992c,d, 1996, Borg et al. 1992, Roux-Dosseto et al. 1992, Scorilas et al. 1999), whereas many other studies do not find such a correlation (Table 1; Deming et al. 2000). Reports on the prognostic value of overexpression of c-myc mRNA or protein are not only inconsistent but also conflicting (Table 1). While many other studies do not find any association between c-myc expression and prognosis (Mizukami et al. 1991, Spaventi et al. 1994), several investigations find that a higher expression level correlates with a poorer outcome (Guerin et al. 1988, Pertschuk et al. 1993, Mimori et al. 1998). However, a recent study shows that higher c-myc mRNA levels in breast cancer are correlated with better survival (Bieche et al. 1999). This conclusion is consistent with the earlier immunohistochemical studies on protein levels, but probably is correct only for the axillary lymph node negative tumors (Pietilainen et al. 1995). Similar findings that higher levels of c-myc expression reflect better prognosis have also been reported for patients with other types of malignancy, such as testicular cancer (Watson et al. 1986), colorectal cancer (Smith & Goh 1996), uveal melanoma (Chana et al. 1998, 1999), and probably also ovarian cancer (Diebold et al. 1996, Tanner et al. 1998). Many other studies that do not involve analysis of survival also show that higher levels of c-Myc proteins are discerned in better differentiated cancer in testis (Sikora et al. 1985), colon (Sikora et al. 1987, Watson et al. 1987b, Royds et al. 1992), ovary (Watson et al. 1987a, Polacarz et al. 1989), and bile ducts (Voravud et al. 1989), which is also likely to be coupled with a better outcome. Moreover, ectopic expression of c-myc in lung cancer cell lines has recently been shown to suppress tumor development from cells injected into nude mice (Barr et al. 2000). Several studies show that benign breast lesions such as fibroadenomas and fibrocytic disease express C-Myc at levels as high as seen in breast cancer (Whittaker et al. 1986, Spandidos et al. 1987). This property leads to a consideration that c-Myc may be involved in the early development of the cancer and could be used as a marker for the pre-malignancy or for the risk of the cancer.

The controversial prognostic implications for c-myc overexpression should not be surprising, for a few reasons. First, c-Myc proteins may direct cells to proliferation, differentiation, or apoptosis; in the two latter cases the c-Myc level may correlate with a better outcome. Secondly, c-Myc may suppress expression of VEGF in the tumor (Barr et al. 2000), which may also be associated with a favorable prognosis. Thirdly, as mentioned above, the c-myc gene product engenders different proteins that may have different, and even opposite functions, and it is currently unknown which of the c-Myc protein isoforms is expressed in which in vivo situation. Moreover, c-myc may induce cell proliferation, but proliferating cells are usually more sensitive to chemotherapy. Indeed, colonic cancer with low levels of c-myc gene amplification has been reported to respond to adjuvant chemotherapy much better than that without the gene amplification (Augenlicht et al. 1997). This may explain why higher c-myc expression levels are correlated on the one hand with larger sizes of breast tumors but on the other hand with a better survival (Bieche et al. 1999).
In our c-myc transgenic mouse model, we have observed that c-myc expression in the transgenic mammary tumors is actually attenuated in the highly proliferating, less apoptotic tumor foci (Liao et al. 2000). Interestingly, these specific foci show high expression levels of cyclins D1 and E, whereas their adjacent tumor areas do not express these cyclins. Hyperplastic mammary glands in the c-myc mice do not show expression of these cyclins either. Since in vitro studies have demonstrated that c-Myc can suppress expression of cyclin D1, it is likely that the decrease in c-Myc expression in the foci enables the overexpression of these cyclins. Thus, one possible explanation for the inverse correlation between c-myc level and prognosis is that rises in both G1 cyclins, D1 and E, may lead to an unfavorable outcome (Scott & Walker 1997, Wilcken et al. 1997, Nielsen et al. 1998, Lin et al. 2000), whereas high levels of c-Myc prevent their surges.

However, why do high levels of c-Myc associate with an unfavorable outcome in other cases? Attempting to answer this question, we compared the tgfα, c-myc, and tgfα/c-myc transgenic models. This is because not only cyclins D1 and E (Jansen-Durr et al. 1993, Gillett et al. 1996, Nielsen et al. 1996, Sasano et al. 1997, Trudel et al. 1997), but also TGFα and several other growth factors (Panico et al. 1996) or their receptors (Dickson & Lippman 1995) that are known to be survival factors for c-Myc-overexpressing cells (Hoffman & Liebermann 1998, Prendergast 1999), have all been reported to be overexpressed in most cases of human breast cancer (Auvinen et al. 1996). In contrast to the suppressive effects of c-Myc, TGFα may induce cyclin D1, as suggested by the observation that many cyclin D1-positive cells appear in the hyperplastic mammary epithelium from tgfα transgenic mice (Liao et al. 2000). In tgfα/c-myc double transgenic tumors, many cyclin D1-positive cells also appear, but they are spread randomly throughout the whole tumors, without forming any specific foci or showing reciprocal relation to the c-myc expression (Liao et al. 2000). Collectively, these data suggest that in the double transgenic model, TGFα induces expression of cyclin D1, which overrides the suppression by c-Myc. Cyclin E is also overexpressed in a way similar to the expression of cyclin D1 in the tgfα/c-myc tumors (Liao et al. 2000). Thus, three types of tumor tissue, i.e. the major tumor areas of c-myc tumors, the foci in c-myc tumors, and the tgfα/c-myc tumors, manifest different patterns of relationship between c-Myc and G1 cyclins, respectively: (1) c-Myc overexpression without rises in G1 cyclins, (2) loss of c-myc expression with overexpression of G1 cyclins, and (3) high expression levels of both c-Myc and G1 cyclins under the condition of a concomitant increase in TGFα. As described above, the latter two patterns are associated with a higher proliferative rate and a lower apoptotic rate, compared with the former one.

These three patterns of relationships among c-Myc, G1 cyclins, and growth factors provide one possible explanation for the dual prognostic values of c-myc overexpression. It seems that constantly high levels of c-Myc as seen in the major c-myc tumor areas tend to commit the cells to apoptosis, unless this trend is converted to that of proliferation by one of two conditions: (1) the c-Myc level drops to allow G1 cyclins to increase, as seen in the c-myc tumor foci, or (2) the level of TGFα (or other survival factor) is concomitantly increased to rescue G1 cyclins from suppression by c-Myc and to cause their induction. If a breast cancer manifests constant overexpression of c-Myc without concomitant increase in TGFα (or other survival/proliferation-promoting growth factors) or G1 cyclins, the tumor may undergo apoptosis and be more sensitive to apoptotic stimuli or to chemotherapy, which may be reflected in a better prognosis. Under this situation, antagonism of c-myc expression, such as by utilization of c-myc antisense oligonucleotides that has been proposed as a strategy for gene therapy of cancer, may actually prevent apoptosis of the tumor cells and may risk a rise in G1 cyclins and development of a more aggressive tumor phenotype. On the other hand, should the tumor show a high level of c-Myc with concomitant increase in growth factors or G1 cyclins, it may be more aggressive. In this latter case, neutralization of the cyclins and/or survival factors, alone or together with antagonism of expression of c-myc gene by factors such as antisense treatment, may be a more appropriate way to commit the cells to apoptosis. Since a cancer usually contains heterogeneous tumor cell populations, all these patterns of relationship among c-Myc, cyclins and growth factors are likely to exist concomitantly. Thus, the challenge is that one single therapeutic regimen may kill some cancer cells while allowing some other cells to evolve to more aggressive forms.

Special biological properties of the mammary gland

As already pointed out by many other investigators, reports on the roles of c-Myc in proliferation, apoptosis, differentiation, and growth of a cell are often conflicting. Part of the reason may be that the function of c-Myc is cell type-specific and dependent on experimental conditions. However, most of our understanding of the mechanisms for the functions of c-Myc derives from experiments with in vitro systems and with fibroblasts or cells of hemopoietic or lymphopoietic origins. These types of cells differ from epithelium in many respects.

There are, indeed, some studies on c-Myc using epithelial tissues that are not primary targets of sex steroids and which differ greatly from mammary gland, such as liver and kidney. Growth stimuli to liver can be divided into the compensatory type, which is associated with significant cell loss, and the non-compensatory type (Coni et al. 1993,
Columbano & Shinozuka 1996). In response to the compensatory-growth stimulus such as partial hepatectomy or treatment with necrogenic agents, hepatocytes quickly enter the cell cycle from the quiescent G0 phase, characterized by increased expression of immediate-early genes such as \textit{c-fos}, \textit{c-jun} and \textit{c-myc}. The cells proliferate to restore the normal number of hepatocytes and normal size of liver, followed by cessation of proliferation, probably triggered by contact-inhibition between hepatocytes. Non-compensatory growth stimuli such as treatment with phenobarbital, estrogen, growth factors, or many other agents that are not necrogenic to the liver, also induce hepatocytes to proliferate, leading to the enlargement of the liver. However, the proliferated cells tend to undergo apoptosis, especially after withdrawal of the growth stimulus, probably because the liver must reduce its size to normal. Ductal epithelial cells of the kidney normally rest at the G1 phase, and are refractory to various proliferating stimuli (Norman et al. 1988). Unirenalectomy, which causes a 50% loss of kidney tissue, mainly causes hypertrophy of the remnant kidney, i.e. growth in the cell size of the ductal epithelium, with little cell proliferation, although \textit{c-myc} and other immediate-early genes are activated by the operation (Norman et al. 1988).

Unlike liver, kidney, and other epithelium-dominant organs, the mammary gland is not fully developed until the females deliver and nurse their offspring. After weaning, most of the developed glands will undergo atrophy, mainly via apoptotic pathways. More unusually, the glands are embedded inside a fat pad before their growth during development or their induction by various stimuli. The meaning of this property may be much more profound than scientists have realized. For instance, the glands may proliferate freely in response to growth stimulation, extending into the fat pad with no strict size control, although there may still exist contact-inhibition among neighboring epithelial cells. If so, there may not exist the non-compensatory type of growth stimuli for the mammary gland; its proliferation may not be coupled with a tendency for apoptosis to limit the gland size, as may be the case in the liver. The apoptotic process after weaning may differ in its mechanism from the apoptosis of hepatocytes after withdrawal of non-compensatory stimulus. Moreover, the property of ‘growth within a fat pad’ may also make the stromal-epithelial interaction more involved than in other organs. Other unanswered questions regarding mammary gland biology include the determination of which cell cycle stage (G0 or G1) the majority of mammary epithelial cells normally rest. What is clear is that mammary gland epithelium has its distinct biological features; special precautions should be taken when applying knowledge from studies on other non-epithelial and epithelial systems to the mammary gland.

**Perspectives**

Data pertaining to several fundamental questions on the functions of \textit{c-Myc} are still very confusing. For instance, do \textit{c-Myc} proteins play a role in all early and advanced stages of the carcinogenic process? Are tumor cells in a cancer that express high levels of \textit{c-Myc} proteins more malignant or more benign? Can overexpression of \textit{c-Myc} in a spontaneously occurring tumor trigger an apoptotic process as can ectopically expressed \textit{c-Myc}? What does a cytoplasmic localization of \textit{c-Myc} protein suggest to us? Superficially, the answers seem to be that \textit{c-Myc} should be a nuclear protein, as it is a transcription factor, and that it should contribute to all stages of the carcinogenic process to make a normal cell progressively more malignant (Garte 1993), as it may cause genetic instability. Thus, a positive immunohistochemical staining should be observed in the nucleus of tumor cells and should reflect an unfavorable outcome.

However, data on the \textit{c-Myc} expression in various types of human malignancy and data from our transgenic models are often inconsistent with this line of inference. We now consider that, while the above inference may still be correct on many occasions, under certain circumstances \textit{c-Myc} protein may play a ‘hit-and-run’ game during the whole carcinogenic process. In these special, as yet undefined situations, \textit{c-Myc} may just contribute to the tumor onset and early growth. Once a tumor is formed and has grown to a certain size, high levels of \textit{c-Myc} may cause some additional effects that are unfavorable for the survival and continuous growth of the tumor. These effects may include the tendency to apoptosis or the high sensitivity to apoptotic factors, as well as the inhibition of VEGF, G1 cyclins, Her2/neu, etc. Accumulating experimental evidence is now suggesting that \textit{c-Myc} may be an important transcriptional repressor. Some of the genes suppressed by \textit{c-Myc} may be required for the survival or growth of the tumor cells in a hostile environment, such as the hypoxia that often occurs when a tumor becomes large and lacks a sufficient blood supply. Under these circumstances, certain \textit{c-Myc}-signaling pathways may be shut off. Examples of this are the attenuation of \textit{c-myc} expression and the prevention of its entry into the nucleus. These lines of thinking need further validation by experimental and clinical studies.

**References**


Liao and Dickson: c-Myc in breast cancer


Blyth K, Terry A, O’Hara M, Baxter EW, Campbell M, Stewart M, Donehower LA, Onions DE, Neil JC & Cameron ER 1995...


Liao and Dickson: c-Myc in breast cancer

Felsher DW & Bishop JM 1999 Transient excess of MYC activity can elicit genomic instability and tumorigenesis. *PNAS* 96 3940–3944.
Grad JM, Dai JL, Wu S & Burnstein KL 1999 Multiple androgen response elements and a Myc consensus site in the androgen receptor (AR) coding region are involved in androgen-mediated up-regulation of AR messenger RNA. *Molecular Endocrinology* 13 1896–1911.


Liao and Dickson: c-Myc in breast cancer


Musgrove EA, Lee CS & Sutherland RL 1991 Progestins both stimulate and inhibit breast cancer cell cycle progression while...
increasing expression of transforming growth factor alpha, epidermal growth factor receptor, c-fos, and c-myc genes. Molecular and Cellular Biology 11 5032–5043.


Perez-Rover I, Kim SH, Griffiths B, Sewing A & Land H 1999 Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). EMBO Journal 18 5310–5320.


Prall OW, Rogan EM, Muskrogue EA, Watts CK & Sutherland RL 1998 c-Myc or cyclin D1 mimics estrogen effects on cyclin E-Cdk2 activation and cell cycle reentry. Molecular and Cellular Biology 18 4499–4508.


Stewart TA, Pattengale KL & Peder L P 1984 Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MT/Myc fusion genes Cell 38 627–637.


Vos CB, ter Haar NT, Rosenblum C, Peterse JL, Cleton-Jansen AM, Cornelisse CJ & van de Vijver MJ 1999 Genetic alterations on chromosome 16 and 17 are important features of ductal carcinoma in situ of the breast and are associated with histologic type. British Journal of Cancer 81 1410–1418.


Watson JV, Stewart J, Cox H, Sikora K & Evan GI 1987b Flow cytometric quantitation of the c-myc oncoprotein in archival

www.endocrinology.org


Wu M, Bellas RE, Shen J, Yang W & Sonenshein GE 1999 Increased p27<sup>Kip1</sup> cyclin-dependent kinase inhibitor gene expression following anti-IgM treatment promotes apoptosis of WEHI 231 B cells. *Journal of Immunology* 163 6530–6535.


