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c-*myc* Oncogene Expression in Estrogen-Dependent and -Independent Breast Cancer

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We demonstrate that c-*myc* gene expression is essential for growth of breast cancer cells. It also plays an important role in the progression of human breast cancer. c-*myc* gene amplification may be important for cancer cell invasion, but perhaps not essential for nodal metastasis. We also provide compelling evidence that the c-*myc* oncogene is an estrogen target gene in hormone-responsive breast cancer. Hormonal progression of breast cancer could be brought about by the enhanced expression of the c-*myc* gene, with gene amplification and enhanced c-*myc* mRNA stability being two major mechanisms involved.

Tumor progression encompasses multiple changes including such characteristics as hormone dependence, growth rate, invasiveness, and metastatic potential (*1*). Our own efforts in the past decade have focused on the molecular basis of some of the above variables as they relate to the progression of human breast cancer. In particular, we have focused our attention on the possible role of the cellular oncogene c-*myc* in mediating the well-known tumor-promoting effects of the steroid estrogen. These studies have led us to hypothesize that c-*myc* plays a critical and central role in the progression of breast cancer from hormone dependency to hormone independency; the aberrant expression of c-*myc* may also play a role in the transition from in situ to invasive carcinoma.

c-*myc* is the cellular counterpart of the viral transforming gene v-*myc*. Its encoded protein *Myc* (two molecular forms, p64 and p67) is a member of the leucine-zipper class of nuclear transacting factors. The activity of *Myc* is thought to occur through binding to another nuclear protein, *Max* (c-*myc*-associated protein x) (*2*). Although its function is not known, *Myc* is believed to be an essential factor controlling cell proliferation.

The importance of c-*myc* in breast tumorigenesis was first demonstrated when transgenic mice overexpressing *Myc*, under the transcriptional control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) enhancer/promoter, developed mammary tumors after a relatively long latent period (*3*). Overexpression of the c-*myc* gene as a result of its translocation, mutation, or amplification has been implicated in the genesis and progression of a variety of human tumors, including Burkitt lymphoma (*4*), small cell carcinoma of the lung (*5*), leukemia (*6*), and breast cancer (*7*). An assessment of primary human breast tumor specimens found amplification of the c-*myc* gene in 17–33% of the patients (*7, 8*) a value similar to that for the amplification of c-*erbB-2* (*9*). In human breast cancer, c-*myc* amplification and increased expression are associated with high-grade tumors (*10*) and the most aggressive form of breast cancer, inflammatory carcinoma (*8*). Furthermore, according to one study, c-*myc* amplification is a better prognostic marker than is c-*erbB-2* (*11*). Thus, alteration in c-*myc* gene expression may contribute to the rapid progression of human breast tumors.

The above studies of c-*myc* amplification in breast cancer involved the use of primary tumor samples, which were often heterogeneous with respect to cellular and stromal elements. A given primary tumor frequently contains, in addition to various nontumor elements such as normal epithelial cells, a range of neoplastic elements. These elements are easily identified by pathological examination and include in situ carcinoma, consisting of cells still contained within the mammary ducts or lobules; invasive carcinoma, consisting of cells that have already escaped the original sites and have invaded the surrounding tissue elements; and metastatic carcinoma. Metastasis of breast cancer cells to regional lymph nodes is frequently found during initial diagnosis.

c-*myc* Amplification in Breast Cancer Cells

To gain insight into the role of c-*myc* gene amplification in breast tumor progression, we used a quantitative polymerase chain reaction (PCR) to measure the degree of c-*myc* amplification in cells from the various pathological stages of breast tumors from a given patient: normal epithelium, in situ carcinoma, invasive carcinoma, and lymph node metastasis. We first identified those tumors having c-*myc* amplification as determined by Southern analysis of total DNA extracted from frozen primary specimens. We observed c-*myc* gene amplification in ~10% of a relatively small number (100) of primary breast tumors. Next, we examined the paraffin-embedded pathology archival blocks corresponding to the frozen specimens with c-*myc* amplification. A thin histological section from each block was examined to identify and mark the boundaries of the various cell components. This examination was followed by the preparation of 20-μm-thick sections, which we microdissected to yield subregions containing the different cell components. DNA extracted from these microdissected regions were subjected to quantitative PCR. For each DNA sample, c-*mos*, a gene on the same arm of chromosome 8 as c-*myc* but which is not amplified in human breast cancer, was always co-amplified by PCR to serve as a control for DNA input. The c-*myc* gene copy number observed for a given DNA sample was standardized with

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respect to c-*mos*. This subregional analysis revealed a similar level of c-*myc* amplification in both in situ and invasive carcinoma. The unexpected finding was that c-*myc* amplification is infrequent (<50%) in lymph node metastases of the same patients. The quality of the DNA samples isolated from all the subregions was similar, as judged by their similar c-*mos* signals.

This preliminary study strongly suggests that c-*myc* amplification occurs before the invasion process and that in some tumors c-*myc* overexpression may be a prerequisite to tumor invasion. However, in these same tumors, infrequent occurrence of c-*myc* amplification in distal metastasis suggests that c-*myc* amplification does not confer an advantage to the metastatic phenotype in breast cancer. This observation does not preclude the possibility that the c-*myc* gene may still be overexpressed by other mechanisms in those metastatic cells lacking c-*myc* amplification. This latter hypothesis can be tested by immunohistochemical quantification of *Myc* or by subregional analysis for concentrations of c-*myc* mRNA by the reverse transcription-PCR protocol with fresh or frozen tumor specimens.

c-*myc* Expression and the Effects of Estrogen

We next studied the role of c-*myc* oncogene expression in the estrogen-induced growth of breast cancer and in the progression of breast cancer from a hormone-dependent to a hormone-independent, autonomous state. About 40% of breast cancer patients have estrogen-dependent tumors, tumors that require estrogen for growth. These patients benefit from hormonal therapy involving either the inhibition of estrogen action by antiestrogenic drugs such as tamoxifen or the inhibition of estrogen production. Endocrine therapy rarely achieves long-term effectiveness because most, if not all, estrogen-dependent tumors progress to an estrogen-independent state. As a prerequisite to understanding the molecular basis of hormonal progression, it is necessary to identify those genes that are activated early in response to estrogen.

In estrogen-target cells such as breast cells, estrogen binds to its receptor, a nuclear-transacting factor capable of activating a variety of target genes involved in cell replication. Tamoxifen, a synthetic antiestrogenic substance, competes with estrogen for the receptor site, forming an inactive DNA-binding complex. Our search for early estrogen-responsive genes in estrogen-responsive human breast cancer cell lines MCF-7 and T-47D utilized tamoxifen to arrest cell proliferation. Cell growth resumed when tamoxifen-arrested cells were treated with estrogen. Also, there was a rapid induction of the expression of the c-*myc* gene in the estrogen-treated cells (*12, 13*). In vitro nuclear run-on transcription assays revealed a major increase in c-*myc* gene transcription within 2–3 min of estradiol rescue, reaching a maximum of 11-fold induction of c-*myc* transcription by 20 min, followed by a gradual decline to ~3-fold above that of arrested cells by 1 h. c-*myc* mRNA accumulation revealed by Northern analysis followed a similar biphasic pattern, except that the maximum mRNA accumulation, 12-fold, was achieved at 1 h after estradiol rescue. The induction of the c-*myc* protein, revealed by Western immunoblot, compares favorably with that of the c-*myc* mRNA (*14*). Also, c-*myc* induction was specific for estradiol: several other steroid hormones tested had no effect on c-*myc* gene expression.

In contrast, estradiol did not induce the expression of several other cellular genes (e.g., those for actin and glyceraldehyde-3-phosphate dehydrogenase) and oncogenes including c-*H-ras* (*12*). c-*fos*, an estrogen-responsive gene in the rat uterus (*15*), was less sensitive to estrogen treatment in these human breast cancer cells, although >10-fold induction of c-*fos* by insulin was seen in these cells (Watson and Shiu, unpublished; *16*). Thus, c-*myc* is by far the most estrogen-sensitive growth-response gene in human breast cancer cells. Only the *pS2* gene exhibits similar estrogen sensitivity in MCF-7 cells; T-47D cells do not express the *pS2* gene (*17*). Unlike *pS2*, c-*myc* expression is inducible in all hormone-responsive human breast cancer cell lines so far tested. Thus, c-*myc* is probably the important gene whose expression mediates the mitogenic effect of estrogen in hormone-responsive breast cancer cells. Our recent transient expression studies with chimeric DNA constructs of 5′-flanking c-*myc* sequences ligated upstream of a reporter gene showed that the estrogen receptor is a transacting factor of the human c-*myc* gene (*18*).

Having established that the c-*myc* gene is a hormone-responsive gene in breast cancer, we next wanted to compare its expression in hormone-independent breast cancer cells. Such study could provide insight into the role that c-*myc* plays in the progression of breast cancer from hormone-dependency to hormone-independency. We compared and contrasted c-*myc* expression in the hormone-responsive MCF-7 human breast cancer cell lines and the hormone-insensitive, autonomous MDA-MB-231 line. The growth of the latter cells was totally insensitive to estrogen or antiestrogen; they grew with the same rate under these different conditions (*12, 13*). The pattern of c-*myc* expression in these two cell lines under various culture conditions mirrored that of cell growth (*12*). Thus, in the hormone-responsive MCF-7 cells, estradiol stimulated and tamoxifen inhibited accumulation of c-*myc* mRNA. In contrast, c-*myc* expression in the hormone-independent MDA-MB-231 cells was constitutively high; the amount of c-*myc* mRNA produced in these cells in the absence of estrogen was similar to that found in estrogen-stimulated MCF-7 cells. Neither estrogen or tamoxifen affected c-*myc* expression in the MDA-MB-231 cells. This observation supports the notion that the high c-*myc* expression confers the hormone-independent, autonomous growth behavior of the MDA-MB-231 cells.

What then is the mechanism responsible for the increased c-*myc* gene expression in the hormone-independent MDA-MB-231 cells, given that the c-*myc* gene in these cells is not amplified? An enhanced transcriptional rate and (or) an increased stability of c-*myc* mRNA could contribute to the increase in c-*myc* mRNA in the MDA-MB-231 cells. However, the c-*myc* gene

transcription rates for the two cell lines, as determined by nuclear run-on assays, were similarly low in the absence of estrogen (*13*). Thus, transcription cannot account for the enhanced amount of c-*myc* mRNA in the MDA-MB-231 cells.

The stability of the c-*myc* mRNA in the two cell lines was then compared (*13*). We used actinomycin D to arrest RNA synthesis and determined by Northern analysis the amounts of c-*myc* mRNA at various times after actinomycin D addition. In the estrogen-treated MCF-7 cells, the c-*myc* mRNA decayed with a half-life of ~20 min, a value similar to that reported for a variety of cell types and generally accepted as the "normal" half-life for c-*myc* mRNA. In contrast, the half-life of the c-*myc* mRNA in the hormone-independent MDA-MB-231 cells was close to 1 h, indicating that the c-*myc* mRNA in the hormone-independent cells was about three times as stable as that in the hormone-responsive cells and most other cells and tissues. This threefold increase in half-life is probably sufficient to account for the observed enhanced amounts of c-*myc* mRNA in the MDA-MB-231. Thus, we have described another novel mechanism by which c-*myc* gene expression can be enhanced in human breast cancer.

Mechanisms for Expression

There are at least three different mechanisms by which human breast cancer can achieve increased c-*myc* gene expression: transcription activation by estrogen, gene amplification, and increased mRNA stability. Hormone-responsive breast cancer cells, irrespective of their c-*myc* gene copy numbers, require estrogen for c-*myc* activation and growth. Hormone-independent breast cancer cells can achieve high c-*myc* gene expression through gene amplification, increased mRNA stability, or both. We have therefore proposed (*14*) that the hormonal progression of human breast cancer involves an alteration of c-*myc* gene expression and that enhanced c-*myc* expression may be sufficient to activate downstream cellular events necessary for cell growth.

If this hypothesis is correct, one would expect that cell growth would be arrested if c-*myc* gene expression alone is inhibited. We therefore selectively inhibited c-*myc* expression by the use of c-*myc* mRNA sequence-specific antisense phosphorothioate oligonucleotides (*14*). A 15-mer c-*myc* sequence-specific antisense phosphorothioate oligonucleotide at 10 μmol/L inhibited the estrogen-induced c-*myc* protein synthesis by >90% in MCF-7 cells. It was similarly effective in suppressing c-*myc* protein in the MDA-MB-231 cells. A 15-mer sense oligonucleotide used as the control was without effect on c-*myc* protein synthesis. As an additional control, we used a 15-mer *pS2* sequence-specific antisense phosphorothioate oligonucleotide, which blocked estrogen-induced *pS2* protein production by ~80%.

The c-*myc*-specific antisense oligonucleotide blocked not only the estrogen-induced cell growth in MCF-7 but also the autonomous growth of the hormone-insensitive MDA-MB-231 cells. Neither the c-*myc*-specific sense oligonucleotide nor the *pS2*-specific antisense oligonucleotide affected the growth of these cell lines. These results lend further support to the notion that c-*myc* expression alone is sufficient to provide the necessary growth stimulus to both hormone-responsive and non-responsive breast cancer cells. Because the growth of the MCF-7 cells was not impeded, despite the >80% blockade of pS2 synthesis by the *pS2*-specific antisense oligonucleotide, we conclude that *pS2* is not essential for growth of breast cancer.

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