TRANSFECTION OF THE BREAST CANCER CELL LINE MDA-468 WITH ANTISENSE RNA TO $P21^{CIP1}$ IN ORDER TO INVESTIGATE THE MECHANISM OF EGF-MEDIATED G_1 ARREST IN THESE CELLS

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by

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ABSTRACT

The human breast cancer cell line MDA-468 is characterized by an overexpression of the epidermal growth factor (EGF) receptor. As a result of this overexpression, MDA-468 cells have the unique characteristic of undergoing arrest of cell cycle in G_1 when treated with pharmacological concentrations of EGF. While the ability of EGF to cause arrest of this cancer cell line is well documented, the effectors that mediate this arrest remain unknown. Since the tumour suppressor gene, $p21^{Cip1}$, is a potent inhibitor of cyclin dependent kinases with a well-defined ability to confer G_1 arrest on tumour cells, it is proposed that $p21^{Cip1}$ may play a role in the EGF-mediated arrest of these cells. An antisense against $p21^{Cip1}$ expression was transfected into MDA-468 cells to test this hypothesis. Single-clone cell lines were derived and examined for (i) an elimination of $p21^{Cip1}$ mRNA and protein, and (ii) a reduction of the ability of these cells to undergo G_1 arrest when treated with EGF. Three antisense-transfected clones demonstrated that antisense expression caused a reduction of $p21^{Cip1}$ with a significantly decreased capacity to undergo G_1 arrest. In these cell lines an absence of $p21^{Cip1}$ reduced the ability of EGF-treatment to cause growth arrest. This correlation may indicate that $p21^{Cip1}$ is an important player in the EGF-mediated growth arrest of MDA-468 cells.

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TABLE OF CONTENTS

LIST OF ABREVIATIONS vii

LIST OF TABLES viii

LIST OF FIGURES viii

1. INTRODUCTION 1

i) The Cell Cycle 1

ii) Stages of the cell cycle 2

iii) Cyclin Dependent Kinases 3

a. Cdks in yeast 3
b. Cdk in mammalian cells 5

iv) Mammalian cyclins 5

a. Cyclin D 6
b. Cyclin E 6
c. Cyclin A 6
d. Cyclin B 7

v) Regulation of Cdks by phosphorylation 7

vi) Checkpoint control of the cell cycle 8

vii) Cell-cycle control by Rb 9

viii) The tumour suppressor gene p53 10

ix) The $p21^{Cip1}$ tumour suppressor gene 11

x) Cdk-inhibitory proteins 12

a. $p27^{Kip1}$ and $p57^{Kip2}$ 13
b. The Ink4 family 13

xi) The MDA-468 breast cancer cell line 14

xii) Possible role for $p21^{Cip1}$ in EGF-mediated growth arrest 15

2. MATERIALS AND METHODS 18

i) The MDA-468 cell line 18

ii) Bacterial cells and plasmid growth 18

a. Transformation of bacteria 18
b. Transformation of bacterial cells 19
c. Large scale prep of purified plasmid 19

d. Small scale prep of bacterial plasmid 20

iii) Construction of expression vectors 21

a. Vector plasmid (pcDNA-neo) 21
b. cDNA insert of $p21^{Cip1}$ 22
c. Ligation of $p21^{Cip1}$ into vector 23
d. Identification of clones bearing ligated insert 23
e. Identification of orientation of ligated inserts 24

iv) Stable transfection of MDA-468 Cells 24

a. Calcium phosphate transfection 24
b. Freezing cells under liquid nitrogen 25
c. Generation of single clone cell lines 25

v) Northern Blot Analysis 26

a. Isolation of RNA 26
b. Preparation of Northern blot 27
c. Generation of probes for Northern blot analysis 28
d. Random primer labelling 28
e. Hybridization of probe to RNA blot 29

vi) Western blot analysis 29

a. Whole cell lysate (denaturing) 29
b. Immunoprecipitation (non denaturing) 30
c. SDS-PAGE and electroblotting of proteins 31
d. Immunodetection of protein blots 31

vii) EGF-mediated growth arrest 32

3. RESULTS 34

i) EGF induces upregulation of p21Cip1 34

ii) Analysis of $p21^{Cip1}$ protein in MDA-468 cells 38

iii) Construction of a $p21^{Cip1}$ antisense expression vector 42

iv) Screening of ligated plasmids 42

v) Determining the orientation of ligated insert 44

vi) Transfection of plasmid into MDA-468 cells 45

vii) Isolation of single clones of transfected MDA-468 48

viii) Cell growth experiments 49

ix) Growth of sense-transfected cells 55

x) Northern Analysis of transfected cells 55

xi) Analysis of $p21^{Cip1}$ protein in single clones 59

xii) Time course of EGF effects on growth 61

4. DISCUSSION 70

i) Upregulation of $p21^{Cip1}$ mRNA in MDA-468 cells prior to G_1 arrest. 70

ii) Western analysis of $p21^{Cip1}$ in MDA-468 cells. 70

iii) Antisense to $p21^{Cip1}$ 70

iv) Effect of antisense expression on $p21^{Cip1}$ mRNA 70

a. Short length antisense molecules 70
b. Targeting antisense to the untranslated regions of target mRNA 70

v) Effect of antisense expression on $p21^{Cip1}$ protein levels. 70

vi) Growth analysis of antisense-transfected cells 70

vii) Growth of sense transfected single clones. 70

viii) Inducible expression of antisense. 70

ix) Time course of EGF on the growth of MDA-468 and single clones 70

x) EGF-mediated arrest in A431 cells: A comparative analysis. 70

xi) Antisense to $p21^{Cip1}$ in A431 cells 70

xii) Involvement of other Cdk inhibitory proteins. 70

xiii) Proposed mechanisms for EGF-mediated upregulation of $p21^{Cip1}$ 70

a. Possible role for p53 in MDA-468 G_1 arrest. 70
b. Possible activation of $p21^{Cip1}$ by STAT proteins. 70

5. CONCLUSION 70

6. REFERENCES 70

LIST OF ABREVIATIONS

BSA	Bovine Serum Albumin
CMV	Cytomegalovirus
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediamine tertraacetic acid
FACS	Flouresence-activated cell sorter
FBS	Fetal Bovine Serum
GTE	Glucose-Tris-EDTA buffer
HBS	Hepes-buffered saline
HRP	Horse radish peroxidase
mRNA	messenger RNA
MOPS	3-[N-morpholine]-propane sulfonic acid
NaOH	Sodium hydroxide
PAGE	polyacrylamide gel electrophoresis
PMSF	Phenyl methyl sulfonyl fluoride
SDS	Sodium doceyl sulphate
SSC	Standard saline citrate
SSB	SDS-sample loading buffer
SSPE	Standard saline buffered EDTA
STE	Sodium-Tris-EDTA buffer
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TBST	Tris-buffered saline with Tween
dATP	Deoxyadenosine triphosphate
dTTP	Deoxythimidine triphosphate
dGTP	Deoxyguanine triphosphate
dCTP	Deoxycytosine triphosphate

LIST OF TABLES

Table 1: Effect of EGF on the growth of MDA-468 and transfected cell lines. 50

Table 2: Time course of the effect of EGF on growth over 8 days. 65

LIST OF FIGURES

Figure 1: Northern blot analysis of the dose response and long term effect of EGF on p21Cip1 expression in MDA-468 cells 35

Figure 2: Northern blot analysis of the effect of EGF on $p21^{Cip1}$ expression during onset of G_1 arrest. 37

Figure 3: Northern blot analysis of $p21^{Cip1}$ expression upon short-term EGF treatment and removal from EGF 39

Figure 4: Western blot analysis of $p21^{Cip1}$ protein in MDA-468 cells. 41

Figure 5: Diagram of the plasmids pcDNA-neo and pBS-Cip1(T7) and result of *Xho I* digest. 43

Figure 6: Ethidium bromide stain of two gels used to screen cloning of the antisense and sense $p21^{Cip1}$ expression vectors. 46

Figure 7: Diagram of ligation products and diagnostic digest with *BglII/Xho1*. 47

Figure 8: Percent change in growth of EGF-treated MDA-468 and antisense transfected cells. 52

Figure 9: Untreated growth of MDA-468 and antisense clones over 8 days. 54

Figure 10: Percent change in growth of EGF-treated MDA-468, sense-transfected, and antisense-transfected. 56

Figure 11: Northern blot analysis of the effect of EGF on $p21^{Cip1}$ expression in MDA-468, antisense and sense transfected cells 58

Figure 12: Histogram of intensities of band in Figure 11A. 60

Figure 13: Western blot analysis on the expression of $p21^{Cip1}$ after 72 hours treatment for MDA-468, the two antisense-transfected cell lines: 6-25 and 6-26, and the sense-transfected cell line 7-6. 62

Figure 14: Western blot analysis on the expression of $p21^{Cip1}$ after 72 hours treatment for MDA-468, the antisense-transfected cell line 6-19 63

Figure 15: Time course effect of EGF treatment on growth. (1) 67

Figure 16: Time course effect of EGF treatment on growth. (2) 68

Figure 17: Time course effect of EGF treatment on growth. (3) 69

1. INTRODUCTION

The disease of cancer is characterized by cells which have acquired the ability to proliferate indefinitely. Under normal growth conditions, cells divide when proper growth signals exist and likewise cease proliferation when inhibitory signals prevent division. Tumour cells, on the other hand, are cells which have undergone mutations such that their proliferation is no longer regulated leading to neoplastic or uncontrolled growth. With further mutational events, a neoplastic cell can adopt an aggressive or malignant phenotype which is ultimately fatal to the organism (Levine, 1993).

To better understand the process of cell division investigators have sought to determine what components of the cell triggers DNA synthesis and eventual cell division, what signals cause the cells to be mitotic at times and quiescent at others, and most importantly, what causes the loss of this control, ultimately leading to the formation of deadly tumours.

i) The Cell Cycle

To acquire a better understanding of the process of tumourigenesis, investigators focused on the central machinery of cell division to determine what factors govern normal growth and what events foster de-regulation to allow malignant cells to develop. The result of this search has developed what is referred to as the cell cycle. The cell cycle is a sequence of events or steps a cell must complete in order to divide into two new daughter cells. The critical elements of the cycle involve duplication of the chromosomes, assembly of the

mitotic spindle, and the segregation of chromosomes into two new cells (Earnshaw, 1988 ; Nicklas, 1988).

To prepare for a mitotic event, a great deal of coordination is required to ensure that each step in the cycle is initiated and completed sequentially. For example the process of chromosome duplication consists of DNA synthesis, followed by chromosomal condensation and then attachment to the mitotic spindle (Wolniak, 1988). Completion of each step involves the timely synthesis, assembly and activation of a host of proteins to mediate these functions in proper sequence. Equally important is that key enzymes are inactivated in proper sequence for the process of division to proceed from one stage to the next.

The coordination of these events is referred to as cell cycle control. Normal dividing cells can be thought of as those cells that make their way through the cycle only when proper growth promotion signals exist (Murray *et al.*, 1989a). Tumour cells, on the other hand, pass through stages of the cycle continually and are unresponsive to growth inhibitory signals. In other words they have escaped cell cycle control (Hartwell *et al.*, 1994).

ii) Stages of the cell cycle

The cell cycle is defined by 5 different stages in the preparation of a mitotic event. A new daughter cell is usually quiescent or in G_0 phase. At this stage the cell is dormant in terms of proliferation and depending on the cell type, G_0 may last a few hours, a few days or an infinite amount of time. When stimulated to divide by growth-promoting signals, such as growth factors, cells exit G_0 and enter a preparatory stage of the cycle known as G_1 stage in

which the cell activates gene products required for the initiation of DNA replication. At the end of G_1, the cell initiates the replication of chromosomal DNA and is said to have entered S phase. Following the complete replication of the DNA in S phase, the cycle enters a second preparatory stage, G_2, in which the components required for the process of cytokinesis are assembled and activated. At the end of G_2, the cell cycle culminates in M phase when the cell segregates the chromosomes and physically separates into two new daughter cells (Nicklas, 1988).

The last decade has seen the emergence of a theory of cell cycle control that involves regulation by phosphorylation. The phosphorylation of a host of regulatory proteins modify their activity, assembly, stability, or subcellular location to mediate progress through a given stage of the cycle (Ducommun, 1991). The class of kinases most responsible for cell-cycle specific phosphorylation are the cyclin-dependent kinases (Cdks) (Hunter *et al.*, 1994).

iii) Cyclin Dependent Kinases

a. Cdks in yeast

The cyclin dependent kinases are a highly conserved family of serine/threonine kinases whose activity is critical for progression through the cell cycle. These proteins of roughly 33kDa were initially characterized in yeast as the gene product cdc2 (cell division cycle) in *Schizosaccharomyces pombe* and cdc28 in *Saccharomyces cerevisae* (Reed, 1991). Through the use of temperature sensitive mutants of cdc2 and cdc28, it was revealed that actively dividing cells became cell cycle arrested at either G_1 or G_2 when grown at the nonpermissive temperature (Rowley *et al.*, 1993). This indicated that the

activity of cdc2 and cdc28 was essential for continued progression through the G_1 and G_2 phases of the cell cycle (Wittenberg *et al.*, 1991). In the absence of cdc2 or cdc28 activity the cells could no longer progress through the cell cycle and become arrested.

The activity of cdc2 and cdc28 is dependent on the binding of regulatory proteins named cyclin proteins. The cyclin proteins briefly appear in the cell cycle and bind and activate cdc2 or cdc28 in a cell cycle-specific manner (Hadwiger *et al.*, 1989). Due to obligatory binding of cyclin proteins to achieve activity, cdc2 and cdc28 were named cyclin-dependent kinases (Cdks).

The observation that temperature sensitive mutants of the Cdks encoded by cdc2 and cdc28 caused arrest at both the G_1/S and G_2/M boundaries, indicated that in yeast cells a single Cdk molecule is required for progression through both the G1/S and G2/M boundaries. This dual activity is achieved by means of two temporally expressed subsets of cyclins which bind and activate the kinase, the G_1 cyclins and the G_2 cyclins. In preparation for S phase, the G_1 cyclins are expressed and activate cdc2 or cdc28 in G_1 and direct these kinases to targets that mediate preparation of DNA synthesis (Reed, 1991). Upon completion of S phase the level of G_1 cyclins drop significantly and the level of G_2 cyclins rise. Hence at G_2/M, the G_2 cyclins bind the Cdk to activate the components of the cell responsible for the preparation of mitosis. Thus, in yeast cells, a single Cdk has differential activity at two different phases of the cell cycle mediated by binding different cyclin partners.

b. Cdk in mammalian cells

In mammalian cells, cell cycle regulation is more complex as there are at least seven identified Cdks numbered Cdk1 to Cdk7. These proteins share similar size and homology and are stably expressed throughout the cycle. In addition there are at least eight types of mammalian cyclin proteins known (A to H) which form complexes with one or more of the Cdks. In mammalian cells the timing of Cdk activation is defined by the cyclin partner to which they bind (Hunter *et al.*, 1994).

iv) Mammalian cyclins

The cyclin proteins are so named because of their cyclic appearance at specific times in the cell cycle. While Cdks are stably expressed throughout the cycle, the cyclin proteins have a short duration of expression mediated by both a brief period of transcription and by a rapid turnover rate of the protein (Sherr, 1993). For example, in G_1, the G_1 cyclins (D and E) are briefly expressed and the proteins undergo rapid degradation due to a sequence on their C-terminal which confers instability (Murray *et al.*, 1989b). In contrast the G_2 cyclins, A and B, become expressed in late S or G_2 phase (Pines *et al.*, 1989) and are relatively stable throughout G_2 and M. These cyclins become rapidly degraded at the conclusion of mitosis by ubiquitin-dependent degradation (Glotzer *et al.*, 1991) through a conserved region in cyclin A and B known as the destruction box which promotes ubiquitin conjugation and subsequent degradation (Minshull *et al.*, 1989). Thus by these mechanisms mammalian cyclins are present at high levels only at specific stages of the cycle. Consequently, they are present to activate their Cdk partners only at these defined times.

a. Cyclin D

The first cyclin to be expressed in the cycle is cyclin D. D-type cyclins are most closely linked with signal transduction as their expression is dependent on the presence of growth factors (Sherr, 1993). Stimulation of cells by growth factors induces the expression of cyclin D which signals that the cell cycle has exited G_0 to enter G_1 phase (Baldin *et al.*, 1993). In mammalian cells, D-type cyclins bind and activate Cdk2 (Xiong *et al.*, 1992), Cdk4 (Matsushime *et al.*, 1992) and Cdk 6 (Meyerson *et al.*, 1994) in G_1. Withdrawal of growth factors during G_1 stops transcription of cyclin D and due to the rapid degradation of the protein its levels rapidly decline and the cycle halts (Won *et al.*, 1992). This indicated that the continued expression of cyclin D in G_1 is necessary for the cycle to progress to S phase. In fact, cyclin D has been identified as an oncogene as there are tumour cell lines with constitutively elevated levels of cyclin D which continuously promote progression through G_1 (Quelle *et al.*, 1993).

b. Cyclin E

In late G_1, cyclin E is expressed and reaches peak levels at the G_1/S transition and sharply declines as cells progress through S phase (Koff *et al.*, 1992). Cyclin E binds to Cdk2 and this complex is responsible for the initiation of DNA synthesis signaling entry of the cycle into S phase (Ohtsubo *et al.*, 1995). Inactivation of cyclin E by microinjection of antibodies to cyclin E block DNA synthesis and cause G_1 arrest of the cycle.

c. Cyclin A

Following the initiation of S phase, cyclin E is degraded and cyclin A levels increase and bind to Cdk2 (Rosenblatt *et al.*, 1992). Evidence suggests that cyclin A/Cdk2 activity is

necessary for the completion of DNA replication yet is not necessary for the G_1/S transition. In the absence of cyclin A, DNA synthesis can be initiated but does not continue to completion. Hence, progress through S phase is regulated by the cyclin E/Cdk2 complex which is required for the initiation of DNA synthesis and cyclin A/Cdk2 activity which is required for DNA synthesis to reach completion.

d. Cyclin B

In G_2 and M phase, cyclin A and B are present and are bound to Cdk1. Cdk1 activity is required for the breakdown of nuclear lamina in G_2 and signals for preparation of cytokinesis (Heichman *et al.*, 1994). At the end of M phase termination of Cdk1 function is required for complete division to occur. Its function is terminated by ubiquitin-mediated degradation of cyclins A and B. With inactivation of Cdk1, mitosis is completed.

v) Regulation of Cdks by phosphorylation

In addition to the binding of a cyclin partner, cyclin-dependent kinases are also regulated by phosphorylation. All Cdks require phosphorylation of a conserved threonine-161 (Thr-160 in Cdk2) to become active. Phosphorylation of this threonine residue causes a conformational shift in the cyclin-Cdk complex necessary for substrate binding (Morgan, 1995).

Cyclin dependent kinases are also negatively regulated by phosphorylation at residues tyrosine-15 and threonine-14 (McGowan and Russell, 1993). There are two separate kinases that are responsible for the initial phosphorylation of residues tyr-15 and thr-14. These kinases have been characterized in yeast as wee1 and mik1 (Lee *et al.*, 1994 ;

McGowan *et al.*, 1993). It is thought that phosphorylation of Cdks by these kinases constitute the first post-translational modification of the Cdk (Poon *et al.*, 1995).

The enzymes that catalyze the removal of these inhibitory phosphates are the cdc25 family of phosphatases (cdc25a, cdc25b, and cdc25c) Each enzyme of this family acts at a different phase of the cell cycle and is responsible for activating the corresponding Cdk complexes formed at those times.

vi) Checkpoint control of the cell cycle

It is well accepted that it is in G_1, prior to DNA synthesis, that the cell maintains the majority of control on the Cdks and consequently on the cell cycle (Hartwell *et al.*, 1989). In mammalian cells this checkpoint is referred to as the restriction point, or the R point, which occurs in late G_1 prior to initiation of S phase (Tsai *et al.*, 1993). At this point in the cycle, dividing cells maintain a mechanism to monitor the state of the cell before proceeding to S phase. Should cell division be undesirable the cell remains at G_1 and the cell cycle is said to have arrested (Sherr, 1996).

The loss of these critical checkpoints can lead to an increase in mutation and eventual transformation of the cell (Hartwell *et al.*, 1994). In the context of the cell cycle, tumourigenesis results when cells no longer respond to inhibitory signals and continuously proceed through checkpoints in the cycle. In the event of damage to the DNA, the loss of checkpoints allow mutations to accumulate in the genome ultimately leading to further deregulation of growth and the development of aggressively growing tumour cells (Cox *et al.*, 1995). With the awareness that cancer cells evolve by escaping growth controls, it is

obvious that a good understanding of both the growth-promoting and growth-inhibitory signals which control the function of Cdks at these checkpoints is necessary.

vii) Cell-cycle control by Rb

The chief means by which the G_1 checkpoint is maintained is through the regulation of the retinoblastoma protein (Rb) by Cdks (Sherr, 1994). Rb and its two homologues, p107 (Ewen *et al.*, 1992), and p130 (Hannon *et al.*, 1993), are phosphoproteins which act as repressors of the cell cycle in G_1. Rb and its homologues, bind and inactivate the E2F family of transcription factors which are responsible for transactivating a number of genes involved in DNA synthesis such as DNA polymerase-α (DeGregori *et al.*, 1995), dihydrofolate reductase (Slansky *et al.*, 1993), thymidylate synthase (Johnson *et al.*, 1994) and cyclins D (Herber *et al.*, 1994) and E (Ohtani *et al.*, 1995). However, when bound to Rb the E2F proteins are prevented from transactivating these S phase genes and the cell cycle remains in G_1. In addition, Rb-E2F complexes act as repressors of transcription at the promoters of these genes (Slansky *et al.*, 1996).

However, the Rb protein can only bind E2F proteins when in a hypophosphorylated state (Nevins, 1992). In early G_1 Rb protein is in this low phosphorylated state and thus is found in complexes with E2F proteins. Upon stimulation of cdk4 and cdk6 by cyclin D and, later in G_1, Cdk2 by cyclin E, the activated Cdks phosphorylate the Rb protein converting it to a hyperphosphorylated state. As a result of this phosphorylation by the Cdks, Rb is released from the E2F proteins which are then free to transactivate the genes required for the cycle to enter S phase. Hence the ability of Cdks to phosphorylate Rb is an important

function for Cdks to signal progression through the cell cycle. For cells to maintain a G_1 checkpoint, phosphorylation of Rb by Cdks needs to be kept in check.

viii) The tumour suppressor gene p53

Another significant regulator of G_1 Cdks in terms of checkpoint control is the tumour suppressor p53. This protein of 53 kDa has a well-documented role in tumour development. To date, roughly 70% of all primary tumours indicate a loss of p53 function due to either a point mutation or deletion on one or both of the chromosomes (Greenblatt *et al.*, 1994) and its absence in tumours of all tissue types suggests a universal role for p53 in the management of normal cell growth.

Currently it is believed that p53 regulates normal cell growth by way of monitoring DNA integrity. When cells encounter DNA damage, especially in the form of DNA breaks, the levels of p53 protein increase and induce G_1 arrest of the cell cycle (Kastan, 1993).

Arrest of the cell cycle by p53 is thought to serve two critical functions in the prevention of tumourigenesis. Firstly, by halting the cell cycle prior to S phase, the cell is prevented from propagating errors in the DNA onto progeny cells. The arrested cell is given time to activate the repair machinery and remove mutations in the DNA to ensure genomic integrity is maintained (Smith *et al.*, 1995a). Secondly, in cases where DNA damage is too severe, the cell cycle arrest also allows the cell to activate programmed cell death (apoptosis) (Lowe *et al.*, 1993). Activation of apoptosis in the face of severe DNA damage is an evolved safety mechanism whereby it is more favorable to trigger the death of the cell rather than allow propagation of errors in the cell's DNA (Kastan *et al.*, 1995). Obviously, cells

lacking functional p53 lose this critical response to DNA damage and permit the accumulation of mutations in the genome ultimately contributing to tumourigenesis (Smith *et al.*, 1995b).

ix) The $p21^{Cip1}$ tumour suppressor gene

With the successful cloning of a full length p53 cDNA, functional analysis revealed that this tumour suppressor functions as a transcription factor (Zambetti *et al.*, 1992). The search for genes transactivated by p53 discovered GADD45 (Chin *et al.*, 1997a), mdm-2 (Perry *et al.*, 1993) and cyclin G (Smith *et al.*, 1998). The most significant gene mediated by p53 in the regulation of Cdks following DNA damage was a 21kDa protein which would increase upon p53 activation.

Investigators focusing on p53 as a transcription factor, named the p21 protein WAF-1 (wildtype p53 activated fragment) or $p21^{waf1}$ (el Deiry *et al.*, 1993). Sequence analysis showed that it was identical to a 21-kda protein simultaneously being studied for its interaction and inhibition of the cyclin dependent kinases. Investigators focusing on this aspect of p21, termed the protein CiP1 (cyclin dependent kinase inhibitory protein-1) or $p21^{cip1}$ (Harper *et al.*, 1993). $p21^{cip1}$ has been shown to bind and inhibit all G_1 cyclin/Cdk combinations. Upregulation of $p21^{cip1}$ by p53 halts the function of all cyclin/Cdk complexes causing arrest of the cell cycle (Xiong *et al.*, 1993).

The discovery of $p21^{Cip1}$ provided direct evidence for the role of p53 in the inhibition of Cdk activity in maintaining a G_1 checkpoint. In normal cells, stimulation of p53 *in vivo* by DNA damaging agents causes a dramatic increase in the levels of $p21^{cip1}$ (el-Deiry *et al.*,

1994). The $p21^{cip1}$ protein binds and inactivates Cdk-cyclin activity. With the inactivation of Cdks in G_1, Rb remains in a hypophosphorylated state and remains bound to E2F proteins with the consequence of cell cycle arrest in G_1 (Dulic *et al.*, 1994). In addition, overexpression of $p21^{Cip1}$ in the absence of activation by p53 could cause growth arrest when transfected in established tumour cell lines (Chen *et al.*, 1995 ; Michieli *et al.*, 1996).

In cells lacking p53 function, the basal expression of $p21^{cip1}$ is dramatically lower and upregulation of $p21^{cip1}$ by U.V., gamma-irradiation or other DNA damaging agents is not observed (Cox *et al.*, 1995). Functional Cdks accumulate, phosphorylate Rb, and signal progression through the cell cycle. With the loss of this checkpoint, mutations within the DNA remain unrepaired and are duplicated and propagated in S phase ultimately contributing to a malignant phenotype (Cox *et al.*, 1995). This led to the hypothesis that Cdks could escape normal growth controls due to a lack of inhibition by what are now called Cdk-inhibitory proteins.

x) Cdk-inhibitory proteins

Following the discovery of $p21^{Cip1}$ as an inhibitor of Cdk activity, other proteins with similar function have been identified (Elledge *et al.*, 1994). These regulatory proteins bind either directly to the Cdk molecule or to a cyclin-Cdk complex to inhibit kinase activity. Once bound, the Cdk activity is inactivated and progression through the cell cycle is halted. These proteins, generally referred to as Cdk inhibitory proteins, are divided into two families, the Cip1/Kip1 family and Ink4 family.

a. $p27^{Kip1}$ and $p57^{Kip2}$

The proteins $p27^{Kip1}$ (Polyak *et al.*, 1994) and $p57^{Kip2}$ (Lee *et al.*, 1995) have a high degree of homology with $p21^{Cip1}$ and, like $p21^{cip1}$, repress Cdk activity by binding cyclin-Cdk complexes. In particular they inactivate cyclin D and cyclin E complexes with Cdk2, Cdk4, and Cdk6 in G_1. Thus, transfection of these Cdk inhibitory into established cell lines induces G_1 arrest. While $p57^{Kip2}$ is tissue restricted (Lee *et al.*, 1995), $p27^{Kip1}$ appears to have a universal role in management of G_0. The level $p27^{Kip1}$ are highest throughout G_0 and early G_1 (Pagano *et al.*, 1995). The current model for the action of $p27^{Kip1}$ is that it provides a threshold of Cdk inhibition which must be overcome by mitogen activation. In fact, elimination of $p27^{Kip1}$ with antisense removes the requirement of growth factors for cells to divide (Coats *et al.*, 1996). Although $p27^{Kip1}$ and $p57^{Kip2}$ are related to $p21^{cip1}$ they are not regulated by p53 nor do they participate in a G_1 checkpoint in response to DNA damage.

b. The Ink4 family

The Ink4 family of Cdks inhibitors differ from the $p21^{Cip1}$ family in that they inhibit Cdk function by binding directly to a single Cdk molecule to form an inactive heterodimer (Serrano *et al.*, 1993). These Cdk inhibitors prevent cyclin binding and retain the Cdk in an inactive state. Members of this family include $p16^{Ink4}$ (Serrano *et al.*, 1993), $p15^{Ink4b}$ (Hannon *et al.*, 1994), $p18^{Ink4c}$ (Guan *et al.*, 1994), and $p19^{Ink4d}$ (Chan *et al.*, 1995) and bind to Cdk4 and Cdk6 in G_1. They were named Ink4 for their observed ability to inhibit Cdk4 (Sherr *et al.*, 1995).

Each of the Ink4 inhibitors can cause G_1 arrest in tumour cells and their loss is implicated in tumourigenesis. Their role in the inhibition of Cdk4 and Cdk6 appears to

specifically modulate phosphorylation of Rb. Upon transfection into established cell lines, the INK4 proteins bind to Cdk4 and Cdk6, which prevents phosphorylation of Rb, thereby causing G_1 arrest (Sherr *et al.*, 1995).

However, unlike the $p21^{Cip1}$-family of Cdk inhibitors, the INK4 proteins are unable to induce G_1 arrest in cell lines that lack Rb despite forming complexes with Cdk4 or Cdk6. This provided evidence that Rb is the principle substrate for Cdk4 and Cdk6 and in cells lacking Rb, Cdk4 and Cdk6 activity may be redundant (Lukas *et al.*, 1995).

With the awareness that cancer cells evolve by escaping growth control it is obvious that a good understanding of the mechanism of growth arrest merits investigation. In the study presented here the role of $p21^{Cip1}$ in G_1 arrest is examined using the cell line MDA-468.

xi) The MDA-468 breast cancer cell line

The breast cancer cell line MDA-468 is a unique cell line characterized by an overexpression of the epidermal growth factor (EGF) receptor (Filmus *et al.*, 1985). While other cells of mammary origin express 10^4-10^5 EGF receptors, MDA-468 cells express in the order of 10^6 receptors per cell, resulting from a 20-fold duplication (i.e. twenty copies) of the gene for the receptor (Kawamoto *et al.*, 1984). Furthermore, MDA-468 is unusual in that upon exposure to EGF at pharmacological concentrations (ie.10^{-8}M), cells *in vitro* respond by undergoing reversible growth arrest within 24 hours (Filmus *et al.*, 1985). Cell cycle analysis by FACS showed that the arrest occurs in the G_1 phase of the cell cycle and upon removal of EGF from the media the cells re-enter the cell cycle (Church *et al.*, 1992).

The ability of EGF to induce growth arrest is a departure from its well-documented role as a potent mitogen (Carpenter, 1984). In the case of MDA-468 cells, it would seem that growth arrest by EGF is likely the consequence of signaling through the overexpressed receptors on its surface. Indeed another well studied cell line A431, an epidermoid cancer cell line, also overexpresses the EGF receptor to the same degree as MDA-468 (Merlino *et al.*, 1984) and undergoes EGF-mediated G_1 arrest in the same manner (Barnes, 1982). However, there are cell lines that overexpress the EGF receptor which do not undergo EGF-mediated growth arrest but adopt an proliferative phenotype (Pandiella *et al.*, 1988). Hence EGF receptor overexpression alone is not a prerequisite for growth arrest and arrest is therefore most likely due to an altered signal transduction pathway within these cells. The exact mechanism by which EGF transduces a signal for cell cycle arrest in MDA-468 cells remains unknown.

xii) Possible role for $p21^{Cip1}$ in EGF-mediated growth arrest

Since targeting cancer cells to cease proliferation is a goal of cancer research, the mechanism by which EGF can cause a transformed cell line to undergo cell cycle arrest may be of clinical significance. Initial studies sought to examine a possible role for p53 in the EGF-induced G_1 arrest of this cell line (Prasad *et al.*, 1997a). Given its well defined role in growth arrest, it was postulated that signal transduction via the activated EGF receptor could increase p53 function thereby inducing cell cycle arrest.

It was postulated that EGF signaling in MDA-468 might cause an induction of p53 activity by post-translational modification, most likely by phosphorylation, such that this

induction of activity resulted in G_1 arrest. The conformational flexibility of p53 from an inactive to active state lends well to this idea (Halazonetis *et al.*, 1993). Indeed, EGF causes changes in p53 phosphorylation, protein conformation and subcellular localization in these cells (Prasad *et al.*, 1997a). The p53 protein expressed in MDA-468 cells is from a single allele with a mutation at codon 273 where an asparagine residue is substituted by histidine (Nigro *et al.*, 1989). Although this is a mutant p53 molecule, this particular mutation, $p53^{273.his}$, has a curious phenotype.

The p53 mutant at codon 273 (arg to his) has been dubbed a "pseudo-wild type" mutant. Transfection of this molecule into various cells has shown the capacity to mediate both oncogenic and, in some cases, growth suppressive activity (Gollahon *et al.*, 1996). While most p53 mutants lose their ability to bind DNA (Kern *et al.*, 1991), $p53^{273.His}$ clearly is able to bind to the p53 consensus sequence (p53CON) and can transactivate reporter constructs with p53CON while other mutants of p53 do not (Park *et al.*, 1996). Most significantly the $p53^{273.His}$ mutant is capable of transactivating a CAT reporter construct containing the p53CON element when transfected into MDA-468 cells. In addition expression of this reporter construct was increased when the cells were treated with EGF (Prasad *et al.*, 1997b).

$p53^{273.His}$ also has the unique characteristic of being recognized by both wild type specific anti-p53 antibodies as well as mutant specific antibodies. This observation indicates that this peculiar mutant may retain a certain degree of wild type conformation which may explain its ability to perform wild type functions. In the context of MDA-468 cells, treatment with EGF causes a shift in the proportion of p53 molecules detected by these

antibodies such that the protein becomes predominantly recognized by the wild type specific antibody (Prasad *et al.*, 1997a). Concurrent to the observed shift to wild type conformation, the protein migrates almost exclusively to the nucleus prior to the onset of growth arrest of MDA-468 by EGF. These observations may indicate a gain of function and localization to the nucleus where it can act as a transcription factor of the genes which mediate the growth arrest. Presumably, $p21^{Cip1}$ might be one of them.

The study presented here focuses on a possible role for $p21^{cip1}$ in this EGF-mediated growth arrest. Given its potent ability to inactivate the G_1 Cdks, it was logical to pursue a role for $p21^{Cip1}$ in mediating the growth inhibitory response of these cells to EGF. Thus it was proposed that EGF-mediated upregulation of $p21^{cip1}$ would lead to a saturating amount of $p21^{cip1}$ in G_1 Cdk complexes to cause growth arrest.

The specific goal of this study is to use antisense to $p21^{cip1}$ to remove it from a putative role in EGF-mediated growth arrest. Theoretically, antisense technology "knocks out" the expression of an endogenous gene by presenting the complementary RNA strand which binds to the endogenous message. The resulting double stranded complex becomes a substrate for degradation by the ribonuclease RNAse H (Bonham *et al.*, 1995). By this manner the levels of an endogenous mRNA can be reduced or abolished, consequently eliminating or reducing the amount of message which can be translated into protein.

Should removal of $p21^{cip1}$ from these cells eliminate or diminish the ability of EGF to induce arrest then it could be suggested that $p21^{cip1}$ plays a role in the signal transduction pathway by which EGF causes growth arrest in these cells.

2. MATERIALS AND METHODS

i) The MDA-468 cell line

The cell line used in this study was the breast cancer cell line MDA-468 obtained from Dr. Ron Buick of the Ontario Cancer Institute. This immortalized cell line of breast epithelial origin is maintained in Leibovitz medium (L15) at 37°C in Forma Scientific tissue culture incubators. Media was prepared by filter sterilization through 0.22μ filter (Nalgene) to which was added fetal bovine serum (Gibco BRL) to 10%, penicillin to 10 mg/ml and streptomycin to 50 mg/ml (Gibco BRL). Media was stored at 4°C and heated to 37°C prior to cell culture use. Cells were maintained in 75 cm^2 (Costar) flasks until reaching 80% confluence whereupon they were trypsinized and replated at a concentration of $1x10^5$ cells.

ii) Bacterial cells and plasmid growth

a. Transformation of bacteria

Bacterial cells (JM109 or MC1061/P3) were inoculated in 5 ml of LB media and grown overnight at 37°C with constant shaking. The next day the cell suspension was diluted 1:200 and placed in a 500 ml flask. Over 3 to 4 hours 1 ml samples of the suspension were taken to determine the concentration of cells by measuring optical density using a Beckman DU-64 spectrophotometer at a wavelength of 600 nm. Upon reaching an absorbance of 0.400 the cell suspension was poured into 50 ml Oakridge tubes and placed on ice for 10 minutes, then centrifuged at 4000 rpm for 10 minutes at 4°C. The cell pellet was rinsed in an ice-cold solution of 0.1M $CaCl_2$ and spun again at 4000 rpm for 10 minutes at

4°C. The final pellet of cells was resuspended in 2 ml of ice-cold 0.1M $CaCl_2$ and left at 4°C for 24 hours and used for transformation with purified DNA or was added glycerol to a final concentration of 15% to store as a stock of transformationally competent cells at -70°C.

b. Transformation of bacterial cells

In all cases plasmids were transfected into bacterial cells made transformationally competent by the calcium chloride method described above. Competent cells were thawed on ice and 25-50 ng of plasmid DNA (max. of 10 μl) was added to the cell which were then left on ice for an additional 30 minutes. Cells were heat shocked at 42°C for 90 seconds and chilled on ice for 2 minutes. 0.8 ml of LB minimal broth was added to the cells which were then incubated at 37°C for 45 minutes. Each sample was plated in triplicate on LB agar plates containing ampicillin (50μg/ml) and tetracycline (1μg/ml) and incubated at 37°C overnight.

c. Large scale prep of purified plasmid

Bacterial cultures, from frozen stocks or from colonies growing on agar plates, containing plasmids of interest were grown individually overnight (18hr) at 37°C in 200 ml of LB with selectable antibiotic. Recovery and purification of plasmid DNA was performed using the alkaline lysis method [Sambrook et al., 1990]. Briefly, overnight cultures were centrifuged in four 50 ml centrifuge bottles at 5000 rpm for 15 minutes at 4°C. The pellet was rinsed in 25 ml of STE buffer (0.1M NaCl, 10mM Tris (pH 8.0) and 1mM EDTA) and centrifuged again at 7000 rpm for 15 minutes at 4°C. The pellet was resuspended in 6.5 ml of GTE buffer (50mM glucose, 25mM Tris (pH 8.0), 10mM EDTA) on ice, after which was

added 10 ml of alkaline lysis buffer (0.2M NaOH/1.0% SDS) and left on ice for 5 minutes. The solution was then neutralized by mixing with 7 ml of ice-cold 5M potassium acetate for 10 minutes on ice. Cellular debris and chromosomal DNA was removed by centrifugation at 12,000 rpm for 15 minutes at 4°C. The supernatant was removed and precipitated at -20°C with 0.5 volumes of isopropanol for 30 minutes. Nucleic acid precipitate was collected by centrifugation at 8000 rpm for 10 minutes at 4°C, then washed with 70% ethanol and left to air dry. The pellet was resuspended in 4.2 ml of 50mM Tris (pH 8.0), 1mM EDTA. To generate a cesium chloride gradient, 4.7g of cesium chloride and 0.5ml of ethidium bromide (10 mg/ml) was added to the DNA suspension. This mixture was spun at 8000 rpm for 10 minutes at 4°C and the supernatant was transferred into Beckman #342412 Quick Seal ultracentrifuge tubes and sealed. Tubes were centrifuged at 45,000 rpm in a Vti 65.2 rotor overnight (~18hr) at 20°C. At this point the plasmid band was visible by exposure to long wavelength ultraviolet light and was extracted by a syringe inserted into the tube where the plasmid band had settled. Plasmid DNA was extracted with an equal volume of butanol and then diluted to 3 volumes with 50mM Tris (pH 8.0), 1mM EDTA and then precipitated in 95% ethanol at -20°C overnight. The precipitate was centrifuged at 8000 rpm for 30 minutes, washed with 70% ethanol and resuspended in sterile dH_2O. Estimation of DNA concentration was done by spectrophotometer readings at 260 nm. Plasmids were then stored at 4°C.

d. Small scale prep of bacterial plasmid

Five ml of LB with antibiotics were inoculated under sterile conditions with cells picked from a colony on an LB agar plate and grown overnight with shaking at 37°C. The

next day, a 1.5 ml aliquot was taken and placed in a sterile 1.5 ml Eppendorf tube. The remainder of the cell suspension was placed on ice and kept if needed or stored at -70°C as a glycerol stock. The 1.5 ml aliquot was centrifuged at 5000 rpm for 2 minutes at 4°C. The supernatant was removed and cells were resuspended in 100 µl of GTE buffer. To this was added 200 ml of alkaline lysis solution. The suspension was then mixed for 5 minutes at room temperature, neutralized with 150 µl of 5M potassium acetate and placed on ice for 10 minutes. This mixture was centrifuged at 14,000rpm for 5 minutes at 4°C to remove chromosomal DNA and cellular debris. The supernatant was transferred to a new Eppendorf microcentrifuge tube and extracted with one volume of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was then precipitated with 2 volumes of 95% ethanol and stored at -20°C for at least an hour. The tubes were centrifuged at 14,000rpm for 20 minutes at 4°C yielding a pellet which was washed with 1 ml of 70% ethanol, centrifuged again and left to air dry. The plasmid was resuspended in 150 µl of sterile dH_2O for analysis by restriction enzyme digestion.

iii) Construction of expression vectors

a. Vector plasmid (pcDNA-neo)

The vector chosen to deliver $p21^{Cip1}$ anti-sense expression into MDA-468 cells is the eukaryotic expression vector pcDNA1-neo (Invitrogen). This widely used expression system makes use of a cytomegalovirus (CMV) promoter sequence, an SV40 poly-adenylation tail, and the gene expressing resistance to neomycin to act as a selectable marker for transfected cells. This vector also contains a multiple cloning site adjacent to the CMV

promoter to allow for efficient and specific ligation of inserts (Fig.5A). This plasmid was donated by Dr. Laura Gillespie and was transfected and stored in the bacterial cell line MC1061/P3 by the method described above.

b. cDNA insert of $p21^{Cip1}$

The antisense expression vector was constructed using a cDNA copy of the $p21^{Cip1}$ gene. This was obtained by generous donation from Dr. David Harper who kindly offered the $p21^{Cip1}$ cDNA cloned into the plasmid pBS-Cip1(T7) (Fig.5B) which was transfected into the bacterial cell line JM109 for storage.

The cDNA insert encoding $p21^{Cip1}$ was cut from pBS-Cip1(T7) with *Xho I* and purified from an agarose gel following electrophoresis. In this procedure the entire digestion reaction was loaded onto a 1% agarose gel in TAE containing 1 μg/ml of ethidium bromide and run at 80 volts in 1xTAE buffer. The excised fragment was visualized by exposure to low wavelength U.V. and cut from the gel. The gel slice was then placed in dialysis tubing containing 0.4 ml of 1xTAE. The ends of the tubing were sealed and immersed in a mini-gel electrophoresis apparatus. The DNA fragment was then electroeluted at 80V for 1 hour from the gel slice into the buffer within the dialysis tube. The buffer within the tubing was removed and purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and precipitation in two volumes of 95% ethanol, 0.1 volumes of 3M sodium acetate and 2 μl of glycogen (Boehringer). Precipitation was done at -20°C overnight. Following resuspension in sterile dH_2O the concentration of the band was estimated by running various amounts on a

1% agarose gel with 1 μg/ml ethidium bromide and comparing the intensity of the staining with that of a 1kb DNA ladder marker.

c. Ligation of $p21^{Cip1}$ into vector

For ligation of the insert, the vector plasmid pcDNA-Neo-I was linearized by digested with *Xho I* overnight and purified by phenol:chloroform extraction and ethanol precipitation as described above. 100 ng of linearized plasmid was ligated with 500 ng of $p21^{Cip1}$ cDNA (isolated as above) with the T4 ligase enzyme (Gibco BRL) in a total volume of 500μl at 37°C for 2.5 hrs.

10 μl of each ligase reaction was transfected into competent bacterial cells MC1061/P3 by the method described above. Transfected cells were plated on agar containing ampicillin and tetracycline and grown overnight. Single colonies of transfected bacterial cells were selected to identify colonies containing the expression vector with ligated $p21^{Cip1}$ cDNA. This was done by mini-prep of bacterial plasmid. This isolation of plasmid by mini-prep is so named as it involves the same method of bacterial plasmid isolation as the maxi prep described above but is quicker and deals with much smaller quantities of plasmid. This is suitable for screening a large number of positive bacterial colonies on a plate.

d. Identification of clones bearing ligated insert

Identification of bacterial clones bearing pcDNA-neo with the $p21^{Cip1}$ fragment was done by digestion of mini-prep plasmid with *Xho 1*. The plasmid samples from each colony of cells were digested and the products of digestion were run on a 0.7% agarose gel

containing 1 μg/ml of ethidium bromide. The bands were visualized by exposure of the gel to ultraviolet light. Plasmids containing the insert will release a 2.0kb insert as a result of this digestion. Plasmids without the insert will appear as a single band ~6.75 kb.

e. Identification of orientation of ligated inserts

To identify the orientation of the insert within the multiple cloning site of pcDNA-neo, bacterial plasmid was digested with both *Bgl II* and *Hind III*. The products of the digestion were run on a 0.7% agarose gel and visualized as described above. The antisense expression construct was labeled p68CiP1-AS (Fig.7A), and the sense expressing construct was labeled p74CiP1-S (Fig.7B). Where positive results were confirmed the resulting plasmid constructs were transfected into MC1061/P3 and propagated to yield high quantities by maxi-prep isolation described above.

iv) Stable transfection of MDA-468 Cells

a. Calcium phosphate transfection

MDA-468 cells were grown to 80% confluency in 10 cm dishes (Fisher) and transfected by the calcium phosphate method (Chen, 1988). A fine precipitate was formed in 1xHBS using 100 ng of vector by slowly adding (dropwise) a solution of 2M $CaCl_2$ to a final concentration of 0.125M. This mixture was added to the cells in 5 ml L15 media and left at 37°C for 3 hours. The media was removed and 15% glycerol in 1xHBS was added to the cells and left for 1 minute. Cells were washed twice with PBS and given fresh media. The following day, the cells were trypsinized and replated in media containing 0.6 mg/ml of G418 (Gibco BRL) to select transfected clones. (From this point on all transfected cells are

grown in media containing 0.6 mg/ml of geneticin to maintain selection.) Cells were washed and fresh media with geneticin was added every two days to remove dead cells and keep selection stringent. After eight days of selection numerous surviving colonies were visible. These cells were pooled together to form a mixed population of positive transfectants, portions of which were frozen under liquid nitrogen.

b. Freezing cells under liquid nitrogen

In cryogenic vials (Nalgene) to 1 ml suspension of cells was added 0.1 ml FBS and 0.1 ml of DMSO. Vials were placed at -70°C in an isopropanol container for 2hrs and then immersed in liquid nitrogen.

c. Generation of single clone cell lines

From the mixed population of transfected cells, single clones were obtained using 96 microwell plates (Fisher). Cell suspensions were diluted to a concentration of 0.9 cells per 200 μl aliquot to increase the probability of generating a colony of cells from a single cell origin. After a week of growth only those wells containing colonies of a single origin were maintained and developed into a single clonal cell line. Upon growing to adequate numbers (~$1x10^6$cells) stocks of each single clone cell line were frozen in liquid nitrogen. The remainder were propagated and used to study the effect of EGF-mediated arrest on the transfected cells.

v) Northern Blot Analysis

a. Isolation of RNA

Total RNA was isolated from cells using Trizol solution (Gibco BRL). This solution containing phenol and guanidium thiocyanate was added directly to the cells to lyse and isolate nucleic acids. Cells were plated at $1.5x10^6$ cells in 10 cm dishes (Fisher) and grown in the presence or absence of EGF (10^{-8}M) for specified time periods. Following incubation the media was removed, and 1.0 ml of Trizol solution was added directly to the dish at room temperature. After five minutes the cells were scraped using a cell scraper and the resulting lysate was transferred to 1.5 ml Eppendorf tubes (Fisher). The lysate was pipetted up and down for 30 seconds to aid homogenization and left to stand at room temp for 5 minutes. 200 µl of chloroform was added and vortexed for 15 seconds and left to stand for 5 minutes at room temperature to promote phase separation. Samples were then centrifuged at 10,000 rpm for 15 minutes at 4°C. The clear upper phase was carefully removed avoiding the protein interface, and placed in new eppendorf tubes. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, briefly vortexed and centrifuged at 12,000 rpm for 5 minutes at 4°C. The upper phase was then precipitated with 0.5 ml of isopropanol and stored at -20°C. The RNA precipitate was collected by centrifugation at 14,000 rpm at 4°C for 25 minutes. The pellet was washed with 70% ethanol, centrifuged again and then left to air dry for 15 minutes. The RNA was resuspended in 100 µl of DEPC-treated water containing 0.5% SDS and heated to 55°C for ten minutes to aid resuspension. The RNA concentration was determined

spectrophotometrically (Beckman DU-64 Spectrophotometer) at wavelengths 260 nm and 280 nm. Samples were then stored at -20°C.

b. Preparation of Northern blot

To measure expression of $p21^{Cip1}$ in all cell lines, total RNA was isolated and transferred to Gene Screen membranes (DuPont) for Northern blot analysis. Fifteen to twenty micrograms of total RNA from specific time points (+/- EGF treatment) were mixed in a loading buffer containing 50% (v/v) formamide, 0.25M formaldehyde, 1xMOPS (3-[N-morpholino]propanesulfonic acid: 0.2M MOPS pH 7.0, 0.5M sodium acetate, 0.01M EDTA) and 4 μg/ml ethidium bromide. Samples were run in a 1.2 % agarose gel prepared in DEPC-treated dH_2O containing 1.25M formaldehyde and 1X MOPS. Samples were run for four hours at 80 volts in 1 X Mops running buffer. After running the gel the large bands of ribosomal RNA were visualized by brief exposure to low wavelength U.V. and photographed as a means to assess equivalent loading of RNA. The gel was immersed in RNAse-free water for 10 minutes to remove excess formaldehyde while the Gene Screen membrane was immersed in RNAse-free dH_2O for 1 minute and then in 10xSSC (1.5M NaCl 0.15M trisodium citrate $2H_2O$) for 15 minutes. A capillary transfer apparatus was set up to facilitate overnight transfer of the RNA to the membrane using 10xSSC as buffer. Upon disassembly of the transfer apparatus, the gel was exposed to U.V. once again to assess transfer of the RNA from the gel to the membrane. A photograph of this exposure was taken as well. The membrane was washed in 2xSSC solution and then placed on Whatman paper

to air dry for 10 minutes. Once dry the RNA was fixed to the membrane by baking in an oven at 80°C for 2 hrs under vacuum.

c. Generation of probes for Northern blot analysis

All Northern blots were probed with 2.0kb *XhoI/HindIII* fragment of $p21^{Cip1}$. Five micrograms of the plasmid were digested overnight at 37°C. The reaction was run on a 1xTBE gel containing 1 μg/ml ethidium bromide and the 2.0kb fragment was gel purified by gel electro-elution (as described above).

d. Random primer labelling

Using a random priming labelling kit (Gibco BRL), the 2.0kb fragment was labelled with α-[^{32}P]-dCTP(DuPont). Twenty-five to fifty nanograms of the fragment diluted to 20 μl was boiled for 5 minutes and placed on ice. 15 μl of random prime buffer (Gibco BRL) and 2 μl (0.050mM) each of dGTP, dATP, and dTTP were added. 5 μl of α-[^{32}P]-dCTP (50uCi) was added and then 1 μl of Klenow enzyme (3.0 Units/μl) began the labelling reaction which proceeded at room temperature for 1.5 hrs. To terminate the reaction and to separate the labelled probe from unincorporated nucleotides, 50 μl of 1xTE was added and the entire reaction mixture was applied to a 1 ml column of G-50 Sepharose. The labelled fragment was collected by brief centrifugation of the column and a 1 μl sample was added to 3 ml of Amersham BDS scintillant to measure incorporated radioactivity by scintillation counting (Beckman LS-3801). In all cases only probes of specific activity greater than $1x10^8$ cpm/μg were used for hybridization to the Northern blots.

e. Hybridization of probe to RNA blot

Prior to hybridization RNA blots were placed in sealable plastic bags and incubated in hybridization solution for 3 hours at 42°C. The solution contained 100 μg/ml sonicated salmon sperm DNA, 5XDenhardts (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 1mg/ml BSA), 10% dextran sulphate, 1% SDS, 10xSSC, and 50% formamide. Following this incubation the solution was removed and fresh hybridization solution containing $0.5x10^6$ cpm/ml of boiled radiolabelled fragment was added and incubated overnight at 42°C.

Blots were washed with 2xSSC/0.5%SDS at 42°C and heated to 60°C. A final wash was done at 0.5xSSC/0.5%SDS at 60°C for 15 minutes. Following this wash the blots were resealed in plastic and exposed to XAR film (Kodak) for one to five days at -70°C. Densitometric scan of the films were done on an LKB Bromma, Enhanced Laser Densitometer to quantify the intensity of the resulting bands.

vi) Western blot analysis

For protein analysis cells were lysed by two methods:

a. Whole cell lysate (denaturing)

$1x10^6$ cells (+/- EGF treatment) were lysed in 1.0ml of denaturing buffer heated to 80-90°C. This buffer contained 0.5M Tris (pH 6.8), 2mM EDTA, 10% glycerol, 5% β-mercaptoethanol, and 10% SDS. Cells were scraped and placed in 1.5 ml Eppendorf tubes and incubated at 80°C for another 5 minutes. Samples were then briefly sonicated to break up genomic DNA and any insoluble material was removed by centrifugation at 12,000 rpm for 15 minutes at 4°C. The protein concentration of these samples were assayed by a Bio-

Rad detection kit. 200 µl of a diluted sample (~1:10) was added to 0.8ml of concentrated Bio-Rad detection reagent. The sample was mixed and then read by spectrophotometer at wavelength A=595 nm. Concentrations were determined by a comparison to a standard curve using a 10 mg/ml stock of BSA (bovine serum albumen) protein. Samples were then stored at -70°C or prepared in SDS-sample buffer (SSB) (15% (v/v) glycerol, 0.125M Tris-Cl, pH6.8, 5mM Na_2 EDTA, 2% (w/v) SDS, 0.1% (w/v) bromphenol blue, 1% (v/v) 2-mercaptoethanol) and loaded onto a 12% SDS separating gel using 15 µg of total protein per sample.

b. Immunoprecipitation (non denaturing)

$1.5x10^6$ cells (+/-EGF) were lysed in 1 ml non-denaturing lysis buffer containing 50mM Tris-HCl (pH 7.4), 150mM NaCl, 1% Nonidet-P40, 5mM EDTA, 1 µg/ml PMSF (Phenylmethyl sulfonylflouride), 2 µg/ml aprotinin and 1 µg/ml leupeptin. Cells were left at 4°C for 30 minutes and then scraped into Eppendorf tubes. Samples were clarified by centrifugation at 10,000 rpm for 15 minutes at 4°C. The supernatant was transferred to clean tubes to which was added 2 µg of anti-$p21^{Cip1}$ antibody (Oncogene Science) and left rotating overnight at 4°C. The following day, to the immune complex was added 50 µl of a 50% solution protein G-sepharose beads (Pharmacia) and rotated for 1hr. This complex was brought down by brief centrifugation (10 seconds). The pellet was washed three times with lysis buffer (as above) and then washed twice with 150mM NaCl. The protein was resuspended in 35µl of 2xSSB and stored at -70°C.

c. SDS-PAGE and electroblotting of proteins

Fifteen micrograms of protein was prepared in SDS-sample buffer and run on a 12% SDS-PAGE in Gibco BRL mini-gel apparatus and then electroblotted to nitrocellulose. Protein samples were boiled for 5 minutes and then loaded onto the gel using a Hamilton syringe. Samples containing immunoprecipitates were resuspended in 35 µl of 2xSSB buffer and the entire sample was loaded onto the gel. Samples from whole cell lysate were resuspended as 15 µg of protein in one volume of 2xSSB. Included with the protein samples were low-range pre-stained molecular weight markers (Bio-Rad) and biotinylated markers (Bio-Rad). Gels were run at 15 mA for 30 minutes and then 30 mA for 60 minutes in an SDS-Page running buffer consisting of 0.125M Tris, 0.3M glycine, and 0.1% SDS. At the end of the run, the gel was soaked in transfer buffer containing 0.025M Tris (pH 7.4), 0.20M glycine, and 20% methanol for 15 minutes. Nitrocellulose (Amersham, Hybond-ECL) was also soaked in transfer buffer. Gel and membrane were assembled in a sandwich and placed in a gel apparatus to transfer the proteins from the gel to the nitrocellulose at 100 V for 1.5 hour at 4°C. The transfer buffer was changed at 45 minutes to keep the buffer cold. The membrane was then placed in a solution of TBST (1M Tris, 5M NaCl, 0.1% Tween) until needed. To assay proper transfer the gel was stained with Coomasie Blue and the blots were reversibly stained with Ponceau-S dye.

d. Immunodetection of protein blots

Protein blots were blocked for non-specific binding with TBST containing 5% milk powder for 1hr. The primary antibody, anti-$p21^{Cip1}$ (Oncogene Science), diluted to 2 µg/ml

in TBST containing 5% milk powder and 0.02% azide was incubated with the blot with gentle rocking overnight at room temperature. The blots were then washed in TBST for one hour with vigorous shaking. The secondary antibody, goat anti-mouse whole antibody conjugated to biotin (Amersham), [and in one case it was sheep anti-mouse whole antibody conjugated to horseradish peroxidase (HRP)], was diluted to 1:1000 in TBST and added to the blot and rocked for 1hr. Blots were washed as above. Where the secondary antibody was conjugated to biotin, streptavidin-horseradish peroxidase (Amersham) was added at a dilution of 1:500 in TBST and rocked for 1hr. Blots were washed again and then 1.0 ml of detection reagent was added (Amersham, ECL-detection kit). After 1 minute blots were placed between two sheets of plastic page protectors and taped down in a film cassette (Fisher). Chemiluminescent-sensitive film (Amersham; Hyper-film-ECL) was exposed to the blot to get sufficient detection of protein bands (5 minutes to an hour). When required, blots were stripped in 100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl (pH 6.7) for 30 minutes in shaking 70°C water bath and rinsed extensively in TBST before storing at 4°C. Effectiveness of stripping was assayed by adding detection reagents and exposing to film.

vii) EGF-mediated growth arrest

MDA-468 cells or transfected single clones were plated in triplicate in 60 mm dishes (Nunclon) at a concentration of $2x10^4$ cells. Epidermal growth factor (Collaborative Research; natural mouse, culture grade) was mixed with L15 media to a concentration of 10^{-8}M and added to cells to undergo EGF-treatment; L15 alone was added to control cells. After EGF treatment for a given period (i.e. four or eight days) cells were trypsinized in 1.0

ml of 5% trypsin in PBS and resuspended in 0.5 ml of media to be counted by haemocytometer. Cells treated for longer than four days had their media changed on day four (washed once with PBS then fresh media with or without EGF).

3. RESULTS

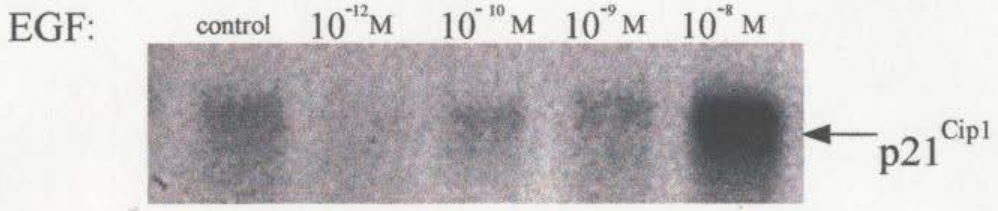
i) EGF induces upregulation of p21Cip1

To elucidate a role for $p21^{Cip1}$ in the EGF-mediated growth arrest of MDA-468, it was of interest to examine if EGF treatment alone affected the expression of $p21^{Cip1}$ during the course of G_1 arrest. Should EGF treatment foster a change in the expression of $p21^{Cip1}$ concurrent with growth arrest, then a role for $p21^{Cip1}$ in that arrest could be suggested.

To measure the effect of EGF on $p21^{Cip1}$ expression, MDA-468 cells were treated with EGF and analysed by Northern blot analysis using probes to detect $p21^{Cip1}$ mRNA. Cells were serum starved for 48 hours to remove growth factors from the media and then incubated in serum free media supplemented with EGF at concentrations ranging from 10^{-12}M to 10^{-8}M for 12 hours. Control cells were also serum starved for 48 hours but with subsequent addition of media containing 10% FBS to give an indication of the expression of $p21^{Cip1}$ during normal cell culture conditions.

Figure 1A shows that EGF can induce the expression of $p21^{Cip1}$ mRNA and does so in a dose dependent manner. Clearly the expression of $p21^{Cip1}$ is dramatically induced when cells are exposed to EGF at a concentration of 10^{-8}M, the concentration which induces G_1 arrest in these cells (Prasad and Church 1992). At lower concentrations which do not induce G_1 arrest, (10^{-10}M or 10^{-9}M), the level of $p21^{Cip1}$ mRNA is not significantly increased above control levels. At an EGF concentration of 10^{-12}M no expression of $p21^{Cip1}$ is seen. Since

A.



B.

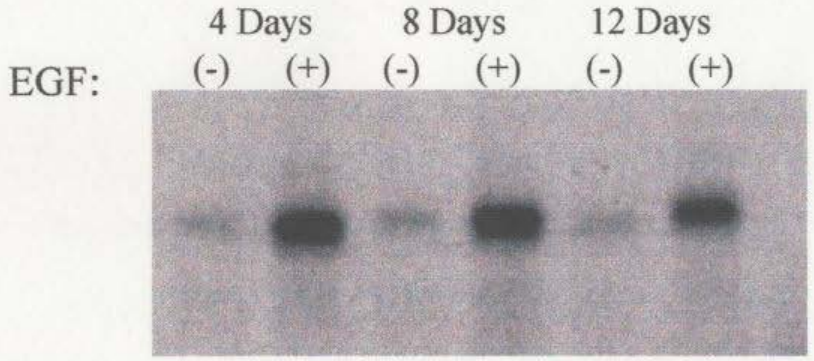


Figure 1: Northern blot analysis of the dose response and long term effect of EGF on p21Cip1 expression in MDA-468 cells

(A) **Northern blot analysis of the dose response of $p21^{Cip1}$ mRNA to EGF.** MDA-468 plated at $1x10^6$ and exposed to EGF at concentrations ranging from 10^{-12}M, 10^{-10} M, 10^{-9} M, and 10^{-8} M for 12 hours. After exposure the cells were lysed for RNA isolation using Trizol solution (Gibco BRL). Twenty micrograms of total RNA was run on a formaldehyde gel, transferred to GeneScreen membranes, and probed with a radiolablelled fragment of $p21^{Cip1}$ cDNA. Labelled blot was washed and then exposed to Kodak X-OMAT film overnight.

(B) **Northern blot analysis on the long-term effect of EGF on $p21^{Cip1}$ expression.** MDA-468 cells were grown in the presence or absence of EGF at 10^{-8} M for 4, 8, and 12 days. After exposure the cells were lysed for RNA isolation using TriZol solution (Gibco BRL). Twenty micrograms of total RNA was run on a formaldehyde gel and detected as described above.

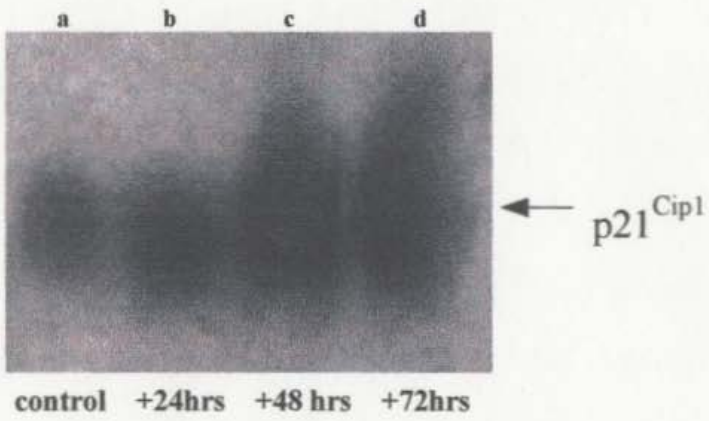
the levels of $p21^{Cip1}$ mRNA significantly increase when the cells are exposed to the growth inhibitory concentration of EGF (i.e. 10^{-8}M), further references to EGF treatment in this text are assumed to be at 10^{-8}M.

Induction of $p21^{Cip1}$ mRNA in these cells can also be demonstrated upon long term exposure to EGF. Figure 1B shows a Northern blot of cells treated with EGF (at 10^{-8}M) for 4, 8 and 12 days. Clearly, strong upregulation of $p21^{Cip1}$ is maintained in the continued presence of EGF while levels remain low in its absence.

In the presence of EGF at 10^{-8}M, MDA-468 cells begin to accumulate in G_1 within 48-72 hours (Prasad *et al.*, 1991). Figure 2A demonstrates that induction of $p21^{Cip1}$ also occurs within this time frame. In cells treated with EGF from 12, 24, 48, and 72 hours, expression of $p21^{Cip1}$ increases with the duration of exposure to EGF, reaching a maximum at 72 hours.

To examine the immediate effect of EGF on the expression of $p21^{Cip1}$, MDA-468 cells were exposed to EGF from 2 to 15 hours (Fig. 3A). Surprisingly, the early effect of EGF on $p21^{Cip1}$ is quite dramatic. Within two hours of treatment there is a rapid increase in the level of mRNA (Fig. 3A). This initial response remains high for the first six hours then begins to decline, yet remains above basal levels. Previous studies have revealed that the G_1 arrest initiated by EGF in these cells is reversible upon removal of EGF from the media. Upon removal of EGF from the media, the cells were able to return to their proliferative growth rate (Prasad *et al.*, 1991). The Northern blot analysis of Figure 3B demonstrates that induction of $p21^{Cip1}$ message by EGF is also reversible upon removal of

A.



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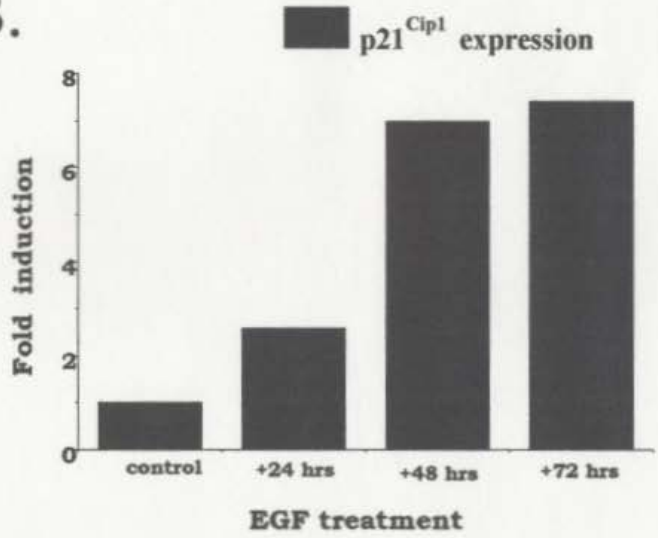


Figure 2: Northern blot analysis of the effect of EGF on $p21^{Cip1}$ expression during onset of G_1 arrest.

(A) **Northern blot analysis of $p21^{Cip1}$ mRNA prior to onset of EGF-induced growth arrest.** MDA-468 cells were grown in the presence or absence of EGF at 10^{-8} M for 24, 48, and 72 hours. After exposure the cells were lysed for RNA isolation using TriZol solution (Gibco BRL). Twenty micrograms of total RNA was run on a formaldehyde gel and detected as described above.

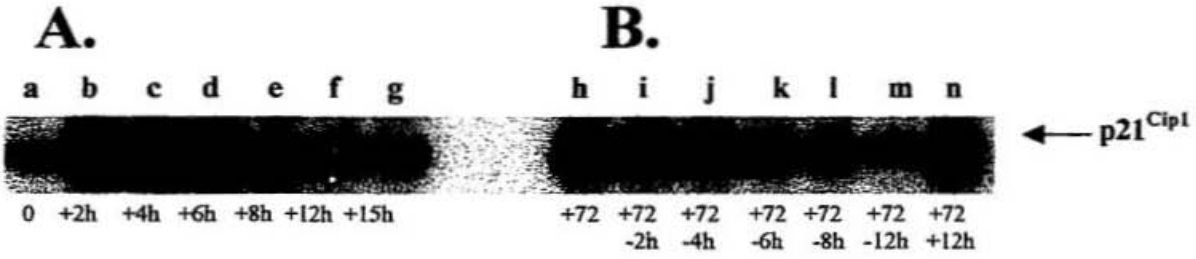
(B) **Histogram of intensities of band in Figure 2A.** The film of the blot in Figure 2A was scanned by laser densitometry and the values presented as fold induction over the signal from untreated MDA-468 cells.

EGF from the media. In each lane of Figure 3B all cells were grown in the presence of EGF for 72 hours to initiate a strong induction of $p21^{Cip1}$. Then the cells were washed with PBS and incubated in media lacking EGF for an additional 2, 4, 6, 8, or 12 hours before lysis for RNA isolation. Clearly it can be seen that upon removal of EGF, induction of $p21^{Cip1}$ ceases and its expression decreases within 12 hours. Lane n of Figure 3B depicts cells which were not washed with PBS but remained in the continuous presence of EGF for another 12 hours. In this case significant $p21^{Cip1}$ expression is maintained.

Thus it can be seen that the level of $p21^{Cip1}$ mRNA is dramatically upregulated by EGF, occurs prior to onset of G_1 arrest, and that high expression of $p21^{Cip1}$ is maintained only in the continued presence of EGF. The histogram of Figure 3C is generated from a scan of the continuously-treated samples from figure 3A,B (ie. Lanes a-g, and h and n). This histogram shows that EGF induction of $p21^{Cip1}$ is somewhat bi-phasic in nature. Initially, there is a rapid increase in $p21^{Cip1}$ mRNA in the first four hours, which quickly drops off. However a second phase of induction occurs between 15 and 72 hours.

ii) Analysis of $p21^{Cip1}$ protein in MDA-468 cells

Having demonstrated an induction of $p21^{Cip1}$ mRNA by EGF it was of interest to examine the effect of EGF on the level of $p21^{Cip1}$ protein. Western blot analysis was done using a mouse monoclonal antibody to $p21^{Cip1}$ (Oncogene Science) as a primary antibody and chemilumenescent detection was done using either a biotin-conjugated secondary antibody (Fig.4A) or an HRP-conjugated secondary antibody (Fig.4B). The isolation of protein from MDA-468 was by using either a whole cell lysate or using protein samples



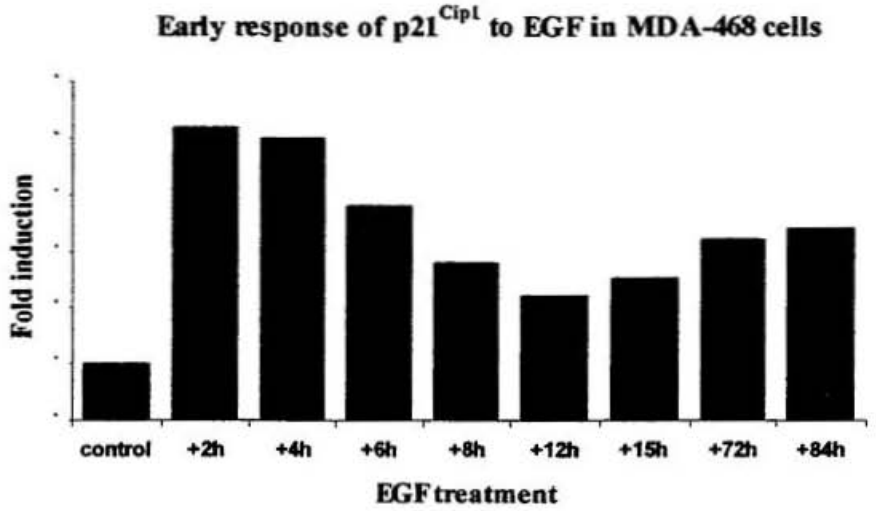


Figure 3: Northern blot analysis of $p21^{Cip1}$ expression upon short-term EGF treatment and removal from EGF

(A) **Northern analysis on the early effect of EGF on $p21^{Cip1}$ expression.** MDA-468 cells were exposed to EGF at 10^{-8} M for either 2, 4, 6, 8, 12 or 15 hours. After exposure the cells were lysed for RNA isolation using TriZol solution (Gibco BRL). Twenty micrograms of total RNA was run on a formaldehyde gel and detected as described previously.

(B) **Northern analysis on time course of $p21^{Cip1}$ expression following removal of EGF from media from cells pre-exposed to EGF at 10^{-8} M for 72 hours.** MDA-468 cells were exposed to EGF at 10^{-8} M for 72 hours at which time the media was replaced with media lacking EGF and cells were incubated for a further 2, 4, 6, 8, or 12 hours. One sample of cells remained in the presence of EGF for 12 hours.

(C) **Histogram of the intensities of continuously EGF-treated samples from the Northern blot of Figure 3A,B.** The film of the blot in Figure 3A,B was scanned by laser densitometry and the values presented as fold induction over the signal from untreated MDA-468 cells.

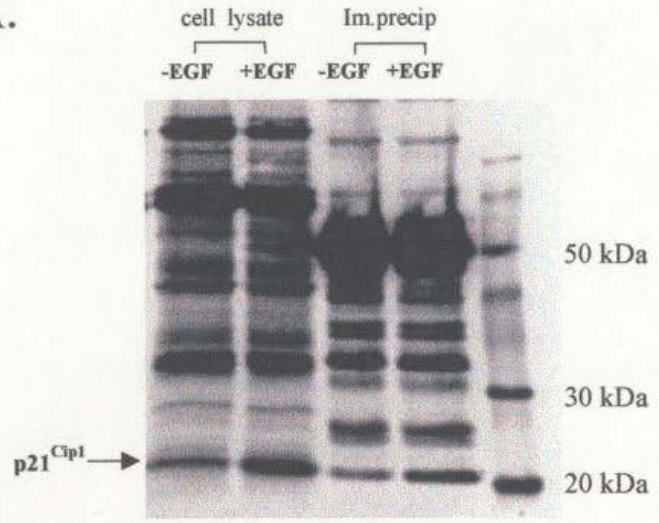
collected by immunoprecipitation of a nondenaturing cell lysate with an anti-$p21^{Cip1}$ antibody. To analyse the effect of EGF on $p21^{Cip1}$ protein, the cells were treated with EGF for 72 hours prior to lysis and protein isolation to be compared with protein samples from untreated cells.

In Figure 4A, a 21 kDa protein is detected and this 21kDa band increases in intensity following EGF-treatment while other non-specific bands do not. Whether samples were from whole cell lysate or from immunoprecipitated samples, 72 hours EGF treatment caused a 2 to 3-fold induction of $p21^{Cip1}$. Most notable in Figure 4A is the presence of many bands other than that of $p21^{Cip1}$. This was expected as the detection in this Western blot uses a biotin-conjugated secondary antibody which tends to be oversensitive and result in a high degree of background. Even when the protein samples came from immunoprecipitation with an anti-$p21^{Cip1}$ antibody, a significant degree of non-specific bands persists.

Figure 4B depicts a Western blot where the detection uses a sheep anti-mouse secondary conjugated to horseradish peroxidase which is less sensitive in its detection. In Figure 4B the detection of $p21^{Cip1}$ is much weaker yet the samples purified by immunoprecipitation specifically detect $p21^{Cip1}$ without an apparent interference by background.

For the immunoprecipitated lanes of figure 4B, one can see the $p21^{Cip1}$ band at 21kDa, the heavy chain of the immunoprecipitating antibody at 50kDa, and that of the light chain at 25kDa. While the amount of heavy and light chain remain the same in both lanes,

A.



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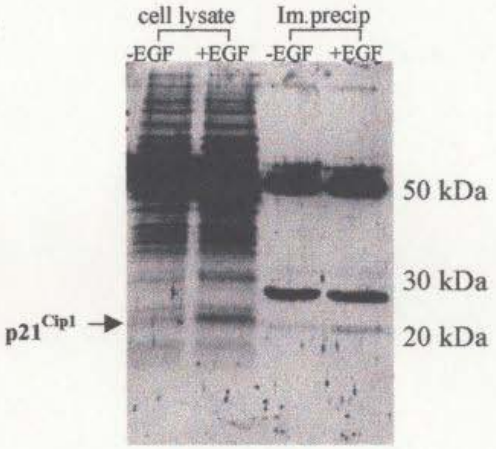


Figure 4: Western blot analysis of $p21^{Cip1}$ protein in MDA-468 cells.

(A) **Western blot analysis of $p21^{Cip1}$ protein in EGF-treated MDA-468 cells.** The detection of $p21^{Cip1}$ protein by Western blot analysis was derived from samples of MDA-468 cells incubated in the absence or presence of EGF at 10^{-8} M for 72 hours. Isolation of protein was either by whole cell lysate or by a non-denaturating lysate immunoprecipitated with mouse anti-$p21^{Cip1}$ antibody. 100µg of whole cell lysates or resuspended immunoprecipitates were loaded and run on a 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. The detection of protein was done using the ECL chemilumenescent kit from Amersham. The murine anti-$p21^{Cip1}$ antibody was used as a primary antibody while a biotin-conjugated sheep anti-mouse antibody was used as a secondary. Following the addition of streptavidin-horse radish peroxidase the ECL substrates were applied to the blot and the blot was exposed to Kodak X-OMAT film for 5 to 10 minutes.

(B) Western detection was similar to that described above, however, the secondary antibody used was a sheep anti-mouse HRP-conjugated antibody.

the specific induction of $p21^{Cip1}$ following EGF treatment is clearly visible. Having confirmed that a specific detection of $p21^{Cip1}$ could be achieved in the immunoprecipitated protein samples, it was decided that further detection of $p21^{Cip1}$ protein would use the biotin-conjugated secondary antibody (Fig. 4A). Despite the increased background, this method is more sensitive in the detection of the $p21^{Cip1}$ band. (Fig 4A).

iii) Construction of a $p21^{Cip1}$ antisense expression vector

To provide direct evidence for the role of $p21^{Cip1}$ in the growth arrest of MDA-468 cells, the goal of this study was to transfect these cells with an antisense to $p21^{Cip1}$ and examine the subsequent result on the ability of these cells to undergo EGF-mediated growth arrest. Hence, the full length cDNA of the $p21^{Cip1}$ cDNA was inserted in the inverse orientation into the eukaryotic expression vector pc-DNA-neo (Invitrogen) (Fig.5A). The cDNA of $p21^{Cip1}$ was excised from the pBS-cip1(t7) plasmid (Fig.5B) by cleavage with *Xho 1* and the 2.0kb fragment was retrieved by gel purification. This fragment was then ligated into the *Xho 1* site of pc-DNA-neo with T4 ligase and transfected into MC1061/P3 bacterial cells. Bacterial clones were selected on ampicillin/tetracycline plates and screened for plasmids bearing the 2.0kb $p21^{Cip1}$ cDNA insert.

iv) Screening of ligated plasmids

To screen positive clones bearing the $p21^{Cip1}$ insert in pc-DNA-neo bacterial plasmid was isolated from clones by mini-prep isolation and digested with *Xho 1*. Digestion of positive clones should produce two bands: the vector at 6.75kb and the $p21^{Cip1}$ insert at

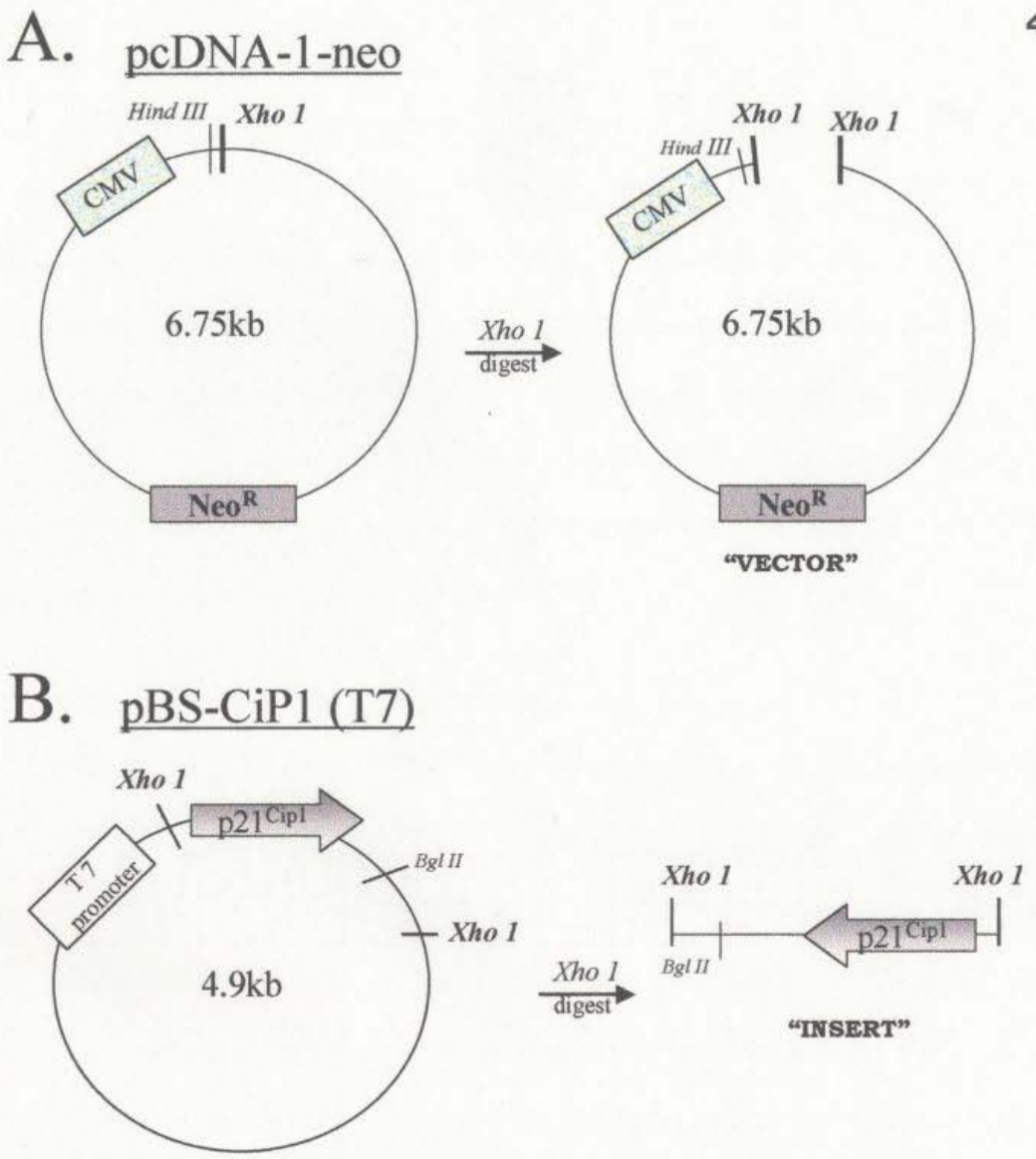


Figure 5: Diagram of the plasmids pcDNA-neo and pBS-Cip1(T7) and result of *Xho 1* digest.

(A) **Plasmid pcDNA1-neo.** The 6.75bp expression vector was digested with *Xho 1* to generate a linear band to function as the vector for ligation with the $p21^{Cip1}$ insert.
(B) **Plasmid pBS-CiP1(T7)** The plasmid pBS-CiP1(T7) was digested with *Xho 1* to generate a 2.0kb band to function as the $p21^{Cip1}$ insert.

2.0kb. Clones bearing pcDNA-neo without an insert would be linearized by *Xho I* and produce a single band at 6.75kb. Figure 6A shows a picture of numerous digests run on a 0.7% agarose gel and stained with ethidium bromide to visualise the DNA. Lanes a-d are labelled to indicate digestions where a 2.0kb fragment was detected in four of the colonies, thus identifying these bacteria as containing the $p21^{Cip1}$ insert ligated into the vector. Other lanes of this figure show cases where ligation of the insert into the vector did not occur. The positive transfectants shown in this Figure are from bacterial colonies that were labelled 6.8 (lane a), 7.4 (lane b), 8.1 (lane c) and 8.2 (lane d).

v) Determining the orientation of ligated insert

Since the insert generated by this cloning stratagy of *Xho I* overhangs at both ends, it was possible that the $p21^{Cip1}$ cDNA insert could ligate into pcDNA-neo in both a sense or antisense orientation relative to the CMV promoter. To determine the orientation of the insert in the positive clones, plasmid from these colonies were also digested with *Bgl II* and *Hind III*. As depicted in Figure 7, the two possible orientations would produce two different pattern of bands from this double digest. For inserts ligated in the antisense direction relative to the CMV promoter, the digestion reactions will give three products of 5030bp, 3350bp, and 360bp [Fig. 7A]. Alternatively, pcDNA-neo containing the $p21^{Cip1}$ fragment in the sense direction, would produce three fragments of 5030bp, 2030bp, and 1680bp size [Fig. 7B].

Figure 6B shows two examples of plasmids which contain the $p21^{Cip1}$ insert in pcDNA-neo with opposing orientations. Lane a, shows the 1kb marker, lane b, the

BglI/HindIII digest of plasmid from clone 6.8 and lane c, that from clone 7.4. The pattern of bands in lane b indicate a plasmid bearing the $p21^{Cip1}$ cDNA ligated in an antisense orientation. This lane shows fragments at 5.0kb, 3.3kb and 360bp. (The 360 bp fragment is not visible in the photograph shown, yet clearly identifiable on the original).

The pattern of digestion in lane c fits the pattern predicted for a plasmid with the $p21^{Cip1}$ insert in a sense direction relative to the CMV promoter. As a result, two expression vectors with the $p21^{Cip1}$ gene in the antisense and sense direction relative to the CMV promoter were created. For the purposes of nomenclature the positive clone containing $p21^{Cip1}$ in antisense direction was found in colonly 6-8, thus the antisense bearing plasmid was named p68-Cip1(AS) (Fig. 7A) and likewise the sense containing plasmid was labelled p74-Cip1(S) (Fig. 7B). The expression constructs were transfected into bacteria (MC1061/P3) to generate large quantities and to store as glycerol stocks at -70°C.

vi) Transfection of plasmid into MDA-468 cells

The antisense (Fig. 7A) and sense (Fig.7B) expression vectors were stably transfected into MDA-468 cells as well as the empty vector (pcDNA-neo without the $p21^{Cip1}$ insert) to act as a control. Five micrograms of each plasmid were transfected into the cells by calcium phosphate precipitation and then selected in media containing 0.6 mg/ml of G418 (neomycin). Untransfected MDA-468 cells were also treated with G418 to act as a

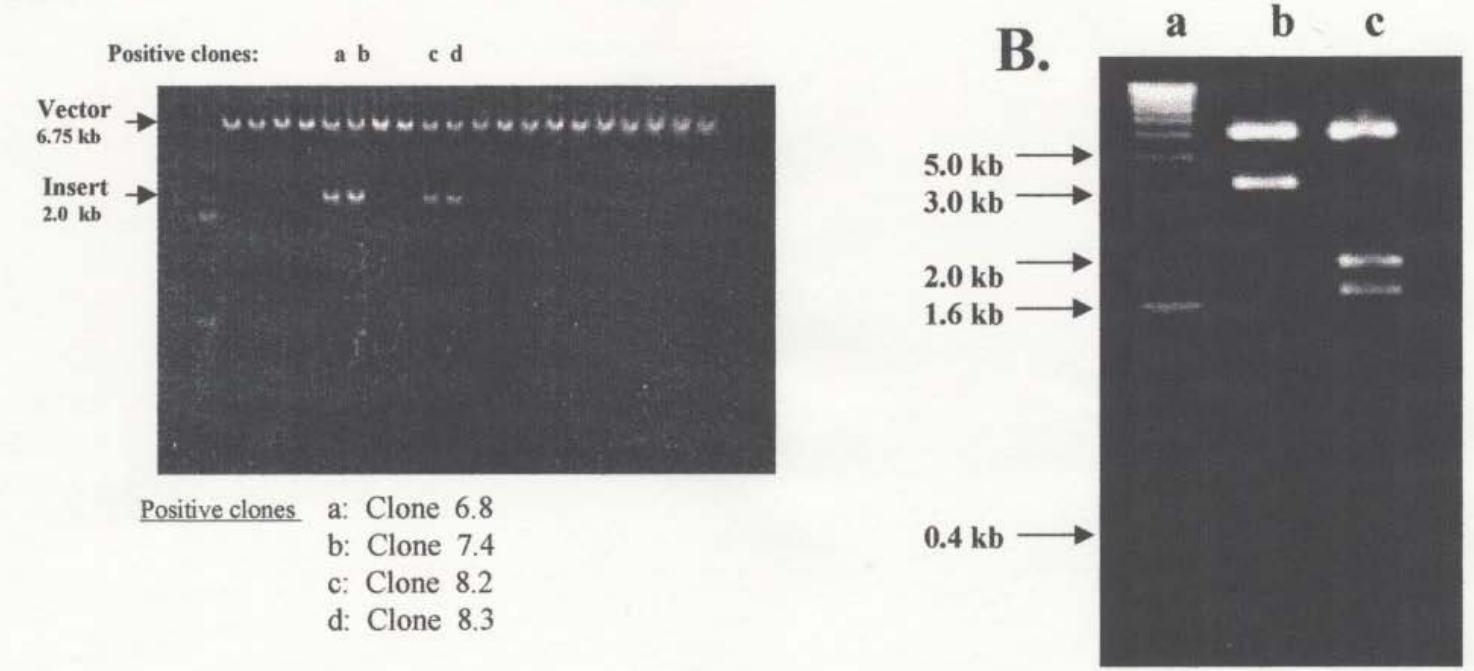


Figure 6: Ethidium bromide stain of two gels used to screen cloning of the antisense and sense $p21^{Cip1}$ expression vectors

(A) **Agarose gel of *Xho 1* digested mini-prep DNA from bacterial clones.** The ligation of the $p21^{Cip1}$ insert into pcDNA1-neo vector and transformed into mC1061 bacteria and isolated by mini-prep plasmid isolation. 1 ug of bacterial plasmid was digested with *Xho 1* for one hour and then run on a 0.7% agarose gel containing 10ug/mL of ethidium bromide. The gel was exposed to ultraviolet light and photographed. Band size were determined by the 1 kb ladder (Gibco BRL).
(B) **Screening of positive clones for orientation within vector.** Plasmids screened for the presence of the $p21^{Cip1}$ insert were digested in *BglII/HindIII* to determine the orientation of ligated insert in the vector. 2ug of bacterial plasmid was digested with *BglII/HindIII* for one hour and then run on a 0.7% agarose gel containing 10ug/mL of ethidium bromide. The gel was exposed to ultraviolet light and photographed. Band size were determined by the 1 kb ladder (Gibco BRL).

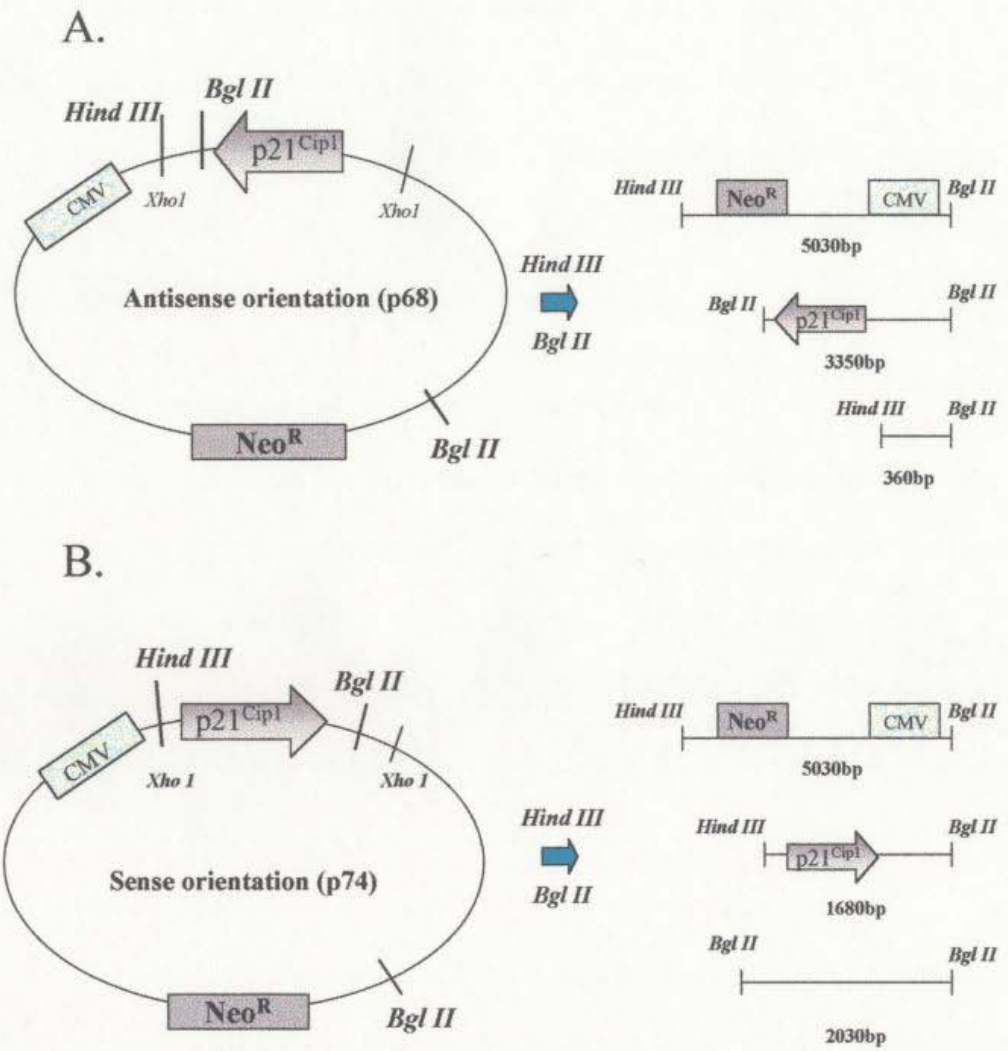


Figure 7: Diagram of ligation products and diagnostic digest with *BglII/Xho1*.

(A) **Antisense orientation of $p21^{Cip1}$ expression vector.** Ligation products containing $p21^{Cip1}$ in and antisense direction relative to the CMV promoter can be identified by a double digest of *HindIII/BglII* giving the fragments of 5340bp, 3350bp, and 360bp in size.
(B) **Sense orientation of $p21^{Cip1}$ expression vector.** Ligation products containing $p21^{Cip1}$ in and antisense direction relative to the CMV promoter can be identified by a double digest of *HindIII/BglII* giving the fragments of 5340bp, 2030bp, and 1680bp in size.

control for drug selection.

During the initial selection, cell death became evident within 5 days and selection was maintained for an additional 8 days at which time the untransfected cells had died off and the surviving transfected cells were pooled together to form a mixed population of positive transfectants. To maintain selection, all subsequent cell culture with these transfected cells was done using media containing G418.

vii) Isolation of single clones of transfected MDA-468

To generate single clone cell lines from the mixed population of transfected MDA-468 cells, the antisense and sense transfected cells were each plated on 96-microwell plates to generate colonies derived from a single cell. All single clones derived from the transfection with p68-Cip1(AS) were labelled 6-n (where n=1,2,3...) to signify clones of MDA-468 transfected with the antisense construct to $p21^{Cip1}$. Those cell lines transfected by plasmid p74-Cip1(S) were labelled 7-n (where n=1,2,3...) to signify clones containing the sense construct of $p21^{Cip1}$.

An interesting observation was that relatively few single clones generated by the sense transfection survived to form single clone cell lines. While close to 30 antisense transfected single clone cell lines could be propagated, only 5 sense transfected single clone cell lines could be generated. This may not be surprising given the potent tumour suppressive activity seen when $p21^{Cip1}$ is ectopically expressed in tumour cells (Yang *et al.*, 1995).

viii) Cell growth experiments

The aim of transfecting MDA-468 cells with the antisense to $p21^{Cip1}$ is ultimately to test for a reduced ability of the cells to undergo G_1 arrest in the presence of EGF. Hence an important initial screen for the effectiveness of the antisense was to observe the growth of single clones when treated with EGF.

To assay for EGF growth arrest, a fixed number of cells were plated and grown in the presence or absence of EGF for a duration of eight days at which time the cells were counted. A comparison between the difference in cell numbers of treated and untreated cells would give an indication of the degree of growth arrest for each cell line. With a longer incubation the difference in cell number between treated and untreated is more significant, hence the cells were incubated in the presence or absence of EGF for 8 days. In all cases each cell line was counted in triplicate (i.e. three dishes were treated, and three dishes were untreated) and each cell line examined in at least three independent trials. Table 1 shows the result of these growth experiments.

The growth of MDA-468 cells in the presence of EGF serves as a benchmark to which antisense-transfected cell lines were compared. After 8 days incubation, the untreated MDA-468 cells grow to a final number of $44x10^4$ while those treated with EGF number only reach $16x10^4$. The fact that cell division persists in the treated group is due to the observation that a small proportion of the mixed population that make up MDA-468 cells do not undergo G_1 arrest (Prasad *et al.*, 1991).

An initial observation from the data in Table 1 is that MDA-468 cells treated with EGF for 8 days grow to a number that is consistently 60% less than the

Table 1: Effect of EGF on the growth of MDA-468 and transfected cell lines.

Cell Line	-EGF ($x10^4$ ± Sd)		+EGF ($x10^4$ ± Sd)		% Arrest
MDA-468	44.3	± 9.4	16.0	±1.4	64%
6-2	25.4	± 2.9	11.1	± 5.5	57%
6-4	41.2	± 11.7	18.8	± 1.7	54%
6-6	19.3	± 9.9	8.5	± 3.3	56%
6-7	32.3	± 6.4	16.9	± 4.6	48%
6-8	36.7	± 7.6	15.3	± 4.2	58%
6-10	21.0	± 4.6	9.7	± 3.7	54%
6-11	25.1	± 4.1	16.0	± 3.6	36%
6-12	29.0	± 0.3	19.1	± 0.3	34%
6-15	16.6	± 8.2	6.3	± 1.0	62%
6-16	20.1	± 1.8	10.8	± 1.2	46%
6-17	20.4	± 2.3	5.0	± 1.4	76%
6-18	36.6	± 9.7	14.9	± 3.6	59%
6-19	35.7	± 9.9	27.7	± 7.0	22%
6-20	28.2	± 9.2	14.3	± 5.1	49%
6-22	21.1	± 2.5	14.1	± 2.0	33%
6-23	45.6	± 12.2	29.8	± 12.6	35%
6-25	43.2	± 7.0	32.4	± 5.2	25%
6-26	31.7	± 6.3	25.1	± 7.2	21%
7-6	39.9	± 6.0	21.0	± 1.9	46%
7-7	34.8	± 2.7	22.9	± 1.9	36%
7-9	35.7	± 5.2	22.0	± 5.1	38%
7-17	27.6	± 3.8	21.6	± 6.7	21%
7-19	21.9	± 8.6	10.9	± 4.3	50%

antisense

sense

All cell lines were plated at $2x10^4$ in 60mm dishes in triplicate and grown for eight days in the absence or presence of EGF at 10^{-8} M. On day 4 of incubation the media was removed and replaced with fresh media with or without EGF. On day 8 all cells were trypsinized and the total number of cells counted by haemocytometer. The cell numbers presented in this table represent the number of cells $x10^4$ and are the average cell numbers from three repeats of this procedure.

% arrest = (#cells with EGF (day 8) - ## cells without EGF(day 8)) / # cells without EGF(day8)

untreated cells. Presumably expression of antisense to $p21^{Cip1}$ would interfere with the ability of EGF to cause growth arrest, thus an even greater proportion of treated cells would escape G_1 arrest over this time. Thus, any reduction of growth arrest due to the antisense to $p21^{Cip1}$ would result in a growth difference which is less than 60%. In cases where the percent difference in growth for a cell line is below 60%, it could be said that growth arrest has been attenuated.

The histogram of Figure 8 presents the percent difference between treated and untreated cells for MDA-468 and the antisense-transfected cell lines. The profile of figure 8 indicates that antisense-transfection appears to have made some reduction in growth arrest when compared to MDA-468. However, the majority of these cell lines demonstrate a growth difference between 40 and 60%, This may not be significantly different from the average of 60% observed in MDA-468, suggesting that a significant degree of growth arrest is still occurring in these cells.

However there are a few notable exceptions where the treated cells continue to proliferate somewhat similar to untreated cells. The cell lines 6-11, 6-12, 6-19, 6-22, 6-23, 6-25, and 6-26 demonstrate only a 22-36% reduction in growth for the EGF-treated group. Of these, the cell lines 6-19, 6-25 and 6-26 stand out as having a dramatically reduced ability to arrest.

Table 1 also reveals that the single clones each grow at different rate from one another, even in the untreated case. Each cell line, though plated at $2x10^4$ cells, after 8 days without treatment grow to a final number as low as $16X10^4$ (6-15) or as high as $45x10^4$ (6-23). Between these two cell lines there is a 3-fold difference in basal growth rate. This is

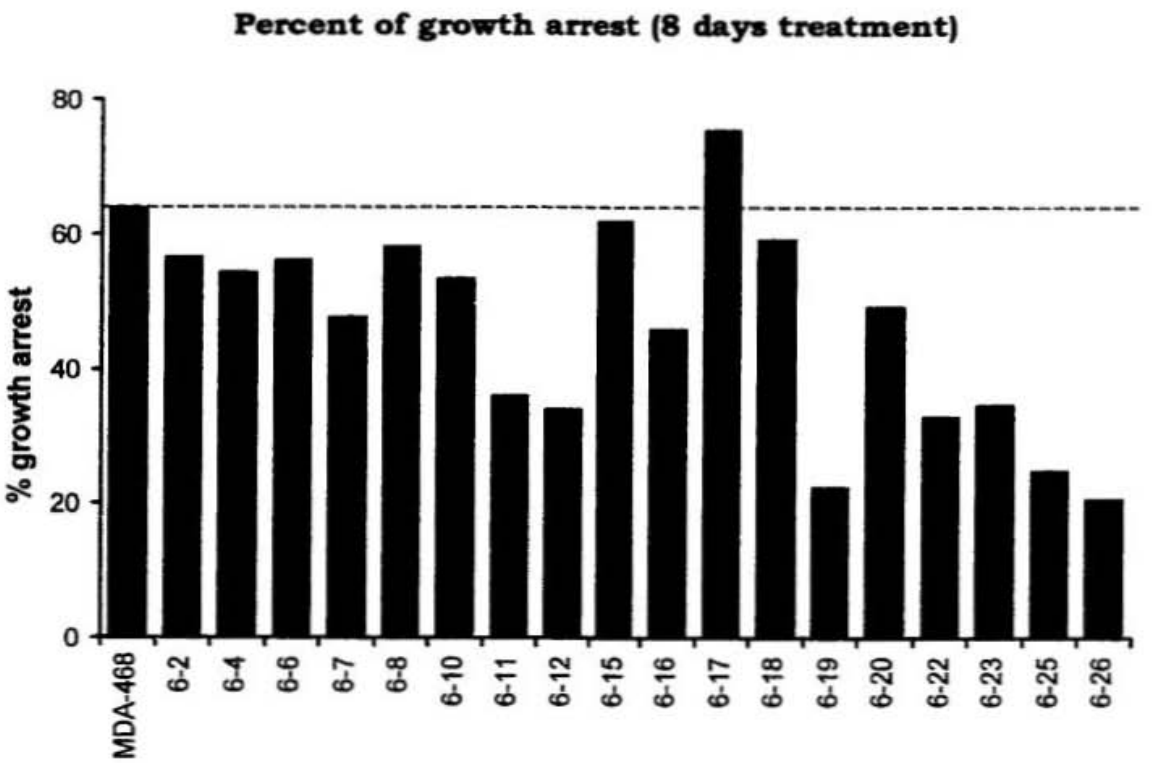


Figure 8: Percent change in growth of EGF-treated MDA-468 and antisense transfected cells.

Percentages are derived from the data of Table 1 and refer to the difference in total number of cells between treated and untreated cells for each given cell line. Treatment of cells is as described in legend of Table 1.

not surprising since the MDA-468 cell line is a mixed population of cells such that isolation of unique clones would result in a variety of growth rates. When individual single clones are derived, each of these can have their own growth characteristics which will inevitably differ from clone to clone.

Figure 9 presents the variability in basal growth rates among the single clones (ie. growth in the absence of EGF). Due to this variability the actual numbers of cells from one cell line to another cannot be used to compare an ability to undergo growth arrest (Table 1). Clearly there are some cell lines such as 6-2, 6-6, 6-10, 6-15 etc. which, in relation to the parental cell line, are quite slow growers. Hence, only the percent difference in growth can be compared from cell line to cell line.

This variability adds complexity to the comparison of the single clones. A cell line that initially has a slow growth rate may not show a significant degree of growth difference since the untreated cells may not significantly out pace the treated group. As a result, this will appear as a diminished percentage difference in growth arrest (i.e. less than 60%) as measured in figure 8. More importantly, slow growing cell lines the degree of growth arrest may appear to be less than 60% but not because of transfection with an antisense to $p21^{Cip1}$ but because the untreated cells do not grow at a rate such that a difference with the treated cells would have been achieved to begin with.

Therefore, the cell lines which show reduced growth arrest yet are relatively slow growers may give a false positive if a decrease of growth arrest is presumed to be the result of antisense to $p21^{Cip1}$. Therefore these slow growing cell lines identified in figure 9 may make for poor comparison with MDA-468.

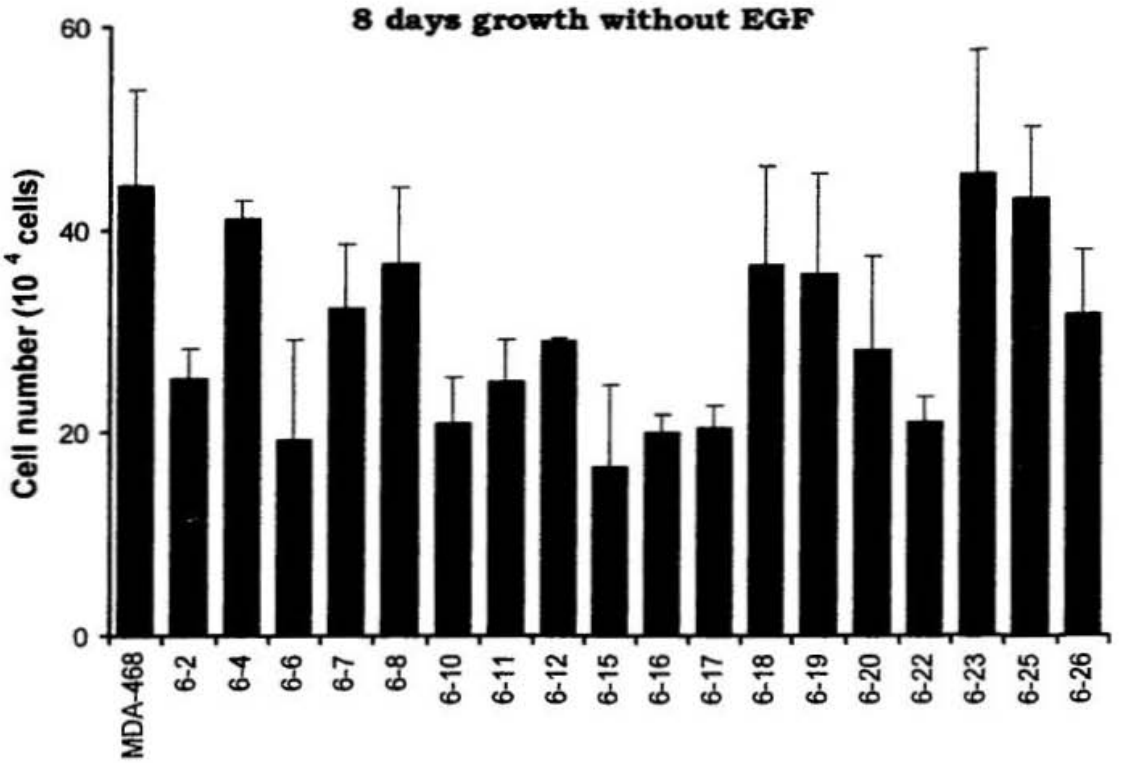


Figure 9: Untreated growth of MDA-468 and antisense clones over 8 days.

An examination of the growth of MDA-468 and single clones in the absence of EGF for 8 days. Data generated in Table 1 (-EGF).

Hence it was decided to focus on those clones that had growth rates similar to the basal rate of growth for MDA-468. The cell lines 6-19, 6-25 and 6-26 were chosen for molecular analysis as they displayed the greatest reduction in growth arrest (Fig.8) yet grow at rates similar to MDA-468 (i.e. within one standard deviation) (Fig.9). The cell line 6-23, although fitting this criteria, was not included in the molecular analysis but was examined in the EGF-time course experiment (see below).

ix) Growth of sense-transfected cells

Transfection of MDA-468 with a sense expressing construct of $p21^{Cip1}$ (Fig.7B) was done to act as a control to demonstrate that reduced growth arrest in antisense-transfected cells was specific to antisense expression. However, figure 10 shows that even the sense transfected cell lines (those that are labeled 7-n) demonstrate a difference between treated and untreated cells which is less than 60%. It appears that these cells also undergo growth arrest to a degree even less than that of MDA-468. This is certainly a very curious and unexpected result. Nevertheless, these sense-transfected cells were useful as controls for the molecular analysis which follows

x) Northern Analysis of transfected cells

To assess the ability of antisense $p21^{Cip1}$ RNA to downregulate the expression of $p21^{Cip1}$, selected single clones were analyzed by Northern blot probing for $p21^{Cip1}$. Previous work (Fig. 2) showed that strong expression of $p21^{Cip1}$ and G_1 arrest occurred after 72 hours of EGF treatment. Hence, the level of $p21^{Cip1}$ in the single clones

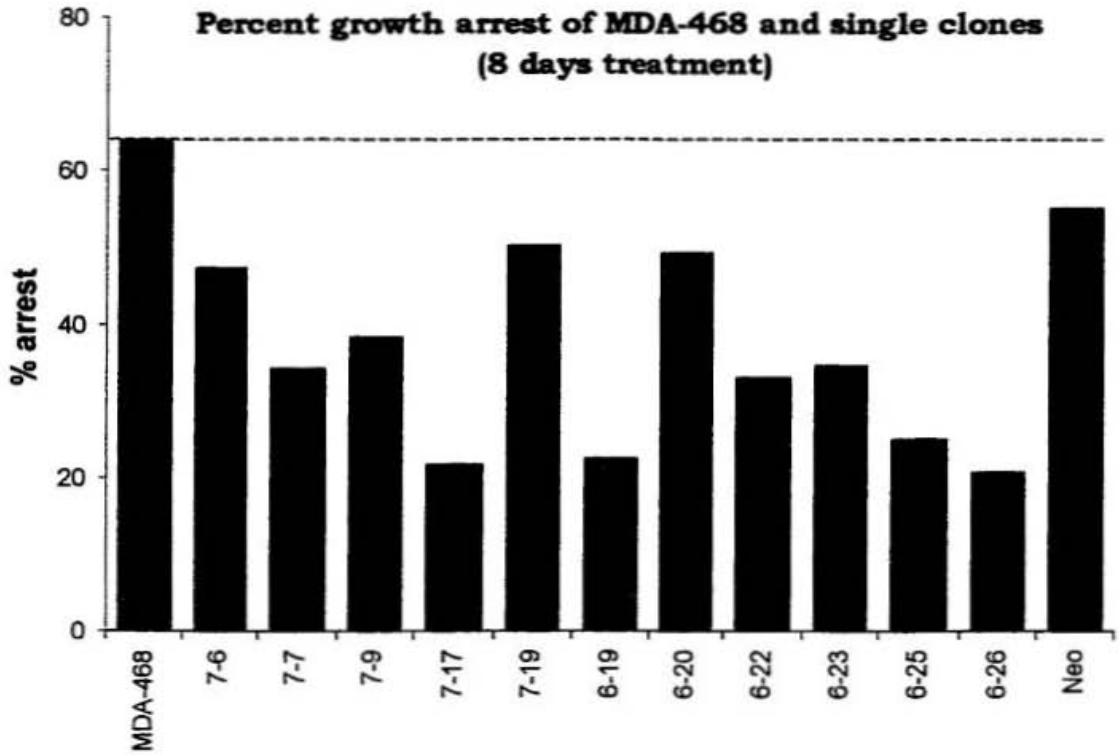


Figure 10: Percent change in growth of EGF-treated MDA-468, sense-transfected, and antisense-transfected.

Percentages are derived from the data of Table 1 and refer to the difference in total number of cells between treated and untreated cells for each given cell line.

was best analysed at this point in its upregulation by EGF. Using the expression of $p21^{Cip1}$ in untransfected MDA-468 cells, sense transfected cells and empty vector transfected cells (Neo) as controls, the relative expression of $p21^{Cip1}$ in the antisense-transfected single clones could be determined. Figure 11 shows a Northern blot probed for $p21^{Cip1}$ mRNA from cells grown in media with or without EGF for 72 hours. The lane showing EGF-treated MDA-468 (Fig. 11a, lane b) does not show as dramatic an increase in $p21^{Cip1}$ levels as seen in the sense expressing cell lines (lane f, n, p) or the neo cells (lane r) or seen in previous Northerns (Fig 1,2, and 3). However this was expected since an ethidium bromide stain of the RNA gel which produced this blot shows a significant decrease in the amount of total RNA loaded in that particular lane. This is seen by the decreased intensity of the 28S and 16S RNA bands of ribosomal RNA (Fig 11B, lane b).

To confirm the disparity in the loading of RNA, the blot was also stripped and probed for β-actin, a constitutively expressed message and reliable indicator of the relative amounts of RNA in a given lane. The reduced expression of β-actin in the MDA-468 EGF-treated lane (Fig. 11C, lane b) confirms that the lack of signal seen when probed for $p21^{Cip1}$ can be attributed to reduced RNA loading for that lane. Hence, the expression of MDA-468 in this blot is not the best control to compare with the antisense bearing cells. In this case they are better compared to the sense-transfected cells lines and the control vector transfected cells (Neo) (Fig.11A).

As a result of probing for relative RNA content in each lane, the relative intensities of the $p21^{Cip1}$ bands in each lane can be normalised against the β-actin bands and presented

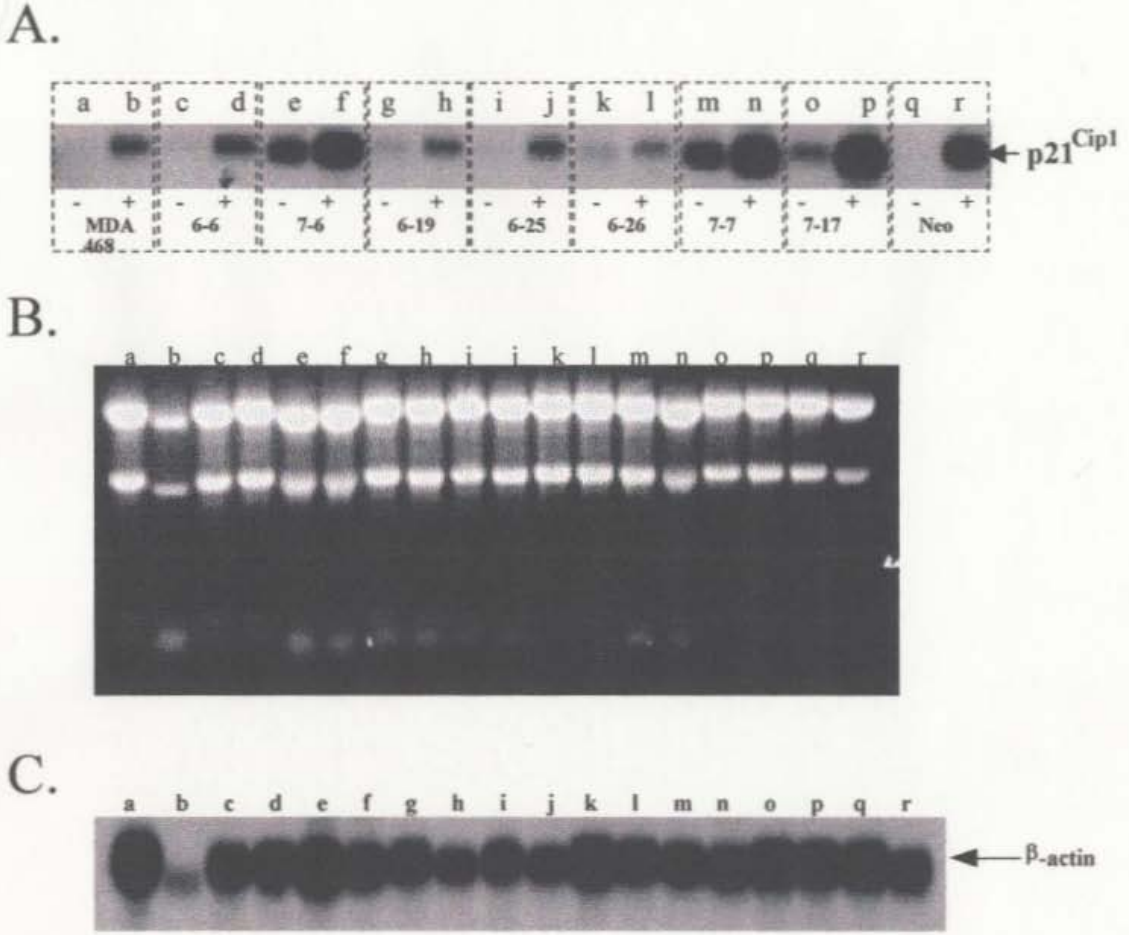


Figure 11: Northern blot analysis of the effect of EGF on $p21^{Cip1}$ expression in MDA-468, antisense and sense transfected cells

(A) **Northern blot analysis for the expression of $p21^{cip1}$ in MDA-468, antisense and sense transfected single clones.** MDA-468, antisense, sense, and control transfected cells were grown in the absence or presence of EGF at 10^{-8} M for 72 hours. After the exposure the cells were lysed for RNA isolation using TriZol solution (Gibco BRL). Twenty micrograms of total RNA was run on a formaldehyde gel, transferred to GeneScreen membranes, and probed with a radiolabelled fragment of $p21^{Cip1}$ cDNA. The labeled blot was washed and exposed to Kodak X-OMAT film overnight.

(B) **Ethidium bromide stain of formaldyhde gel for the Northern blot of Figure 11A.** Prior to the blotting of RNA to the membrane probed in Figure 11A, the gel containing ethidium bromide was exposed to ultraviolet light and photographed.

(C) **Expression of β-actin in blot of Figure 11A.** Following exposure to film the blot of Figure 11A was stripped in 90°C Tris-EDTA and re-probed for β-actin using a radiolabelled fragment of the β-actin cDNA. The labeled blot was washed and exposed to Kodak X-OMAT film overnight.

as a histogram shown in Figure 12. This figure clearly demonstrates that EGF induces $p21^{Cip1}$ mRNA in MDA-468, sense and neo transfected cells yet is downregulated where the cells are transfected with antisense. In the cell lines 6-6, 6-19, 6-25, and 6-26 this reduced expression is obvious. Interestingly, the presence of antisense does not completely abolish the endogenous mRNA, nor does it completely prevent a slight induction of the message by EGF. The sense-transfected cell lines (7-6, 7-7, and 7-17) express $p21^{Cip1}$ just as well as MDA-468 and demonstrate an increase of message due to the overexpression of the gene.

xi) Analysis of $p21^{Cip1}$ protein in single clones

Since the goal of this antisense approach is the removal of the $p21^{Cip1}$ protein, measuring the protein content of $p21^{Cip1}$ in the antisense-transfected clones is a definitive experiment to asses the effectiveness of antisense expression.

To examine the ultimate effect on $p21^{Cip1}$ protein, the cell lines 6-19, 6-25 and 6-26 were selected for immunoprecipitation and Western blot detection. In addition, MDA-468 cells and the sense transfected cell line 7-6 were also immunoprecipitated for $p21^{Cip1}$ to serve as controls. Figure 13 shows that the protein levels of $p21^{Cip1}$ for the cell line 6-25 and cell line 6-26 are dramatically lower than that of MDA-468 cells and especially lower than that seen in the overexpressing cell line 7-6. While some induction of $p21^{Cip1}$ protein can still be seen in the EGF treated samples of 6-26, the end result is a dramatic downregulation of the levels of $p21^{Cip1}$ in cell lines 6-25 and 6-26. This Figure (13A) represents the longest exposure of the blot to film (1 hour) in order to confirm the lack of signal in the cell line 6-25 and 6-26. Shorter exposures showed no bands in the lanes containing 6-25 and 6-26.

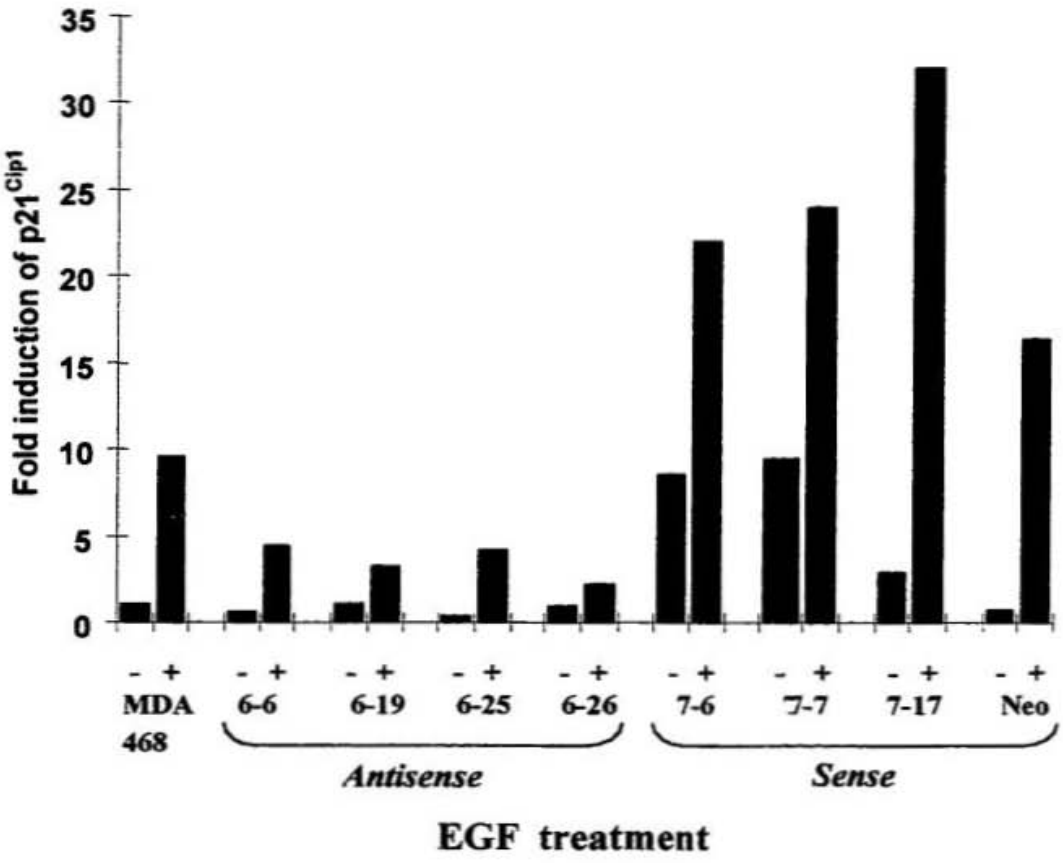


Figure 12: Histogram of normalized intensities of band in Figure 11A.

The film of the blot in Figure 11A was scanned by laser densitometry and the values for each band was normalized to the intensity of the β-actin within the same lane in Figure 11C. The relative intensities are then presented as fold induction over the signal from untreated MDA-468 cells.

Figure 13B and 13C present the intensities of the bands of Figure 13A as histograms. In each case the fold induction is normalised against the intensity of the light chain of the immunoprecipitating antibody (not shown). This figure further demonstrates the significant reduction of $p21^{Cip1}$ levels in the cell lines 6-25 and 6-26.

In another Western blot (Fig.14), the levels of $p21^{Cip1}$ in MDA-468 and the cell line 6-19 are compared. Using immunoprecipitates from cells treated or untreated with EGF for 72 hours. As with the cell lines 6-25 and 6-26, the cell line 6-19 shows a reduction of $p21^{Cip1}$ protein when compared to MDA-468. Induced levels of $p21^{Cip1}$ in 6-19 are only 50% that of induced levels in treated MDA-468. This, however, is less than the reduction seen in cell line 6-25 where induction of $p21^{Cip1}$ less than 30% that of the parental cell line.

xii) Time course of EGF effects on growth

Having demonstrated reduction of mRNA and protein for $p21^{Cip1}$ in the cell lines 6-19, 6-25 and 6-26, a more in depth analysis of the growth of these cell lines in EGF was examined. The preceding examination of growth of these cell lines was limited to a single measurement of cell number following 8 days +/- EGF treatment. To obtain more data about the growth of these cells during the course of EGF-treatment, the cell lines MDA-468, 6-19, 6-23, 6-25, 6-26, 7-6 and Neo-transfected cells were grown and counted on days 2, 4, 6, and 8 of EGF-treatment.

As with previous growth experiments, the cell lines were plated in triplicate in 60mm dishes at a density of $1x10^4$ cells/dish for each condition and duration of treatment. Each set of cells were trypsinized and counted on days 2,4,6, and 8. The results from this growth

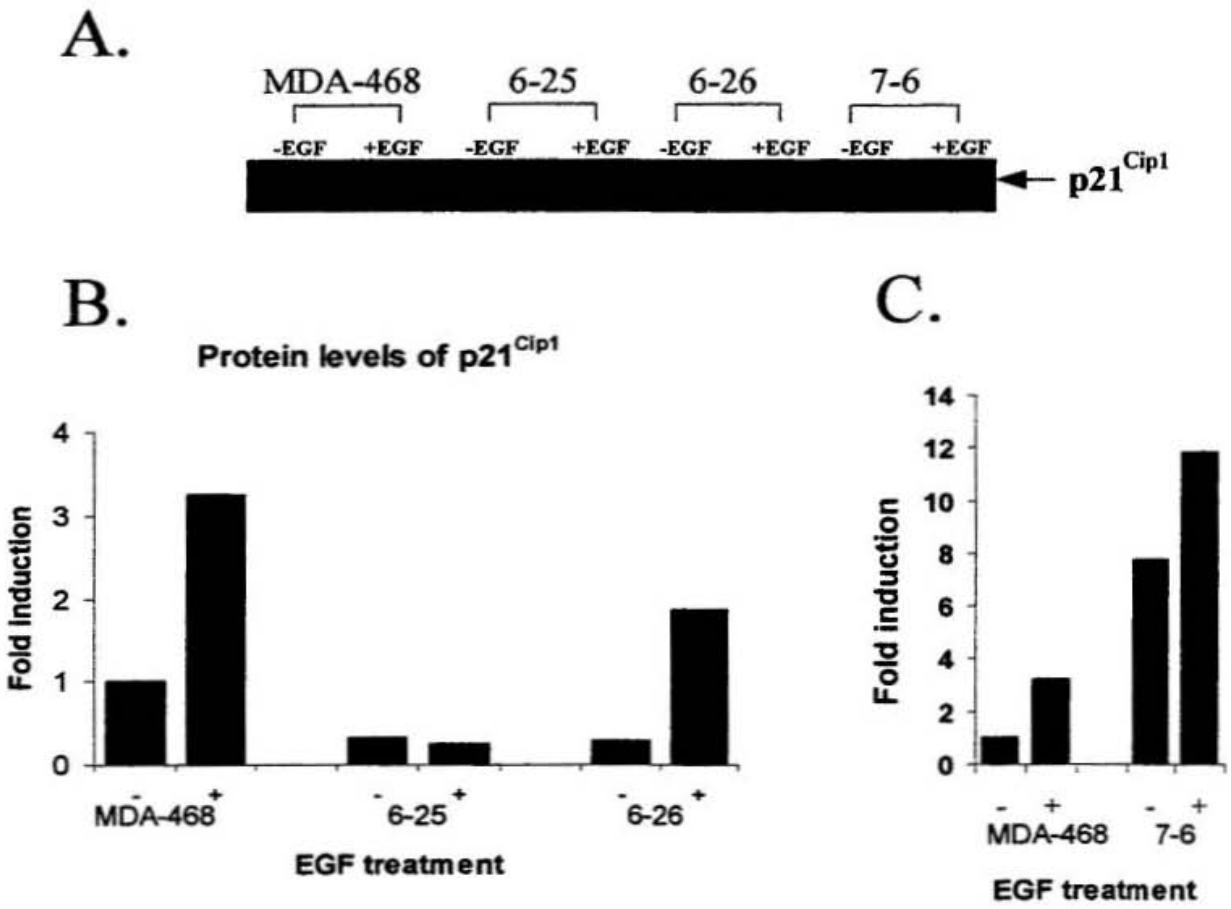


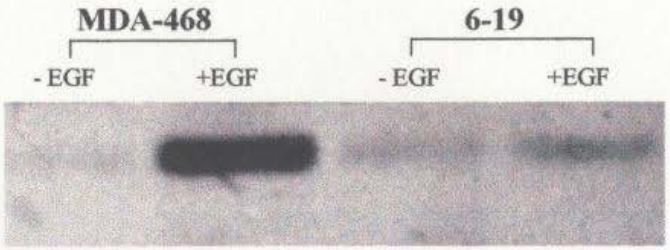
Figure 13: Western blot analysis on the expression of $p21^{Cip1}$ after 72 hours treatment for MDA-468, the two antisense-transfected cell lines: 6-25 and 6-26, and the sense-transfected cell line 7-6.

(A) **Western blot analysis of MDA-468, 6-25, 6-26 and 7-6 for the $p21^{Cip1}$ protein following 72 hours treatment with EGF at 10^{-8} M.** The cell lines MDA-468, 6-25, 6-26, and 7-6 were grown in 10cm dishes in the presence or absence of EGF for 72 hours. The cells were lysed in non-denaturing solution and immunoprecipitated with mouse anti-$p21^{Cip1}$ antibody overnight. The immunoprecipitates were resuspended in SDS sample buffer and run on a 12% SDS-polyacrylamide gel, blotted onto nitrocellulose. The detection of $p21^{Cip1}$ was done as previously described.

(B) **Histogram of intensities of band in Figure 13A.** The film of the blot in Figure 13A was scanned by laser densitometry and the values for each band presented as fold induction over the signal from untreated MDA-468 cells.

(C) **Histogram of the intensities of the cell line 7-6 and MDA-468.**

A.



B.

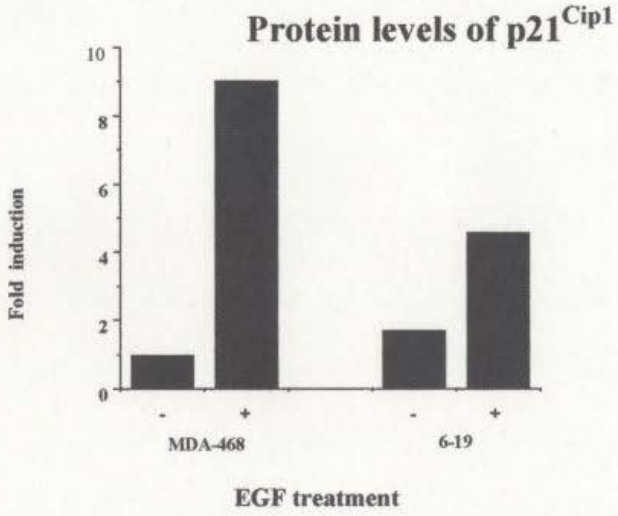


Figure 14: Western blot analysis on the expression of $p21^{Cip1}$ after 72 hours treatment for MDA-468, the antisense-transfected cell line 6-19

(A) **Western blot analysis of MDA-468, 6-19 for the $p21^{Cip1}$ protein following 72 hours treatment.** The cell lines MDA-468, 6-19 were grown in 10cm dishes in the presence or absence of EGF for 72 hours. The cells were lysed in non-denaturing solution and immunoprecipitated with mouse anti-$p21^{Cip1}$ antibody overnight. The immunoprecipitates were resuspended in SDS sample buffer and run on a 12% SDS-polyacrylamide gel, blotted onto nitrocellulose. The detection of $p21^{Cip1}$ was done as previously described.
(B) **Histogram of intensities of band in Figure 14A.** The film of the blot in Figure 14A was scanned by laser densitometry and the values for each band presented as fold induction over the signal from untreated MDA-468 cells.

experiment are presented in Table 2 and in graphs which compare the growth of a given cell line with MDA-468 (Fig.15-17).

Figure 15A shows the profile of growth for the cell line 6-25 against that of MDA-468. Most striking from this figure is that even within 2-4 days the 6-25 cells treated with EGF do not demonstrate significant growth arrest while at the same time difference between treated and untreated MDA-468 cells is obvious. Clearly a reduced ability for the cell line 6-25 to undergo growth arrest is occurring within the first few days of treatment. Figure 15B shows the profile of 7-6 against MDA-468. In this case the rate of growth between 7-6 and MDA-468 throughout this experiment is roughly similar as both cell lines respond to EGF in the same manner. Although the 7-6 cell line shows a slight decrease in growth arrest, the difference is not very significant throughout the course of EGF treatment. The profile of growth for the cell lines 6-26 and 6-19 and (Fig. 16A,B) also indicate a significant reduction in response to EGF within 6 days of treatment. Although untreated 6-26 grows somewhat slower than MDA-468 (Fig. 16A), there is negligible growth arrest occurring in this cell line by this time. Between 6 and 8 days, a difference between treated and untreated 6-26 cells becomes noticeable yet EGF-treated 6-26 treated cells clearly outgrow EGF-treated MDA-468 cells. The profile of growth for cell line 6-19 (Fig. 16B) also shows negligible growth arrest by 6 days treatment.

Figure 17A shows that empty vector transfected cells (Neo) grow to numbers that are virtually identical to MDA-468 cells. The profiles of growth for these two cell lines

Table 2: Time course of the effect of EGF on growth over 8 days.

	MDA-468 ($x10^4 \pm Sd$)		6-25 ($x10^4 \pm Sd$)		7-6 ($x10^4 \pm Sd$)		6-26 ($x10^4 \pm Sd$)	
Days	*-EGF*	*+EGF*	*-EGF*	*+EGF*	*-EGF*	*+EGF*	*-EGF*	*+EGF*
2	**2.9** ±0.3	**3.0** ±0.3	**4.9** ±0.1	**5.1** ±0.6	**2.5** ±0.3	**2.0** ±0.2	**2.8** ±0.5	**2.8** ±1.0
4	**9.9** ±1.4	**6.1** ±0.6	**13.7** ±1.0	**12.7** ±0.6	**8.2** ±0.6	**7.5** ±0.8	**7.8** ±0.4	**7.0** ±1.2
6	**25.7** ±1.7	**12.5** ±2.2	**30.7** ±1.1	**28.9** ±1.7	**21.4** ±2.2	**13.0** ±1.8	**18.5** ±1.0	**17.1** ±1.1
8	**49.1** ±1.8	**20.0** ±1.4	**52.7** ±1.4	**39.0** ±1.1	**45.5** ±1.0	**22.7** ±0.8	**41.6** ±2.2	**29.7** ±2.2

	6-19 ($x10^4 \pm Sd$)		Neo ($x10^4 \pm Sd$)		6-23 ($x10^4 \pm Sd$)	
Days	*-EGF*	*+EGF*	*-EGF*	*+EGF*	*-EGF*	*+EGF*
2	**4.3** ±0.5	**4.3** ±0.3	**2.9** ±0.3	**2.3** ±0.9	**4.4** ±0.1	**4.4** ±0.2
4	**12.2** ±0.6	**9.9** ±0.4	**8.6** ±1.1	**7.4** ±0.6	**8.0** ±0.4	**7.8** ±0.1
6	**23.9** ±1.0	**20.5** ±0.6	**21.6** ±2.5	**13.0** ±1.0	**21.4** ±1.6	**17.6** ±1.7
8	**46.8** ±1.7	**34.9** ±0.8	**40.2** ±1.8	**18.0** ±2.3	**52.2** ±6.9	**42.3** ±3.3

The cell lines MDA-468 and single clones were plated in 60 mm dishes at $1x10^4$ and grown in either the absence or presence of EGF. At days 2, 4, 6, and 8 three dishes for each condition were trypsinized and ennumerated for total cell number. Cells incubated for 6 and 8 days received fresh media with or without EGF.

virtually superimpose one another. Finally, the cell line 6-23 is compared to the parental cell line in Figure 17B. Although this cell line was not examined by Northern or Western blot analysis, its growth characteristics seen in this time course of growth is quite dramatic. Once again even in the short term (i.e. 6 days) there is virtually no difference in growth between the treated and untreated cells of this antisense transfected cell line.

Hence, the time course of growth in the presence of EGF reveals a greater attenuation of G_1 arrest in the antisense-transfected cells than in the previous examination of growth. In correlation with a decrease in $p21^{Cip1}$ mRNA and protien for the cell lines 6-19, 6-25, and 6-26 there is an obvious reduction of the ability of these cell lines to undergo significant G_1 arrest within 6 days of EGF-treatment.

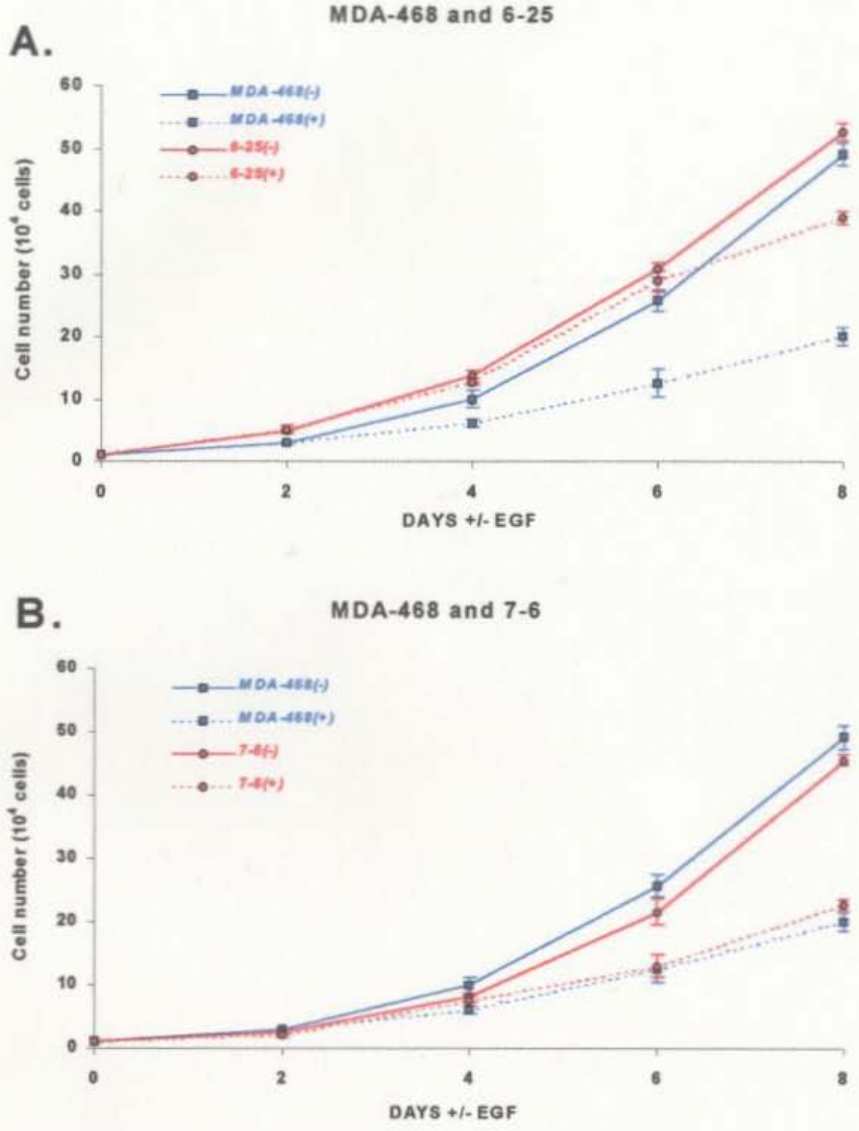


Figure 15: Time course effect of EGF treatment on growth. (1)

(A) **Time course of the effect of EGF on the growth of MDA-468 and 6-25 cell lines.** Data from Table 2
(B) **Time course of the effect of EGF on the growth of MDA-468 and 7-6 cell lines.** Data from Table 2

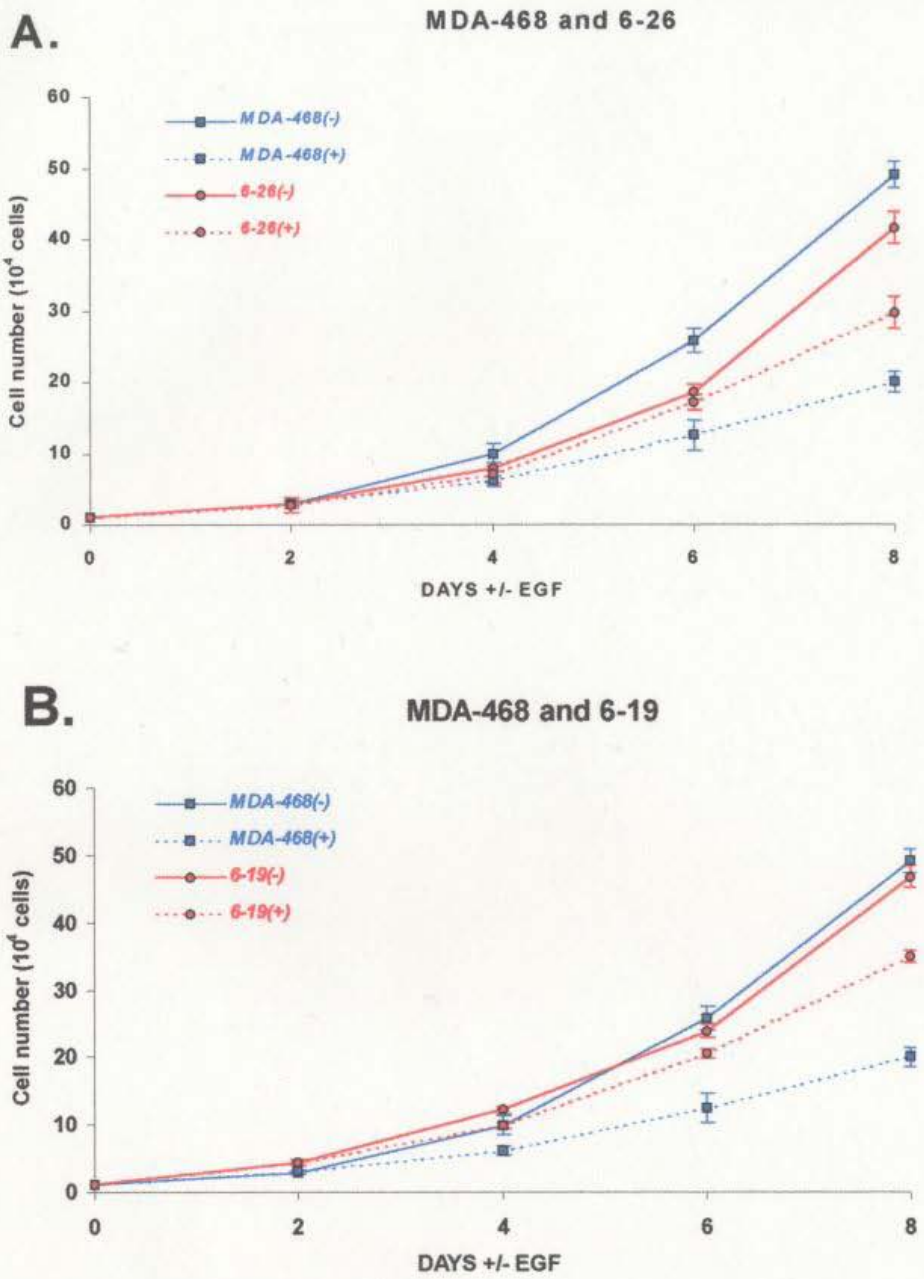


Figure 16: Time course effect of EGF treatment on growth. (2)

(A) **Time course of the effect of EGF on the growth of MDA-468 and 6-26 cell lines.** Data from Table 2
(B) **Time course of the effect of EGF on the growth of MDA-468 and 6-19 cell lines.** Data from Table 2

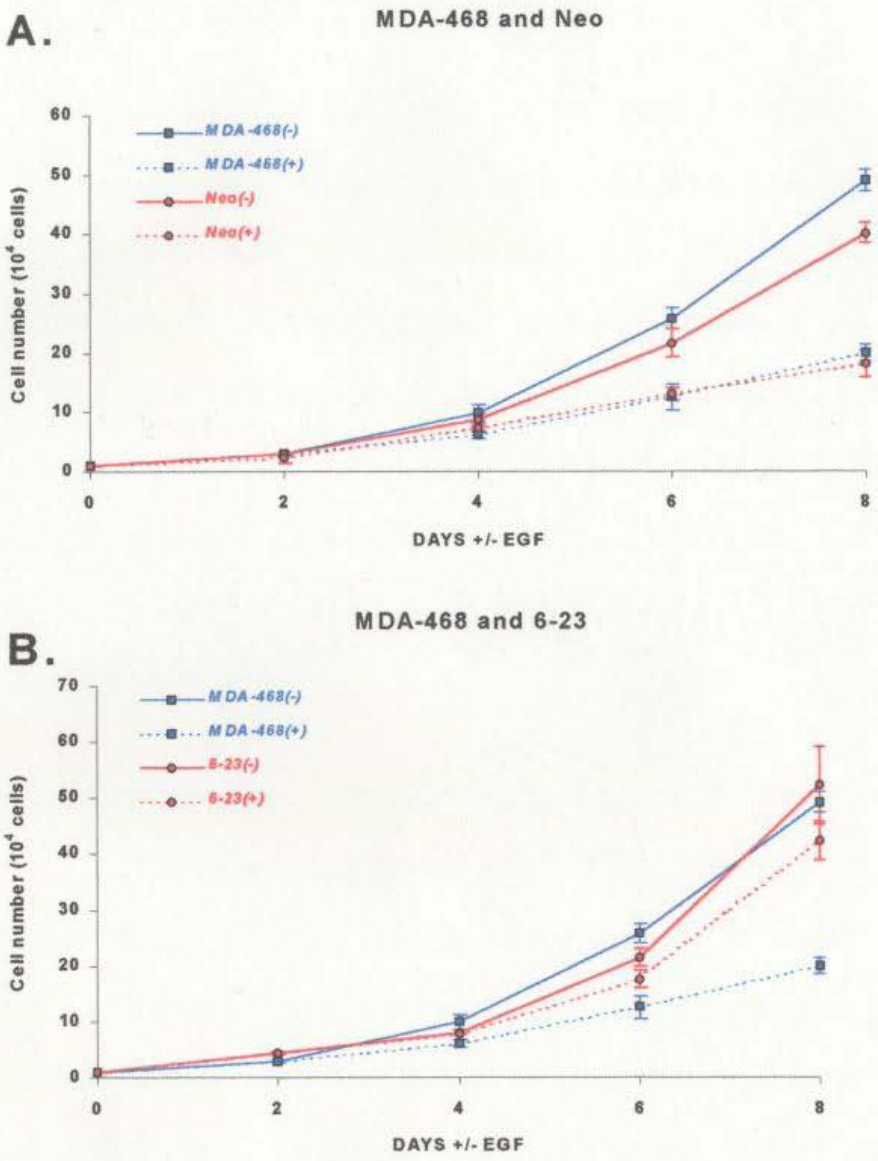


Figure 17: Time course effect of EGF treatment on growth. (3)

(A) **Time course of the effect of EGF on the growth of MDA-468 and neo cell lines.** Data from Table 2
(B) **Time course of the effect of EGF on the growth of MDA-468 and 6-23 cell lines.** Data from Table 2

4. DISCUSSION

The human breast cancer cell line MDA-468 has the unique characteristic of undergoing G_1 arrest when treated with pharmacological concentrations of EGF. While the ability of EGF to cause this cancer cell line to arrest is well documented (Prasad *et al.*, 1991), the effectors that mediate the arrest are unknown. Since the tumour suppressor gene $p21^{Cip1}$ is a potent inhibitor of cyclin dependent kinases (Sherr *et al.*, 1995) and has a well characterized ability to confer G_1 arrest on tumour cells (Xiong *et al.*, 1993), it is reasonable to suggest that activation of $p21^{Cip1}$ may play a role in EGF-mediated arrest of MDA-468 cells. To indicate a role for $p21^{Cip1}$ in this growth arrest the ability of EGF to upregulate $p21^{Cip1}$ expression needed to be examined.

i) Upregulation of $p21^{Cip1}$ mRNA in MDA-468 cells prior to G_1 arrest.

In MDA-468 cells, treatment with EGF for 12 hours caused a dose-dependent induction of $p21^{Cip1}$ mRNA (Fig.1A). Most importantly the concentration of EGF which induces G_1 arrest (10^{-8}M) is the concentration which causes the most dramatic increase in $p21^{Cip1}$ message (Fig.1A). This induction of $p21^{Cip1}$ mRNA occurs quite rapidly with a 5-fold increase within 2-4 hours exposure to EGF (Fig.3A), continues to be elevated after 72 hours (Fig.3B, lane n), and remains high in the continued presence of EGF for more than eight days (Fig.1B). Elevated expression of $p21^{Cip1}$ was specific to EGF since upon removal of EGF the level of $p21^{Cip1}$ message gradually returned to basal levels within 12 hours (Fig.3B).

The fact that $p21^{Cip1}$ mRNA undergoes a rapid increase immediately following EGF treatment is critical for the hypothesis that $p21^{Cip1}$ plays a role in G_1 arrest. Since the induction of $p21^{Cip1}$ occurs well in advance of G_1 accumulation (Prasad *et al.*, 1991), it can be stated that upregulation of $p21^{Cip1}$ might be a cause of this arrest rather than a consequence of G_1 arrest.

The biphasic profile of $p21^{Cip1}$ mRNA induction by EGF (Fig.3C) is a rather curious phenomenon. The initial increase of message occurs very quickly (less than four hours) probably due to a sudden decrease in mRNA turnover, while the longer, sustained increase may be due to increased transcription or both. This kind of response to EGF in MDA-468 cells is not unique to $p21^{Cip1}$ mRNA. EGF signaling also causes a biphasic induction of IP_3 production within 72 hours of treatment (Wahl *et al.*, 1987) and as a result induces a biphasic release of calcium in these cells (Kremer *et al.*, 1989). The data presented here does not address this curious pattern of response to EGF. In addition, RNA analysis was not done between 15 and 72 hours of treatment to give a more complete profile of $p21^{Cip1}$ mRNA induction. Nevertheless, it is clear that exposure to EGF at 10^{-8}M causes a significant upregulation of $p21^{Cip1}$ mRNA in these cells prior to G_1-arrest.

ii) Western analysis of $p21^{Cip1}$ in MDA-468 cells.

The increase of $p21^{Cip1}$ mRNA by EGF ultimately causes an increase in $p21^{Cip1}$ protein as measured by Western blot analysis. After 72 hours treatment $p21^{Cip1}$ protein has increased by 3-4 fold over untreated cells [Fig 4A,B]. This induction is seen when samples are from either whole cell lysate or from immunoprecipitates.

From these observations it is reasonable to suggest that EGF treatment of these cells causes an increase in $p21^{Cip1}$ mRNA followed by an elevation of $p21^{Cip1}$ protein levels such that the Cdks of MDA-468 become bound and inactivated by this inhibitor with the consequence of G_1 arrest. The ability of EGF to induce $p21^{Cip1}$ expression in MDA-468 cells has recently been confirmed elsewhere. Two independent studies demonstrated that EGF-treatment increased the level of $p21^{Cip1}$ mRNA and protein within 24 hours and was proposed to be the mediator of G_1 arrest in these cells (Reddy *et al.*, 1999 ; Thomas *et al.*, 1999).

The possibility that EGF also causes a biphasic induction of $p21^{Cip1}$ protein was not examined. Protein analysis was done on cells exposed to EGF for 72 hours to observe a change in $p21^{Cip1}$ at a time when growth arrest is most evident (Prasad *et al.*, 1991). Analysis of the content of $p21^{Cip1}$ protein earlier in EGF-treatment might reveal that $p21^{Cip1}$ protein levels increase as rapidly as the increase in message. While this data would provide more information on the levels of $p21^{Cip1}$ throughout an exposure to EGF, the goal of the work presented here was focused on the elimination or reduction of $p21^{Cip1}$.

iii) Antisense to $p21^{Cip1}$

Should $p21^{Cip1}$ play a vital role in EGF-mediated arrest, then downregulation by antisense expression would reduce the ability of EGF to cause arrest in these cells. On the other hand, if $p21^{Cip1}$ is not a player in this particular G_1-arrest, then reducing the expression of $p21^{Cip1}$ by antisense expression would be redundant and the ability of EGF to cause G_1-arrest would be unchanged.

Hence, MDA-468 cells were transfected with an antisense expression construct by calcium phosphate precipitation using neomycin as a selective agent. Since integration into the genome is a random event, resulting in different degrees of expression within individual clones, it was important to obtain single clone cell lines following transfection and selection. Thus a population of single clones expressing an antisense to $p21^{Cip1}$ (Fig.7A) and a population of single clones expressing the sense transcript of $p21^{Cip1}$ (Fig.7B) were generated. These were compared to the parental cell line, MDA-468, and to a mixed population of MDA-468 cells transfected with the empty vector, pcDNA-neo.

When targeting a gene for downregulation by an antisense molecule there are primarily two methods to assess the downregulation of the target gene. A reduction of expression can be assessed by either Northern blot analysis to measure a reduction of mRNA or by Western blot to demonstrate the elimination of the protein.

iv) Effect of antisense expression on $p21^{Cip1}$ mRNA.

Figure 11 presents the level of $p21^{Cip1}$ mRNA in MDA-468, antisense, and sense transfected cell lines in both the presence or absence of EGF. The goal of this experiment was to identify those cell lines where the antisense vector caused the most significant reduction of $p21^{Cip1}$ mRNA.

Using the CMV promoter it was hoped that a saturating amount of antisense message would be produced and result in the complete elimination of the endogenous message. In figure 11 all of the antisense-transfected cells display a persistence of $p21^{Cip1}$ message and the levels can be slightly induced by EGF. Thus, the effectiveness of the

$p21^{Cip1}$ antisense used in this study is questionable. It would appear that expression of the antisense molecule reaches a steady state level that significantly reduces the levels of endogenous $p21^{Cip1}$ message but does not completely eliminate its expression, indicating that a better construct for antisense could have been used.

a. Short length antisense molecules

For this study, the reverse orientation of full-length $p21^{Cip1}$ cDNA was used to generate the antisense molecule (Fig.7A). Although full-length antisense are commonly used in the design of antisense molecules, an antisense of this size can be problematic. As the length of a single stranded nucleic acid increases, so to does the probability that stem loop structures can form within the molecule due to intramolecular base-pair binding. Not only will these secondary structures interfere with the binding of the antisense to the target message, but can also render the antisense molecule a substrate for degradation by RNAse H.

An alternative approach to generating full-length antisense is to use short oligonucleotide sequences. Short antisense molecules, which are typically 18 to 22 bases in length, are more stable than longer full-length strands as they have less chance of intramolecular base-pair binding. Despite their short sequence, antisense constructs of this size have been shown to be highly specific to target and eliminate the expression of a target gene.

For instance, in one study a 20-mer (20-base) $p21^{Cip1}$ antisense was generated to eliminate $p21^{Cip1}$ levels in esophageal carcinoma cells. Expression of this construct significantly reduced $p21^{Cip1}$ protein levels and eliminated the cell's ability to undergo

growth arrest when exposed to γ-irradiation. On the other hand, a 20-mer oligonucleotide of random sequence did not reduce $p21^{Cip1}$ levels and did not prevent cell cycle arrest (Rigberg *et al.*, 1999).

b. Targeting antisense to the untranslated regions of target mRNA

In addition, it has recently been determined that short antisense molecules which bind the 5' untranslated region (5'UTR) (Mologni *et al.*, 1998) or the 3' untranslated region (3'UTR) (Petrukhin *et al.*, 1998) of an mRNA can be both specific and effective in the elimination of a target gene. The 5' UTR lies on the mRNA molecule upstream of the ATG start of translation site and the 3'UTR is downstream of the mRNA stop codon. These regions, despite occurring outside the coding sequence, maintain a high degree of sequence specificity for a given gene. In a study using 3 different 18-mer antisense molecules to eliminate cyclin D expression targeting the 5'UTR was more effective than using an antisense to the coding region.(Hung *et al.*, 1996). Hence short antisense sequences targeted to either the 5'UTR or 3'UTR of $p21^{Cip1}$ might have been more effective in the elimination of endogenous expression of $p21^{Cip1}$ in this study.

However, despite the persistence of $p21^{Cip1}$ message in antisense-transfected cells (Fig.11A), clearly a reduction of $p21^{Cip1}$ mRNA has been achieved in these cells (Fig. 12). When comparing the levels of expression in the antisense-transfected cells with MDA-468, there is a 2-3 fold decrease in induced message. Yet, when compared to the Neo-transfected control cells the level of $p21^{Cip1}$ mRNA in the antisense-transfected cells are significantly

reduced. Thus the antisense-expressing construct has made some significant reduction in $p21^{Cip1}$ mRNA, furthermore, full-length antisense to $p21^{Cip1}$ has been shown to be equally effective in reducing its expression and function(Freemerman *et al.*, 1997).

v) Effect of antisense expression on $p21^{Cip1}$ protein levels.

While Northern blot analysis revealed a limited effectiveness in the elimination of $p21^{Cip1}$ mRNA, the Western blot analysis of selected clones provides convincing evidence for a reduction of $p21^{Cip1}$. In figures 13 and 14, MDA-468, 6-19, 6-25, 6-26 and 7-6 cell lines were grown in the absence or presence of EGF for 72 hours and immunoprecipitated for $p21^{Cip1}$. Clearly the presence of $p21^{Cip1}$ in the cell line 6-25 appears to be virtually eliminated (Fig.13). Despite a persistence of $p21^{Cip1}$ message in the cell line 6-25 (Fig.12), the Western blot indicates a significant reduction of $p21^{Cip1}$ protein with almost negligible increase after 72 hours treatment. In addition, the cell lines 6-19 (Fig.14) and 6-26 (Fig.13) also demonstrate a reduction of $p21^{Cip1}$ protein levels following EGF treatment. Clearly, the protein data gives a more relevant indication of downregulation by the antisense construct, especially in the cell line 6-25.

vi) Growth analysis of antisense-transfected cells

With the aim of proposing a role for $p21^{Cip1}$ in the EGF-mediated growth arrest of MDA-468 by using antisense to $p21^{Cip1}$, it is necessary to correlate a reduction of its expression with a reduction of G_1 arrest during EGF treatment. While the molecular analysis was not done on all of the single clone cell lines, each cell line was assayed for an

ability to undergo EGF-mediated growth arrest (Table 1). In this experiment growth arrest is measured by the percent difference of cell number following 8 days growth in the presence or absence of EGF.

The percent growth difference of antisense-transfected cells is presented in figure 8. Here it is seen that the parental cell line MDA-468 shows a difference in growth of roughly 60% when treated with EGF. The majority of the antisense transfected cell lines show a growth difference between 40% and 60% (Fig.8). This indicates that a significant amount of arrest is still occurring in these cell lines. This was expected given the possible variability of expression of antisense in each clonal cell line. Hence, it is possible that only with significant removal of $p21^{Cip1}$ by the expression of antisense will a reduction of growth arrest be measurable. Presumably in the clones in which the percent growth difference remains 40% or greater the reduction of $p21^{Cip1}$ by antisense is below a significant threshold. However, since Northern and Western blot analysis was not done on all cell lines this remains speculative.

Molecular analysis of these other cell lines may show growth inhibition persists despite reduced $p21^{Cip1}$ expression. For example, the cell line 6-6 has a percent difference in growth of 56% (Fig.8), yet demonstrates some reduction of $p21^{Cip1}$ message (Fig.12). Hence the level of $p21^{Cip1}$ mRNA and protein in those cell lines that lack significant growth arrest (Fig.8) need to be examined before making a difinitive conclusion that antisense to $p21^{Cip1}$ impairs EGF-mediated growth arrest in these cells.

However the purpose of the growth experiments was to screen for those cell lines where antisense expression was most effective in reducing growth arrest. The cell lines 6-

19, 6-25 and 6-26 in additon to having similar basal growth rates as MDA-468 demonstrate less than a 25% difference between treated and untreated cells. Given that these cell lines also demonstrate a reduced expression of $p21^{Cip1}$, it is tempting to suggest that growth arrest is attenuated by the reduction or absence of $p21^{Cip1}$ in these single clones.

vii) Growth of sense transfected single clones.

In addition to transfecting MDA-468 cells with antisense to $p21^{Cip1}$, the sense expressing $p21^{Cip1}$ construct was also transfected into these cells to possibly serve as controls. Unexpectedly, the growth of sense-transfected cells in the presence of EGF also shows a decreased ability to undergo growth arrest as measured by cell counts (Fig.10). Five sense-transfected cell lines (7-6, 7-7, 7-9, 7-17, and 7-19) display a degree of growth arrest around 40%, and slow growth does not explain the reduced percent arrest of the sense-transfected cells (Table 1).

An examination of the molecular data for three of these cells lines (Fig.11A) shows that sense transfected cell lines 7-6, 7-7, and 7-17 significantly over express $p21^{Cip1}$ when compared to MDA-468 and demonstrate a dramatic upregulation of $p21^{Cip1}$ mRNA when treated with EGF (Fig.11,12). In addition, the Western blot of Figure 13C shows that $p21^{Cip1}$ protein levels in the cell line 7-6 is significantly overexpressed.

However, due to the strong antiproliferative effect of $p21^{Cip1}$ on tumour cells validity of using sense-transfected cells as controls for cell growth is questionable. Transfection of tumour cells with $p21^{Cip1}$ almost invariably causes irreversible growth arrest typically followed by cell death by necrosis or in some cases by apoptosis (Yang *et al.*, 1995). A

study by Sheikh et al. (1995) demonstrated that transfection of $p21^{Cip1}$ into breast cancer cell lines caused the cells to swell to 50 to 100-fold in size. These cells, referred to as giant cells, persist for several days and eventually undergo apoptosis. At the same time a number of small drug-resistant clones persisted for up to a week and eventually all cells died by necrosis (Sheikh *et al.*, 1995). These observations are very similar to what was seen when MDA-468 cells were transfected with sense $p21^{Cip1}$. Shortly following transfection, many large cells were observed and within 2 weeks many drug-resistant clones spontaneously died within 2-3 passages. This death was seen after elimination of untransfected cells with neomycin had been completed, thus this spontaneous death is not to be confused with death due to selection with neomycin. Within two weeks post-transfection, only 5 out of 30 single clone sense-transfected cell lines remained viable. On the other hand, cells transfected with antisense or empty vector did not form these giant cells and did not spontaneously die off following selection.

Thus, it is curious that five cell lines expressing sense $p21^{Cip1}$ in fact do survive and continue to propagate for months after transfection. It is difficult to explain how cells overexpressing $p21^{Cip1}$ continue to grow, however it is possible that transfection of MDA-468 with sense $p21^{Cip1}$ strongly selects for those cells in which $p21^{Cip1}$ is not linked to growth. While this is a purely speculative suggestion, there is evidence of cells with mutant Cdks that no longer bind Cdk-inhibitory proteins. Despite transfection and overexpression of these inhibitors, these particular mutant Cdks maintain activity (Shapiro *et al.*, 1998) and cell proliferation is maintained. Perhaps in this experiment, cells containing these mutant

Cdks have been selected for, especially cell line 7-6 which continue to grow despite an overexpression of $p21^{Cip1}$ protein (Fig.13C).

While it was hoped to gain some single clones which overexpress the $p21^{Cip1}$ protein, it is quite possible that all sense-transfected cell lines should have died due to the overexpression of $p21^{Cip1}$ (Yang *et al.*, 1995). Having survived long term expression of $p21^{Cip1}$ these cells may no longer be good controls for growth given that overexpression of $p21^{Cip1}$ creates its own dramatic selective pressure. One would expect that a cell line surviving constitutive $p21^{Cip1}$ expression would have a slower rate of growth. It appears that in the few sense-transfected cell lines which have survived, $p21^{Cip1}$ is being ignored by the cell-cycle.

It should be noted that when antisense molecules are used to downregulate the expression of a gene, the overexpression of the sense copy is normally not used as a control. In most cases, either the empty expression vector or a random oligonucleotide sequence is used as a control. In this study the use of sense $p21^{Cip1}$ introduces its own dramatic effect on cell viability and it could be argued that it does not generate a suitable control for growth in this experiment. Given these complications, the best controls in this study are the parental MDA-468 and the pcDNA-neo transfected cells.

viii) Inducible expression of antisense.

A possible improvement in the construction of both the antisense and sense expression vectors for this study could have included the use of an inducible promoter to regulate expression of these vectors in a timely manner. Rather than use the CMV to

constitutively express sense or antisense $p21^{Cip1}$, a promoter under the control of an inducible element such as the steroid inducible promoter (Mader *et al.*, 1993), the metallothionine promoter (Suzuki *et al.*, 1994), or the currently popular tetracycline induction system (Chen *et al.*, 1995b) might have provided a more controlled expression of these constructs. In fact, tetracycline control of the $p21^{Cip1}$ promoter activity has been previously demonstrated (Chen *et al.*, 1995) in addition to the use of metallothionine to control expression of antisense to $p21^{Cip1}$ (Nakanishi *et al.*, 1995).

In this study, the inducible expression of either sense or antisense to $p21^{Cip1}$ in MDA-468 cells might have provided some interesting data. Firstly, with an inducible system, the immediate effects of expression can be assessed, thus avoiding the long-term selection and propagation of clones in which the sense or antisense RNAs are constitutively expressed prior to treating the cell lines with EGF. While both the constitutive and inducible expression of $p21^{Cip1}$ antisense and sense should have the same end result of either downregulating or overexpressing the target gene, an inducible system prevents any non-specific effects that long term expression the constructs may produce. Secondly, the effect of antisense expression on growth can be examined within a single clone cell line rather than across different single clones. Each single clone in the absence of induction can act as its own negative control. This would control for differences in basal growth rate currently seen when comparing different single clones.

Most importantly the ability to activate expression of sense or antisense molecules can give an immediate indication of the effect of these molecules on the cell's response to EGF. For instance, it would be of interest to see if inducible expression of

$p21^{Cip1}$ antisense can reverse an ongoing G_1 arrest. The presence of $p21^{Cip1}$ in G_1 Cdk complexes could be assessed at time points following induction of antisense to measure a lack of Cdk inhibition by $p21^{Cip1}$ or FACS analysis of induced antisense could indicate an immediate changes in the proportion of cells in G_1. Obviously there are many advantages to using an inducible expression in this study.

ix) Time course of EGF on the growth of MDA-468 and single clones

The final experiments performed for this study involved a time-course treatment of EGF examining cell numbers after 2, 4, 6, and 8 days treatment. Figures 15, 16, and 17 demonstrate the growth of cell lines 6-19, 6-23, 6-25, 6-26, 7-6 and Neo in comparison with MDA-468. This data provides the most convincing evidence that antisense expression of $p21^{Cip1}$ reduces the ability of these cells to undergo G_1-arrest. Where the previous examination of cell growth for the cells was derived from a single time point in treatment (i.e.8 days), the time course of EGF treatment provides a better indication of the growth of these cell lines during treatment.

The most striking observation from this data is that after 6 days in the presence of EGF the cell lines 6-19 (Fig.16B), 6-23 (Fig. 17B), 6-25 (Fig.15A), and 6-26 (Fig.16A) show virtually no growth arrest in comparison with the parental cell line. Yet over the same time period the cell lines MDA-468, 7-6 (Fig.15B), and Neo (Fig.17A) indicate a significant difference in EGF treated samples during treatment. The profile of growth presented in these figures is quite convincing in demonstrating an inability of cells transfected with antisense to $p21^{Cip1}$ to undergo growth arrest. This data indicates that the earlier analysis of

growth, following 8 days of treatment (Table 1), may have been too long an incubation to witness an effect of antisense to $p21^{Cip1}$ on the growth. This suggests that the presence of an antisense to $p21^{Cip1}$ may have a maximum effect in preventing growth arrest within the first 6 days of EGF treatment. After which continuous signaling through the EGF receptor causes an accumulation of $p21^{Cip1}$ protein to overcome any attenuation of growth arrest.

x) EGF-mediated arrest in A431 cells: A comparative analysis.

The most comprehensive data to corroborate a role for $p21^{Cip1}$ in the EGF-mediated arrest in MDA-468 cells comes from the epidermoid cell line A431. A431 is remarkably similar to MDA-468 in that it overexpresses the EGF receptor and undergoes G_1 arrest in the presence of EGF (Gill *et al.*, 1981). As with MDA-468, overexpression of the EGF receptor in A431 cells is due to an amplification of the gene for the receptor and the receptor retains normal function (Merlino *et al.*, 1984). Hence these two cell lines display the same phenomenon associated with EGF treatment and its unique ability to cause G_1 arrest.

Similar to the results presented here, treatment of A431 with growth inhibitory concentrations of EGF (10^{-8}M) causes a rapid increase in $p21^{Cip1}$ mRNA and protein and cells subsequently accumulate in G_1 (Johannessen *et al.*, 1999). When exposed to lower concentrations of EGF, which are growth proliferative, there was no increase in $p21^{Cip1}$ and G_1 arrest does not occur. Similar to the data presented here in Figure 3A and 1B, these investigators demonstrated that EGF induction of $p21^{Cip1}$ in A431 reaches a maximum at 4 to 6 hours and remained above basal levels for up to 5 days.

These investigators also provide direct evidence that $p21^{Cip1}$ plays a role in EGF-mediated arrest of A431 cells by immunoprecipitation analysis of the G_1 cdks. In A431 cells, EGF-treatment caused a dramatic increase of $p21^{Cip1}$ protein in the G_1 Cdk complexes and *in vitro* kinase assays showed that with increased $p21^{Cip1}$ binding, the Cdks have significantly reduced kinase activity.

For MDA-468 cells, an examination of increased $p21^{Cip1}$ binding of G_1-Cdks following EGF treatment has not been reported. Obviously this evidence would significantly justify a role for $p21^{Cip1}$ in this arrest. If indeed $p21^{Cip1}$ is a chief mediator of arrest in MDA-468 cells, then the majority of the G_1 Cdks of EGF-treated cells should be found in a inhibitory complex with the $p21^{Cip1}$ protein. In addition, immunoprecipitation of the G_1-cdks in cell lines such as 6-25 could have provided a more direct method of demonstrating an effect of antisense to $p21^{Cip1}$ on the Cdks of MDA-468 cells.

Although the interaction of $p21^{Cip1}$ with G_1-Cdks of MDA-468 cells treated with EGF has not been examined, exposure of these cells to other agents has been reported. In two separate studies MDA-468 cells were treated with either silymarin, a flavonoid antioxidant, (Zi *et al.*, 1998) or with two topoisomerase inhibitors, 10-hydroxycamptothecin and camptothecin (Liu *et al.*, 1998). These agents induced a dramatic increase in $p21^{Cip1}$ mRNA and protein followed by G_1-arrest of the cells. Immunoprecipitation of CDK2 and CDK6 following treatment demonstrated increased $p21^{Cip1}$ binding and inhibition of kinase activity following treatment of MDA-468 with these agents.

xi) Antisense to $p21^{Cip1}$ in A431 cells

The most comparative analysis of A431 involved the use of antisense to $p21^{Cip1}$ in a study which reported results quite similar to that presented here. To determine a putative role for $p21^{Cip1}$ in the arrest of A431, these cells were transfected with an 18-mer antisense to $p21^{Cip1}$ (Ohtsubo *et al.*, 1998). Northern blot analysis indicated that a 50% reduction of $p21^{Cip1}$ mRNA was achieved and the protein was virtually undetectable by Western blot analysis. In addition, FACS analysis showed that A431 cells transfected with $p21^{Cip1}$ antisense did not accumulate in G_1 when subsequently treated with EGF while cells transfected with a random 18-mer continued to arrest in G_1 when treated with EGF. Furthermore, an examination of the growth of these antisense-transfected cells demonstrated that with 72 hours treatment of EGF growth arrest was reduced to 20% of the parental cell line.

These results presented by Ohtsubo et al. (1998), are remarkably similar to the effects of $p21^{Cip1}$ antisense on MDA-468 cells in this study. Similar to figure 11, this group reported a residual amount of endogenous message could persist (50%) and a slight induction by EGF could still be detected despite antisense expression. Yet, as seen here, despite persistent mRNA detection, protein analysis indicated almost 90% reduction in $p21^{Cip1}$ protein. In addition, analysis of growth revealed that transfection with the antisense to $p21^{Cip1}$ reduced growth arrest to roughly 20%, similar to what was reported here with the cell lines 6-19, 6-25, and 6-26.

xii) Involvement of other Cdk inhibitory proteins.

While there is evidence that $p21^{Cip1}$ upregulation in MDA-468 cells correlates with G_1-arrest, it might be suggested that other Cdk inhibitors are involved in the arrest, such as the $p21^{Cip1}$ related proteins $p27^{Kip1}$ and $p57^{Kip2}$ or the INK4 family of Cdk inhibitors. Each of these has a demonstrable role of inducing G_1 arrest through the binding and inactivation of Cdks (Morgan, 1995).

An analysis of EGF causing the induction of other Cdk inhibitors in MDA-468 cells has not been reported. However, EGF treatment in A431 cells does not induce the expression of $p27^{Kip1}$, nor the $p16^{Ink4a}$ inhibitor. Only an increase of $p21^{Cip1}$ protein was found in Cdk2 and Cdk6 within 24 hours of EGF-treatment (Fan *et al.*, 1997)

In addition, in the context of MDA-468 cells, the INK4 family, consisting of the inhibitors p15, p16, p18, and p19, might possibly be ruled out since MDA-468 cells lack the Rb gene product (Ang *et al.*, 1988). The INK4 family of Cdk inhibitors are responsible in preventing the phosphorylation of Rb by Cdk4 and Cdk6. In cells lacking Rb, the overexpression of INK4 inhibitors does not cause growth arrest as the binding and inactivation of Cdk4 and Cdk6 by these inhibitors is redundant. $p21^{Cip1}$ on the other hand, efficiently inhibits Cdk2 as well as Cdk4 and Cdk6 and can cause growth arrest in Rb-deficient cells (Almasan *et al.*, 1995). If this is the case then it could be argued that for MDA-468 cells, inhibition of Cdk2 by $p21^{Cip1}$ is most likely the mechanism of G_1-arrest in this Rb-lacking cell line.

However the Rb homologues, p107 and p130, have been shown to maintain some growth inhibitory function in cells that lack Rb (Moberg *et al.*, 1996). If so, then these

homologues to Rb may require inhibition of Cdk4 and Cdk6 by INK4 proteins to block E2F function (Schlessinger, 1988). Since the state of expression and activity of p107 and p130 in MDA-468 is unknown, a role for INK4 inhibitors in preventing the phosphorylation these proteins cannot be ruled out as a mechanism of G_1-arrest in these cells. Especially given that TGF-β has been shown to upregulate $p15^{INk4b}$ as well as induce G_1 arrest in MDA-468 cells (Sandhu *et al.*, 1997).

xiii) Proposed mechanisms for EGF-mediated upregulation of $p21^{Cip1}$

a. Possible role for p53 in MDA-468 G_1 arrest.

If indeed upregulation of $p21^{Cip1}$ by EGF leads to G_1 arrest in MDA-468 cells, the mechanism by which the activated EGF-receptor signals an increase in $p21^{Cip1}$ expression remains to be examined. Previous investigators examined a possible role for the tumour suppressor gene p53 as part of the signal transduction pathway ultimately leading to the arrest of these cells (Prasad and Church, 1997a,b). It was postulated that EGF signaling in MDA-468 might cause an induction of p53 activity by post-translational modification, most likely by phosphorylation, such that this induction of activity resulted in G_1 arrest. It has been demonstrated that EGF-treatment not only alters the $p53^{273.His}$ to a wild-type conformation, but also causes the nuclear localization of this transcription factor prior to the onset of growth arrest (Prasad *et al.*, 1997a). Furthermore, EGF-treatment induced the expression of a CAT-reporter under the control of the p53-consensus element p53CON (Prasad *et al.*, 1997b). These observations suggest a gain of function and localization to the nucleus where it can transactivate the genes which mediate growth arrest, such as $p21^{Cip1}$.

However, the p53 binding site within the $p21^{Cip1}$ promoter, is slightly different from the p53 consensus sequence (p53CON) to which $p53^{273.His}$ can bind (Abarzua *et al.*, 1995). In addition, it has been reported that the $p53^{273.His}$ mutant is unable to bind or transactivate the $p21^{Cip1}$ promoter (Pocard *et al.*, 1996). While it has been shown that expression of a CAT reporter with the p53 consensus binding site can be upregulated in MDA-468 cells with EGF treatment (Prasad *et al.*, 1997b), the parallel studies using the $p21^{Cip1}$ promoter elements have not been done. Transfection of this construct into MDA-468 cells would give a good indication of whether or not the EGF-induced changes in $p53^{273.His}$ ultimately lead to the upregulation of $p21^{Cip1}$. However even if $p53^{273.His}$ does not upregulate $p21^{Cip1}$, it may participate in G_1 arrest through another downstream effector.

b. Possible activation of $p21^{Cip1}$ by STAT proteins.

Another possible mechanism whereby EGF signaling might induce $p21^{Cip1}$ is through activation of the STAT pathway. STAT proteins (Signal Transducers and Activators of Transcription) are cytoplasmic transcription factors which are phosphorylated by activated tyrosine kinase receptors (Leaman *et al.*, 1996). When receptors for IFN-γ, the interleukins, TGF-β, and EGF (Quelle *et al.*, 1995) are activated by their ligands, the activated receptor phosphorylates STAT proteins which causes their dimerization and translocation to the nucleus. In the nucleus these dimers transactivate genes with STAT binding elements in their promoters (Leaman *et al.*, 1996). The $p21^{Cip1}$ promoter region

contains binding sites for STAT-1 and STAT-3 and its expression has been shown to be upregulated by these proteins (Chin *et al.*, 1996).

The suggestion that $p21^{Cip1}$ levels increase in EGF-treated MDA-468 cells due to activation of STAT proteins is quite reasonable given the evidence of STAT participation in EGF-treated A431 cells. A431 treated with EGF have a dramatic increase in the phosphorylation and activation of STAT-1 (Chin *et al.*, 1996), and more importantly, STAT-1 activation by EGF subsequently induces $p21^{Cip1}$ and leads to G_1 arrest (Johannessen *et al.*, 1999). Given the remarkable similarities between the cell lines MDA-468 and A431, it is tempting to suggest that a similar mechanism of G_1 arrest exists. In fact, a recent study has demonstrated that both A431 and MDA-468 cells activate STAT-1 when treated with EGF (Chin *et al.*, 1997b).

To confirm the similarities between these cell lines, further work on the mechanism of EGF-induced arrest of MDA-468 cells can be done as in A431. The experiment linking EGF treatment to STAT activation to $p21^{Cip1}$ upregulation to growth arrest in MDA-468 cells has not been reported. This question is reasonably answered by the transfection of these cells with a reporter gene under control of the STAT-1 promoter element or even with the 5' promoter elements of $p21^{Cip1}$, luciferase and examine the effect of EGF on expression. In fact making use of a reporter construct with both the STAT binding element and the p53 binding element, deletion analysis might resolve whether $p21^{Cip1}$ upregulation by EGF is mediated by p53 activation or by the activation of STAT proteins.

5. CONCLUSION

The aim of this study was to determine if the Cdk-inhibitor protein $p21^{Cip1}$ functioned as a mediator of EGF-mediated G_1 arrest of MDA-468 cells. The approach was to express antisense to $p21^{Cip1}$ in order to reduce its expression and remove it from a putative role in this arrest. Of the antisense-transfected cells, three of the single clones provide good evidence of a reduction or elimination of $p21^{Cip1}$ correlated with an attenuation of G_1 arrest. Before a definitive conclusion can be made from this data, it would be of interest to examine the level of $p21^{Cip1}$ in those antisense-transfected clones in which a reduction of G_1 arrest was not observed. Should these cell lines demonstrate a persistence of $p21^{Cip1}$ coupled with persistent G_1 arrest then this evidence would further support the observation that antisense to $p21^{Cip1}$ reduces EGF-mediated growth in these cells.

Certainly there are other experiments which would provide more conclusive evidence to the effect of $p21^{Cip1}$ antisense on growth. In this study growth arrest was determined by the enumeration of cells following a given incubation. A more precise method of comparing of growth arrest between the cell lines could have involved FACS analysis to examine the change in proportion of cells in G_1. In addition a more definitive examination of $p21^{Cip1}$ function could have been done by the direct analysis of the G_1 Cdks in MDA-468. If indeed EGF-mediated growth arrest is occurring due to a significant increase in the binding and inactivation of Cdks by $p21^{Cip1}$, then demonstrating

that a reduction of $p21^{Cip1}$ in these complexes is correlated with an attenuation of G_1 arrest would suggest a major role for $p21^{Cip1}$ in this arrest.

The evidence presented here and reported elsewhere clearly demonstrate that EGF-treatment of MDA-468 cells causes and upregulation of $p21^{Cip1}$ mRNA and protein. The mechanism by which EGF activates this upregulation remains unknown. It would be of interest to determine if in fact the changes in p53 structure and localization by EGF lead to activation of $p21^{Cip1}$ expression. Likewise an analysis of STAT proteins in the upregulation of $p21^{Cip1}$ may also provide clues to the signal transduction pathway by which EGF induces the expression of $p21^{Cip1}$ and potentially G_1 arrest in these cells.

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