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13. ABSTRACT ***(Maximum 200 Words)***

c-Myc has been implicated in breast cancer as the oncoprotein is overproduced in nearly 80% of the breast cancer cells. c-Myc constitutes a transcription factor, modulating transcription of cell cycle-related target genes, and facilitating cell cycle progression. Deregulated c-Myc also promotes genomic instability with unknown mechanism(s). Genomic instability has been implicated as a driving force for the tumorigenesis. For the creation of permissive conditions for the gene amplification, a form of genomic instability, the abrogation of cell-cycle checkpoint controls is recognized as a prerequisite. Checkpoint control arrests cells with DNA damage, such as broken chromosomes, that are important intermediates in gene amplification. The focus of the current study is investigating if c-Myc abrogate the checkpoint to DNA damage, creating the permissive conditions for gene amplification. With a carefully controlled set of cell lines, established by retroviral transfection of c-Myc and c-MycS (N-terminally truncated c-Myc), we identified that both Myc-Box I and II are required to alter the checkpoint at the G1/S boundary. This should help finding a novel target for the prevention of the gene amplification and arrest of proliferation of tumor cells with the phenotype. The current research provides me an invaluable experience for both conceptual and technical training.

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J. Shaw 7/20/2000

PI - Signature Date

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For Annual Report Grant #DAMD17-99-1-9205
"A Gene Amplification Phenotype in c-Myc-induced Mammary Tumor Cells"

This Annual Summary Report addresses Grant # DAMD17-99-1-9205, a Pre-Doctoral Training Fellowship, covering research conducted by the principal investigator Joon-Ho Sheen (an Ph.D. student at the Lombardi Cancer Center, Georgetown University Medical Center), entitled "A Gene Amplification Phenotype in c-Myc-Induced Mammary Tumor Cells."

INTRODUCTION:

The *c-myc* oncogene is particularly important for the breast cancer, since the oncogene *per se* is amplified in the approximately 30% of the human breast tumor cases (Bonilla et al., 1988; Escot et al., 1986). Furthermore, the c-Myc oncoprotein is overproduced or deregulated in nearly 80% of the disease. Therefore, it suggests that the importance of the oncogene in the genesis and/or progression of breast cancer. The oncoprotein c-Myc is a 439 amino acid protein, constituting nuclear transcription factor that interacts with DNA, when heterodimerized with the partner MAX. This heterodimerization, through the interaction of c-terminal leucine zipper and basic helix-loop-helix motifs, is required for c-Myc-mediated cell cycle progression, cell growth, cellular transformation, and apoptosis (Dang et al., 1989). However, in addition to those already established roles of c-Myc, recent studies have suggested novel role of the oncoprotein, a c-Myc-induced genomic instability phenotype. The development of the malignant breast cancer phenotype is thought to depend upon the stepwise accumulation of genetic changes, as is the case in other types of tumors (Nowell, 1976; Vogelstein and Kinzler, 1993). The genomic instability in the tumor cells has been proposed and investigated as a driving force for the accumulation of necessary genetic changes (Loeb, 1991; Tlsty et al., 1995). Therefore, understanding the mechanisms of genomic instability phenotype would provide important insights into the progression of breast cancer and into potential ways to prevent it. Specially, genomic instability phenotype at the level of gene amplification has been implicated in the development of drug resistance and metastatic potential (Lucke-Huhle, 1994). Since the major causes of morbidity of breast tumors are metastasis and drug resistance, it is crucial to know how normal mammary epithelial cells acquire the gene amplification phenotype for the prevention and the treatment of these malignant phenotypes. Gene amplification is a complex mechanical process, including chromosomal breakage, losses of chromosome region, and gains of chromosome region (Stark, 1993). A widely accepted explanation for the gene amplification phenotype is based on the altered cell cycle, resulting from the abrogation of a cell cycle checkpoint(s) (Hartwell, 1992; Hartwell et al., 1994; Paulovich et al., 1997). Checkpoint control is a feedback signaling for the ordered cell cycle progression, insuring the completion of one process prior to initiating a downstream process (Hartwell, 1992; Hartwell et al., 1994; Paulovich et al., 1997). Mutations of the feedback-signaling pathway will therefore predispose for the disarray of the cell cycle. Among the various checkpoints, the DNA-damage checkpoint has been implicated crucially in the prevention of gene amplification (Ishizaka et al., 1995; Wahl et al., 1997; Wright et al., 1990). Failure in detecting various genomic alterations and in the subsequent arrest of cells at the checkpoint could create permissive conditions for the accumulation of genetic changes, such as broken chromosomes. Furthermore, the inappropriate entry of damaged DNA into S-phase eventually amplifies DNA damage such as the double-strand DNA breakage during unprepared DNA replication (Almasan et al., 1995). Broken chromosomes are involved in the gene amplification phenotype as a central intermediate, gene amplification could not occur in normal cells with intact checkpoints. Moreover, broken chromosomes resulting from the double-strand DNA breakage, possibly induced by ionizing radiation and/or DNA-damaging chemotherapeutic drugs, have been by far the most effective inducers for the gene amplification process (Smith et al., 1992; Smith et al., 1995; Windle et al., 1991). However, again, in normal cells with intact checkpoint controls, broken chromosomes are monitored and eliminated. Cells with DNA damage are arrested and destined to undergo apoptosis. This checkpoint detects even a single double-strand break and permanently arrests cells containing DNA damage. Therefore, abrogation of checkpoint controls is a prerequisite for a gene amplification phenotype (Di Leonardo et al., 1993). Interestingly, my preliminary study with the c-Myc-induced mouse mammary carcinoma cells indicates an increased frequency of *CAD* gene amplification as well as alterations of several checkpoint controls. I thus proposed that deregulated c-Myc creates permissive conditions for the gene amplification phenotype through bypassing cell cycle checkpoints. Of protein structure of c-Myc, the oncoprotein has highly conserved domains among the species, so called MB-I (Myc-Box I) and MB-II (Myc-Box II), in its N-terminal transactivation

region. Furthermore, previous studies showed that both MB-I and MB-II are required for the c-Myc-induced transactivation. However, only MB-II is required for the c-Myc-induced repression. Interestingly, a naturally truncated form of c-Myc, named c-MycS, has only MB-II in the transactivation region since its translation starts at the internal AUG initiation codon, located between MB-I and MB-II domain (Spotts et al., 1997; Xiao et al., 1998). Therefore, we may dissect the c-Myc-induced checkpoint alteration phenotype whether c-Myc-induced transactivation or c-Myc-induced transrepression mediates it. Currently, I am studying the alteration of cell cycle checkpoint control at the cellular and molecular levels in human mammary epithelial cells (HMEC) with human c-*myc* and c-*mycS* constructs. Genomic instability phenotype has been implicated in tumor initiation as well as in the malignant progression to more advanced malignancies. Therefore, this study would provide useful information about the development of mammary tumors induced by deregulated c-Myc. Molecular detection of an alteration in a specific tumor-related gene, such as c-*myc*, may be used to diagnose the pre-malignant cells with the potential to develop the malignancy. This pre-doctoral research project therefore provides me with an invaluable opportunity to think seriously about how a specific oncogene promotes tumor progression in the mammary tissues at the molecular level. This knowledge will be used to uncover novel targets for the prevention of the mammary tumor progression by inhibiting the genomic instability phenotype. The proposed study also allows me to learn cellular and molecular genetic research tools such as the recombinant DNA technology, retroviral transfection for cell line establishment, and flow cytometry. These conceptual and technical advances will lead me to a Ph.D. degree and provide me an invaluable background experience for my future as an independent scientist focusing on breast tumorigenesis. Finally, this proposed study has been preparing me for this goal in the excellent environment of the Lombardi Cancer Center, an NCI-designated Comprehensive Cancer Center.

BODY OF SUMMARY REPORT:

This Annual Summary Report of Training and Research Accomplishments covers the first year (the period between July 1, 1999 and June 30, 2000) of 3 year pre-doctoral training grant # DAMD17-99-1-9205 for Joon-Ho Sheen. Please note that a revised statement of work will be presented within this summary discussion.

A revised specific aim #1 is now requested to emphasize more the study of c-Myc-induced checkpoint alteration (original specific aim #1D). We believe that the study of possible alteration(s) of the DNA damage-dependent checkpoint(s) by c-Myc is very important and further study is urgently needed to elucidate the fundamental molecular mechanism(s) for gene amplification phenotype. Furthermore, to address directly the role of c-Myc in the background of human mammary epithelial cells (HMEC), we established an experimental system consisting of a set of HMECs transfected with human c-*myc* and c-*mycS* constructs.

I. Original Specific Aim #1: To characterize a gene amplification phenotype in c-Myc-induced tumor cells *(Months 1-18)*.

A. To measure the prevalence of the drug resistant cells in c-Myc-induced mammary carcinoma cells.
B. To determine whether drug resistant, tumor cell subclones have the amplification of specific indicator genes.
C. To determine whether the gene amplification phenotype in c-Myc-induced mammary carcinoma cell is chromosomal locus-specific.
D. To characterize the altered cell cycle checkpoint controls in c-Myc-induced mammary carcinoma cells.

I. Revised Specific Aim #1: To characterize a c-Myc-induced checkpoint alteration at G1/S in Human Mammary Epithelial Cells (HMEC) *(Months 1-18)*.

A. To establish an experimental system consisting of a set of HMECs transfected with c-Myc and c-MycS.
B. To characterize the cell cycle checkpoint controls at G1/S boundary responding to γ-irradiation-induced DNA damage in the above HMEC system through the flow cytometric analysis.

Training and Research Accomplishments for Specific Aim #1:

As a first step to investigate c-Myc-induced DNA damage-dependent checkpoint control, we investigated the response of an isogenic pair of HMEC lines, 184A1N4 (hereafter termed A1N4, a human non-transformed mammary epithelial cell) and A1N4-Myc (a human mammary epithelial cell overexpressing c-Myc), to the DNA damage induced by γ-irradiation. The reason for changing our DNA damaging agents from chemotherapeutic drugs to γ-irradiation is that the ionizing radiation could provide more clearly defined effects of inducing DNA strand breaks. Unlike γ-irradiation, chemotherapeutic drugs, such as Adriamycin, provides the broad side effects, including the inhibition of RNA transcriptions, besides the DNA strand breaks. It is important for this project to use a more clearly defined DNA damaging agent for the precise interpretation of the results. The altered response to DNA damage has been identified only in A1N4-Myc cell **(Figure 1)**. In contrast, A1N4 control cells were arrested in response to the DNA damage induced by γ-irradiation. A1N4-Myc has a deregulated murine c-Myc, ectopically expressed through the MMLV (moloney mouse leukemia virus)-LTR promoter. The degree of overexpression has been previously determined as 3-4 fold. Since both parental A1N4 and A1N4-Myc cells are easily synchronized at G0/G1 phase by an EGF (Epithermal Growth Factor) withdrawal technique, we could determine the response of G1-synchronized cells to the DNA damage induced by ionizing γ-irradiation. First, EGF was removed from the culture medium for 72 hrs. After overnight plating for the attachment, the culture medium was changed with the medium without EGF. Following EGF withdrawal, cells were released by refreshing with complete medium containing EGF. Then, after initial 3 hrs of recovery for re-entering the cell cycle, cells were then irradiated with dosage of 12 or 24 Gray (1 Gray = 100 rad). Samples for flow cytometry were prepared at 24hr post-IR (Ionizing Radiation) according to the standard FACS protocol. The time-point of 24hr has been selected to cover roughly one cell cycle period of A1N4 and

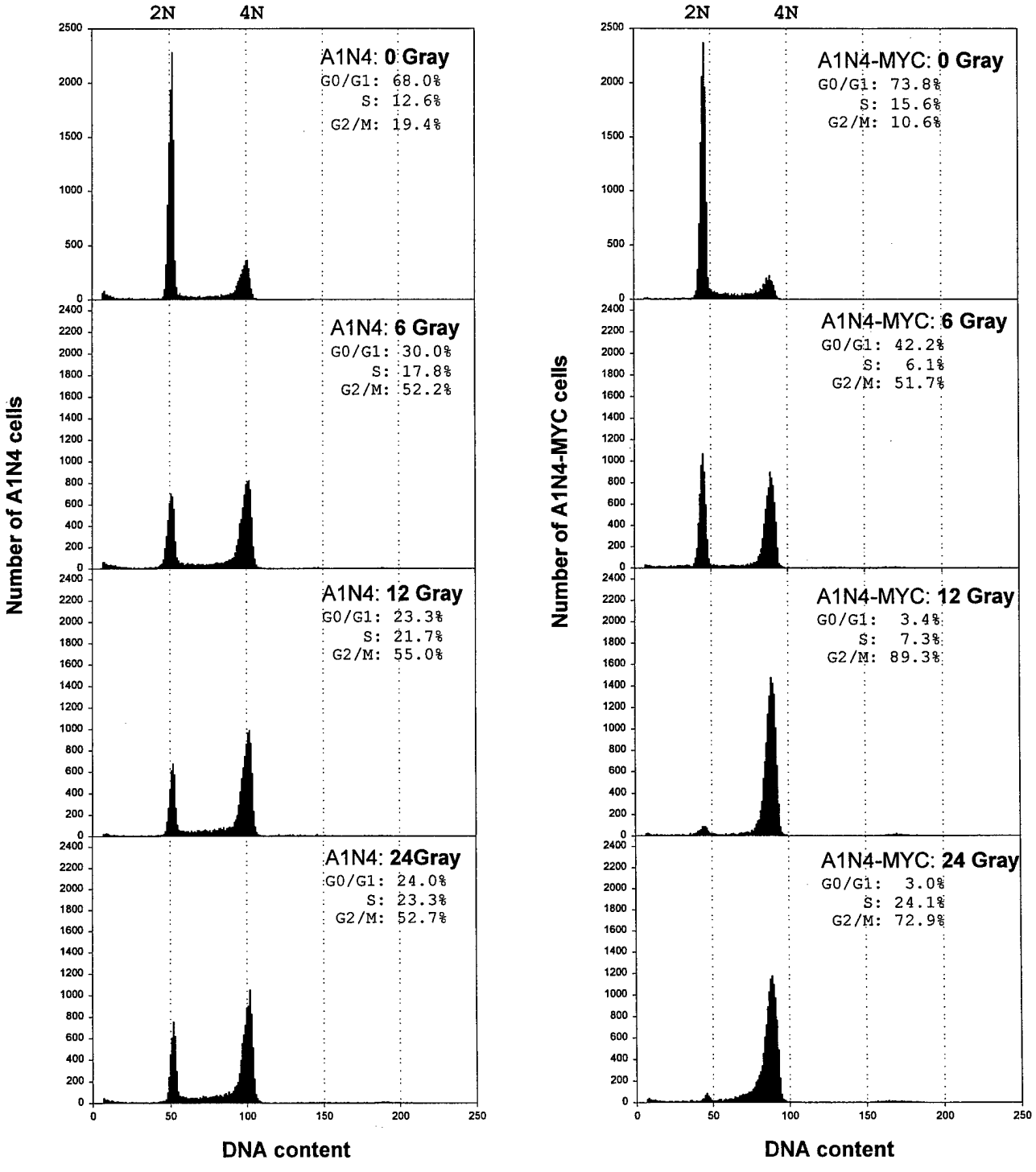
A1N4-Myc. After 24 hrs of release, both non-irradiated A1N4 and A1N4-Myc showed cell cycle progression and a gradual loss of synchrony. γ-Irradiated A1N4 cells were clearly arrested at G0/G1 (~23% of population arrested) and G2/M phase irrespective of irradiation with 12 Gray or 24 Gray. However, γ-irradiated A1N4-Myc cells were clearly not arrested at G0/G1 phase (~3% of population arrested) and most were arrested at G2/M phase. This result clearly indicates that A1N4-Myc cells have an abrogated DNA damage-dependent G1/S checkpoint.

To investigate further the G1/S arrest in the background of independently derived HMECs, we tested the response of MCF10A HMECs to the γ-irradiation-induced DNA damage. As expected, MCF10A cells were easily arrested at both G1 and G2/M phase with dramatically reduced S-phase fraction (from 22.9% to 1-3%) after the 4-12 Gray of γ-irradiation **(Figure 2)**. Based on this predicted wild-type response to the DNA damage of MCF10A cells, we established a set of MCF10A cells retrovirally transfected with human c-*myc* and c-*mycS* constructs (Miller and Rosman, 1989). After receiving a human c-*myc* cDNA construct (a generous gift from Drs. M. Stampfer and P. Yaswen at The Lawrence Berkeley National Laboratory, Berkeley, CA), a c-*mycS* construct has been created by deleting an *Eco*V fragment of 5' end of the original human c-*myc* cDNA with a restriction endonuclease. This partial restriction digestion deletes the first and second translation initiation codons of c-Myc **(Figure 3)** and leaves the third internal translation initiation codon for the initiation of protein translation. As a result, full-length c-Myc (64 Kd.) has both Myc-Box I (MB-I) and Myc-Box II (MB-II) and c-MycS (46Kd.) has only Myc-Box II. As mentioned in the introduction, both MB-I and MB-II are required for the c-Myc-induced transactivation. However, only MB-II is required for the c-Myc-induced repression. Therefore, we could dissect the underlying molecular mechanism(s) of c-Myc-induced checkpoint alteration by using these constructs. Since we used human cDNA to transfect HMECs, it is nearly impossible to distinguish foreign human c-Myc from the endogenous human c-Myc in HMEC lines. Although the endogenous expression of human c-Myc is tightly regulated upon the cell cycle progression, it is also high in expression in exponentially growing cells. Therefore, to check the exogenous expression of human c-Myc, we also transfect the mouse embryonic fibroblast line, NIH3T3 cells, with human c-*myc* and c-*mycS* cDNA. With the availability of anti-human c-Myc monoclonal antibody (clone #9E10), we could detect the expression of our transfected genes in MCF10A cells and NIH3T3 cells **(Figure 4)**. Obviously, in NIH3T3-c-Myc and NIH3T3-c-MycS cells, anti-human c-Myc antibody detects only exogenous human c-Myc and c-MycS, not endogenous mouse c-Myc. Although the level of c-Myc is not dramatically high in MCF10A-c-Myc cells, c-Myc has been known to have negative feedback mechanism for its expression. When its expression is high, it represses its own transcription. Therefore, we interpreted that the majority of signal is coming from expression of the exogenous c-*myc*. This interpretation is consistent in the MCF10A-c-MycS cells. In the equally loaded samples, the endogenous level of c-Myc is inhibited by the exogenous c-MycS expression when it is compared to the level of c-Myc in MCF10A-LXSN, a vector-only transfected MCF10A HMECs. Again, c-MycS, an internally initiated, N-terminal truncated c-Myc, has been studied showing that it has only the capacity of transcription repression. Therefore, one purpose of using c-MycS is to dissect the molecular mechanism of c-Myc-induced checkpoint alteration. By using this c-MycS, "a repression-only c-Myc", we wanted to study if c-Myc-induced checkpoint alteration is mediated by c-Myc-induced trans-activation of it downstream targets or if c-Myc-induced repression of target genes is sufficient for. In addition to c-MycS, recently we added a positive control for the altered G1/S checkpoint in HMECs. We transfected p53DD (a C-terminal fragment of p53 protein that is acting dominant negative; the cDNA was kindly provided by Dr. M. Oren, Weizmann Institute, Israel) into MCF10A cells. The role of p53 in the DNA damage-dependent checkpoint control has been well established. Therefore, we wanted to know the response of HMECs without p53 function to the γ-irradiation-induced DNA damage. The flow cytometry result is quite interesting, although the degree of checkpoint abrogation by c-Myc is less than the effect of p53DD **(Figure 5)**. After the 12 Gray of γ-irradiation-induced DNA damage, both MCF10A-LXSN and MCF10A-MycS showed a dramatic decrease (from 26% to ~5%) in S-phase fraction. In contrast, MCF10A-Myc cells have a ~16% of cells in S-phase fraction and MCF10A-p53DD cells have a ~21% of cells in S-phase fraction. Furthermore, it is clearly demonstrated that the full-length c-Myc is required for alteration of the G1/S checkpoint. Therefore, we concluded that both MB-I and MB-II are required and possibly c-Myc-induced transactivation of some target genes is essential, for the checkpoint alteration phenotype.

Synchronized
A1N4, A1N4-MYC.

Figure 1.
Army report.

Dose-dependent effects of γ-irradiation on the cell cycle of c-Myc-overexpressing HMEC



The effects of c-Myc on DNA-damage-dependent cell cycle checkpoint control in HMEC: time-course study using G0/G1-synchronized A1N4 & A1N4-MYC cells

72hr -EGF synchrnized at G0/G1
3hr +EGF recovery/ γ-irradiation with 0-24Gray
24hr +EGF post-irradiation cell cycle progress

Unsynchro MCF10A.

Figure 2.
Army report

99-09-16 FACS/
Response of MCF10A to γ-irradiation

The response of MCF10A cells to DNA damage induced by γ-irradiation

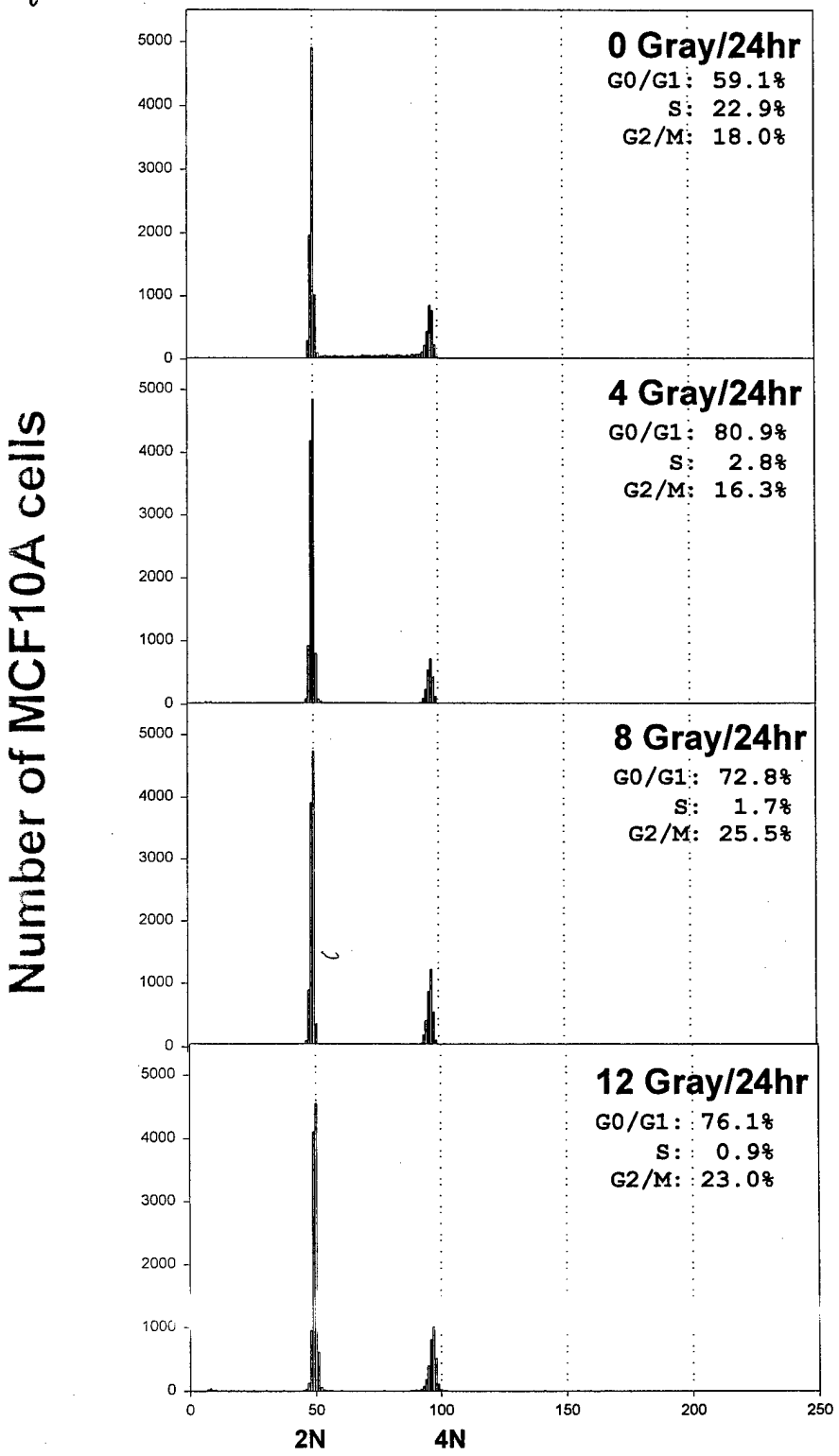
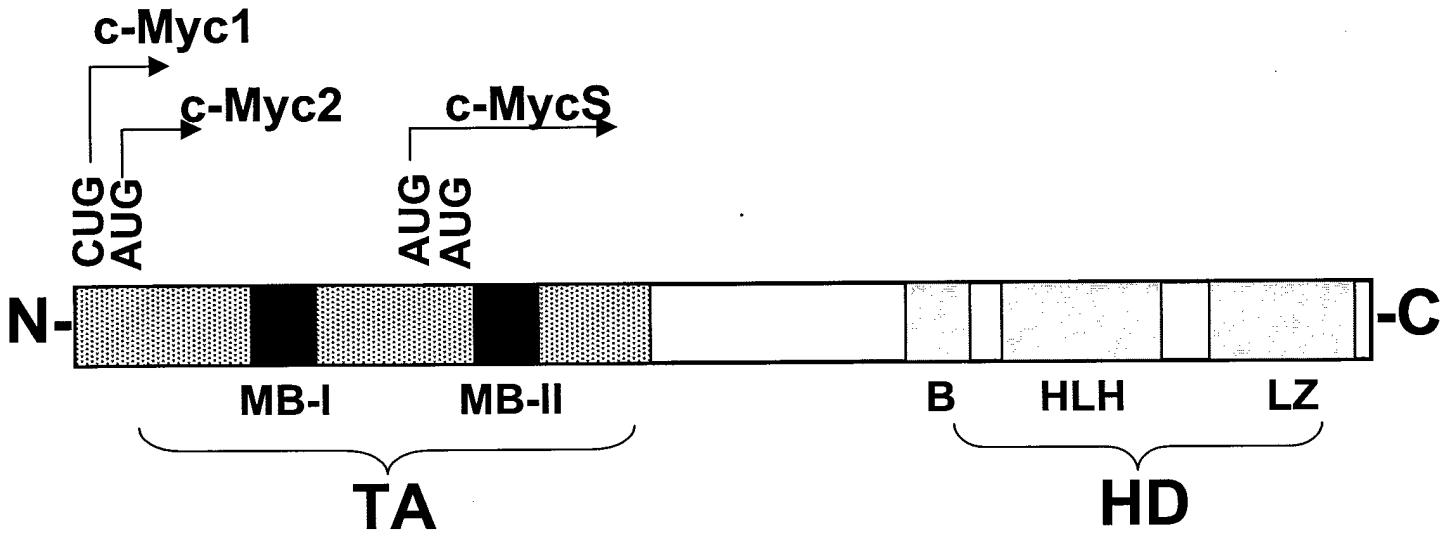


Figure 3.
Army report

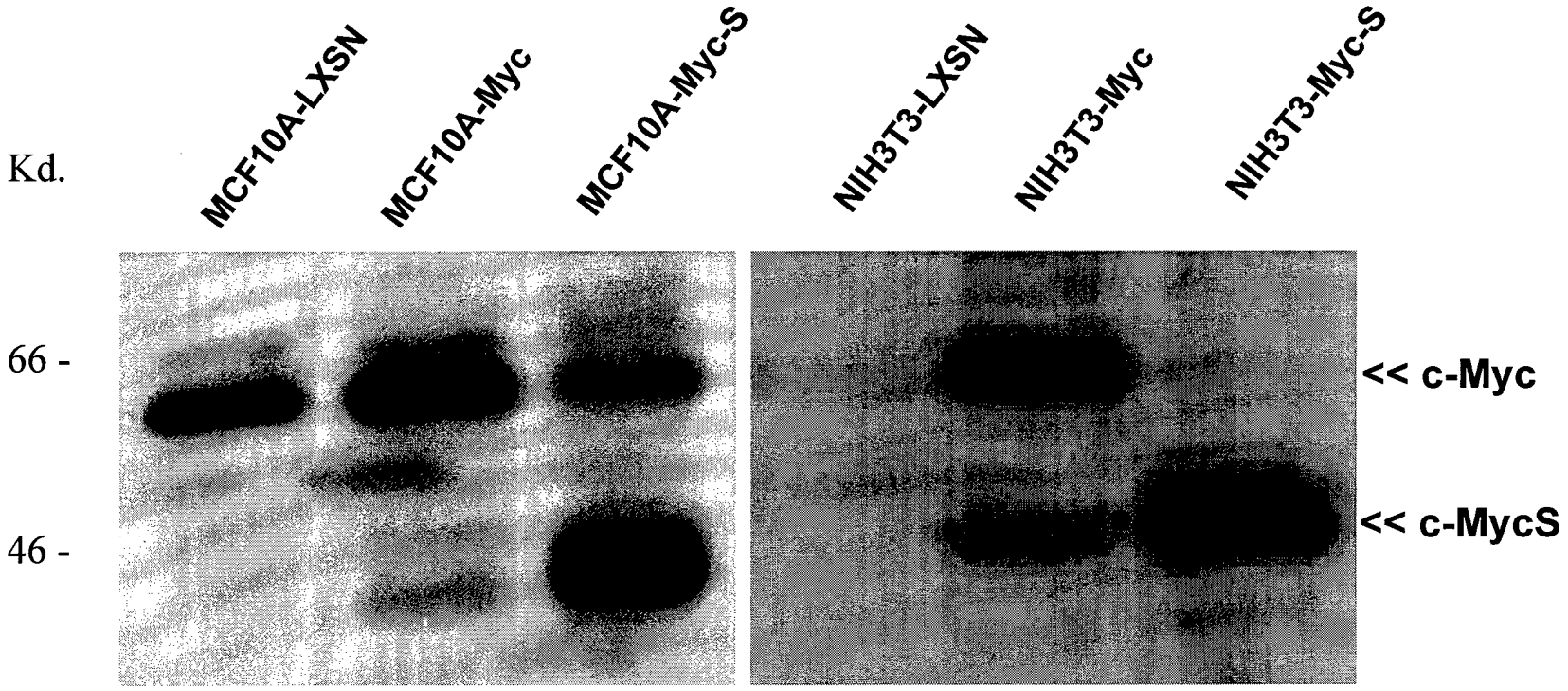
Protein structure of c-Myc



TA:Transactivation domain
HD:Heterodimerization domain
B:Basic region
HLH:Helix-loop-helix
LZ:Leucine zipper

Figure 4.
Army report

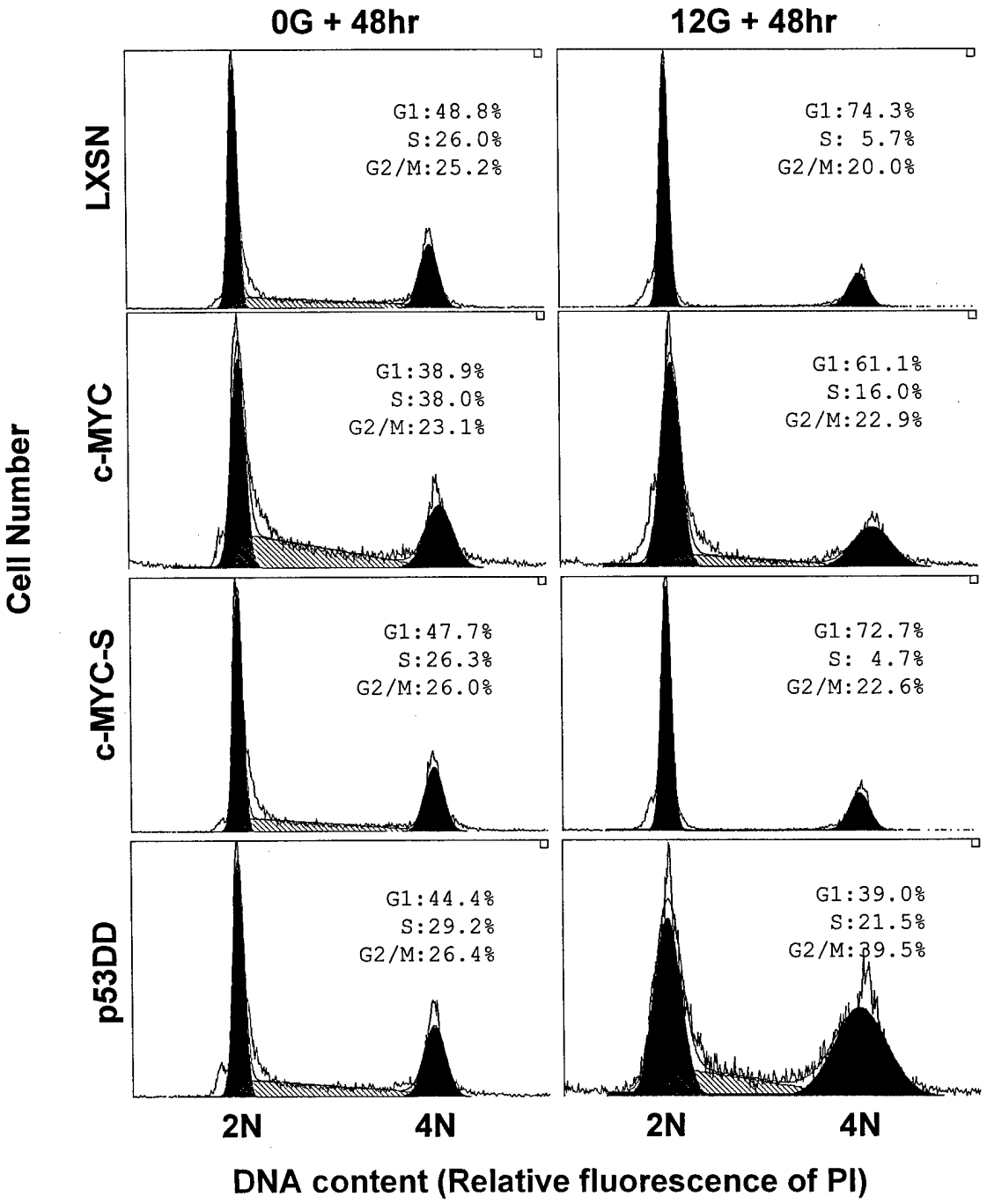
MCF10A HMEC and NIH 3T3 mouse fibroblast transfected with human c-Myc and c-MycS



Western probed with anti-c-Myc Ab (9E10).

Figure 5.
Army report

Effects of c-Myc on the G1/S checkpoint in HMEC



*Cell cycle analyzed by Modfit program for flow cytometry

KEY RESEARCH ACCOMPLISHMENTS for GRANT # DAMD17-99-1-9205:

- Characterization of DNA damage-dependent G1/S checkpoint control in A1N4 cells, an HMEC line, by using γ-irradiation, a clearly defined DNA damaging agent.
- Identification of abrogated DNA damage-dependent checkpoint at G1/S boundary in A1N4-Myc cells, a c-Myc-overexpressing HMEC line.
- Characterization of G1/S checkpoint in MCF10A cells, an independently derived HMEC line.
- Establishment in MCF10A, a system of overexpression of human c-Myc or c-MycS by using retroviral transfection techniques.
- Confirmation of c-Myc-induced G1/S alteration in c-Myc-overexpressing MCF10A cells.
- Identification of two specific regions of the c-Myc protein (MB-I and MB-II) that are required to provide the checkpoint alteration phenotype.

REPORTABLE OUTCOMES LIST for GRANT # DAMD17-99-1-9205:

Abstract Presentations:

1. Joon-Ho Sheen and Robert B. Dickson. Deregulated c-Myc alters DNA damage-dependent G1/S checkpoint. Georgetown University Medical Center and Lombardi Cancer Center Research Poster Day. February 2000.

- Copy of Abstract Attached in the Appendix.

2. Joon-Ho Sheen and Robert B. Dickson. Deregulated c-Myc induces alteration of DNA damage-dependent G1/S checkpoint in human mammary epithelial cells. Abstract #2793. 91st Annual Meeting of American Association for Cancer Research. April 2000, San Francisco, CA.

- Copy of Abstract Attached in the Appendix.

CONCLUSIONS:

During this first year of work on the pre-doctoral training grant # DAMD17-99-1-9205 by Joon-Ho Sheen, an interesting phenotype of abrogated G1/S checkpoint control has been identified in A1N4-Myc cells, c-Myc-overexpressing HMECs. As expected, the G1/S checkpoint is intact in the parental HMECs, arresting cells at G1 phase in response to the γ-irradiation-induced DNA damage. As a follow-up of this first series of experiments, we established a carefully designed HMEC system using MCF10A, an independently derived HMEC line, through retroviral transfection of human c-*myc* and c-*mycS* cDNA constructs. In this improved system, we were able to confirm that the DNA damage-dependent G1/S checkpoint is altered by deregulated c-Myc. Furthermore, we determined that the c-Myc-induced checkpoint alteration requires both the MB-I and MB-II, highly conserved domains in the N-terminal of c-Myc protein. MycS, which does not have the MB-I domains, is not sufficient to allow c-Myc-induced checkpoint alteration. For the trans-activation of c-Myc targets, both MB-I and MB-II domains are known to be required. However, just MB-II domain is required to the c-Myc-mediated trans-repression. Based on the current findings, we propose that the c-Myc-mediated transactivation of its downstream targets is required to the G1/S checkpoint alteration. Additionally, this principal investigator has acquired significant accomplishments over the past year, including the numerous research-oriented skills like the following: cell culture technique, recombinant DNA technology, retroviral transfection technique, flow-cytometry, and immuno-blot analysis.

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APPENDIX

Abstract #1: Joon-Ho Sheen and Robert B. Dickson. Deregulated c-Myc alters DNA damage-dependent G1/S checkpoint. Georgetown University Medical Center and Lombardi Cancer Center Research Poster Day. Feburuary 2000.

Among the recently uncovered effects of c-Myc, genomic instability induced by this oncoprotein may provide one aspect of its oncogenic activity. Genomic instability in cancer cells, coupled with selective pressures, have been considered as driving forces for the accumulation of somatic mutations required for tumor progression. According to previous studies, overexpression of c-Myc induces genomic instability, including gene amplification and chromosomal instability. However, the molecular mechanism(s) for c-Myc-induced genomic instability has not been elucidated. Since abrogation of cell cycle checkpoint has been identified as a prerequisite in production of certain types of genomic instability, we studied the possibility that c-Myc alters checkpoint controls in response to DNA damage. Alteration of DNA damage-dependent checkpoint is thought to promote the accumulation of genetic damage in cells by allowing replication of unrepaired DNA damage in the following S phase. As a first step, to test this hypothesis, we investigated the DNA damage-dependent G1/S checkpoint, since c-Myc is well known to facilitate G1/S progression. In a flow cytometric study, HMEC (Human Mammary Epithelial Cells) and mouse fibroblast cells expressing deregulated c-Myc show a reduced proportion of G1/S arrested cells after γ-irradiation in contrast to the control parental cells. We also determined that both the MBI (Myc-Box I) and MBII (Myc-Box II) domains of c-Myc are required for the alteration of the G1/S checkpoint. These data suggest that transactivation of c-Myc target genes is required for the genetic destabilization effects of c-Myc. This ability of c-Myc to alter a crucial safeguard mechanism for genomic instability may therefore contribute to its role as a potent oncogene when its expression is deregulated. This work is supported in part by Department of the Army Fellowship grant # DAMD17-99-1-9205 to JHS.

Abstract #2: Joon-Ho Sheen and Robert B. Dickson. Deregulated c-Myc induces alteration of DNA damage-dependent G1/S checkpoint in human mammary epithelial cells. Abstract #2793. 91st Annual Meeting of American Association for Cancer Research. April 2000, San Francisco, CA.

Deregulated c-Myc has been implicated in breast cancer, both at the gene and protein levels. Among the recently uncovered effects of c-Myc, genomic instability induced by this oncoprotein may provide one aspect of its oncogenic activity. Genomic instability in cancer cells, coupled with selective pressures, have been considered as driving forces for the accumulation of somatic mutations required for tumor. According to previous studies, overexpression of c-Myc induces gene amplification and chromosomal instability. However, the molecular mechanism(s) for c-Myc-induced genomic instability has not been elucidated. Since abrogation of cell cycle checkpoint has been identified as a mechanism for certain types of genomic instability, we investigated the possibility of c-Myc-induced alteration in cell cycle arrest in response to DNA damage. Interestingly, according to flow cytometric study, HMEC (human mammary epithelial cells) with deregulated c-Myc show a significantly reduced proportion of G1/S-arrested cells after γ-irradiation. In contrast, the control HMEC, transfected with an empty vector, showed a large proportion of cells arrested at G1/S in response to DNA damage induced by γ-irradiation. These results suggest that c-Myc alters the DNA damage-dependent G1/S checkpoint. This alteration predisposes cells for the accumulation of genetic damage by allowing replication of unrepaired DNA damage in the following S phase. Previous studies of tumor suppressors such as p53 and pRB have shown abrogated checkpoint in the absence of the tumor suppressor. However, based on this study, we propose that activated oncogenes such as c-Myc may provide similar effects of altered checkpoint (supported by DOD Pre-doctoral grant to J.H.S.)