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HORMONAL CARCINOGENESIS V

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Identification of Downstream Targets of Estrogen and c-myc in Breast Cancer Cells

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Summary Estrogen (E) plays a pivotal regulatory role in the control of cell proliferation in the normal breast and breast cancer (BC). To identify genes with likely roles in proliferation control that are regulated by E and its downstream target c-myc, we compared transcript profiles of antiestrogens-arrested cells stimulated to reinitiate cell cycle progression by E treatment or c-myc induction. Approximately 2/3 of the probe sets significantly regulated by E (adjusted $p < 0.01$) increased in expression. Half of the E-regulated probe sets were also regulated by c-myc. Genes involved in cell growth, cell proliferation, and cell survival were over-represented in the E-regulated geneset. Analysis of selected candidates has identified a nucleolar protein whose expression is correlated with c-myc expression in BC cell lines. These data indicate that a significant component of E-induced mitogenesis is mediated by c-myc and that selected c-myc target genes may be surrogate markers of c-myc expression in BC.

Introduction

The female sex steroid, estrogen (E), regulates cell proliferation and cell survival in breast epithelial cells and breast cancer (BC). The early events in E-induced mitogenesis include the transcriptional activation of two proto-oncogenes, c-myc and cyclin D1, the expression of which is rate limiting for progression through the G1 to S phase transition of the cell cycle (1). Inducible expression of either c-myc or cyclin D1 can activate cyclin E/cdk2 and over-ride anti-estrogen-induced growth arrest, mimicking the effects of E treatment (2). This has led to the hypothesis that E induces cell cycle progression through two initially independent pathways, one downstream of c-myc and the other involving cyclin D1. However, comparison of publicly available databases (3, 4) reveals that only 20% of the known E target genes are also known targets of c-myc. Thus, recent work in this laboratory has focused on delineating the pathways downstream of c-myc in BC cells and determining their role both in E-induced mitogenesis and in BC.

Overexpression of c-myc occurs at high frequency in both estrogen receptor $(ER\alpha)^+$ and $ER\alpha^-$ BC (5, 6). The rapid decrease in c-myc expression following

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anti-estrogen treatment, and the ability of an antisense oligonucleotide-mediated decrease in c-myc expression to recapitulate many of the effects of anti-estrogen treatment (7), imply that c-myc overexpression could modulate sensitivity to endocrine therapies. Support for this concept is provided by data from laboratories, including our own, demonstrating that inducible or constitutive overexpression of c-myc in E-responsive MCF-7 cells confers resistance to tamoxifen and ICI 182780 in vitro [(5, 8, 9), McNeil et al. in preparation].

The evidence for a pivotal role for c-myc in E-induced cell proliferation and the possibility that this might be associated with endocrine resistance raises the question of which downstream targets of c-myc might be required for these effects. Our goal in this work was to identify those acutely E-regulated genes that are also downstream targets of c-myc, using an experimental model designed to enhance sensitivity to the mitogenic effects of E.

Results

Experimental Model and Transcript Profiling. To study the effects of E and c-myc on cell proliferation, we have utilized the well-characterized E-responsive BC cell line MCF-7. A series of clonal MCF-7 cell lines was developed that expressed wild-type c-myc or c-Zip (a deletion mutant lacking the transactivation domain) under the control of the zinc-inducible metallothionein promoter (Fig. 1). Representative clones with E and antiestrogen responses matched to those of the parental MCF-7 cells were chosen for further experiments. Zinc treatment resulted in increased c-myc or c-Zip expression within 3 h, similar to the timing of E induction of c-myc and consistent with our previous data using this zinc-inducible construct (2). The majority of the cell population synchronously reinitiated cell cycle progression following E treatment. Induction of c-myc also led to reinitiation of cell cycle progression to a degree comparable with the effects of E. However, although cells transfected with c-Zip could respond to E treatment, c-Zip induction did not lead to cell cycle progression (Fig. 1).

RNA for transcript profiling was collected 6 h after E or zinc treatment, within the time-frame during which critical E-dependent events necessary for S phase entry occur (10). Transcript profiling was undertaken in triplicate following E treatment (compared with vehicle treatment) and c-myc or c-Zip induction (compared with zinc-treated empty vector cells), using Affymetrix HG-U133 plus 2.0 arrays. Analysis of the microarray data used Bayesian linear modeling methods in the limma package and the Benjamini and Yuketieli adjustment was applied for multiple-hypothesis comparisons (11, 12). Probe sets that were significantly up- or down-regulated following E treatment compared with vehicle-treated cells were identified (adjusted $p < 0.01$, Fig. 1 and Table 1).

These E-regulated probe sets were further divided into those that were significantly regulated following c-myc but not c-Zip induction, designated "E2 and myc," and the remainder, which we designated "E2 not myc." Approximately

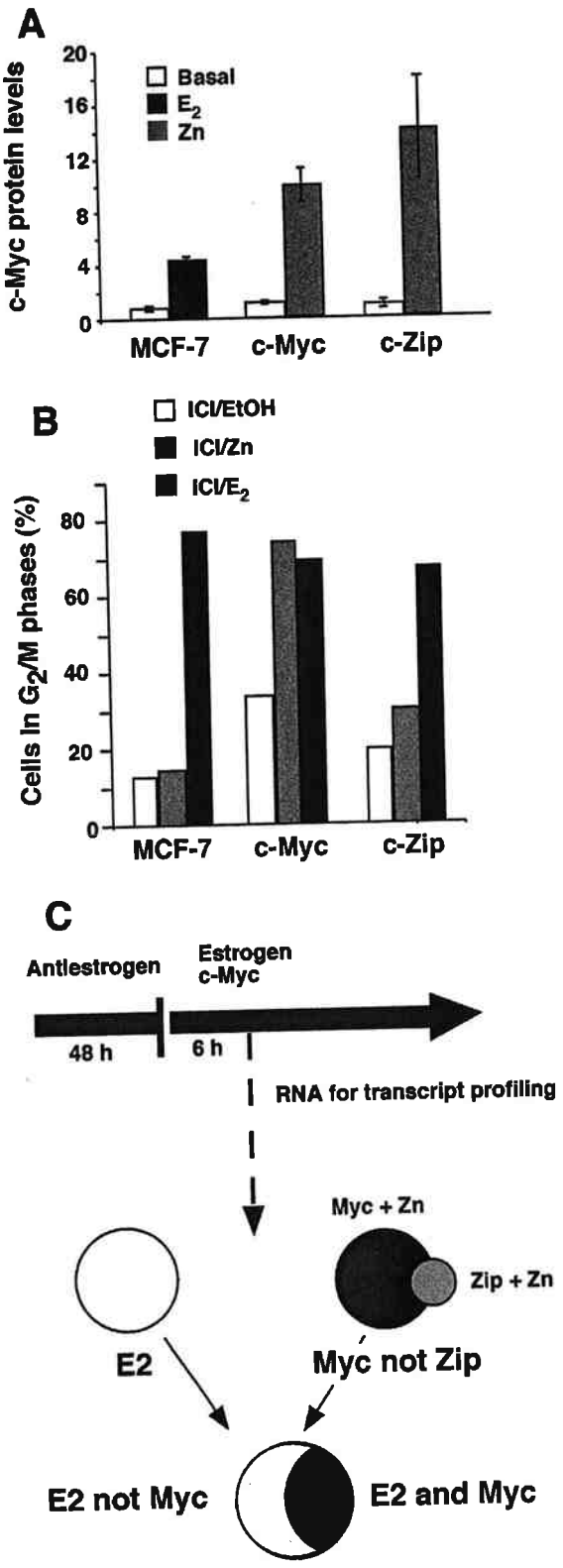

Fig. 1 Experimental model for identifying E-regulated c-myc target genes in BC cells. MCF-7 cells inducibly expressing c-myc (wild type or c-Zip), parental cells, and cells bearing the empty vector were pretreated with 10 nM ICI 182,780 for 48 h. Parental cells were then treated with E (100 nM 17β-estradiol) or vehicle (ethanol), and cells transfected with c-myc, c-Zip or the empty vector were treated with zinc (65 μM). (**A**) Quantitation of Western blots 6 h after E treatment or c-myc-induction in representative MCF-7 clones. (**B**) Cells were additionally treated with nocodazole to block E- or c-myc-stimulated cells in G2/M. Cell cycle phase distribution was determined after 36 h E treatment using flow cytometry. (**C**) RNA was prepared from cells harvested 6 h following treatment for transcript profiling. The E-regulated probe sets were compared with those regulated by c-myc but not c-Zip to identify probe sets regulated by both E and c-myc (but not c-Zip), or by E but not c-myc or c-Zip

two-thirds of the E-regulated genes were upregulated and in total, half of the probe sets significantly regulated by E in this model system were also significantly regulated by c-myc.

Table 1 Proportion of probe sets identified as E-regulated that are known to be E-responsive or targets of c-myc

		# of probe sets	% in ERGDB[a]	% in *myc* database[b]
Up	E_2	635	188 (29%)	172 (27%)
	E_2 not *myc*	303	118 (39%)	43 (14%)
	E_2 and *myc*	332	70 (21%)	129 (39%)
Down	E_2	303	42 (14%)	37 (12%)
	E_2 not *myc*	166	27 (16%)	22 (13%)
	E_2 and *myc*	137	15 (11%)	15 (11%)

[a]http://defiant.i2r.a-star.edu.sg/projects/Ergdb-v2/index.htm (3)
[b]http://www.myc-cancer-gene.org (4)

Publicly available databases of E-responsive genes (*ERGDB*) (3) and c-myc targets (4) were searched to determine what proportion of the probe sets in each category had been previously identified as either E- or c-myc-regulated. This analysis revealed that overall 29% of probe sets significantly upregulated by E were present in the ERGDB, and 27% of those that were also significantly upregulated by c-myc induction were present in the myc target gene database (Table 1). However, relatively few of the probe sets down-regulated in this experimental model were previously-described E or c-myc targets (12–14%, Table 1). The probe sets from the "E_2 not myc" category that increased in expression had the highest proportion of previously-documented E targets (39%, Table 1). Within the E-upregulated probe sets, 118/188 (63%) of the known E targets were regulated by E but not c-myc, consistent with our initial premise that genes regulated by both E and c-myc have been under-represented in previous studies. Although the latter category contains relatively few previously identified E targets, it likely contains a significant number of bona fide targets since a high proportion of the probe sets in the "E_2 and myc" category that increased in expression were present in the myc target gene database (39%).

To identify biological processes that were significantly over-represented in the probe sets regulated by E, we used the data-mining tool Onto-Express (http://vortex.cs.wayne.edu/index.htm). The functions were essentially all related to either cell growth and proliferation or apoptosis. Bioinformatics analysis to identify specific aspects of these processes that may be regulated by both E and c-myc or by E alone is ongoing.

Analysis of Selected Candidates Arising from Transcript Profiling. Sixteen of the most highly-regulated genes in the transcript profiling experiments were examined using semiquantitative RT-PCR to document the time-course of regulation by E and c-myc. All but one displayed regulation consistent with the transcript profiling experiment. One gene that was regulated by both E and c-myc has undergone more detailed characterization and was designated E- and c-myc-upregulated (EMU4). E treatment or c-myc induction both led to increased EMU4 mRNA abundance within 3 h, and this was followed by increased protein abundance. Chromatin immunoprecipitation experiments demonstrated the recruitment of c-myc to the EMU4

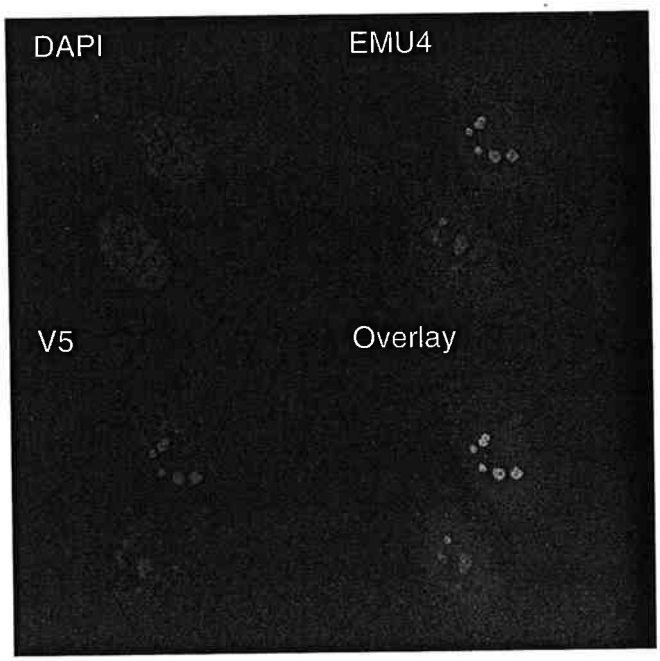

Fig. 2 Localization of EMU4 protein. MCF-7 cells constitutively expressing V5-tagged EMU4 were paraformaldehyde-fixed, permeabilized with Triton X100, and immunostained. The cells were counterstained with the DNA-specific dye DAPI (4,6-diamidino-2-phenylindole) to visualize the nuclei

promoter in response to E treatment (5), suggesting that EMU4 is a direct target of c-myc and that this accounts for its regulation by E. In BC cell lines, EMU4 expression was correlated with c-myc expression (5), (Butt et al. in preparation) and preliminary analysis suggests that expression of these two genes is also correlated in BC specimens.

The EMU4 protein is well conserved in mammals but less well-conserved in flies and worms, with no obvious yeast homologues, and is of unknown function. To gain some insight into possible functions for this protein, we used confocal microscopy to determine the subcellular localization of V5-tagged EMU4 protein. This revealed prominent EMU4 immunoreactivity in the nucleolus, with only faint staining apparent elsewhere in the nucleus (Fig. 2). Since many proteins involved in ribosome biogenesis and cell cycle control are localized to the nucleolus, ongoing experiments are aimed at determining whether EMU4 plays a role in these processes.

Discussion

To better understand the effects of E on BC cells, we have developed an experimental model that is particularly sensitive to E regulation of cell proliferation. Transcript profiling at an early time-point during E stimulation of cell cycle progression has revealed many potential targets for further functional characterization

and determination of their putative roles in E action. Although some are known E targets, the majority are not. In particular, by focusing on the overlap between E-regulated targets and those downstream of c-myc, we have been able to substantially extend the list of genes regulated by both E and c-myc in BC cells. Although increasing data from various cell culture models argues for an important role for c-myc in E-stimulated cell cycle progression, the proportion of genes regulated by E that are also regulated by c-myc is unexpectedly high. It will be of particular interest to determine the roles of these genes in E action, for example by determining which are essential for E-induced mitogenesis or cell survival.

One approach to dissecting these pathways is to undertake functional characterization of selected targets. Our characterization of one novel E- and c-myc-target, EMU4, has revealed it to be a direct c-myc target. The importance of c-Myc in determining EMU4 expression is illustrated by the relationship between c-Myc and EMU4 expression (5), suggesting that irrespective of its role in c-myc action, EMU4 may be a surrogate marker of c-myc expression. There have been surprisingly few studies examining c-myc expression and its relationship to clinicopathological parameters including outcome in BC, probably because of technical difficulties in measuring c-myc in clinical specimens. Thus, any validated surrogate markers of c-myc expression or activity that can be readily assessed in pathological specimens may have particular utility in clinical applications.

Although the approach of analyzing individual genes is expected to yield important novel information that enhances our understanding of E action, it is increasingly apparent that complementary studies using a more "systems biology" approach may be required to give an overall picture of the complexity of the processes regulated by this important physiological hormone. Contemporary bioinformatics tools offer a starting point for such analyses and our initial studies using such tools indicate that, as previously documented, E has major effects on cell proliferation and cell death. A more unexpected finding was the close association of E and c-myc in the regulation of several nucleolar proteins with roles in ribosome biogenesis. The role of these target genes in E-induced RNA and protein synthesis is currently under investigation.

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