Mechanism of Cisplatin Resistance in Human Malignant Melanoma

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To Mom & Dad: for all your sacrifices

Abstract

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It has been more than 25 years since the former United States President, Richard Nixon, has declared war on cancer. Over the past two decades, our knowledge of the genetic mishaps that are associated with many forms of familial and sporadic cancers is mounting, but this has yet to be translated into effective weaponry in this battle. Toxic cocktails of chemotherapeutic drugs remain our main treatment of systemic human malignancies that frequently results in acquired drug resistance in the very cancers they aim to eradicate. Vigorous efforts are continuously made to elucidate the mechanisms of drug resistance in the hope of unveiling novel molecular targets in cancer therapy.

The work described in this thesis has attempted to characterize the cellular and genetic alterations responsible for the resistance to cisplatin in cisplatin-resistant cell lines that were established using retroviral insertional mutagenesis. The cellular changes characterized include: a pattern of cross resistance to other chemotherapeutic agents, the formation of tight three-dimensional spheroids in the cisplatin-resistant cells, the differential induction of stress activated protein kinase (SAPK) activity, and suppression in the induction of apoptosis in the cisplatin-

resistant cell lines. Using a molecular approach, we detected over-expression of the melanocyte-specific gene, *tyrosinase related protein-2 (Trp-2)* in all of the cisplatin-resistant melanoma cell lines studied. The expression of Trp-2 not only correlates directly with sensitivity to cisplatin but has been shown to be, at least in part, responsible for cisplatin resistance *in vitro*.

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List of Abbreviations

ATP.....adenosine triphophate

°C.....degree Celsius

CDK.....cyclin dependent kinase

DEPC.....diethylpyrocarbonate

DHI.....5,6-dihydroxyindole

DHICA.....5,6-dihydroxyindole-2-carboxylic acid

DOPA.....3.4-dihydroxyphenylalanine

DNA..... deoxyribonucleic acid

cDNA.....complementary deoxyribonucleic acid

dCTP.....deoxycytosine triphosphate

DTIC.....dacarbazine

FBS.....fetal bovine serum

GSH.....glutathione

hr.....hour

kDa.....kiloDaltons

IFN.....interferon

IL-2.....interleukin-2

LOH.....loss of heterozygosity

mRNA.....messenger RNA

µg.....microgram

µl.....microliter

μM.....micromolar

MT.....metallothionein

%.....percent

PCR.....polymerase chain reaction

PMSF.....phenylmethylsulfonyl fluoride

RB.....retinoblastoma

RGP.....radial growth phase

RNA.....ribonucleic acid

SDS.....sodium dodecyl sulphate

Na_2VO_3.....sodium vanadate

NaCl.....sodium chloride

SAPK.....stress activated protein kinase

Tris.....tris(hydroxymethyl)-aminomethane

Trp-1.....tyrosinase related protein-1

Trp-2.....tyrosinase related protein-2

UVA.....ultraviolet A

UVB.....ultraviolet B

VGP.....vertical growth phase

CHAPTER I

GENERAL INTRODUCTION

1.1 Human Malignant Melanoma

Melanoma, a malignancy of melanocytic origin, is a neoplasm whose incidence is increasing dramatically in persons with light-colored skin in all parts of the world (Elwood and Koh 1994, Armstrong and Kricker 1994, Buske 1998). In 1997, an estimated 40,300 persons were diagnosed with malignant melanoma in the United States, and an estimated 7,300 will die in the advanced stages of the disease (Parker *et. al.* 1997).

The inverse relationship between risk and latitude is well established and is exemplified by the 19.1 per 100 000 incidence in women seen in Australia compared to an incidence of only 9.4 per 100 000 in Danish women (Elwood and Koh 1994). Phenotypic characteristics of pale skin, the inability to tan, blue eyes and red hair have been shown to increase the risk of melanoma development (Longstreth 1988). The gender differences in anatomical site distribution, with males predisposed toward trunk lesions and a high incidence of cancers occurring on the lower limbs in females, may reflect sunbathing differences between the sexes (Mackie *et al.* 1989). The frequency of nevi is likely to be both a good indicator of future melanoma risk and a short-term biologic marker of the effects of sun exposure (Elwood and Koh 1994). A person who harbors 50 to 100 moles larger than 2mm in width has a fivefold increased risk for the development of melanoma, compared with the population at large (Longstreth 1988). In fact, the relative risk for development of melanoma increases from 1.6 fold for persons with 10 to 25 nevi to 10 fold for persons with a mole count of 100 or more (Longstreth 1988). The clinical behavior of melanoma is considerably more aggressive than any other skin cancer where its five-year survival rate, provided that the lesion is detected early, is

at best around 20% compared to virtually 100% for basal cell carcinoma of the skin (MacNeill *et. al.* 1995).

1.2 Etiology of Malignant Melanoma

Similar to other malignancies, the development of malignant melanoma and its progression involves the progressive accumulation of irreversible alterations in an unknown number of genes in the melanocyte (Vogelstein and Kinzler 1993, Laporte 1998). The genetic and biochemical defects that transform the melanocyte from a cell that rarely proliferates in normal adult skin to one that progresses to a highly invasive and often fatal tumor are unknown.

The dependency on latitude, the predilection for fair-skinned persons, the increased risk after migration to regions nearer to the Equator and the differing body distribution patterns of incidence in each sex, support a role for solar radiation, particularly ultraviolet B (UVB) (280-315nm) irradiation, in the genesis of malignant melanoma (Elmwood and Koh 1994, Longstreth 1988, Laporte 1998, Langley and Sober 1997, Walsh 1997). In recent years, however, the long wavelength ultraviolet light, ultraviolet A (UVA) has also been shown to be a potent inducer of squamous cell carcinoma and melanomas in animal models and epidemiological studies (Osterlind 1993). Although the exact quantitative and qualitative nature of UV exposure is not clear, it has been suggested that intermittent exposures and intense exposures with consequent sunburns in a high-risk phenotype are critical in increasing the risk of developing melanoma (Langley and Sober 1997).

UV exposure may rapidly initiate a complex series of events resulting in a transient but limited number of cell divisions that increases the number of melanocytes

within the epidermis (Rosen *et al.* 1987, Husain *et al.* 1991, Stierner *et al.* 1989). UV radiation may also damage DNA by promoting free radical formation, single-strand breaks, and, most commonly, chemical bond formation between two adjacent pyrimidines, particularly thymidine (Ananthaswamy and Pierceall 1990). Moreover, ultraviolet radiation has been shown to reduce the number of Langerhans cells in the epidermis and modifying their antigen-presenting cell capacity, suggesting a relationship between UV-induced immune suppression and skin cancer (Donawho and Kripke 1991, Meunier *et al.* 1998). The ability of UVB radiation to induce melanocyte proliferation and cause DNA damage makes it a likely contributor to the generation and propagation of gene mutations and eventual chromosomal loss that provide the melanocyte with malignant characteristics.

1.3 Synthesis of Melanin Pigment

Exposure to ultraviolet radiation and enduring potential damage to cellular macromolecules such as DNA is unavoidable particularly as the ozone layer continues to be depleted. What may prevent the accumulation of cellular damage and eventual development of malignant melanoma in all persons exposed to UV radiation is the inherent ability of melanocytes to produce the polymer, melanin (Prota and Thomson 1976).

Early in gestation, from the eighth embryonic week, the nonpigmented precursors, melanoblasts migrate to the meninges, the uveal tract and the epidermis where they mature into melanocytes (Le Douarin 1982). Cutaneous melanocytes reside in the basal layer of the epidermis where their function is to produce melanin pigments that is contained within a discrete cellular organelle, the melanosome which is later secreted via

dendritic processes and taken up by surrounding kertinocytes (Le Douarin 1982). There are two types of melanin polymers, phaeomelanins and eumelanins, both of which contribute to the natural melanin present in the skin (Nicolaus *et al.* 1964, Piattelli *et al.* 1963, Odh *et al.* 1994). A positive correlation has been found between the eumelanin/phaeomelanin ratio and sensitivity to UV radiation, suggesting that high UV sensitivity is associated with high phaeomelanin and low eumelanin levels (Vincensi *et al.* 1998).

The process of melanogenesis is thought to occur through the enzymatic activities of a family of proteins, the tyrosinases, consisting of tyrosinase, tyrosinase related protein-1 (Trp-1), and tyrosinase related protein-2 (Trp-2) (Jimbow *et al.* 1994). Melanin synthesis in mammals proceeds from the amino acid L-tyrosine through a series of enzymatic and chemical events initiated by the rate-limiting step of tyrosine hydroxylation to yield DOPA and DOPA oxidation to L-DOPAquinone (Figure 1.1) (Hearing 1987, Prota 1988). Both reactions are catalyzed by tyrosinase, a melanocyte-specific copper-containing glycoprotein located within the melanosomes (Korner and Pawelek 1982, Hearing and Tsukamoto 1991). L-DOPAquinone is the first and principal branch point in the pathway of melanogenesis determining whether phaeomelanin or the stronger UV absorption, eumelanin is to be produced. In the absence of low molecular weight thiolic compounds, L-DOPAquinone cyclizes rapidly and is converted to L-DOPAchrome (Prota, 1992). L-DOPAchrome is a relatively unstable intermediate and in the absence of Trp-2, it undergoes spontaneous decarboxylation to yield 5,6-dihydroxyindole (DHI) and CO_2, which in turn, can be oxidized either spontaneously or enzymatically by tyrosinase, ultimately leading to the formation of the melanin polymer

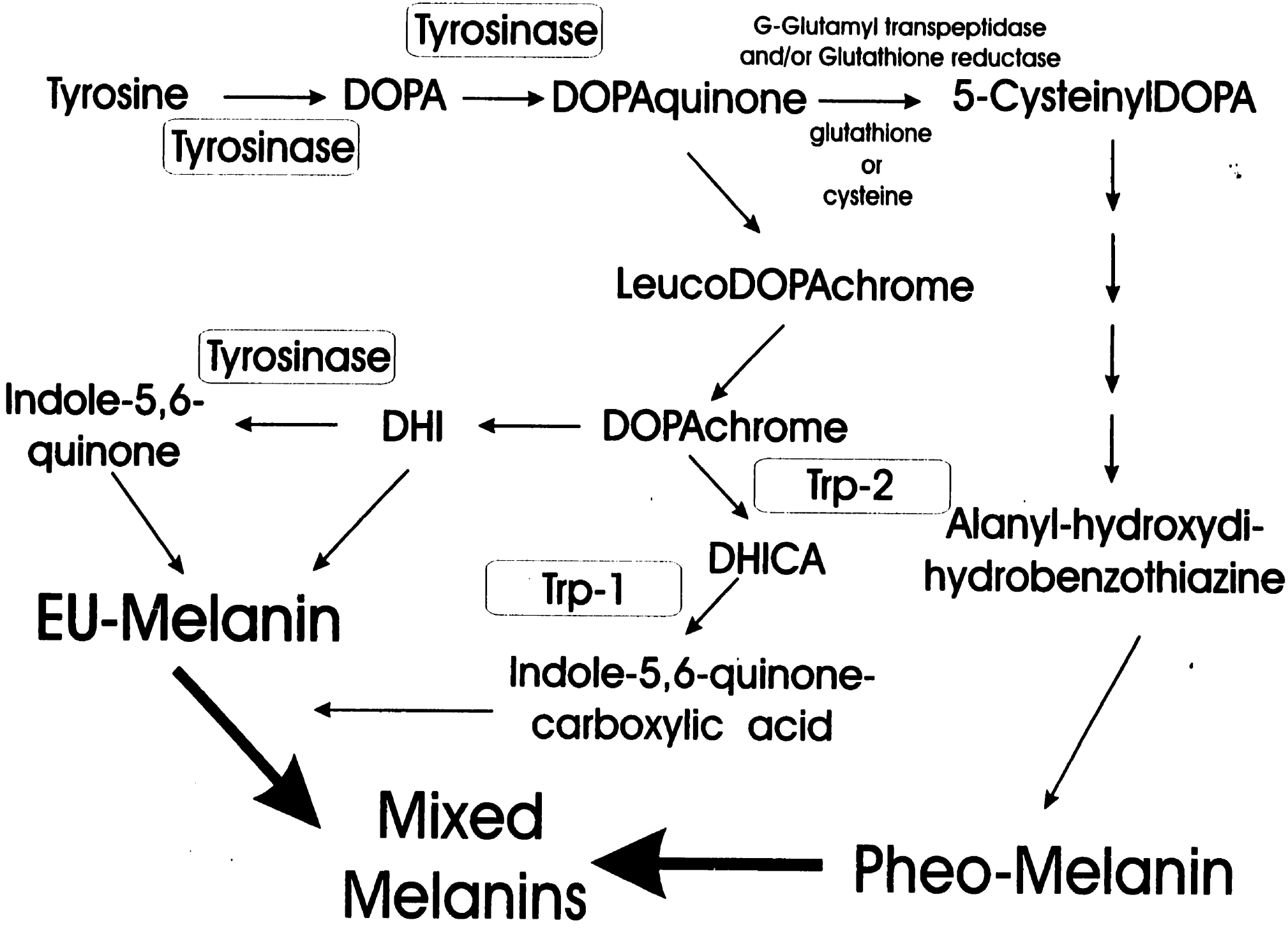


Figure 1.1 Pathway of melanogenesis (modified from Jimenez-Cervantes *et al.* 1994).

(Korner and Pawelek 1982, Winder *et al.* 1994, Kobayashi *et al.* 1994). Therefore, L-DOPAchrome marks the second branch point in the melanogenic pathway where the degree of carboxylation within the melanin polymer is determined by the activity of Trp-2. Trp-2 can efficiently catalyze the non-decarboxylative tautomerization of DOPAchrome to the highly stable carboxylated product 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Leonard *et al.* 1988, Aroca *et al.* 1990, Pawelek 1990, Tsukamoto *et al.* 1992, Jackson *et al.* 1992). Little is known concerning the pathway followed by DHICA prior to its incorporation into the melanin pigment. It has been reported that peroxidase may promote the oxidative polymerization of DHI and DHICA to melanin more effectively than tyrosinase, suggesting that some intramelanocytic peroxidase might be involved in the catalysis of the final steps of the melanin pathway (D'Ischia *et al.* 1991). The structure of melanin has been defined and it is believe that *in vivo*, the melanin polymer is distributed on a lamellar structure within melanosomes due to the polymerization of mainly DHICA, as opposed to DHI (Odh *et al.* 1994). Studies on the structure of natural melanin from various zoological sources have revealed that they contain high concentrations of carboxylic acid residues, and that these groups are derived from the incorporation of DHICA monomers into melanin (Ito 1986, Palumbo *et al.* 1987).

1.3.1 Tyrosinase Family of Proteins

The identification of tyrosinase and Trp-1 was made simultaneously by Kwon and others who demonstrated that mouse melanoma melanosomes contain two distinct proteins, termed HEMT (high electrophoretic mobility tyrosinase) and LEMT (low electrophoretic mobility tyrosinase) (Kwon *et al.* 1988, Ruppert *et al.* 1988). It was later

confirmed that HEMT is the only tyrosinase protein that enzymatically catalyzes tyrosine hydroxylation and DOPA oxidation in the initial steps of melanogenesis (Jimenez-Cervantes *et al.* 1993, Valverde *et al.* 1992). Tyrosinase is encoded by the *c albino* locus on mouse chromosome 7 and mapped to human chromosome 11 (Muller *et al.* 1988, Kwon *et al.* 1988, Shibahara *et al.* 1986). As previously mentioned, it is the principal and rate-limiting enzyme in the melanogenic pathway, catalyzing three important reactions: the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA), the oxidation of L-DOPA to DOPAquinone, and the oxidation of 5,6-dihydroxyindolate (DHI) to indole-quinone. Transfection of wildtype *c locus* cDNA induced tyrosinase activity and melanin synthesis in many cell types, including fibroblasts, amelanotic melanoma cells, albino melanocytes, among others, confirming that the *c locus* indeed encodes tyrosinase.

Jimenez-Cervantes and others showed that LEMT corresponds to Trp-1 in melanocytes and was later assigned to the *brown* locus on mouse chromosome 4 (Jimenez-Cervantes *et al.* 1993, Jackson 1988). A single base alteration in the cDNA of Trp-1 results in an arginine to cysteine amino acid change in the protein that characterizes the dominant 'light' mutation of the *b locus* whereby brown rather than black melanin is synthesized (Johnson and Jackson 1992). This mutation also causes premature melanocyte death and partial loss of hair pigmentation in *brown* mice (Luo *et al.* 1994, Zhao *et al.* 1996, Johnson and Jackson 1992). The human homologue of Trp-1 has 10 amino acids less than the mouse Trp-1 gene product at the carboxyl terminus and the degree of sequence homology is about 93% (Cohen *et al.* 1990). Likewise, melanocytes from human homologue of the murine *brown* mutation have decreased tyrosinase activity, reduced melanin content and brown melanin production (Cohen *et al.* 1990).

Trp-2, also known as dopachrome conversion factor, is a glycoprotein with putative iron binding sites and requires ferrous iron (Fe^{++}) for full activity (Chakraborty *et al.* 1992, Jackson *et al.* 1992, Tsukamoto *et al.* 1992). The initial report of a dopachrome conversion factor present in melanocytes capable of converting orange-red DOPAchrome into a colorless compound was later verified by Korner and Gettings who identified DHICA as the product of the reaction through the use of mass spectroscopy and nuclear magnetic resonance (Korner and Pawelek 1980, Pawelek *et al.* 1980, Korner and Gettins 1985). A gene encoding a protein with this dopachrome tautomerase activity was subsequently determined to be mutated in mice with the *slaty* genotype (sl/sl) (Jackson *et al.* 1992). *Slaty* has a single amino acid mutation of arginine substitution for glutamine in the first copper binding site of the Trp-2 protein. However, this missense mutation does not abolish Trp-2 activity but merely decreases it by approximately 3-fold (Jackson *et al.* 1992). The human Trp-2 has been cloned and is composed of 519 amino acids with a molecular weight of 59kDa and has about 84% identity with the mouse counterpart (Yokoyama *et al.* 1994b, Tsukamoto *et al.* 1992, Jackson *et al.* 1992). There are two regulatory regions in the Trp-2 gene, the 32 base-pair element and the proximal region where they each contain a CANNTG motif that is required for pigment cell-specific expression (Yokoyama *et al.* 1994a). Trp-2 protein contains two alternating domains of cysteine-rich regions with Cu^{2+} binding sites, a signal peptide at the amino terminus and a transmembrane domain at the carboxyl terminus.

1.4 Melanoma Development and Progression

Progression from the normal melanocyte to metastatic melanoma is a multistep, multigenic process (Walch and Goldberg, 1997). While relatively easy to conceptualize,

the stages of melanoma progression are not necessarily discrete, nor are they unambiguously defined at the morphologic or molecular levels. Based on clinical and histopathological features, six steps of melanoma progression have been proposed: common acquired and congenital nevi with structurally normal melanocytes, melanocytic nevi with aberrant differentiation, dysplastic nevus with structural and architectural atypia, early radial growth phase (RGP) primary melanoma, advanced vertical growth phase primary melanoma (VGP) with competence for metastasis, and metastatic melanoma (Clark *et. al.* 1986, Heryln *et. al.* 1987, Herlyn 1990, Meier *et al.* 1998, Thompson 1994).

1.4.1 Step I: Initial Lesion: The Common Acquired Melanocytic Nevus

Normal epidermal melanocytes exist in a relatively quiescent state in the epidermis. The first emergence of atypical nevi or moles occur during the first two decades of life, and are presented as small tan dots, not exceeding 0.2 cm in diameter. This is the first stage in the development of a melanocytic nevus which may be caused by prolonged UVB exposure, resulting in the proliferation of an increased number of hyperpigmented melanocytes within the basal epidermis, contributing to the development of normal and atypical nevi (Herlyn *et al.* 1987). Over a period of three to four years, the melanocytic nevi may enlarge to 4-5 mm in diameter and form clusters at the tips of rete ridges that subsequently migrate into the dermis, forming small nests of cells (Murphy *et al.* 1988). With the passage of time, intraepidermal melanocytic growth ceases and the lesion becomes clearly elevated and demarcated from the surrounding normal tissue (Murphy *et al.* 1988). The dermal component of the melanocytic nevi may differentiate into neuromesenchyme and the cells may evolve along the line of Schwann cells, forming

small structures similar to nerve endings, encompassing the entire dermal component of the lesion (Murphy *et al.* 1988). Most melanocytic nevi will eventually flatten and disappear, with the skin returning to normal.

1.4.2 Step II: Atypical Nevus: Melanocytic Nevus with Aberrant Differentiation

While a majority of melanocytic nevi regress to normal, some lesions persist and may proliferate to greater than 0.5cm in diameter and exhibit focal areas of eccentric melanocytic growth consisting of a flat irregular periphery extending asymmetrically from the parent mole (Heasley *et al.* 1996). Histologically, growth of melanocytes in the basal epidermis may initially appear to be no different from that in the early stages of a common mole (Murphy *et al.* 1988). However, a band of brightly eosinophilic connective tissue can be detected around the rete, where melanocytes grow aberrantly and small clusters of lymphocytes may be detected (Murphy *et al.* 1994). This stage of melanoma development is marked by the identification of melanocytic nevus displaying architectural, but not cytological atypia.

1.4.3 Step III: Melanocytic Nevus with Dysplasia

The appearance of atypical, genetically unstable "dysplastic" nevi exhibiting characteristics of abnormal growth patterns and cytologic abnormalities has been strongly implicated as precursor lesions in melanoma (Elder *et al.* 1993, Heasley *et al.* 1996). These areas of dysplasia are often associated with subjacent lymphocytic infiltrate and distinctive connective tissue changes (Murphy *et al.* 1988). In contrast to normal melanocytes, melanocytic nevi at this stage are histologically characterized by the appearance of nests of cytologically atypical melanocytes proliferating in the epidermis

beyond the dermal component of the nevus that may lead to the development of melanoma *in situ* (Slominski *et al.*1995). Although the relationship between atypical nevi and the development of sporadic malignant melanoma is not well defined, it has been reported that a significant portion of primary invasive malignant melanomas has histopathologic evidence of an associated atypical nevus (Clark *et al.* 1984, Milm *et al.* 1992). Moreover, it has been reported that in individuals with greater than six atypical nevi, the incidence of melanoma is statistically increased (Tucker *et al.* 1993). However, despite positive correlation between atypical nevi and the development of malignant melanoma, the vast majority of atypical nevi retained the ability to regress and never develop into a malignant melanoma lesion.

1.4.4 Step IV: Radial Growth Phase Melanoma (Superficial Spreading Melanoma)

Early malignant melanocytic lesions may have a slightly elevated and palpable border with variability in tone; some parts are unusually black or dark brown, whereas light brown shades are mingled with pink and light blue tints (Elder *et al.* 1993). On microscopic examination, large epithelioid melanocytes are dispersed in nets or may reside as individual cells throughout the entire thickness of the epidermis (Elder *et al.* 1993). Although these melanocytes may be solely confined to the epidermis (*in situ* melanoma), focal extensions into the papillary dermis are generally the rule (Heasley *et al.* 1996). The enlargement of these circular lesions is mostly at the periphery, hence the term radial growth phase (RGP). The radial growth phase of primary malignant melanoma has a distinct propensity for tissue invasion although without metastatic capacity (Clark *et al.* 1975, Elder *et al.* 1993).

1.4.5 Step V: Vertical Growth Phase Melanoma

Within 1-2 years from the onset of radial growth phase, melanocytes may exhibit focal mitotic activity and grow as spheroidal nodules, in a manner similar to the growth of metastatic nodules (Murphy *et al.*1988). The nodules may expand more rapidly than the rest of the tumor in the surrounding papillary dermis and the net direction of growth tends to be perpendicular to that of the radial growth phase (Heasley *et al.*1996). However, properties required for metastases may still be lacking in melanomas that have entered into the vertical growth phase (VGP). Some tumors have little competence for metastasis, whereas others have a greater potential. Cells of the VGP often have distinct characteristics in contrast to lesions of the RGP (Heasley *et al.* 1996, Murphy *et al.* 1988, Clark *et al.* 1975, Clark *et al.* 1986). For example, the cellular aggregate that characterizes the VGP is larger and substantially less pigmented than the clusters of cells that form the intraepidermal and invasive components of the radial growth phase. Moreover, the dominant site of the tumor growth is shifted from the epidermis to the dermis and tumors that extend into the lower half of the reticular dermis are, by definition, in the vertical growth phase. Finally, in contrast to the RGP lesions, the cellular immune response of the host is frequently absent by the end of the vertical growth phase.

1.4.6 Step VI Metastatic Melanoma

Metastatic melanoma, the final stage in tumor progression, arises from the melanocytes of the vertical growth phase. For invasion to occur, melanocytes must disrupt the extracellular matrix of the dermis prior to metastatic spread by instigating the enzymatic degradation of the matrix and developing a directional proteolysis by

producing various enzymes such as matrix metalloproteinases, including type IV collagenase, serine proteases (eg. plasminogen activators), and lysomal proteases (e.g., cathepsins) (Stetler *et al.* 1993, Boike *et al.* 1992, Lauricella-Lefebvre *et al.* 1993, Monsky and Chen 1993, Seftor *et al.* 1993, de Vries *et al.* 1994).). In addition, melanoma cells may alter their ability to adhere and detach from each other and from surrounding stromal cells, which plays a decisive role in metastatic spread (Reeves, 1992).

1.5 Chromosomal Aberrations Associated with Malignant Melanoma

An overview of malignant melanoma would not be complete without a discussion of the chromosomal alterations associated with this disease. Critical to the development of melanoma is the disruption of genomic integrity resulting in sustained genomic instability within the melanocyte (Albino and Fountain 1993, Bastian *et al.* 1998, Koehler *et al.* 1996). Often the identification of DNA aneupolidy (the numeric deviation of one or a few chromosomes) is used to differentiate melanomas *in situ* from nonmalignant atypical nevi, which typically do not harbor such chromosomal abnormalities (Slater *et al.* 1991, Barks *et al.* 1997). Regardless of the measurable degree of aneupolidy, a feature common in melanomas is their inability to precisely replicate genomic DNA (Giaretti 1993). Consequently, those genes intimately involved in the diverse processes of DNA replication and repair, cellular mitosis, and regulation of the cell cycle are likely targets of the disruptive effects caused by genetic instability that lead to malignant transformation (Hartwell 1992).

Cytogenetic studies, loss of heterozygosity (LOH) analyses, and fluorescence in situ hybridization (FISH)-based techniques have been successfully used to detect and visualize regions of DNA loss or amplified in melanocytic lesions (Kallioniemi *et al.*

1992, Dreyling *et al.* 1995). Many studies support the involvement of genes on chromosomes 1, 6, 7, 9, 10, 11 and possibly 22 and Y in the initiation and progression of both sporadic and familial melanomas (Albino and Fountain 1993, Fountain *et al.* 1990, Thompson *et al.* 1995, Boni *et al.* 1998).

1.5.1 Chromosome 1

Genetic linkage analysis on familial melanoma kindreds and LOH studies on sporadic melanoma have determined that loss of the terminal portion of chromosome 1 (1p36, 1p22-1q21) to be the most frequently altered event in melanoma, accounting for 74-82% of all melanoma lesions examined (Albino and Fountain 1993, Fountain *et al.* 1990, Thompson *et al.* 1995, Healy *et al.* 1995, Hussussian *et al.* 1994, Walker *et al.* 1995). However, any attempt to ascribe specific significance to this observation must be tempered by the knowledge that chromosome 1 is altered in almost every solid tumor (Walker *et al.* 1995). Early interest in this chromosome arose from the finding that nine out of 11 advanced melanomas had deletions or translocations of 1p (Balaban *et al.* 1984). Linkage analysis of fourteen large families in the United States, whose members were assessed as having a life-time risk of 100% of developing melanoma suggests that a melanoma susceptibility gene was associated with chromosome 1p near the rhesus blood group locus (Greene *et al.* 1983, Bale *et al.* 1989). Dracopoli and colleagues, using multiple melanoma metastases from the same patients and tumor and lymphoblast samples from a family with hereditary melanoma, indicated that 1p loss occurred late in tumor progression, dismissing the possibility of a tumor suppressor gene (Dracopoli et al. 1989). Recently, studies by Fountain and colleagues have identified a candidate locus on 1p36, PITSLRE, consisting of three tandemly duplicated genes that span 90kb and

encoding a p58 protein kinase which was subsequently shown to play important roles in regulating the cell cycle and involvement with both the susceptibility and progression of malignant melanoma (Albino and Fountain 1993, Fountain *et al.* 1990, Tomlinson *et al.* 1993, Lahti *et al.* 1994). Although over-expression of the PITSLRE proteins can mediate apoptosis, down-regulation augments cell division or growth (Lahti *et al.* 1995a, Lahti *et al.* 1995b, Bunnell *et al.* 1990, Beyaert *et al.* 1997). The majority of melanoma cell lines have been reported to have either loss of PITSLRE or express abnormal protein isoforms due to differential splicing, (Xiang *et al.* 1994, Neilson *et al.* 1996).

1.5.2 Chromosome 6

Over 50% of melanomas have all types of chromosomal rearrangements, including deletions and amplifications, within both p and q arms of chromosome 6 (Thompson *et al.* 1995, Balaban *et al.* 1984). Genetic linkage studies suggest that chromosome 6 is the potential site of a yet to be identified melanoma predisposition locus and deletions and non-reciprocal translocation on 6q are frequently observed in late-stage melanoma lesions prior to deletions of 1p (Fountain *et al.* 1990, Walker *et al.* 1994, Healy *et al.* 1995, Trent *et al.* 1989). Chromosomal deletions in cancer often are indicative of the presence of tumor suppressor genes. Evidence for the involvement of such genes comes not only from studies on allelic loss but also from a large body of data derived from the use of interspecific and intraspecific somatic cell hybrids (Harris 1969, Stanbridge *et al.* 1982). Single chromosome transfer studies involving somatic cell hybrids indicated that chromosome 6 reduced the tumorigenic capacity of the resultant microcell hybrids, consistent with the possibility that genetic information present on chromosome 6 can suppress the malignant capacity of human melanoma cells (Trent *et*

al. 1990). More recently, Robertson and colleagues found that the suppression of tumorigenicity followed the introduction of a normal copy of chromosome 6 into the UACC 903 human melanoma cell line that correlated with increased chromosome 6 dosage (Robertson *et al.* 1996). Subsequently, it was determined that q22-24 region of chromosome 6 is responsible for suppressing primary tumor formation in UACC 903 cells (Robertson *et al.* 1996). It has also been shown that the antioxidant manganese superoxide dismutase (MnSOD) gene located on 6q25 can inhibit the growth of melanoma cells *in vitro* and *in vivo* (Church *et al.* 1993). However, the lack of mutations in the MnSOD gene in either familial or sporadic melanoma suggests that this gene is probably not the primary target for inactivation on 6q.

1.5.3 Chromosome 7

Using a comparative genomic hybridization method, amplification of chromosome 7, particularly 7q32-34, has been consistently detected in over 50% of invasive growth phase melanoma specimens (Koprowski *et. al.* 1985, Collard *et al.* 1987, Fountain *et. al.* 1990, D'Alessandro *et al.* 1997). A recent study showed that chromosomal copy number gain was found in 40.9% of cases, where chromosomal gain is defined as copies with more than 3 spots (Matsuta *et al.* 1997). In a very small sample of patients with VGP melanoma, it was found that a structural abnormality of chromosome 7 correlated significantly ($p<0.001$) with a shortened survival duration relative to patients without such chromosome abnormalities (Trent 1991). In early stage melanoma too, there is an association between chromosome 7 alteration and melanoma development. The increased expression of the epidermal growth factor receptor (EGFR),

mapped to 7p12, correlated with increased copies of chromosome 7p late in early stage disease (Koprowski *et al.* 1985).

1.5.4 Chromosome 9

Loss of function mutations in one or more tumor suppressor genes within the p21 region on chromosome 9 have been implicated in the early development of both sporadic and familial melanoma (Petty *et al.* 1993, Goldstein *et al.* 1994, Isshiki *et al.* 1994, Holland *et al.* 1994, Walker *et al.* 1994b, Ruig *et al.* 1995, Healy *et al.* 1995, Park *et al.* 1998). In sporadic melanomas, 46% to 73% of all specimens harbor deletions within the 9p21 region, compared with approximately 50% of all kindreds found in familial melanomas (comprised of 8-12% of all cases of melanoma) (Albino and Fountain, 1993, Fountain *et al.* 1990, Isshiki *et al.* 1994, Holland *et al.* 1994, Walker *et al.* 1994, Cannon-Albright *et al.* 1992, Soufir *et al.* 1998, Hayward 1996, Marx 1994).

Analysis of the genes within chromosome 9 has lead to the identification of a candidate tumor suppressor gene, the cyclin dependent kinase inhibitor 2 (CDKN2) or p16 mapped to the critical region of 9p21, in the progression of malignant melanoma (Kamb *et al.* 1994a, Kamb *et al.* 1994b, Nobori *et al.* 1994). Hemizygous and homozygous deletions of CDKN2 are common in metastatic melanomas, although their relative frequency is higher in melanoma cell line DNAs (58%) than in uncultured melanoma DNAs (22.5%) (Kamb *et al.* 1994a, Liu *et al.* 1995, Castellano *et al.* 1997). Moreover, intragenic mutations within CDKN2 are also more frequently observed in melanoma cell lines (16% to 40%) than in uncultured metastatic melanoma DNAs (0% to 15%) (Kamb *et al.* 1994a, Flores *et al.* Ohta *et al.* 1994, Pollock *et al.* 1995, Gruis *et al.* 1995). In one recent study, p16 protein was detected by Western blotting in only 10% of

the melanoma cell lines examined and immunoprecipitation of the protein in these lines, followed by Western blotting to detect the coprecipitation of CDK4 and CDK6, revealed that p16 was functionally compromised in 80% of these cell lines (Castellano *et al.* 1997).

CDKN2 (p16) encodes a 16 kDa nuclear protein, located primarily in the nucleus, that is an inhibitor of the cyclin dependent kinase 4 and 6 (CDK4, CDK6) enzymes (Reed *et al.* 1995, Serrano *et al.* 1993). The association of CDKs with their appropriate partner cyclins is an essential event in the cell's progression through the various phases of the cell cycle. For example, the transition from the G_1 to S phase of the cell cycle normally requires the coupling of specific cyclins with CDK4 or CDK6 which in turn phosphorylates the retinoblastoma (Rb) protein, releasing the Rb-bound transcription factors necessary for DNA synthesis and cell division (Weinberg 1995). When p16 replaces cyclins in its association with CDK4 and CDK6, the phosphorylation of Rb is suppressed and the progression from G_1 to S phase is prevented.

The critical importance of eliminating the p16 pathway during malignant transformation of the melanocyte is evident by the number of different ways an evolving tumor may inactivate its function. Merlo and colleagues reported many different alterations such as deletion or mutation of one or both p16 alleles, and p16 gene methylation, underscoring the critical importance of eliminating the p16 pathway in melanomas (Merlo *et al.* 1995, Herman *et al.* 1995, Cairns *et al.* 1995, Gonzalgo *et al.* 1997). A single copy alteration of the p16 gene in melanoma cells will diminish p16 function, compromising the cellular response to stressful stimuli and increasing the probability of the melanocyte to accumulate additional genetic damage on its course towards malignancy. Damage to DNA as induced by UV irradiation may not be repaired

before replication occurs resulting in the inappropriate entry into S phase when p16 is defective or diminished in the cell. Hence even a single allelic alteration in the p16 gene may predispose the cell to genetic damage and the development of genetic instability and tumor progression. Moreover, early loss or mutation of one copy of the p16 gene may result in a stochiometric decrease in p16 protein and a net increase in melanocyte proliferation. This may be followed by the deletion, mutation or methylation of the second p16 allele during tumor progression, which presumably provides the cell with an additional growth or survival advantage. Therefore, the loss or mutation of the p16 gene within the locus is an important initial step in the development of malignant melanoma and an important determinant of melanoma risk (Greene 1997, Haluska and Hodi 1998).

In addition to p16, various studies suggest that there are other genes in close proximity to p16 on 9p21 important in the development of both sporadic and familial melanoma. One likely candidate for a second 9p gene altered in melanoma is p15, another cyclin-dependent kinase inhibitor with structural and functional similarities to p16 (Glendening *et al.* 1995, Petty *et al.* 1993, Puig *et al.* 1995, Flores *et al.* 1995, MacGeoch *et al.* 1994, Wagner *et al.* 1998). Glendening and colleagues detected homozygous deletions of approximately 200-kb region surrounding p15, but not p16 in all six melanoma cell lines examined (Glendening *et al.* 1995). Moreover, detection of p16 and p15 in melanoma cell lines and tumor specimens demonstrated a non-concordant pattern of expression suggesting that the genes are not functionally redundant and that loss of either gene may be important in alleviating strong brakes in unregulated proliferation, thereby promoting malignant melanoma progression (Robinson et al. 1996, Bahuau *et al.* 1998, Matsumura *et al.* 1998).

1.5.6 Chromosome 10

Approximately a quarter of all atypical nevi and early melanoma lesions examined involve chromosomal rearrangements in 10q24-26 region and are thought to be early genetic changes in melanoma (Albino and Fountain 1993, Thompson *et al.* 1995, Fountain *et al.* 1990). 10q24-46 rearrangements have been observed in early atypical nevi, while the progressive loss (32-67%) of the entire q arm of chromosome 10 has been detected in metastatic lesions (Healy *et al.* 1995, Walker *et al.* 1995b, Isshiki, *et al.* 1993, Herbst, *et al.*, 1994, Indsto *et al.* 1998). The presence of two breakage clusters, 10q11 and 10q24 further suggests the possible existence of two genes involve in melanoma development on chromosome 10 (Indsto *et al.* 1998, Isshiki *et al.* 1993). Recently the *MMAC1/PTEN* gene, located at 10q23.3, a candidate tumor suppressor gene that is commonly mutated in gliomas has been reported to be a frequently deleted and mutated event in malignant melanoma (Guldberg *et al.* 1997). 26% of the cell lines and uncultured tumor specimens showed partial or complete homozygous deletion of the *MMAC1/PTEN* gene and 17% harbor a mutation in combination with loss of the second allele, suggesting that disruption of *MMAC1/PTEN* by allelic loss or mutation may contribute to the pathogenesis or neoplastic evolution in malignant melanoma (Guldberg *et al.* 1997). A recent analysis of polymorphic markers demonstrated deletions of both 9p and 10q regions suggesting that the 10q deletion event is likely to impair a pathway other than the cyclin-dependent kinase-mediated phosphorylation of the Rb protein (Indsto *et al.* 1998).

1.5.7 Chromosome 11

Considerable molecular genetic and cytogeneic evidence indicates that chromosome 11 is a target for chromosome breakage, rearrangement, and loss during the development of human malignant melanomas (Robertson *et al.* 1996, Tomlinson *et al.* 1996, Nedosztko *et al.* 1992, Robertson *et al.* 1997). Evidence from studies have shown that the frequency of LOH observed on chromosome 11 in primary melanomas is approximately 17%, but increases to 22-58% in metastatic tumors, suggesting that a tumor suppressor gene on 11q may be a target late in melanoma progression (Healy *et al.* 1995, Walker *et al.* 1995b, Tomlinson *et al.* 1993, Herbst *et al.* 1995). Using PCR-based assay for LOH in normal and tumor tissues from 24 individuals with cutaneous malignant melanoma of various stages, 5 polymorphic microsatellite repeats on 11q were analyzed (Herbst *et al.* 1995). Herbst and colleagues demonstrated a possible tumor suppressor gene localized on 11q23 consisting with the finding of a chromosomal breakpoints cluster on 11q22-23 (Fountain *et. al.* 1990, Herbst *et. al,* 1995, Ozisik, et. al., 1994, Tomlinson *et al.* 1996). Recent evidence using microcell-mediated chromosome transfer by introducing normal copies of human chromosome 11 into human malignant melanoma cell lines resulted in a dramatic reduction in proliferation in culture and tumor forming abilities of the hybrid cells in vivo (Robertson *et al.* 1996). The loss of 11q23 appears to be a late event in melanoma progression and may serve as an indicator for a less favorable clinical outcome (Robertson *et al.* 1996, Tomlinson *et al.* 1996).

1.6 Treatment of Malignant Melanoma

The important obstacles encountered in the use of chemotherapy have been the toxicity to the normal tissues of the body and the presence of mutations that confer resistance to these chemotherapeutic agents, leading to the acquisition of intrinsic and

acquired drug resistance. Resistance has been termed intrinsic when the tumor initially fails to respond to a wide range of anticancer agents. When tumors initially respond only to return refractory to many agents, the resistance is described as acquired. During the past decades, the application of molecular analysis to normal and neoplastic cells has uncovered some of the mechanisms through which chemotherapeutic agents induce cell death, and the changes within these cells that can confer either sensitivity or resistance to these chemotherapeutic agents. Although many malignancies experience a refractory period of growth due to the cytotoxicity of many chemotherapeutic agents, cells from various stages in malignant melanoma progression have been repeatedly documented to be tremendously resistant to chemotherapeutic interventions and are reportedly intrinsically resistant to many anticancer agents.

Patients with metastatic melanoma are treated with systemic therapy unless they have a surgically resectable single site of metastasis, in which case surgical resection offers the best palliation. The chemotherapeutic agent that is most widely used in systemic therapy is dacarbazine (DTIC), which has been in use for the past 20 years. When used as a single agent, dacarbazine has produced response rates of 10% to 15%, with complete remissions occurring in fewer than 5% of patients (Legha 1989). In addition to DTIC, other drugs with significant activity against metastatic melanoma are the nitrosoureas, the vinca alkaloids, and cisplatin (Coates 1992). Combinations of DTIC with tamoxifen, carmustine and cisplatin (the "Dartmouth regimen") or the combination of chemotherapeutic agents with biologicals have demonstrated some evidence of an increased initial response rate between 25% to 40% (Legha 1989, Mc Clay and Mc Clay 1996, Garbe 1993, Nathan and Mastrangelo 1998). However, despite

seeming improved overall response rates, compared with single agent DTIC chemotherapy, the complete remission rates remain less than 20% (Mc Clay and Mc Clay 1996, Hansson 1997). At present, disseminated, macrometastatic melanoma is largely incurable in a majority of cases with systemic chemotherapy.

Modulation of the patient's immune response can be achieved with vaccines, monoclonal antibodies, interleukin-2 (IL-2) and interferons (IFN), as single agents or in combination between themselves or with peripheral blood mononuclear cells or with tumor infiltrating lymphocytes or even with chemotherapy as therapeutic approaches have been studied (Herlyn and Koprowski 1988, Aapro 1993, Rees and Healy 1996). The most widely used cytokines are interferons and interleukin-2. IL-2 acts entirely through immunologic mechanisms and has been tested extensively, either alone, in combination with other cytokines, or with adoptive cellular therapy (Bear *et al.* 1996, Mc Clay and Mc Clay 1996). Alone, it has only modest antitumor activity, even at high doses, but its utility may be greater when combined with immunocompetent cells, especially tumor-sensitized T lymphocytes, in adoptive immunotherapy (Bear *et al.* 1996, Rosenberg, 1992, Garbe 1993). IFN-alpha also has had fairly limited activity in the advanced disease setting, but, on the basis of a recently completed randomized trial, has arguably become the standard of care in the adjuvant setting for patients with high-risk melanoma. particularly node-positive patients (Kirkwood 1997, Rusciani *et al.* 1997, Agarwala and Kirkwood 1998). Clinical and experimental results suggest that the antitumor activity of IFN was mainly related to its antiproliferative effect; immunomodulatory effects were not substantiated in clinical investigations (Garbe 1993). Where interferon and interleukin-2 therapies have yielded response rate of 15-20% on average, the combinations of

immunotherapy and chemotherapy offer response rates as high as 50-60% (Villikka and Pyrhonen 1996, Thatcher 1991,Nathan and Mastrangelo 1998). To date, IFN-alpha given initially intravenously in high doses followed by subcutaneous therapy for 1 year, is the only treatment that has been shown to increase disease-free and overall survival in patients with high-risk melanomas (Cohen and Falkson 1998). Immunotherapy with bacillus Calmette-Guerin (BCG), Corynebacterium parvum, and levamisole and high dose regimens that use autologous bone marrow or peripheral stem cell support have not been successful in achieving the goal of effectively reducing relapse, and improving survival (Agarwala and Kirkwood 1998, Nathan and Mastrangelo 1998).

In summary, single agent chemotherapy with DTIC, which has for many years formed the cornerstone of therapy, has modest effects and results in disease remission in a minority of patients, usually of short duration. Combination chemotherapy, or the combination of chemotherapeutic drugs and cytokines, results in increased response rates and occasionally remissions of prolonged duration (Abbott and Harman 1995). However, conflicting reports regarding improved responsiveness and survival concerning the use of immunotherapy have surfaced, thereby suggesting that larger clinical trials regarding its effectiveness are required (Cohen and Falkson 1998, Nathan and Mastrangelo 1998). Despite new treatment options, the survival rate of patients with metastatic melanoma has not changed significantly over the last 22 years, remaining at less than 20%; their prognosis remains dismal.

1.7 Cisplatin

Since the discovery of compounds that have the capacity to control unregulated cell proliferation, the compilation of chemotherapeutic agents have been classified

according to their mechanism of action into several groups. One such group are the DNA acting agents, of which cis-diamminedichloroplatinum (II) or cisplatin is an example. In 1965, Barnett Rosenberg, in an experiment to examine the effects of an electric field on the growth of bacteria, serendipitously discovered that cell division in *Escherichia coli* was inhibited by the production of cisplatin (Rosenberg *et al.* 1965). An electric field was conducted via platinum electrodes in an ammonium chloride-containing nutrient solution during the experiment; as a result, growth of the bacteria was altered. It was found subsequently that the electrophilic platinum coordination complex, cisplatin, was the active molecule responsible (Harder *et al.* 1970). The observation that cisplatin controlled the growth of bacteria, and the view of cancer as a disease of unregulated growth, suggested that cisplatin should be examined for antineoplastic activity. Further investigation suggested that cisplatin was indeed cytotoxic to cancer cells in culture and subsequent clinical trails established the drug as an important anticancer agent (Harder *et al.* 1970, Rosenberg 1985, Rosenberg *et al.* 1967, Loehrer *et al.* 1984). Although organ toxicity was evident in the patient, the drug's greatest impact has been in the combination chemotherapeutic treatment of testicular and ovarian cancers where the former frequently produces cures and in the latter substantially improves survival (Loehrer *et al.* 1984, Bosl *et al.* 1986). Additionally, the drug has proved to be beneficial in the treatment of head and neck, lung, esophageal, bladder and small cell lung cancer (Loehrer and Einhorn 1984).

1.7.1 Structure of Cisplatin

Cisplatin is a neutral, square planar molecule containing two chlorine leaving groups oriented in a *cis* configuration. It exists in six oxidation states--Pt (I to VI), where

Pt (IV) molecule, $[PtC^{l4}(NH_3)_2]$, the final product of the Rosenberg experiment, was believed to be responsible for inhibiting bacterial cell division (Rosenberg *et al.* 1965). It is now generally accepted that the *cis* conformation is critical for cisplatin activity since the *trans* configuration is a far less potent inhibitor of bacterial cell division and tumor growth (Cohen *et al.* 1979).

1.7.2 Mechanism of Cisplatin Action

Many studies have provided evidence that the cellular toxicity of cisplatin is primarily through its ability to associate with macromolecules within the cell, particularly by covalent interactions with the DNA molecule (Gale *et al.* 1973, Roberts and Thomson 1979, Poirier *et al.* 1982). Initial studies by Rosenberg and colleagues demonstrated that cisplatin induced filamentous growth in bacteria while inhibiting cell division, suggesting that cisplatin interferes with DNA replication with little affect on normal RNA and protein synthesis (Rosenberg *et al.* 1965). Subsequent biochemical studies in cultured cells indicated that cisplatin inhibits DNA synthesis (Harder *et al.* 1970). Furthermore, cell lines derived from patients with the DNA repair-deficient diseases Fanconi's anemia and xeroderma pigmentosum have been shown to be particularly sensitive to cisplatin (Fraval *et al.* 1978, Poll *et al.* 1984).

Covalent interactions of cisplatin with DNA are believed to occur as chloride ions in the cisplatin structure are displaced in an environment of low chloride concentrations to allow the formation of aquated species, which are the reactive forms of cisplatin (Hemminki *et al.* 1986). After intravenous administration of cisplatin, it is relatively less reactive in the extracellular space where the chloride concentration is ~100mM, but on crossing the plasma membrane, it is activated in the intracellular space where the chloride

concentration drops to ~3mM (Howe-Grant *et al.* 1980, Pinto *et al.* 1985). Activated cisplatin is a potent electrophile that will react readily with any nucleophile, including sulfhydryl groups on proteins and nucleophilic groups on nucleic acids.

1.7.3 Formation of cisplatin-DNA Adducts

Cisplatin attacks the N_7 position of guanine and adenine bases in the DNA strand to form three main types of DNA-cisplatin products, including the cisplatin-DNA monofunctional, and bifunctional adducts. The Pt-d(GpG), Pt-d(ApG) and the Pt-d(GpNpG) intrastrand crosslinks, the most predominant lesions produced, occur within a single strand of the DNA molecule and account for 90% of the cross-links produced (Chu1994, Plooy *et al.* 1985, Fichtinger-Schepman 1985, Blommaert *et al.* 1995). The other less frequently observed platinum-DNA adducts include the monoadducts and d(G2)Pt interstrand crosslinks which constitute less than 5% of the total platinum-DNA adducts but have also been implicated in cisplatin cytotoxicity (Plooy *et al.* 1985). X-ray diffraction of the cross-linked dinucleotide *cis*-$Pt(NH_3)_2$(d(pGpG)) reveals that the two guanines are completely destacked, and the deoxyribose sugar of the 5'-deoxyguanosine is in a C_3'-endo pucker (Rubin *et al.* 1983). Thus, the 1,2-intrastrand cisplatin cross-link can produce a severe local distortion in the DNA double helix that leads to unwinding and kinking in the DNA strand which prevents DNA transcription and replication and possibly inhibit the proper function DNA repair enzymes. This *cis* conformation has been hypothesized to be responsible for the cytotoxic effect of cisplatin, which has been supported by the observation that the *trans* isomer (*trans*-diamminedichloroplatinum [II]) exerts comparatively little antitumor activity (Sherman *et al.* 1985, Bellon *et al.* 1991).

1.8 Mechanisms of Cisplatin Resistance

The major limitation to the successful treatment of malignancies with platinum drugs is the emergence of drug-resistant tumor cells. Cellular resistance to these drugs is multifactorial. They include:

1.Decreased cisplatin accumulation
2.Enhanced detoxification mechanisms:
    inactivation by metallothionein association
    inactivation by glutathione conjugation
3.Enhanced cisplatin-DNA adduct repair
4.Tolerance to cisplatin-induced DNA damage

1.8.1 Decreased Intracellular Cisplatin Accumulation

Decreased cellular platinum accumulation is one resistance mechanism that is frequently found in *in vitro* models (Hromas *et. al.* 1987, Waud 1987, Richon *et al.* 1987, Teicher *et al.* 1987, Andrews *et al.* 1988, Kraker and Moore 1988, Kuppen *et al.* 1988, Kelland *et al.* 1992, Johnson *et al.* 1994). Since most cell lines exhibiting decreased cisplatin accumulation show no significant alterations in efflux, this mechanism of resistance is believed to occur as a result of decreased cellular drug uptake. Many cell lines made resistant to cisplatin show decreased accumulation of drug. Bungo and colleagues described a human non-small cell lung cancer line (PC9) with acquired resistance to cisplatin (7-fold) in which a 5-fold lower level of drug was accumulated when compared to the parental PC9 cells (Bungo *et al.* 1990). This general pattern has been also seen in the cisplatin-resistant derivative of L1210 and CHO cells. However, unlike the case with multi-drug resistant drugs where the molecular mechanism for resistance is characterized, little is known about the proteins involved in cisplatin transport. Several reports suggest that cisplatin enters cells through passive diffusion (Gale *et al.* 1973, Gately *et al.* 1993). Evidence for this has been provided by studies in

which cisplatin uptake was shown to be nonsaturable up to its solubility limit and not inhibited by structural analogues (Gale *et al.* 1973, Mann *et al.* 1990). Evidence from other laboratories, however, support an active membrane transporter present in cisplatin-resistant cell lines (Shionoya *et al.* 1986). Kawai and colleagues identified a 200-kDa membrane glycoprotein, distinct from the multidrug resistance-associated P-glycoprotein, in a derivative of murine lymphoma cell line that was 40-fold more resistant to cisplatin than parental cells (Kawai *et. al.* 1990). They showed that the level of expression correlated with reduced cisplatin accumulation. It should be noted that decreased drug accumulation in tumor cells resistant to cisplatin and/or alkylating agents is rarely accompanied by increased levels of the P-glycoprotein, which is a putative energy-dependent drug efflux pump involved in natural product resistance (Fojo *et al.* 1987, Kartner *et al.* 1983, Hospers *et al.* 1988). In other studies, cisplatin accumulation has been shown to be partially energy and sodium dependent, ouabain inhibitable, and affected by membrane potential and cAMP levels (Andrews *et al.* 1991, Andrews and Albright 1991, Mann *et al.* 1991).

The co-administration of cisplatin and calcium channel blockers in *in vitro* and *in vivo* tumor models have resulted in increased antitumor activity (Ikeda et al. 1987, O'Dwyer et al. 1992). There is support for calcium channel blockers altering the membrane potential of resistant cell lines and thereby restoring their sensitivity to cisplatin (Andrews and Albright 1991). To date, these hypotheses have not been tested in human subjects, and so their role in clinical situations is unclear. A few other agents have been shown to increase intracellular levels of cisplatin in tumor models including amphotericin B, dipyridamole, phorbol esters, through protein kinase C activation, and

calmodulin antagonists (Morikage *et al.* 1991, Gately and Howell 1993, Basu *et al.* 1990, Kikuchi *et al.* 1990). The clinical significance of these strategies remains to be elucidated.

1.8.2 Enhanced Cisplatin Detoxification through Metallothionein and Glutathione Conjugation

The ability of platinum-resistant cells to limit DNA damage may also occur by cellular inactivation through an association with the sulfhydryl-rich metallothionein (MT) proteins and the nonprotein thiol glutathione. Glutathione (GSH), the most abundant cellular sulfhydryl molecule, has shown to be involved in many cellular functions, including intracellular detoxification, metabolism of active drug metabolites and protection of oxidative stress (Meister and Anderson 1983). The manner in which GSH promotes cisplatin resistance is unknown; however, possibilities include 1) GSH-mediated transport alterations, 2) spontaneous or enzyme-catalyzed formation of inactive GSH-platinum conjugates, 3) protection of DNA from the formation of potentially cytotoxic platinum adducts, or 4) participation in DNA repair (Behrens *et al.* 1987). The product of the reaction of GSH with cisplatin is a GSH-Pt complex in which platinum is bound to GSH in a 1:2 ratio (Ishikawa *et al.* 1993). Although this reaction occurs relatively slowly *in vitro* which questions its relevance to cisplatin cytotoxicity, the formation of a GSH-Pt complex has been shown to occur in cultured cells treated with the drug (Ishikawa *et al.* 1993). In an extensive study on cross-resistance to many different anticancer agents in a panel of human ovarian cancer cell lines, only intracellular levels of GSH correlated with cross-resistance to these diverse anticancer agents and partial loss of resistance was associated with a marked decrease in glutathione levels (Hamaguchi *et al.*

1993). Several other investigators have reported an association between GSH levels and cisplatin resistance in a large number of unrelated cell lines selected for resistance *in vitro* and in tumor biopsies from patients with ovarian cancer (Godwin *et al.* 1992, Mistry *et al.* 1991, Meijer *et al.* 1992). Eastman demonstrated that GSH can inhibit the formation of cisplatin adducts in DNA at physiologic GSH concentrations and observed the formation of a GSH-platinum-deoxyguanosine cross-link (Behrens *et al.* 1987, Eastman 1987). Subsequently, inhibitors of GSH biosynthesis have been synthesized, the most notable of these being buthionine sulfoximine (BSO), which inhibits the activity of γ-glutamylcysteine synethetase that catalyzes the rate-limiting step in GSH biosynthesis and results in reduced levels of cellular glutathione (Meister 1988). When BSO was used to deplete the intracellular levels of GSH, toxicity to cisplatin-resistant cells was much greater than in the sensitive cells but no greater accumulation in the amount of DNA-cisplatin interstrand cross-links could be detected (Meijer *et al.* 1990). Moreover, in some resistant cell lines, increases in GSH levels were less than increases in cisplatin resistance. For example, a human colon cancer cell line that was 5-fold resistant to cisplatin showed only a 3-fold increase in GSH (Farm *et al.* 1990). Taken together, these studies suggest that while the induction of GSH may be an important mechanism of tolerance to higher concentration of cisplatin, clearly, other modes of resistance may be involved.

Other prevalent sulfhydryl peptides found within cells are the low-molecular-weight, sulfhydryl-rich proteins, metallothioneins (MTs), which are responsible for cellular homeostasis of zinc and copper and possibly heavy metal detoxification (Hamer 1986). Many cisplatin-resistant cell lines have elevated levels of GSH or MTs or both.

Several investigators have reported a direct correlation between the level of MT within the cell and their sensitivity to cisplatin (Kelly *et al.* 1988, Schilder *et al.* 1990, Andrews and Howell 1990). Cisplatin binds to MT stochiometrically *in vitro* where approximately 10 platinum molecules can be sequestered by one molecule of MT (Pattanaik *et al.* 1992). In addition, embryonic fibroblasts isolated from MT-null mice were shown to be hypersensitive to a variety of chemotherapeutic drugs including cisplatin (Kondo *et al.* 1995). Transfection of MT genes has been shown to confer low-level resistance to cisplatin (Kelley *et al.* 1988). By contrast, however, analysis of ovarian tumor samples from 48 patients before and after chemotherapy concluded that MT content was not a major determinant of tumor sensitivity to chemotherapy (Murphy *et al.* 1991). Schilder and colleagues likewise showed a poor correlation of MT content with cisplatin sensitivity in the OVCAR-5, -7, 8 and –10 cells lines obtained from untreated or platinum-refractory ovarian carcinoma patients (Schilder *et al.* 1990). In contrast, immunohistochemical analysis of 33 testicular germ cell tumors prior to chemotherapy did show a correlation of MT levels with response (Chin *et al.* 1993). The role of metallothioneins in mediating cisplatin resistance may thus be tumor type-specific. Currently, there is only limited clinical experience with these intracellular defense systems.

1.8.3 Alterations in DNA Repair Mechanisms

Failure to prevent the formation of cisplatin-DNA adducts by mechanisms such as those described previously leaves cells with no option but to repair or tolerate DNA damage in order to survive. Recently, several investigators provided evidence, which suggests that the repair of DNA adduct, produced by platinum agents may be a major

mechanism of cellular resistance (Zlatanova *et al.* 1998, Fink *et al.* 1998, Crul *et al.* 1997). Increased repair of platinum-DNA adducts has been shown to be associated with cisplatin resistance in many human ovarian cancer and murine leukemia cell lines with repair rates in resistant cells approximately twofold to fourfold higher relative to sensitive cells (Masuda *et al.* 1988, Lai *et al.* 1988, Masuda *et al.* 1990, Parker *et al.* 1991, Eastman 1988, Koberle *et al.* 1997). Repair of cisplatin-induced DNA damage has been reported to occur both within the whole cellular genome or preferentially in frequently transcribed genes of cisplatin-resistant cells (Eastman 1988, Masuda *et al.* 1988, Masuda *et al.* 1990, Lai *et al.* 1988, Parker *et al.* 1991, Bohr 1991). Jones and colleagues demonstrated that cisplatin-DNA adducts were removed more efficiently in transcribed regions of the dihydrofolate reductase and *c-myc* genes than in the non-coding regions of the *fos* gene (Jones *et al.* 1991, Petersen *et al.* 1996). This suggests that more dramatic repair rate differences may exist in specific genomic regions of sensitive and resistant cells. However, the significance of increased repair in actively transcribed genes to cisplatin resistance remains to be determined.

The spectrum of cellular proteins and factors that are involved in the repair of DNA damaged by cisplatin includes DNA-cisplatin recognition factors, proteins involved in the mismatched repair and the nucleotide excision repair pathways (Fink *et al.* 1998, Zlatanova *et al.* 1998, Scanlon et al. 1989a, Scanlon et al. 1989b). This suggests that cisplatin-damaged DNA, that is, the presence of inter- and intra-strand breaks within the DNA architecture may be restored by many of these pathways. Using a gel mobility shift assay, Chu and Chang identified a protein that can specifically recognize cisplatin-modified DNA (Chu and Chang 1988, Chu and Cheng 1990). In addition, several groups

have characterized proteins of 80-130 kDa that specifically bind to DNA damaged by platinum compounds of *cis* but not *trans* stereochemistry (Bruhn *et al.* 1992, Pil and Lipard 1992, Toney 1989). Interestingly, some of these cisplatin-DNA damage-recognition proteins share homology with the DNA binding domain of the high mobility group (HMG) proteins. However, they were observed to be present in similar amounts in cisplatin-sensitive and –resistant cell lines (Bruhn *et al.* 1992). In addition to the identification of potential recognition factors in cisplatin-resistant cells, the removal and repair of platinum-DNA damage is believed to occur by the nucleotide excision repair (NER) pathway (Sancar 1995, Li *et al.* 1997). Evidence that DNA repair may be important in the clinical response to cisplatin comes from several lines of investigation. Increased expression of a few putative DNA repair genes of the NER pathway has been suggested to contribute to the enhanced repair capacity observed in some cisplatin-resistant cells. For example, Dobholkar and colleagues have shown that the RNA expression level of the ERCC1 gene in fresh human ovarian carcinoma tissue correlated with response to platinum-based therapy (Dabholkar *et al.* 1992). In fact, elevated XPA and ERCC1 mRNA levels were observed in ovarian cancer tissues from patients who were clinically refractory to platinum drugs (Dabholkar 1994). However, when the ERCC1 cDNA was transfected into CHO cells, the resulting transfectants contained elevated levels of ERCC1 protein, but exhibited hypersensitivity to cisplatin relative to the control cell lines (Bramson and Panasci 1993). Increased levels of another DNA repair protein, XPE, which recognizes a variety of DNA lesions including platinum-DNA adducts, were observed in tumor cell lines selected for cisplatin resistance (Chu and Chang 1990, Chu and Chang 1988). Despite the high specificity these proteins exhibit

toward platinum-DNA damage, their role in cisplatin sensitivity and resistance is unclear. Cellular regulation of their amounts and activities may elicit competition among one another for damaged DNA, where their ultimate role may be in providing signals for repair events, the initiation of apoptosis or both which may ultimately determine the fate of the cell.

1.8.4 Alterations in Apoptotic Response

Investigators have observed that higher levels of platinum-damaged DNA are often present in cisplatin-resistant cells relative to sensitive cells at equitoxic doses of drug (Blommaert *et al.* 1998). It is unclear how this phenotype occurs but two mechanisms of tolerance that have been proposed are enhanced replicative bypass and alterations or defects in the apoptotic response. Enhanced replicative bypass, which allows a cell to continue DNA synthesis past a lesion, could enable a cell to successfully complete DNA synthesis and arrest in the G2 phase of the cell cycle to allow postreplication repair processes to correct the DNA damage before mitosis (Kaufmann, 1989). This process has been demonstrated to occur in two cisplatin-resistant human ovarian cancer cell lines where DNA polymerase B can efficiently bypass a single d(GpG)Pt adduct (Hoffman *et al.* 1995, Mamenta 1994). Alterations or defects in apoptosis may influence the sensitivity of cells to platinum agents since most of the widely used chemotherapeutic drugs utilize apoptosis as the common death pathway (Segal-Bendirdjian and Jacquemin-Sablon 1996, Bold *et al.* 1997). It can be envisioned that platinum-DNA damage recognition is the first step in this process, and a signaling pathway is then activated to transmit this message to the apoptotic machinery. Mutation

or ablation of one or more components of this system could enable cells to continue to survive at higher drug concentrations.

Features common in cells undergoing apoptosis include chromatin condensation, DNA fragmentation, and cellular membrane blebbing leading to cell shrinkage. Some cisplatin-resistant cells do not exhibit any internucleosomal DNA fragmentation or morphological changes such as membrane blebbing, nuclear condensation, typical of apoptosis when exposed to toxic concentrations of cisplatin (Segal-Bendirdjian and Jacquemin-Sablon 1996). However, when some cisplatin-resistant cells are exposed to chemotherapeutic agents to which it is sensitive, DNA fragmentation and morphological features of apoptsis are evident, suggesting that some functional pathway of apoptosis is preserved even in the resistant cells. Currently, the specific apoptotic pathway that mediates cisplatin cytoxicity is unknown.

1.9 Thesis Overview

An understanding of the mechanisms through which chemotherapy acts, and by which genetic alterations can result in resistance to chemotherapy can open new doors to novel paradigms of treatment in molecular, genetic, and biologic therapy of human malignancies. This thesis is an attempt to characterize the cellular and genetic alterations in two kinds of human melanoma cell lines, differing in their sensitivity to cisplatin. Chapter 2 is a description of the cellular changes that markedly distinguishe the retrovirally-derived cisplatin-resistant cell lines from the cisplatin-sensitive, parental WM35 cells. These changes include their differential activation of the stress protein, stress-activated protein kinase (SAPK), their growth properties and morphology in a three-dimensional spheroid context, their differential response to cisplatin as determined

by their cell cycle changes, among others. Furthermore, chapter 2 clearly establishes the role of the melanocyte-specific, tyrosinase related-protein 2 (Trp-2) in cisplatin-resistance, in these melanoma cells. Increased expression of Trp-2 not only correlates with the sensitivity to cisplatin in our cell lines, but exogenous expression of Trp-2 confers resistance to cisplatin in the parental, cisplatin-sensitive, WM35 cells.

CHAPTER 2

Cellular Characteristics and Genetic Alteration of Cisplatin-Resistant Human Melanoma Cells

2.1 ABSTRACT

The incidence of human melanoma is rapidly increasing in North America. Resistance of melanoma cells to both chemotherapy and radiotherapy is one of the main obstacles encountered in the successful treatment of patients with this disease. The alteration of genetic events and cellular mechanisms that accumulate within cancerous cells which ultimately results in this drug resistant phenotype in patients with malignancy are important changes that warrant extensive study. By utilizing the approach of retroviral insertional mutatgenesis, we were able to convert an early-stage and relatively cisplatin sensitive human melanoma cell line, WM35 to resistant variants of this drug. In comparing the cellular changes that are associated with cisplatin resistance, we have identified a pattern of cross-resistance to other chemotherapeutic agents, the absence of G_2/M arrest following cisplatin exposure, a constitutively higher basal level of stress-activated-protein kinase (SAPK) activity and an abrogation of the apoptotic pathways in the cisplatin-resistant cells. A PCR-based subtractive hybridization method utilizing cDNAs extracted from both WM35 and one of the cisplatin-resistant clones, clone E, detected an upregulation of the melanocyte-specific gene, *tyrosinase related protein-2 (Trp-2)*, in all the cisplatin-resistant cell lines. Subsequently, it was demonstrated that Trp-2 expression directly correlates with sensitivity to cisplatin, when a panel of melanoma cell lines was examined. Stable transfection of the full length Trp-2 cDNA into WM35 cells resulted in a decreased sensitivity to cisplatin. These data suggests that an overexpression of Trp-2 can confer at least partial resistance to cisplatin in melanoma cell lines, perhaps by inhibiting the signals required for the induction of apoptosis. These

findings implicate Trp-2 as an important mediator of intrinsic drug resistance in human melanoma and as such, represents what may be a unique lineage-specific mechanism of drug resistance. The targeting of Trp-2 may serve as a novel chemo-sensitization strategy for effective clinical management of human malignant melanoma.

2.2 INTRODUCTION

Although efforts toward utilizing new and novel combinations of chemotherapeutics and cytokines by exploiting the immunologic nature of human malignant melanoma are ongoing, clearly our ability to control the growth and progression of even a small fraction of most early and certainly all late stages of melanoma are failing. At best, the response to chemotherapy in these patients is only 10-20% and treatment rarely prolongs survival, although it can result in an improvement in symptom control. Central to the problem of treating human malignant melanoma and others including pancreatic carcinoma, non-small cell lung cancer and colorectal cancer, is their inherent ability to be intrinsically resistant to nearly all chemotherapeutic agents (Hickson and Carmichael 1997). However, even if the tumor can be "eradicated " with the compendium of anti-cancer drugs, most often, a selected population of resistant cancer cells will arise in due time to repopulate a tumor that is now substantially resistant to multiple chemotherapeutic agents. The amazing potential of tumor cells to adapt to cytotoxic substances continues to be an outstanding challenge for chemotherapy.

Traditionally, anti-cancer drugs have been classified into several categories, according to their pharmacological mechanism of action, as DNA-alkylating agents, topoisomerase II inhibitors, antimetabolites, and others. Cisplatin is a DNA-acting agent where its toxicity is through the direct formation of a spectrum of cisplatin-DNA adducts such as 1,2-intrastrand d(ApG), d(GpG), interstrand and monofunctional cross links, thereby compromising essential cellular functions such as transcription and replication. Since its serendipitous discovery by Rosenberg and colleagues, cisplatin has become an important chemotherapeutic agent for the treatment of a variety of human malignancies,

including head and neck, testicular, cervical cancers and currently malignant melanoma (Coates. 1992, Rosebenger *et al.* 1965).

Cells respond to the presence of cisplatin-DNA adducts by undergoing cell cycle arrest, through the up-regulation of cell cycle protein inhibitors, such as p21, p27 and p53 which prevent the association of cyclin-dependent kinases (CDKs) with partner cyclins required for cell cycle progression. Arrest may be followed by the activation of pathways responsible for DNA repair in the cell so that damaged DNA is not erroneously replicated, or if the damage is too severe and cannot be tolerated, apoptosis is induced. Whether repair of damaged DNA or the activation of apoptotic machinary is to transpire, signaling events must occur to mediate this outcome. The specific signaling cascades activated as part of the cisplatin-induced injury response are poorly understood and most likely involve many of the similar pathways shared by other stress inducing substances. Recent studies have demonstrated that injury response involves activation of the stress-activated protein kinase (SAPK) (also known as JNK) (Kharbanda *et al.* 1995). SAPK is a serine/threoine kinase that is part of the mitogen-activated protein kinase pathway, which contains, in order, PAKp65, mitogen-activated protein kinase kinase 1, SEK1 (also called MKK4), and SAPK (Sanchez *et al.* 1994, Derijard *et al.* 1995, Manser *et al.* 1994). SAPK phosphorylates the three nuclear transcription factors c-Jun, activator of transcription factor 2, and Elk-1 thereby stimulating their transcriptional activities (Hibi *et al.* 1993, Gupta *et al.* 1995, Cavigelli *et al.* 1995). Cisplatin-induced activation of the SAPK pathway is believed to constitute an early step in the generation of signals that coordinate the full injury response, including the initiation of DNA repair and induction of apoptosis.

Whatever signals that mediate the induction of apoptosis, it has been generally accepted that apoptosis proceeds in a stereotypic form that has the characteristics of chromatin condensation and fragmentation, cell shrinkage, membrane blebbing, and the repackaging of membrane-enclosed vesicles (apoptotic bodies). The cellular effectors of apoptosis are numerous. A common, if not universal feature of apoptosis is the activation of one or more members of a family of caspases such as CPP32β (Yamma/apopain/caspase 3) whose activation results in cleavage of PARP (*Mr* 116,000) into a *Mr* 85,000 COOH-terminal fragment and a *Mr* 24,000 NH_2-terminal fragment (Nicholson *et al.* 1995, Martin and Green 1995, Lazebnik *et al.* 1994, Kaufmann *et al.* 1993). Mammalian caspases have conserved substrate binding and catalysis: they cleave their substrates after aspartic acid and are therefore considered as cysteine caspases (Ellis *et al.* 1991, Yuan *et al.* 1993, Alnemri *et al.* 1996). Other caspases, whose activation results in the cleavage of PARP include caspase-6 (Mch2), caspase-7 (Mch3/ICE-LAP3/CMH-1), caspase-8 (FLICE/MACH/Mch5) and caspase-9 (ICE-LAP6/Mch6) (Martins and Earnshaw 1997). Inhibiting the cleavage of PARP, by preventing the activation of caspases, has been shown to confer resistance to many chemotherapeutic agents, including cisplatin (Liu *et al.* 1998, Fuchs *et al.* 1997).

Although numerous pathways have been elucidated that may lead to the induction of apoptosis in cells and should almost guarantee the effectiveness of chemotherapeutic agents in controlling malignant growth, resistance to drugs often occurs. Cellular mechanism of resistance may involve the expression of a cell surface pump which has the propensity to extrude or decrease the entry of active drugs into the cell, the up-regulation of cellular thiols, metallothionein and glutathione, an increase in the repair of cisplatin-

DNA adducts, and/or the presence of defective signaling pathways responsible for the induction of apoptosis. Although many of these mechanisms have been shown to be important in *in vitro* models, and occasionally exist in *in vivo* models, they have not been documented in clinical situations, nor have they been proven to play a role in the clinical management of cisplatin administration. Perhaps one of the problems associated with this type of approach in studying resistance is that resistant cell lines employed in these studies are often established through a series of incremental increases in drug concentrations to levels sometimes greater than 100X the clinically relevant dose. Although there are obvious advantages to this method of study, such as the capability to develop cell lines with varying degrees of resistance relative to the parental cells, a major criticism may be that these models do not adequately reflect the complexity of drug resistance that is observed clinically. In pursuit of uncovering mechanisms of cisplatin resistance that may be clinically important, we were successful in establishing cisplatin-resistant variants of an early, curable, cisplatin-sensitive melanoma cell line, WM35 that are approximately 2-3 fold more resistant to cisplatin (Lu *et al.* 1994). Unlike the common method of increasing cisplatin incubation in selecting resistant variants, we utilized the unique strategy of retroviral insertional mutagenesis, which has the advantage of allowing us to tag and study the genetic events responsible. Northern blot analysis of some of these cisplatin-resistant variants revealed that they over-express a mRNA transcript message of 3.8kb in size (Lu *et al.* 1994). To study the molecular mechanism that is responsible for cisplatin resistance in our system, we have focused our study on the analysis of the characteristic features that differentiate the cisplatin-sensitive and cisplatin-resistant cells. We demonstrate that the cisplatin-resistance cells have numerous

characteristics that are altered, including cross resistance to other chemotherapeutic agents, constitutively high level of SAPK activity, absence of G_2/M phase cell cycle arrest and abrogation of apoptotic pathways. Furthermore, using a subtractive hybridization technique, we detected over-expression of the melanocyte-specific protein, Trp-2 in all the cisplatin-resistant cells. Enforced Trp-2 over-expression in cisplatin-sensitive cells confers resistance to cisplatin. Our data suggests that Trp-2 is at least partially responsible for cisplatin resistance through the altered regulation of downstream events which ultimately lead to a short circuiting of the signals required for apoptosis to occur in the presence of drug challenge. Furthermore, since the only known function of Trp-2 is its involvement in the synthesis of melanin polymer raises the interesting hypothesis that perhaps a mechanism of resistance, specific to melanoma may be involved in the intrinsic resistance of this malignancy.

2.3 RESULTS

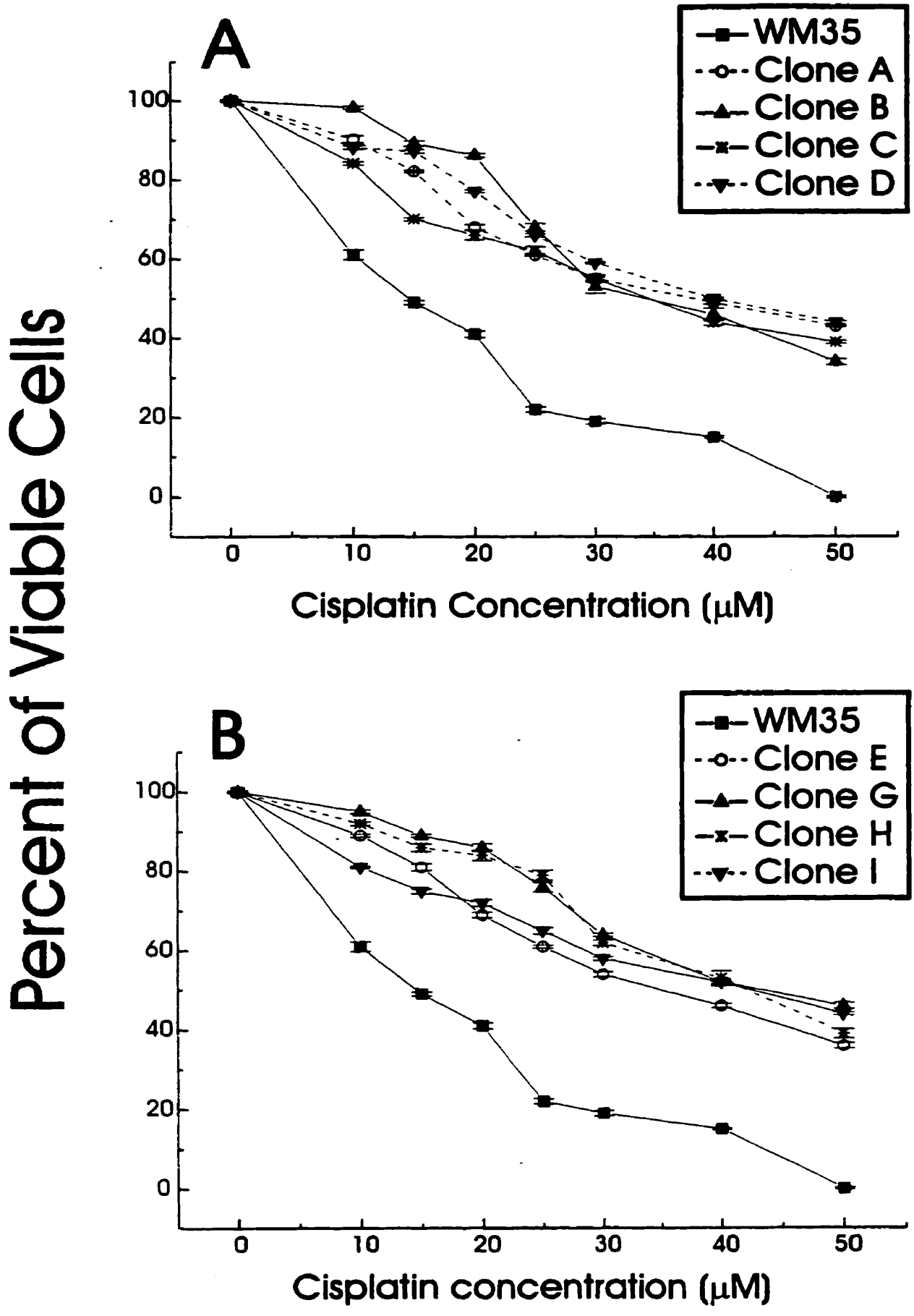
2.3.1 Cisplatin-resistant cells are cross-resistant to carboplatin and methotrexate, but not taxol.

It has previously been determined that clonal cell lines, clones E and G, derived through the strategy of retroviral insertional mutagenesis, formed a greater number of colonies after cisplatin treatment when compared to the parental WM35 cell line (Lu *et al.* 1995). The results of this colony-forming experiment demonstrated that at least two of the retrovirally-derived clones, E and G, displayed a 2-3 fold decrease in sensitivity to the cytotoxic effects of cisplatin at different drug concentrations. To determine whether

this observation is consistently observed in all cell lines derived through retroviral infection. cisplatin-resistant clones A to I, as well as WM35 cells were cultured in 24-well plates, in quadruplicate and treated with 10-50μM of cisplatin for 24 hours. Four hours prior to cell harvesting, a mixture of PMS and MTS reagents were added to the cell culture, and cell viability was determined at 490nm using an ELIZA plate-reader. All eight cisplatin-resistant variants, clones A to I, display decreased sensitivity to the cytotoxic effects of cisplatin under these conditions (Figure 2.1A and 2.1B). In fact, their LD_{50} concentrations, that is the concentration at which only 50% of the cells are still viable, were approximately 2-3 times higher than that of WM35 (Table 2.1).

Intrinsic and acquired resistance to cisplatin occurs in patients as well as in cell culture systems. A striking feature of many cisplatin-resistant cell lines is their cross-resistance to many different agents, including 5-fluorouracil, methotrexate, melphalan, amsacrine, 6-mercaptopurine, bleomycin, taxol, adrimycin, carboplatin, mitoxantrone and UV irradiation (Hamaguchi *et al.* 1993, Singh *et al.* 1995, Parekh and Simpkins 1996). To determine the cross-resistance pattern in our cell lines, we examined their sensitivity to carboplatin, methotrexate and taxol. Carboplatin is classified as a second-generation platinated compound with a similar mechanism of action on DNA as cisplatin. Randomized trials have determined that carboplatin is equivalent to cisplatin in therapeutic efficacy in many types of tumors but was associated with a decreased gastrointestinal, renal and neurologic toxicity (Lokich and Anderson 1998). The efficacy of methotrexate results from its facile uptake by cells, rapid polyglutamylation and virtually stoichiometric inhibition of dihydrofolate reductase (DHFR), a key enzyme in cell replication (Huennekens 1994). Taxol is a new anticancer agent with a novel

Figure 2.1 Viability of WM35 and clones A-I cells following cisplatin treatment. $8X10^3$ cells were cultured in 24-well plates, in quadruplicate for at least 12 hours prior to the addition of cisplatin in a total volume of 200µl 5% FBS-supplemented media and incubated at 37°C, 5%CO_2. Cisplatin was added in a total volume of 100µl. and incubated for 24 hours. Four hours prior to cell harvest, an 20µl mixture of PMS/MTS (100µl/2ml) was added to the drug-containing media. Cell viability was determined at 490nm, using an ELIZA plate-reader. Mean and standard deviation error bars were determined using Origin 4.1.

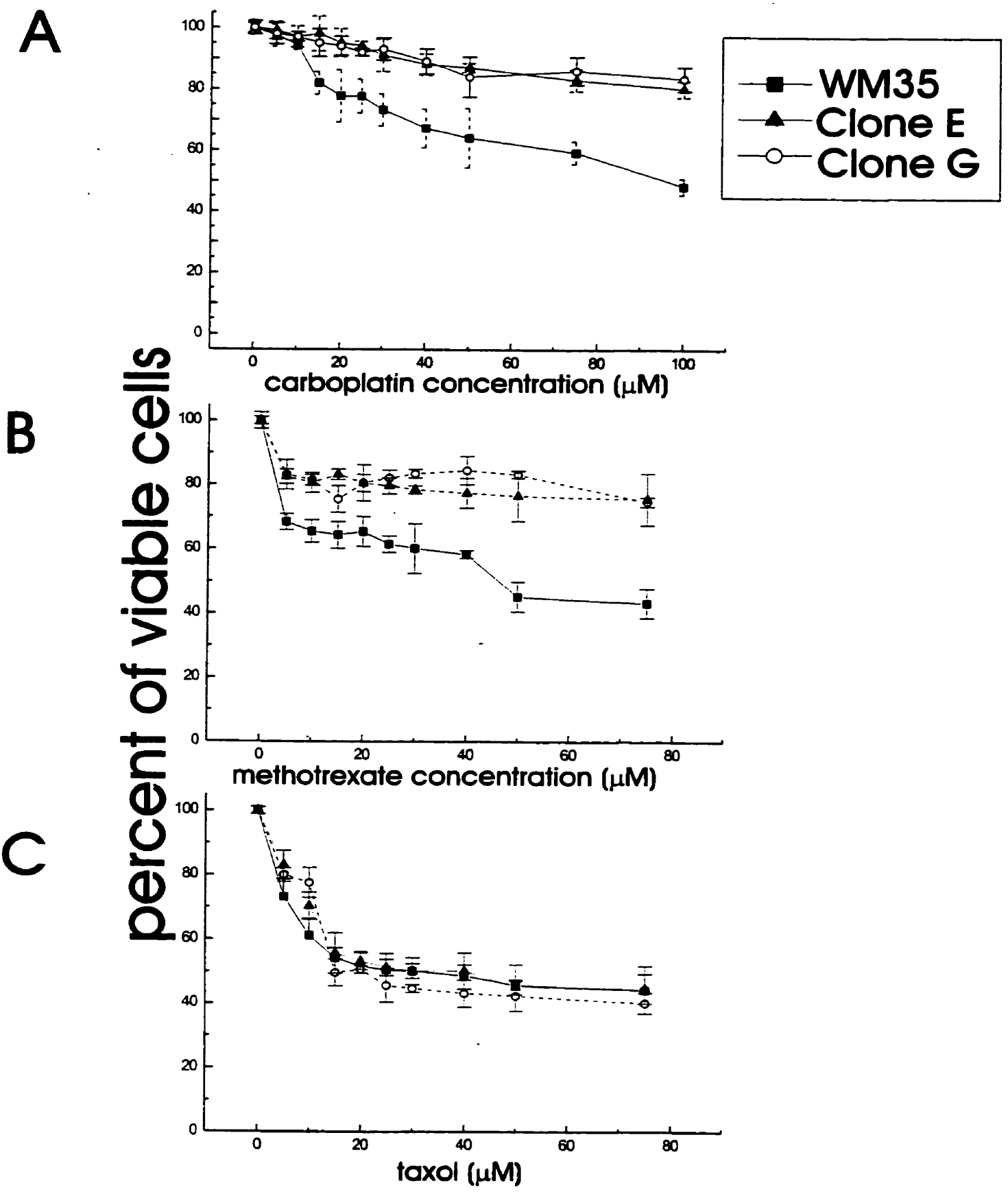

A
WM35
Clone A
Clone B
Clone C
Clone D
Percent of Viable Cells
100
80
60
40
20
0
0
10
20
30
40
50
Cisplatin Concentration (μM)
B
WM35
Clone E
Clone G
Clone H
Clone I
100
80
60
40
20
0
0
10
20
30
40
50
Cisplatin concentration (μM)

Cell Lines	[LD_{50}](μM)
WM35	15±1.2
Clone A	40±0.91
Clone B	36±3.4
Clone C	37±1.3
Clone D	43±0.62
Clone E	40±1.8
Clone G	45±0.8
Clone H	42±0.67
Clone I	44±0.89

Table 2.1 LD_{50} cisplatin concentrations of WM35 and cisplatin-resistant variants, clones A-I. Concentrations were determined from Figure 2.1 as described in results.

mechanism of action. It promotes polymerization of tubulin dimers to form microtubules and stabilizes them by preventing depolymerization and disassembly (Spencer and Faulds 1994). In examining the effects of these chemotherapeutic drugs on our cell lines, we treated WM35 and resistant variants at different concentration ofcarboplatin, taxol, and methotrexate for 24 hours, and measured cell viability with the MTS assay as described earlier. The pattern of sensitivity toward carboplatin and methotrexate in WM35 clones E and G cells is similar to the effects of cisplatin on these three cell lines in which clones E and G are more resistant to both chemotherapeutic agents than WM35 (Figure 2.2A, 2.2B). Although WM35 cells are more sensitive to the effects of carboplatin and methotrexate than clone E and G cells, more than 50% of WM35 cells are viable at concentrations up to 100μM of carboplatin; this is in contrast to 50% of cells viable at 15μM of cisplatin. Similarly, both resistant variants, clones E and G, are more resistant to carboplatin than to cisplatin, where approximately 90% of the cells are viable at concentrations up to 80μM of carboplatin. This is not surprising since carboplatin has been reported to be less toxic on cell lines and exerts less toxicity to organs in patients (Lokich and Anderson 1998). With methotrexate, the LD_{50} methotrexate concentration for WM35 is approximately 44μM, which is higher than the concentration of cisplatin killing 50% of WM35 cells. The differential sensitivity toward carboplatin and methotrexate amongst the three cell lines was not recapitulated on taxol. All three cell lines were equally sensitive to taxol at the various concentrations examined. where 50% of the cells remain viable at approximately 22μM of taxol in all cell lines (Figure 2.2C). These observations obtained using the MTS assay indicate that there is

Figure 2.2 Viability of WM35 and clones E and G cells following carboplatin (A), methotrexate (B) and taxol (C) treatment. $8X10^3$ cells were cultured in 24-well plates, in quadruplicate, for at least 12 hours prior to the addition of above drugs in a total volume of 200µl 5% FBS-supplemented media and incubated at 37°C, 5%CO_2. Drugs were added to the media and incubated for 24 hours. A 20µl mixture of MTS/PMS was added to the cells four hours prior to harvest. Cell viability was determined at 490nm, using an ELIZA plate-reader. Mean and standard deviation error bars were determined using Origin 4.1.


A
B
C
WM35
Clone E
Clone G
percent of viable cells
carboplatin concentration (μM)
methotrexate concentration (μM)
taxol (μM)
0
20
40
60
80
100

cross-resistance in our retrovirally-derived cisplatin-resistant cells to other chemotherapeutic drugs, including carboplatin and methotrexate, but not taxol.

2.3.2 Cisplatin-resistant cells form tight three-dimensional spheroids.

In contrast to monolayer cell cultures, three-dimensional, multicellular spheroids display a reduced growth fraction and resistance to a wide variety of cytotoxic agents (Sutherland 1988, Olive *et al.* 1994). St. Croix and colleagues demonstrated a causal relationship between the ability of cells to form compact spheroids and their resistance to chemotherapeutic agents (St. Croix *et al.*1996). In studies concerning the mechanism of drug resistance, many resistant cell lines were established by selecting for variants of drug sensitive cells following exposure to incremental increase in drug concentration. These drug resistant lines often display similar growth kinetics to their sensitive, parental counterparts when grown in monolayer culture. However, when cultured as three-dimensional or multicellular spheroids using a liquid overlay technique, it has been demonstrated that numerous drug resistant cell lines form tight multicellular spheroids in contrast to the loose three-dimensional spheroid form by their parental counterparts (Graham *et al.* 1994). Since our cisplatin-resistant variants were established using the strategy of retroviral insertion and not through the incubation with higher drug concentrations, it was important to address whether these variants would also demonstrate an ability to form compact multicellular aggregates in three-dimensional cultures. Previously it has been shown that WM35 and clones E and G proliferate at similar rates when grown in monolayer culture (Lu *et al.* 1995). However, when $1X10^5$ cells were cultured using a liquid overlay technique into multicellular spheroids, these three cell lines displayed different growth kinetics, as determined by trypan blue exclusion analysis

(Figure 2.3). WM35 cells grown as multicellular spheroids proliferate at a linear rate, increasing from 20,000 cells to approximately 100,000 cells within 10 days. Morphologically,the appearance of the multicellular spheroids produced by WM35 cells was loose in architecture, with several layers of cells populating the outer edge of the spheroid (Figure 2.4). What began as one translucent layer of WM35 cells initially on day 2, had accumulated to a larger and denser multicellular three-dimensional spheroid by day 10. In contrast, the proliferative rates of clones E and G were much slower than WM35. Under the same growth conditions, both cisplatin-resistant variants displayed an increased intercellular adhesion (or compaction) which was evident visually by day 2 with a decrease in proliferation rate as shown in Figure 2.3. It appears that these resistant variants have virtually growth arrested during this period of 10 days. The spheroids that are formed with WM35 cells are irregular in overall shape, which is in marked contrast to those formed with clones E and G where they are almost perfectly spherical. The differences in the compaction and proliferative rates of WM35 and clones E and G in multicellular culture marks another cellular alteration between these cell lines.

2.3.3 SAPK activity is moderately induced in cisplatin-resistant cells.

Recently, the activity and function of a cellular protein, SAPK (stress-activated protein kinase) or JNK has been of great interest since several recent publications have provided strong evidence that SAPK activation may be of importance in the response to cellular stress, including cisplatin treatment (Zanke *et al.* 1996, Iordanov *et al.* 1998, Wang *et al.* 1998). To determine the activity of SAPK in our cell lines and to ascertain whether the level of activation of this protein may correlate with the degree of sensitivity to cisplatin, the activity of SAPK was determined following incubation with cisplatin by

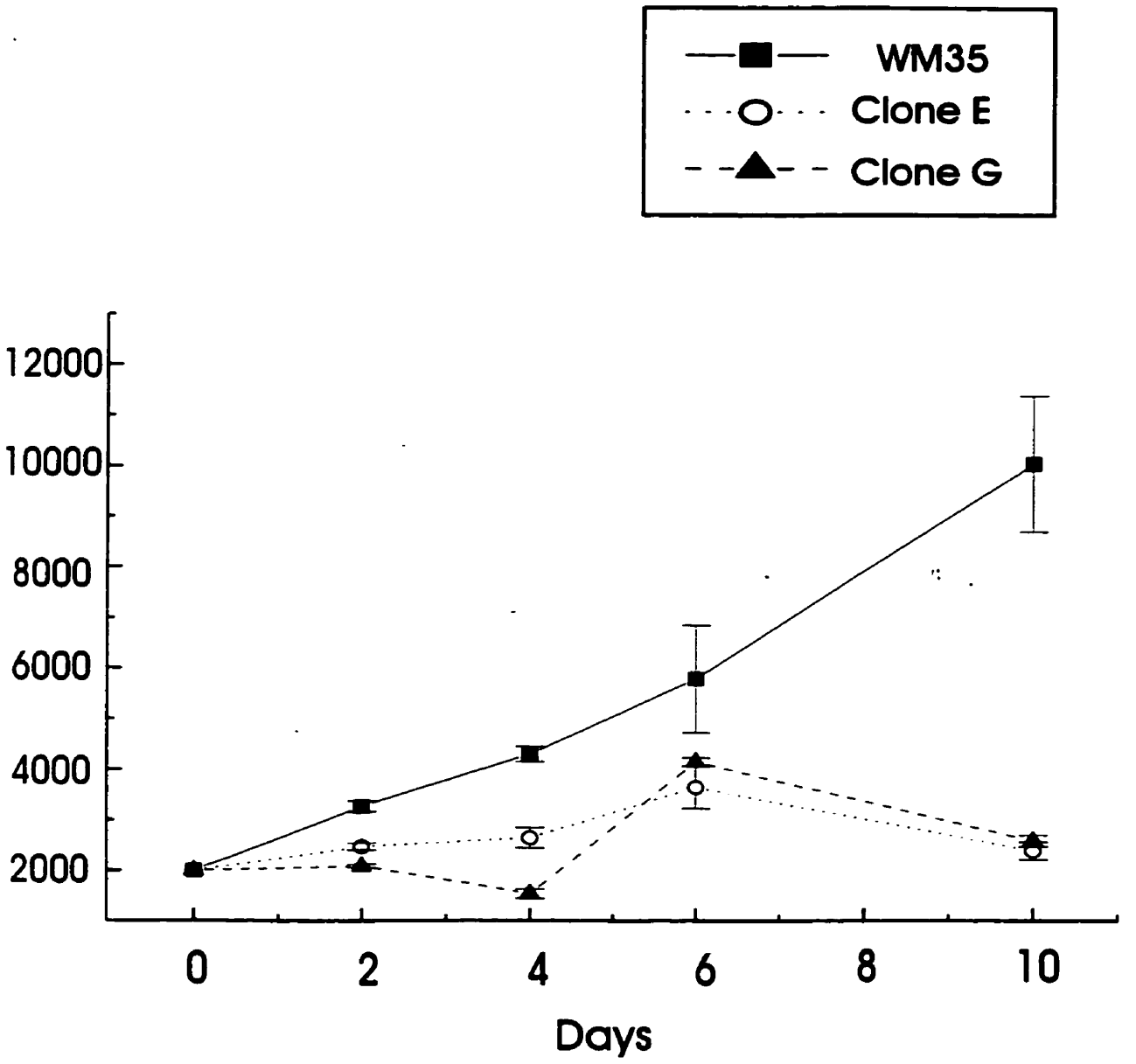
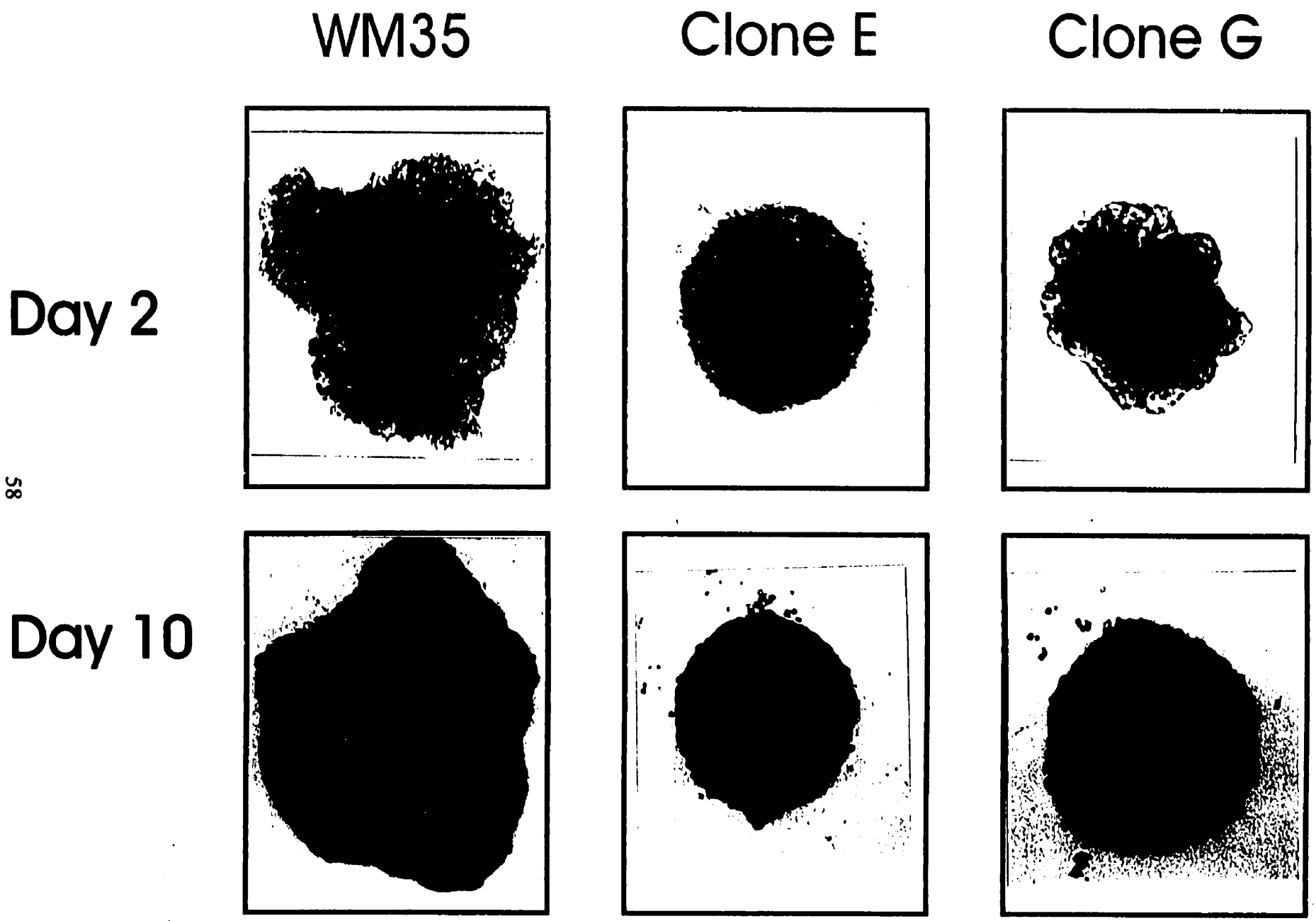


Figure 2.3 Growth kinetics of WM35, clones E and G cells in three-dimensional culture. $2X10^3$ cells were plated using a liquid overlay technique, in triplicates. On the day specified above, the number of viable cells were determined by using typan blue. Statistical analysis was performed using Origin 4.1.

Figure 2.4 Morphology of WM35 and clones E and G cells in three-dimensional spheroid culture. Multicellular aggregates (spheroids) were established by culturing $1X10^5$ cells in 24-well plates previously coated with 1% agarose containing media at 37°C, 5% CO_2. Cisplatin-resistant clones, E and G formed tight, compact and circular aggregates while WM35 cells formed loose, irregular-shaped spheroids.


WM35
Clone E
Clone G
Day 2
Day 10

Determining the amount of GST-c-jun phosphorylation. In our preliminary experiments, we have detected SAPK activation in WM35 when the cells were treated at 15μM cisplatin for 15 minutes, with maximal level of activity detected at 1.5 hour (data not shown). Consequently, WM35 and cisplatin-resistant variants were treated under the same condition as outlined above and SAPK activity was shown to be up-regulated in all three cell lines following cisplatin treatment (Figure 2.5A). The activity of SAPK in WM35 was 10 times greater than its basal levels, compared with a 3- and 2-fold increase in SAPK activity for clones E and G, respectively, as measured by densitometry (Figure 2.5C). SAPK activity may also be induced following UV exposure in WM35 cells with 9mJ/m^2 of UVB for 15 min. Interestingly, although the basal activity of SAPK is minimal in WM35 cells, its activity in both resistant variants is at least 3 fold higher constitutively. In summary this experiment concludes that the activation of SAPK was detectable in both cisplatin-sensitive and –resistant cells. However, the level of SAPK activation was greater in the drug sensitive, WM35 cell line.

2.3.4 Cisplatin-induced apoptosis is altered in cisplatin-resistant cells.

Many chemotherapeutic drugs with different mechanisms of actions ultimately tapped into pathways leading to apoptosis whereby these events are often mediated by the execution of precise checkpoints in the cell cycle. To address the issue of whether an abrogation of apoptotic pathways maybe involved in the acquisition of cisplatin resistance in variants of WM35, cell cycle checkpoints following cisplatin treatment were examined. WM35, clones E and G cells were treated continuously with 15μM of cisplatin and the

Figure 2.5 Induction of SAPK activity in WM35, clones E and G cell following 15μM cisplatin treatment. **(A)** SAPK activity is greatly induced in WM35 cells following cisplatin treatment. (-) denotes untreated cells. (+) denotes cells treated with 15μM of cisplatin for 1 hour. Basal SAPK activity is greater in both clones E and G. **(B)** Amido Black staining of Western Blot demonstrating the integrity of protein used in the experiment. **(C)** Densitometry of SAPK activity determined in all cell lines above. Induction of SAPK activity in WM35 cells is approximately 5-fold, in comparison to 2- and 3-fold in clones G and E respectively.

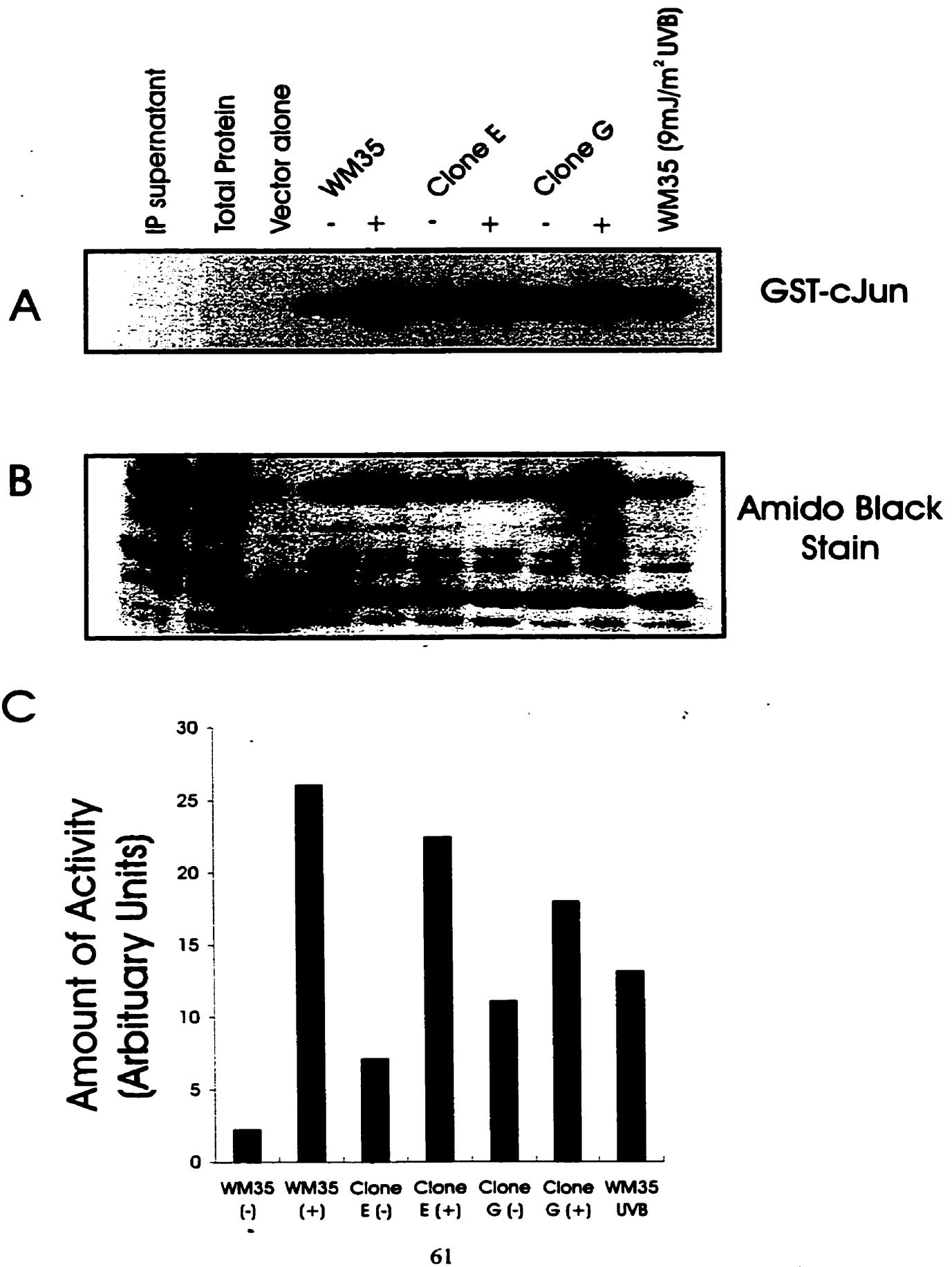
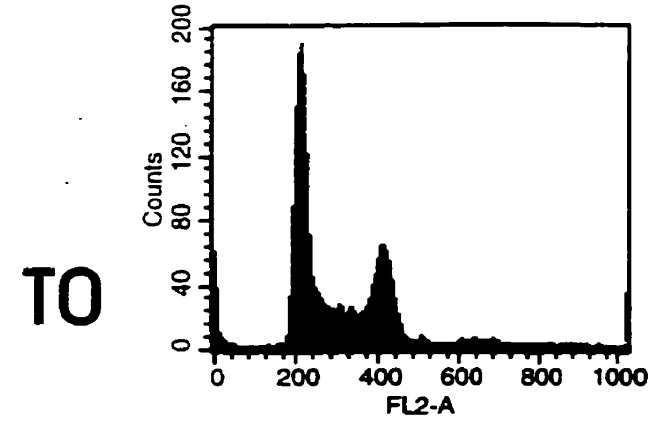
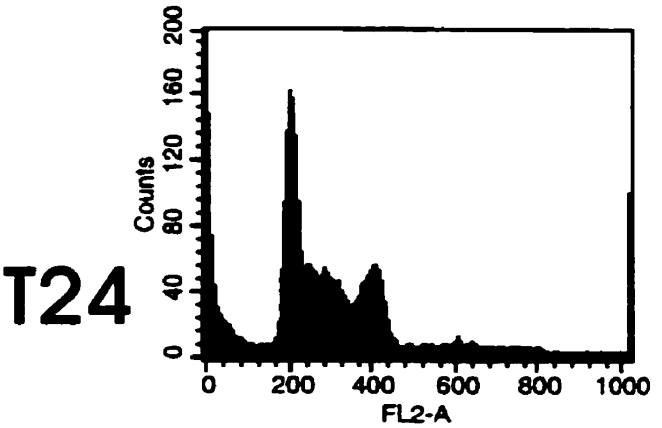
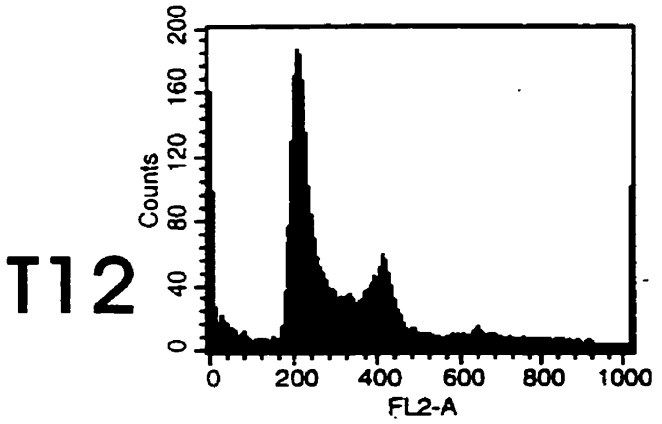

IP supernatant
Total Protein
Vector alone
WM35
Clone E
Clone G
WM35 (9mJ/m² UVB)
- + - + - +
A
GST-cJun
B
Amido Black Stain
C
Amount of Activity (Arbituary Units)
30
25
20
15
10
5
0
WM35 (-)
WM35 (+)
Clone E (-)
Clone E (+)
Clone G (-)
Clone G (+)
WM35 UVB

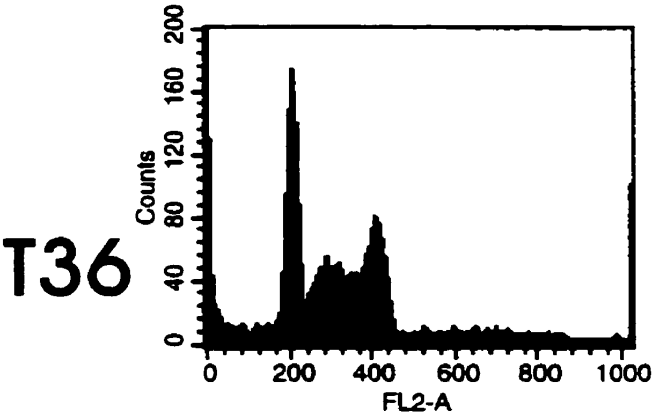
Figure 2.6 Cell cycle profile of WM35 cells treated continuously with 15μM of cisplatin for 48 hours. WM35 cells were cultured to semi-confluency for at least 12 hours prior to exposure to 15μM of cisplatin. At each of the time points indicated, adherent cells were washed, harvested, and stained with propidium iodide in preparation for Fluorescent Cell Sorting Analysis, as described in Materials and Methods. FL2-A denotes DNA content, and y-axis describes the percent of cells in each phase of the cell cycle. Data was analyzed using Modfit. An increased percent of cells cycling through S-phase is evident by 12 hours following cisplatin treatment. At T36 and T48 hours post cisplatin exposure, G_2/M arrest is apparent.

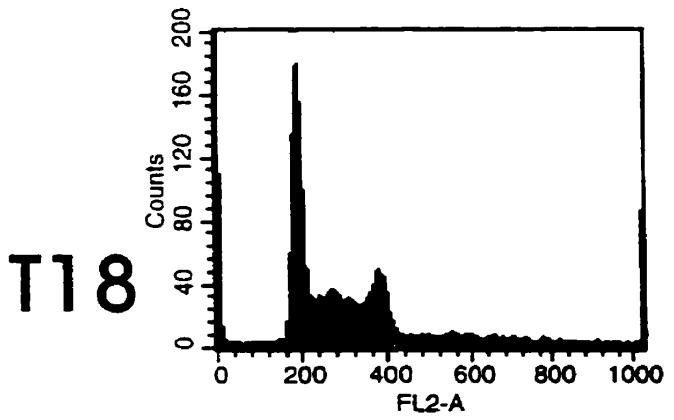
WM35


T0
Counts
0
40
80
120
160
200
0
200
400
600
800
1000
FL2-A


T24
Counts
0
40
80
120
160
200
0
200
400
600
800
1000
FL2-A


T12
Counts
0
40
80
120
160
200
0
200
400
600
800
1000
FL2-A


T36
Counts
0
40
80
120
160
200
0
200
400
600
800
1000
FL2-A


T18
Counts
0
40
80
120
160
200
0
200
400
600
800
1000
FL2-A

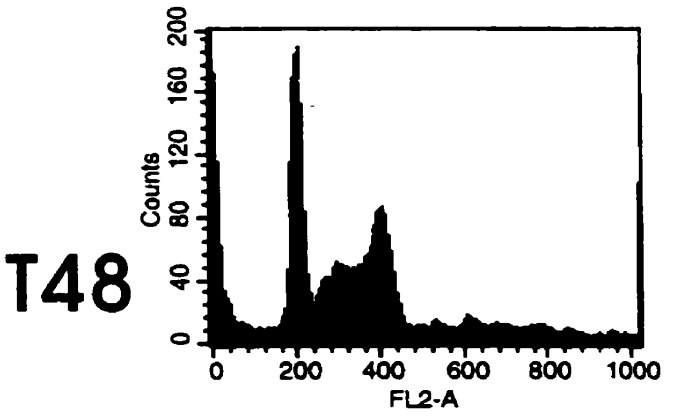
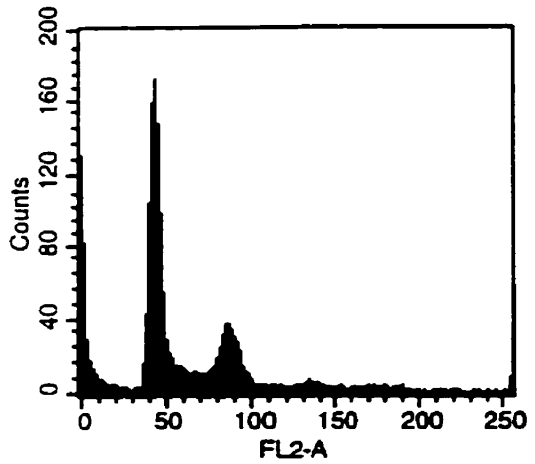

T48
Counts
0
40
80
120
160
200
0
200
400
600
800
1000
FL2-A

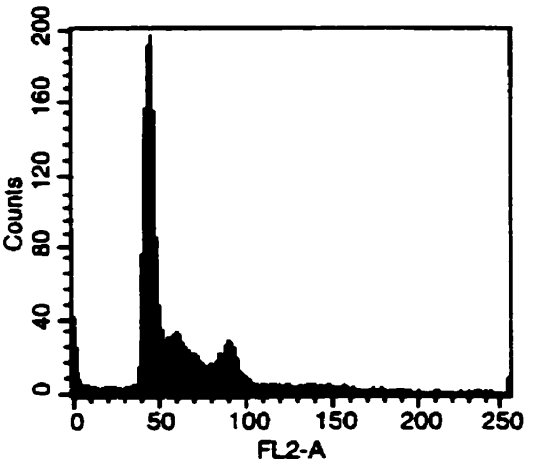
Figure 2.7 Cell cycle profile of clone E cells treated continuously with 15μM of cisplatin for 48 hours. Clone E cells were cultured to semi-confluency for at least 12 hours prior to exposure to 15μM of cisplatin. At each of the time points indicated, adherent cells were washed, harvested, and stained with propidium iodide in preparation for Fluorescent Cell Sorting Analysis, as described in Materials and Methods. FL2-A denotes DNA content, and y-axis describes the percent of cells in each phase of the cell cycle. Data was analyzed using Modfit. An S-phase block is evident by T12 hours, however, this block is only transient as the cell cycle profile returns to normal at T36 and T48 hours.

Clone E

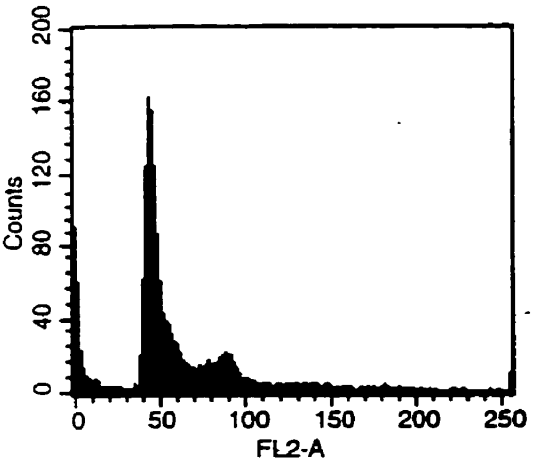
T0



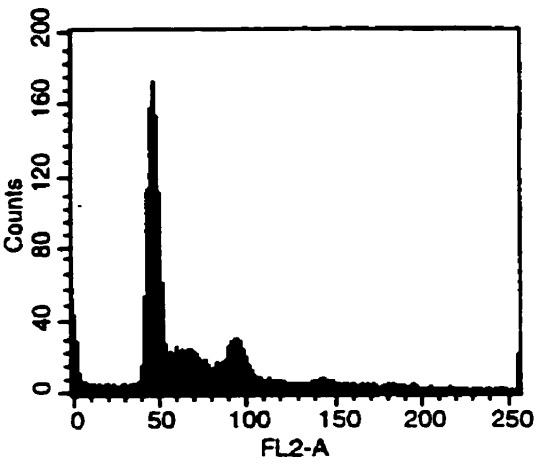
T24



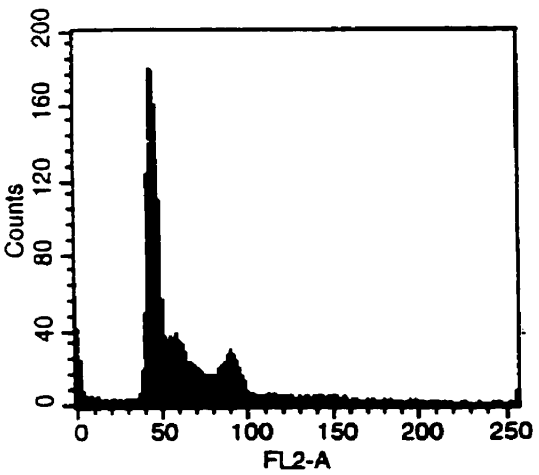
T12



T36



T18



T48

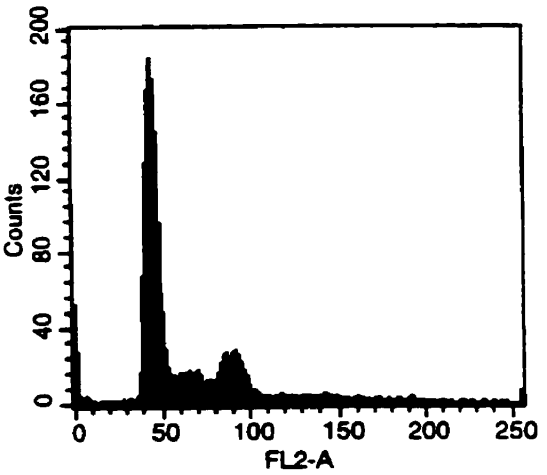
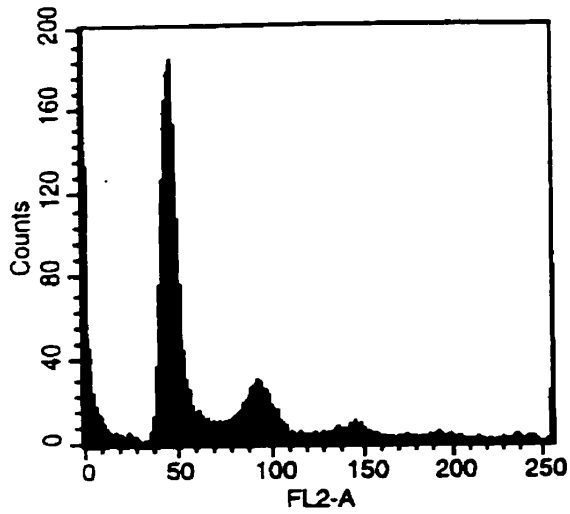


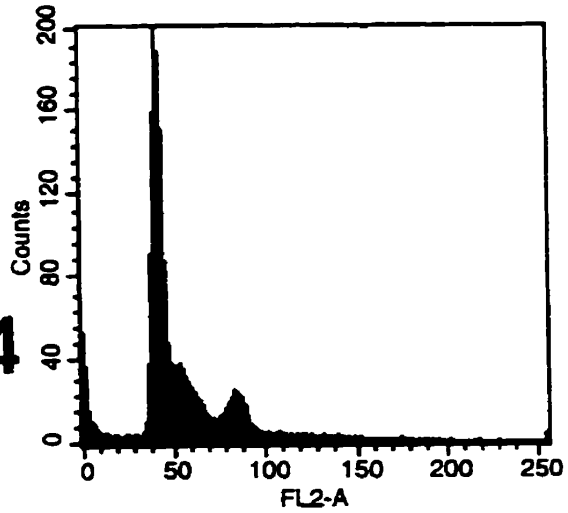
Figure 2.8 Cell cycle profile of clone G cells treated continuously with 15μM of cisplatin for 48 hours. Clone G cells were cultured to semi-confluency for at least 12 hours prior to exposure to 15μM of cisplatin. At each of the time points indicated, adherent cells were washed, harvested, and stained with propidium iodide in preparation for Fluorescent Cell Sorting Analysis, as described in Materials and Methods. FL2-A denotes DNA content, and y-axis describes the percent of cells in each phase of the cell cycle. Data was analyzed using Modfit. An S-phase block is evident by T12 hours, however, this block is only transient as the cell cycle profile returns to normal at T36 and T48 hours

Clone G

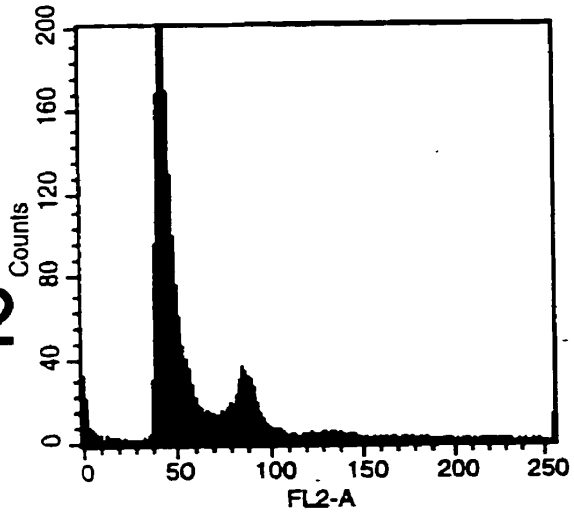
T0



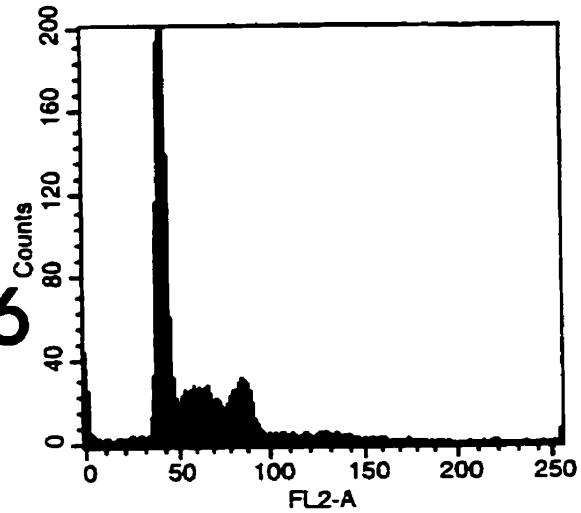
T24



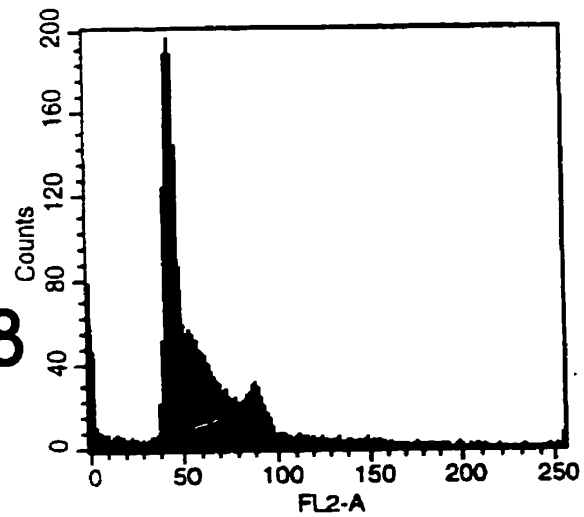
T12



T36



T18



T48

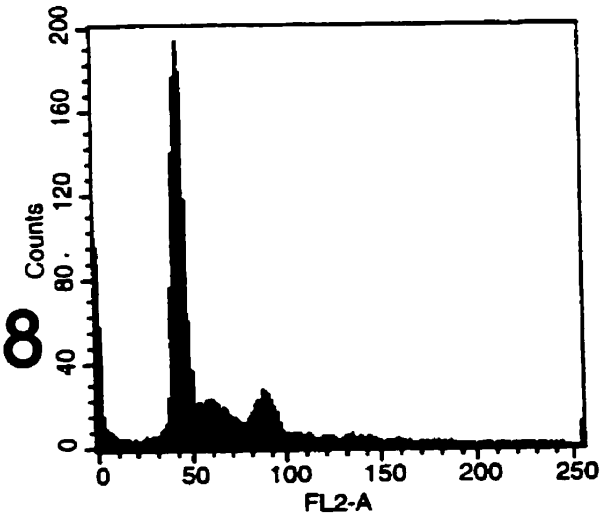
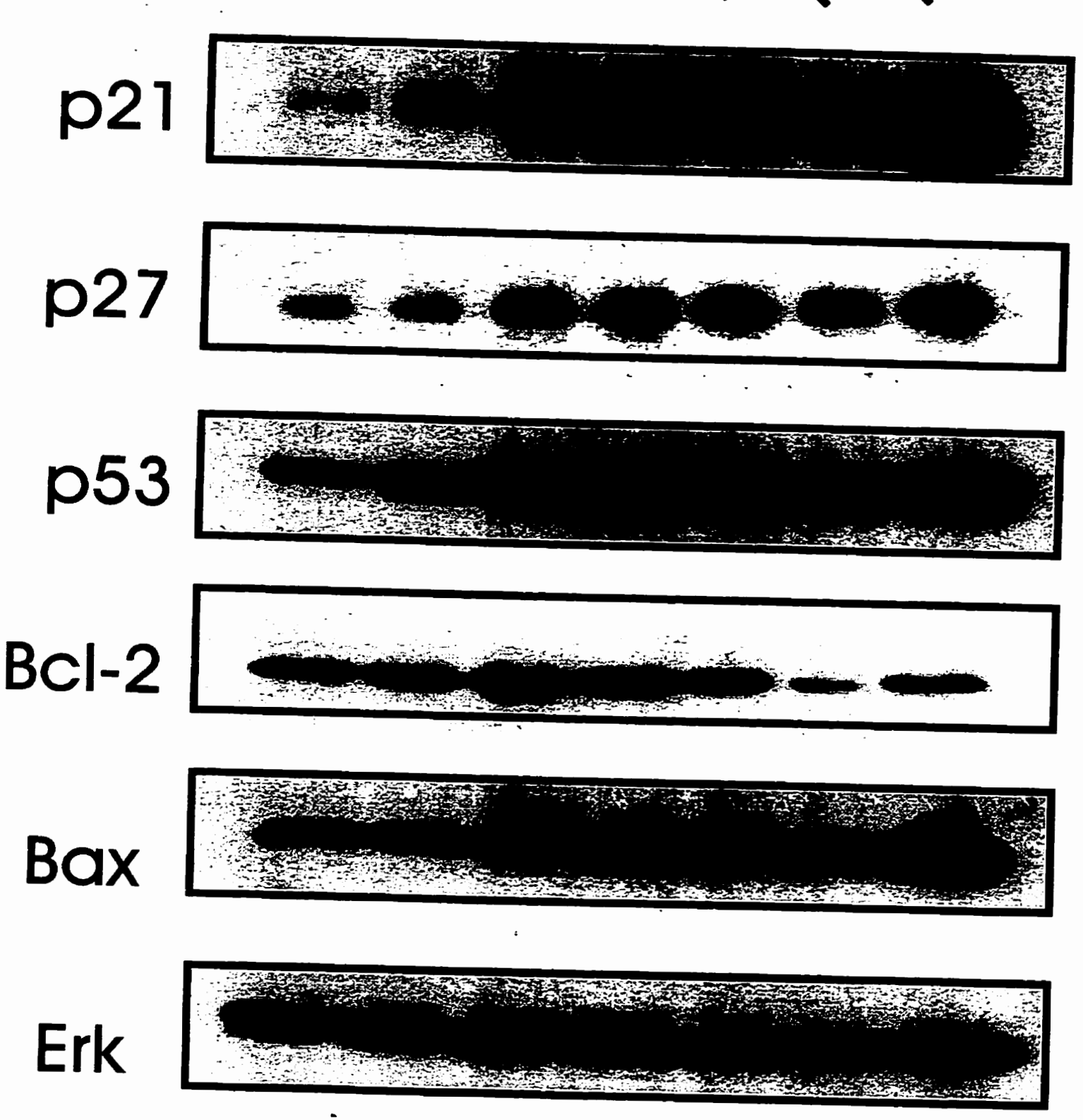


Figure 2.9 Western blot analysis of p21, p27, p53, Bcl-2 and Bax expression in adherent WM35 cells at different time points following exposure to 15μM of cisplatin. Cells were cultured to semi-confluency for at least 12 hours prior to cisplatin treatment. At different time points following treatment, adherent cells were washed, harvested and used for total protein extraction and FACS analysis. Extraction of total protein and western blot analysis were performed according to Materials and Methods. The expression of the three cell cycle proteins, p21, p27 and p53 were strongly up-regulated following treatment. However, there were no significant changes in the level of Bcl-2 or Bax expression following cisplatin treatment.


WM35
T0
T6
T12
T18
T24
T36
T48
p21
p27
p53
Bcl-2
Bax
Erk

p27 expression was minimal in untreated WM35 cells. Six hours following 15μM cisplatin treatment, the expression of p21 increases significantly and by 12 hours post treatment, p27 protein is up-regulated in conjunction with a much higher level of induced p21 expression. Western blot analysis of total cellular proteins extracted from WM35 cells demonstrated that p53 is induced 12 hours following this treatment condition, and protein levels of all three cell cycle inhibitors, p21, p27 and p53 were maintained at the higher induced levels from 12 hours on wards. In contrast to WM35, expression of p21 and p53 in clones E and G was not induced until 18 hours following 15μM of cisplatin treatment, which is a 12 hours delayed from that observed in WM35 cells (Figure 2.10, 2.11). In addition, there is no expression of p21 and particularly p53 proteins in untreated clones E and G cells. However, the constitutive level of p27 protein is significantly higher than observed in WM35 cells. The level of p27 was not induced in either clones E or G cells following cisplatin treatment. Overall, the expression of cell cycle inhibitors was up-regulated at a much earlier time point following cisplatin treatment in WM35 as compared with clones E and G. The expression of bcl-2 and bax proteins in cisplatin treated cells was not significantly altered in WM35, clones E or G cells. Bcl-2 expression was detected in untreated cells, and its level was maintained until approximately 36 hours following cisplatin treatment. The level of bax protein was not induced significantly following cisplatin treatment.

Following cisplatin treatment, most of the adherent cells detached from the extracellular matrix as cells undergo the process of apoptosis. We had observed that a greater number of WM35 "floater" cells could be detected and at an earlier time point, when compared to either cisplatin-resistant clones (data not shown). Not only was the

Figure 2.10 Western blot analysis of p21, p27, p53, Bcl-2 and Bax expression in adherent clone E cells at different time points following exposure to 15μM of cisplatin. Cells were cultured to semi-confluency for at least 12 hours prior to cisplatin treatment. At different time points following treatment, adherent cells were washed, harvested and used for total protein extraction and FACS analysis. Extraction of total protein and western blot analysis were performed according to Materials and Methods. The expression of the three cell cycle proteins, p21, p27 and p53 were strongly up-regulated following treatment, however, at a later time point than in observed in WM35 cells. There were no significant changes in the level of Bcl-2 or Bax expression following cisplatin treatment. Interestingly, p27 protein is constitutively strongly expressed in clone E cells.

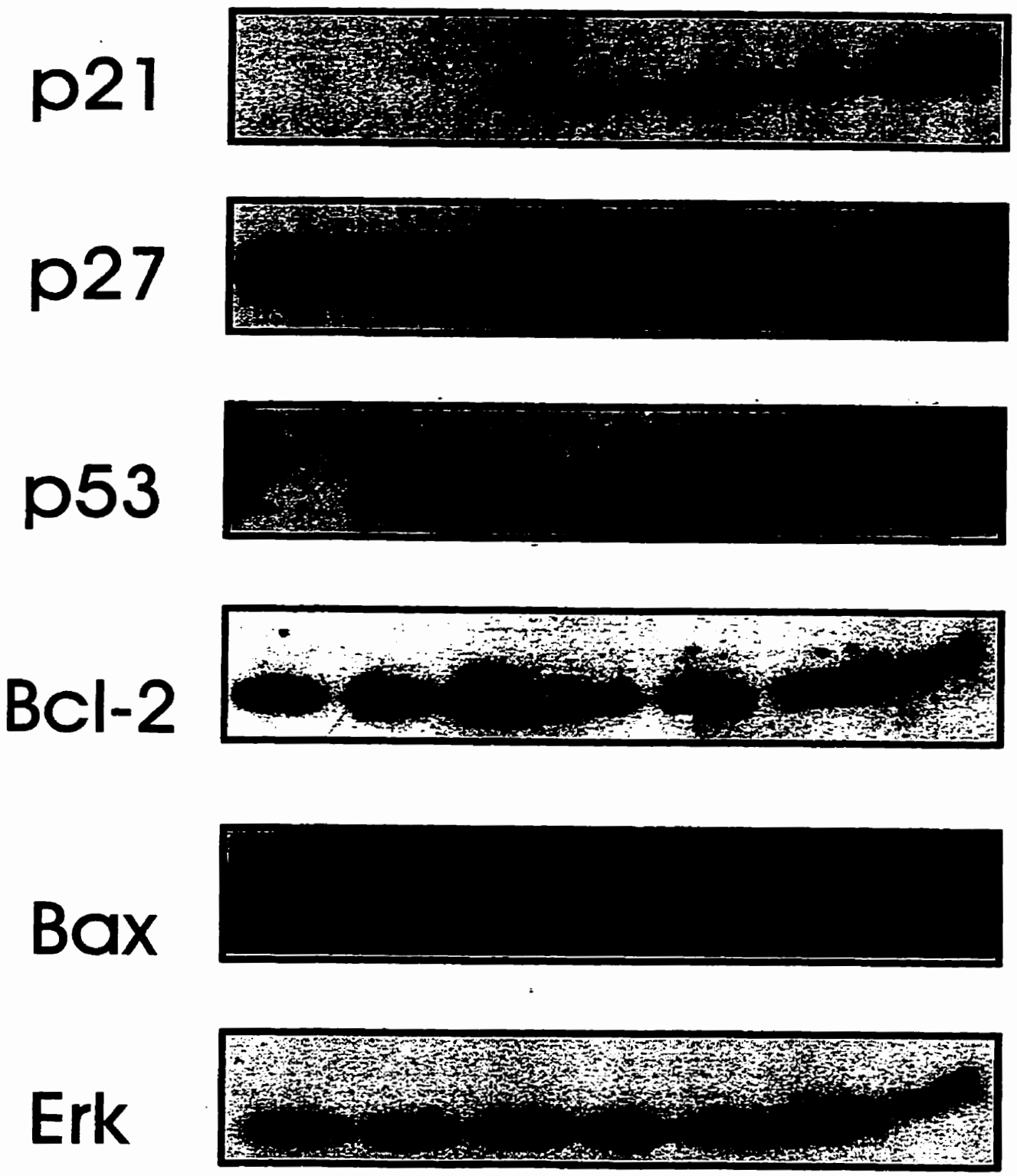
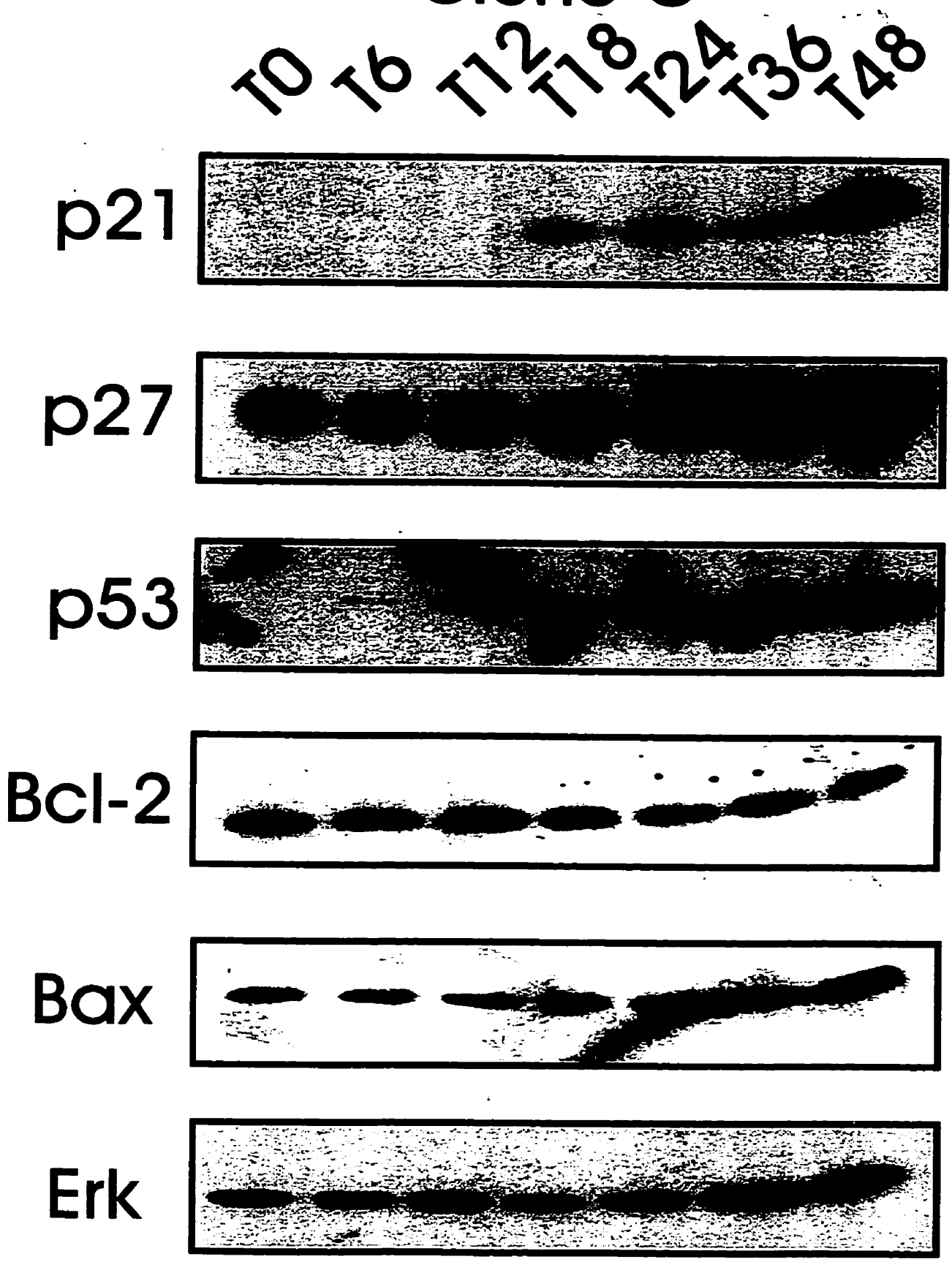

Clone E
T0
T6
T12
T18
T24
T36
T48
p21
p27
p53
Bcl-2
Bax
Erk

Figure 2.11 Western blot analysis of p21, p27, p53, Bcl-2 and Bax expression in adherent clone G cells at different time points following exposure to 15μM of cisplatin. Cells were cultured to semi-confluency for at least 12 hours prior to cisplatin treatment. At different time points following treatment, adherent cells were washed, harvested and used for total protein extraction and FACS analysis. Extraction of total protein and western blot analysis were performed according to Materials and Methods. The expression of the three cell cycle proteins, p21, p27 and p53 were strongly up-regulated following treatment, however, at a later time point than in observed in WM35 cells. There were no significant changes in the level of Bcl-2 or Bax expression following cisplatin treatment. Interestingly, p27 protein is constitutively strongly expressed in clone G cells.


Clone G
T0 T6 T12 T18 T24 T36 T48
p21
p27
p53
Bcl-2
Bax
Erk

number of detached cells different, but the WM35 floater cells appeared as cell debris following cisplatin treatment. This is in contrast to the morphology of the floater population of cisplatin-resistant variants, where they appear to be viable. Therefore, we attempted to analyze the nature of the floater population in these cells to determine their viability using FACS analysis. Because of the differential sensitivity to cisplatin in our cell lines, a significant number of detached cells may only be harvested following 48hours of cisplatin treatment in WM35, clones E and G. All three cell lines were treated continuously with 15μM of cisplatin and equal number of non-adherent population of cells from each cell line were pooled and stained with propidium iodide, and analyzed accordingly to the described materials and methods. As shown in Figure 2.12, where the y-axis denotes amount propidium iodide staining and x-axis represents DNA content, the percentage of viable cells in the floater population of WM35 cells as contained in the boxed region decreased from 71% in untreated cells to approximately 3% at 48hours following 15μM cisplatin treatment. In contrast, at 48 hours post treatment, 21% and 14% of the non-adherent cell population is viable in clones E and G, respectively. In order to further demonstrate that indeed cells within the floater population of clones E and G contain a greater number of viable cells, non-adherent cell populations 48 hours following 15μM cisplatin treatment were harvested, washed and re-cultured in serum containing media. Over a period of 14-21 days, non-adherent cells from both clones E and G re-attached to the extracellular matrix of tissue culture plates, forming colonies of cells. As shown in Figure 2.13, none of the non-adherent cells treated at 15μM cisplatin of WM35 formed colonies after two weeks, in contrast to the numerous colonies observed in clones E and G. This provided further evidence that indeed, the cells within the floater

Figure 2.12 FACS analysis of WM35, clones E and G floater, non-adherent cell populations following 15μM of cisplatin at T0 and T48 hours. Cells were grown to semi-confluency for at least 12 hours prior to cisplatin treatment. At 48 hours following 15μM cisplatin exposure, nonadherent, floater population of cells were harvested, washed and stained with propidium iodide and analyzed with FACS as described in Materials and Methods. FSC-H denotes DNA content and FL3-H describes intensity of propidium iodide staining. Cells contained within the boxed area are viable. Following 15μM of cisplatin treatment, 3%, 21%, 14% of WM35, clone E and clone G cells are viable within the non-adherent population at 48 hours, respectively.

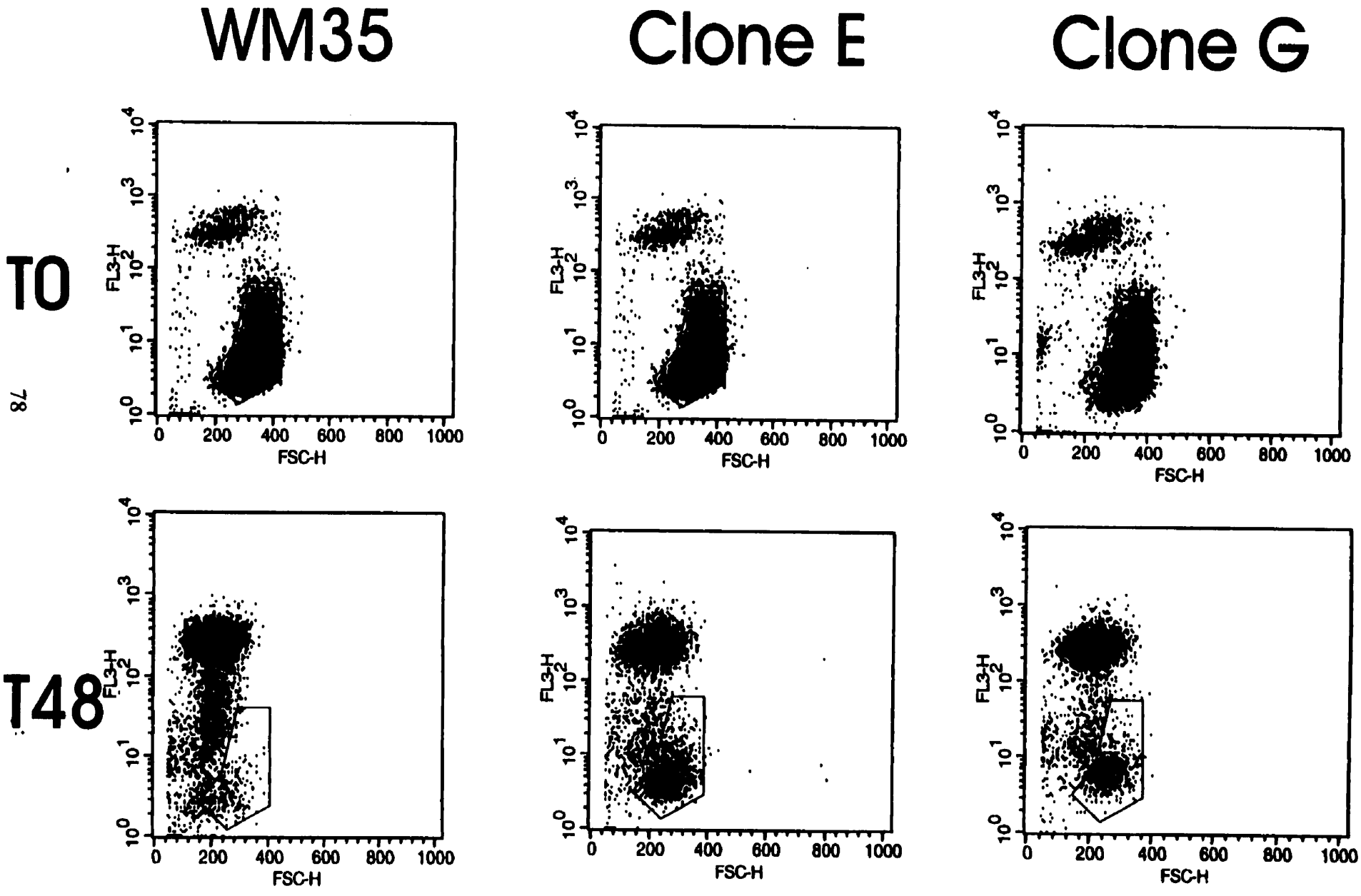

WM35
Clone E
Clone G
T0
T48
FL3-H
FSC-H
0
200
400
600
800
1000
10^0
10^1
10^2
10^3
10^4

Figure 2.13 Floater (non-adherent) population of cells from clones E and G formed colonies when re-cultured in cisplatin-free, serum containing media. Nonadherent cells from WM35, clones E and G were washed, and re-cultured in 5% FBS-supplemented media with media changed every third day. Two weeks following initial exposure to 15μM of cisplatin, floater cells of clones E and G re-attached to extracellular matrix of tissue culture plates and formed colonies, demonstrating that floater cells from these two cisplatin-resistant cell lines were still viable. Colonies were stained as described in Materials and Methods.

WM35

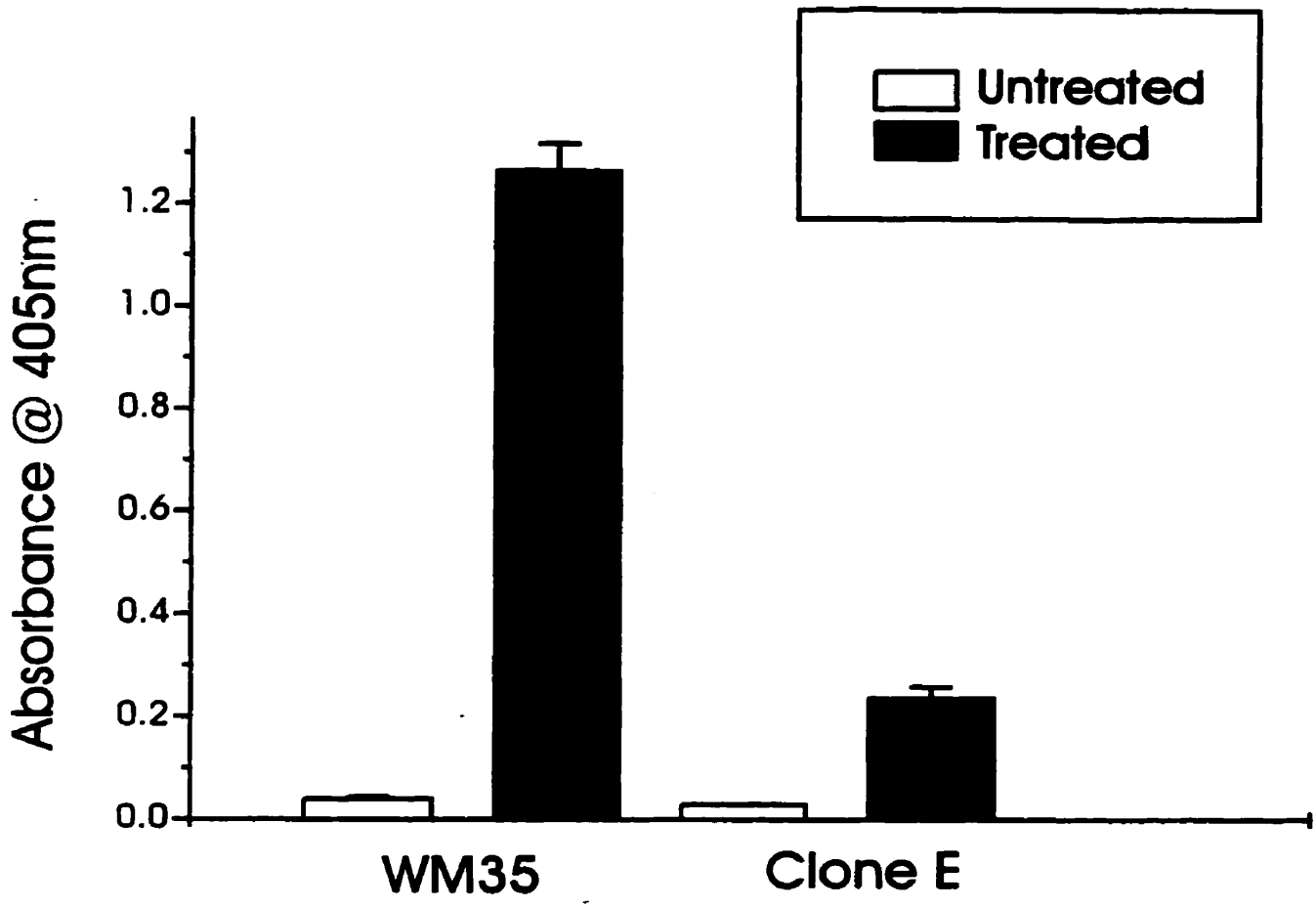
Clone E

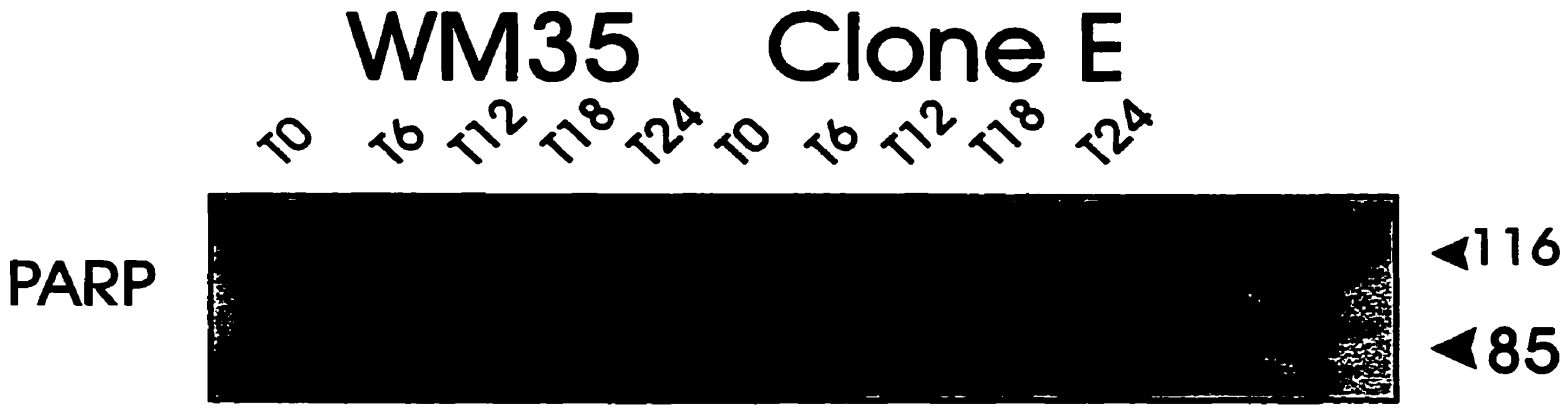
Clone G

population of resistant clones were viable. It is reasonable to suggest that the non-adherent cell population reflects the effect of cisplatin in our cell lines since cisplatin had caused the detachment of adherent cells. Therefore, to demonstrate whether characterized cellular events indicative of apoptosis may be occurring within the adherent cells, we examined a biochemical marker of apoptosis, the cleavage of poly-ADP-ribosyl-polymerase (PARP) in our cell lines following cisplatin treatment. WM35 and clone E were treated with 15μM of cisplatin and total cellular protein was isolated at different times following treatment. As shown in Figure 2.14B, PARP is cleaved in its entirety from 116kDa to 85kDa, beginning at 6 hours following cisplatin treatment in adherent WM35 cells. The intensity of the 85kDa signal increases at later time points in WM35 as a reflection of the greater degree of apoptosis occurring in this cell line. In contrast, the production of the smaller PARP subunit was not evident for up to 24 hours following cisplatin treatment in clone E. Our positive and negative controls for PARP cleavage are MCF-7 cells transfected with OCI-5 gene, resulting in these cells undergoing apoptosis, and the parental MCF-7 cell line, respectively. PARP cleavage detected within the adherent population of WM35 suggests apoptosis is occurring in WM35 and is absent in clone E cells (Gonzalez *et al.* 1998).

To provide further biochemical evidence that only WM35 cells are undergoing apoptosis following cisplatin treatment, we assayed for other biochemical criteria, such as the degradation of cellular DNA fragments into discrete multiples of 180 bp subunits in our cell lines. Using a commerically available cell death detection kit, the Cell Death Detection Eliza, the amount of mono- and oligonucleosomes produced as endonucleases cleave DNA between core histones may be detected in the cytoplasmatic fraction of cell.

Figure 2.14 Suppression of apoptosis in clone E following 15μM of cisplatin treatment. (A) Presence of multiples of 180bp oligomers in the cytoplasm of clone E was approximately 6-fold less than those detected in WM35 cells following 15μM of cisplatin treatment after 18 hours. Both adherent and non-adherent cells were harvested in the analysis, using the Cell Death Eliza Detection Kit as described in Materials and Methods. (B) The cleavage of PARP from 116kDa to the larger 85kDa subunit is detectable by 6 hours following 15μM of cisplatin treatment in WM35 cells. Total protein extract was prepared and analyzed using anti-PARP antibody as described in Materials and Methods. PARP cleavage was not detected in clone E cells.


Untreated
Treated
Absorbance @ 405nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WM35
Clone E


WM35
Clone E
T0
T6
T12
T18
T24
T0
T6
T12
T18
T24
PARP
116
85

WM35 and clone E were treated for 18 hours at 15μM of cisplatin and DNA isolated from the cell was analyzed as described in Materials and Methods. As shown in Figure 2:14A, the amounts of mono- and oliogonucleosomes produced in WM35 following cisplatin treatment were more than 20X the untreated level, as measured by absorbency at 405nm. In contrast, the amounts of nucleosomes detected following cisplatin treatment in clone E were only 4-5 times above the basal level. Taken together, the absence of viable cells within the floater population, the presence of PARP cleavage, and the production of mono- and oligonucleosomes within the adherent population of WM35 strongly argue for the induction of apoptosis in these cells following cisplatin treatment.

2.3.5 Expression of tyrosinase related-protein 2 (Trp-2) is strongly induced in cisplatin-resistant cell lines.

As previously proposed, retroviral insertional mutatgenesis was employed to isolate cisplatin-resistant variants of WM35 (Lu *et. al.* 1995). Lu and colleagues demonstrated by Northern blot analysis that a 3.8 kb mRNA transcript message that is absent in WM35 cells is over-expressed in several cisplatin-resistant variants, including clones E and G. To isolate and characterize the activated genes present in clones E and G, we utilized a commercially available subtraction hybridization kit, PCR-Select cDNA Subtraction, to identify and isolate cDNA fragments that are expressed in clone E but are absent in WM35. As a way to verify the effectiveness and efficiency of the subtraction process, pooled populations of subtracted and unsubtracted cDNAs from WM35 and clone E were electrophoresed on a 1.5% agarose gel, transferred to nylon filters, and subsequently hybridized with a GAPDH probe. GADPH was expressed only in the WM35 and clone E unsubtracted population of cDNAs as expected and was absent in the subtracted pools,

indicative of a clean and effective subtraction procedure (data not shown). The 89 clones identified by using this strategy were later subcloned into the vector pCR2.1 are shown in Table 2.2. cDNA inserts (approximately 0.2-1.2kb) were PCR-amplified, and labeled as probes to screen Northern Blots containing total RNA from WM35, and clones E and G. Table 2.2 is a summary of the result from Northern Blot Analysis showing that most of these clones contained cDNA sequences that were equally expressed in all cell lines. However, we were able to identify three clones using Northern Blot Analysis, clones 65, 80 and 83, that are minimally expressed in WM35 but highly expressed in clones E and G (Figure 2.15). Sequence analysis of the subtracted positive clones revealed clone 80 to be highly homologous to retroviral sequences when sequence comparison was analyzed using GenBank. Interestingly, both clones 65 and 83 were 100% homologous to the 3' translated region of a melanocyte-specific protein, tyrosinase-related protein 2 (Trp2) (Figure 2.16). To determine whether the expression of Trp-2 is augmented in all of our retrovirally-established cisplatin-resistant clones, total RNA and protein was isolated from clones A to H and their expression level of Trp-2 was analyzed and compared with that of WM35. As shown in Figure 2.17, the expression of Trp-2 was up-regulated in all of our cisplatin-resistant variants by Northern and Western Blot Analysis when compared with WM35. To ascertain whether the over-expression of Trp2 in clone E and G was the direct result of strong transactivation by the long-terminal repeats in the viral promoter due to retroviral insertion, possible Trp-2 gene rearrangement was examined using Southern Blot Analysis of genomic DNA from WM35, and clones E and G digested with various restriction endonucleases. As shown in Figure 2.18, the patterns of Trp-2 gene fragments produced by the four restriction enzymes. *BamH*I, *Eco*RI, *Hind*III and *Xba*I

Table 2.2 Results of subtracted clones from the PCR-Select Subtractive Hybridization between clone E and WM35. Clones were isolated on the basis of hybridization between WM35 and clone E cells, as described in Materials and Methods. These cDNA fragments were subcloned into the pCR2.1 vector, PCR amplified and were subsequently used to screen Northern blots containing total RNA of WM35, clones E and G. > denotes where the level of expression was greater than the subsequent cell line. Three clones, 65, 80, 83, shown in bold were found to be up-regulated in both clones E and G.

Clones	PCR Amplified	Size of Insert (Kb)	Expression (Size of Message)
1	Yes	0.5	WM35,E,G (1.0)
2	Yes	0.5	???
3	Yes	0.6	No
4	Yes	None	WM35>E>G (4.0)
5	No	0.4	No
6	Yes	0.4	WM35,E,G (4.0)
7	Yes	0.4	WM35,E,G (4.0)
8	No	0.4	No
9	Yes	0.5	WM35,E,G (1.0)
10	Yes	0.4	No
11	No	None	No
12	Yes	0.4	No
13	Yes	0.5	WM35,E,G (2.5)
17	Yes	0.5	No
18	Yes	0.45	WM35,E,G (1.0)
19	Yes	0.3	No
20	Yes	0.5	No
21	Yes	0.35	No
22	No	None	No
23	Yes	0.5	WM35,E,G (3.8)
24	No	None	No
25	Yes	0.6	WM35,E,G (2.0)
26	Yes	0.35	E,G (0.5)
27	Yes	0.4	WM35,E,G (3.5)
28	No	None	No
29	Yes	0.5	WM35,E,G (3.5)
30	Yes	0.7	WM35,E,G (4.0)
32	Yes	0.3	WM35,E,G (3.5)
33	Yes	0.7	WM35,E,G (0.8)

Clones	PCR Amplified	Size of Insert (Kb)	Expression (Size of Message)
35	Yes	0.3	WM35,E,G (1.0)
36	Yes	0.5	No
37	Yes	0.55	WM35,E,G (2.0)
38	Yes	0.4	WM35,E,G (2.0)
39	Yes	0.3	WM35,E,G (2.2)
40	No	None	No
41	Yes	0.2	No
42	Yes	0.5	WM35,E,G (2.0)
43	Yes	0.3	WM35,E,G (4.0)
44	Yes	0.2	WM35>E>G (3.5)
45	Yes	0.35	WM35,E,G (4.0)
46	Yes	0.55	WM35,E,G (2.0)
47	No	None	No
48	No	None	No
49	Yes	0.4	WM35,E,G (4.0)
50	Yes	0.4	No
51	Yes	0.5	WM35,E,G (4.0)
52	Yes	0.7	WM35,E,G (2.0)
53	Yes	0.4	No
54	Yes	0.3	WM35,E,G (1.0)
55	Yes	0.25	WM35,E,G (4.0)
56	Yes	0.25	WM35>E>G (4.0)
57	Yes	1	WM35,E,G (4.0)
58	Yes	0.25	WM35,E,G (1.5)
59	Yes	0.25	No
60	Yes	0.6	WM35,E,G (3.8)
61	Yes	0.6	No
62	Yes	0.5	WM35.E.G (0.5)
63	No	None	No
64	Yes	0.5	No
65	**Yes**	**0.4**	**E,G (4.0)**
66	Yes	0.25	WM35.E.G(4.0)

Clones	PCR Amplified	Size of Insert (Kb)	Expression (Size of Message)
67	Yes	0.45	WM35,E,G (2.0)
68	Yes	0.3	No
69	Yes	0.25	No
70	Yes	0.3	WM35,E,G (2.0)
71	Yes	0.35	No
73	Yes	0.5	No
74	Yes	0.4	WM35>E>G (1.5)
75	Yes	0.6	No
76	Yes	0.2	WM35,E,G (2.0)
77	Yes	0.25	WM35,E,G (4.0)
78	Yes	0.3	WM35,E,G (2.0)
79	Yes	0.25	No
80	**Yes**	**0.2**	**E,G (3.0)**
81	Yes	0.15	No
82	Yes	0.4	WM35,E,G (2.5)
83	**Yes**	**1.2**	**E,G (4.0)**
84	Yes	0.15	WM35,E,G (2.0)
85	Yes	0.6	WM35,E,G (1.0)
86	Yes	0.2	WM35,E,G (2.0)
87	No	None	No
88	Yes	0.2	No
89	Yes	0.4	No

Figure 2.15 Expression of clones 65, 80 and 83 is strongly induced in clones E and G. 30μg of total RNA was extracted using Trizol, electrophoresed on a 1% agarose gel and blotted onto nitrocellulose. Subtracted clones 65, 80 and 83 were PCR-amplified using nested PCR primers described in the PCR-select Subtractive Hybridization Kit, labeled and used as probes to hybridize the filter. Integrity of RNA was demonstrated with GAPDH.

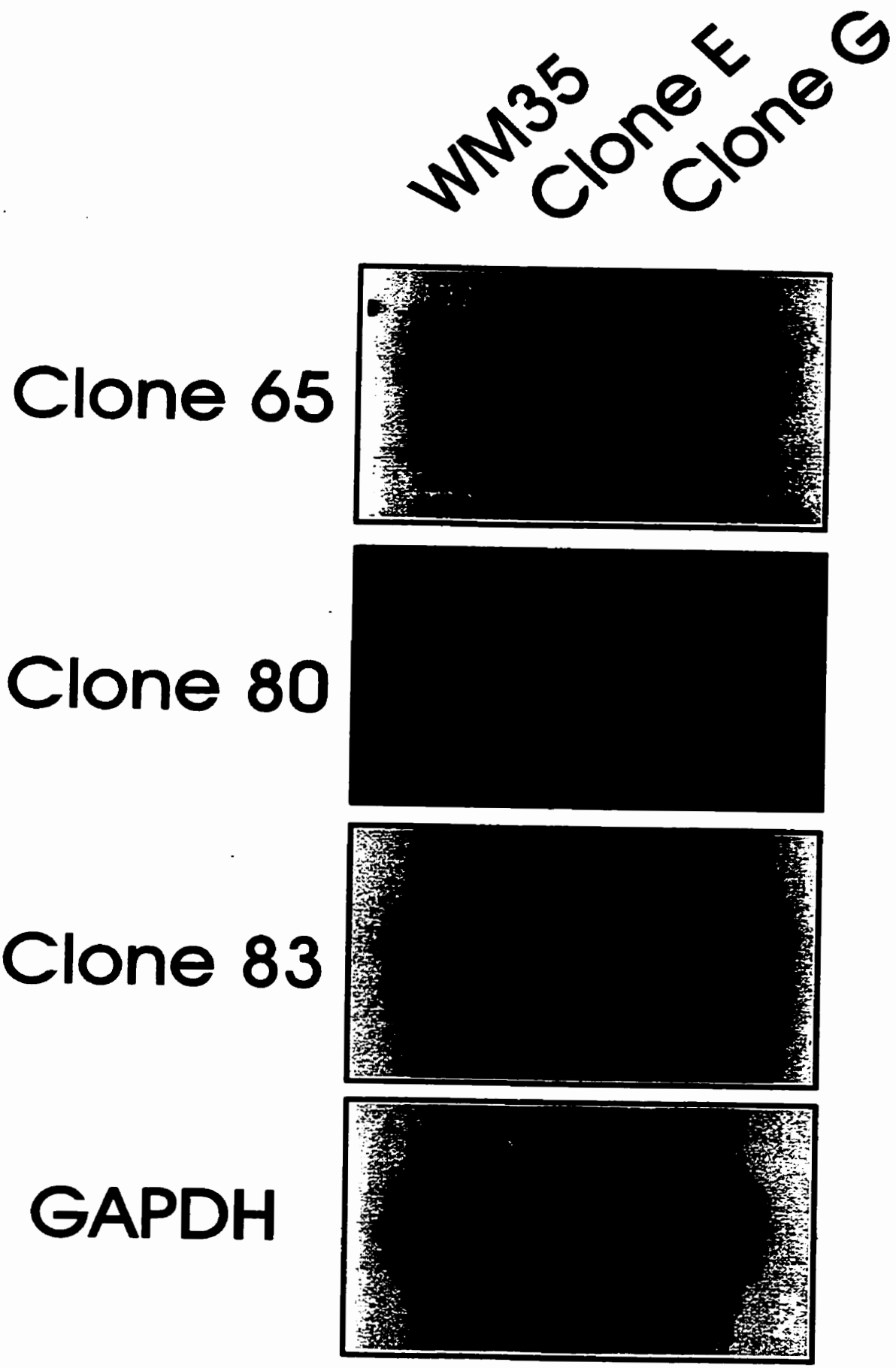

WM35
Clone E
Clone G
Clone 65
Clone 80
Clone 83
GAPDH

Figure 2.16 Clones 65 and 83 are homologous to the tyrosinase-related protein-2 (Trp-2) gene. Subtracted clones 65, 80 and 83 were sequenced using primers complementary to the T7 and T3 primers of the pCR2.1 vector. Clone 80 is homologous to the long-terminal repeats (LTR) sequences of the retrovirus. Both clones 65 and 83 were homologous to the 3' end of tyrosinase related protein-2 (Trp-2).

Tyrosinase related protein-2

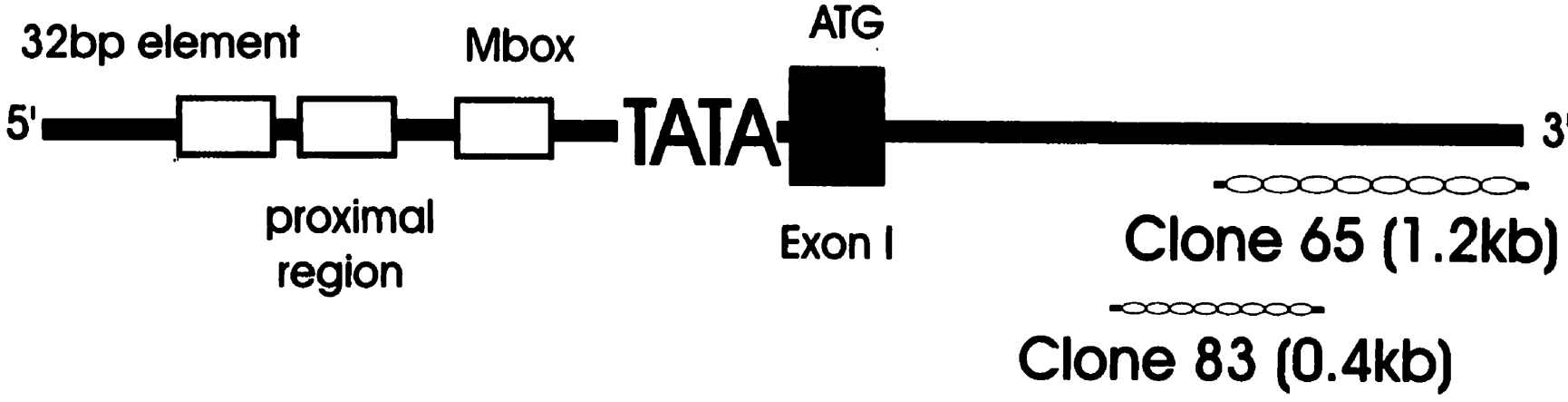


Figure 2.17 Expression of tyrosinase related protein-2 (Trp-2) is strongly induced in all cisplatin-resistant variants of WM35. **(A)** 25μg of total protein from the indicated cells were electrophoresed on a 12% poly-acrylamid gel and blotted onto Immobilon-P membrane. Trp-2 expression was detected using a anti-Trp-2 antibody (PEP 8). Equal protein loading was demonstrated using anti-Erk antibody. **(B)** 30μg of total RNA was extracted using Trizol and subjected to Northern blotting. Trp-2 expression was detected using the 1.6Kb (BamHI/XbaI) Trp-2 fragment as probe. Integrity of mRNA was examined using GAPDH. Trp-2 was strongly induced in all cisplatin-resistant clones, A-I.

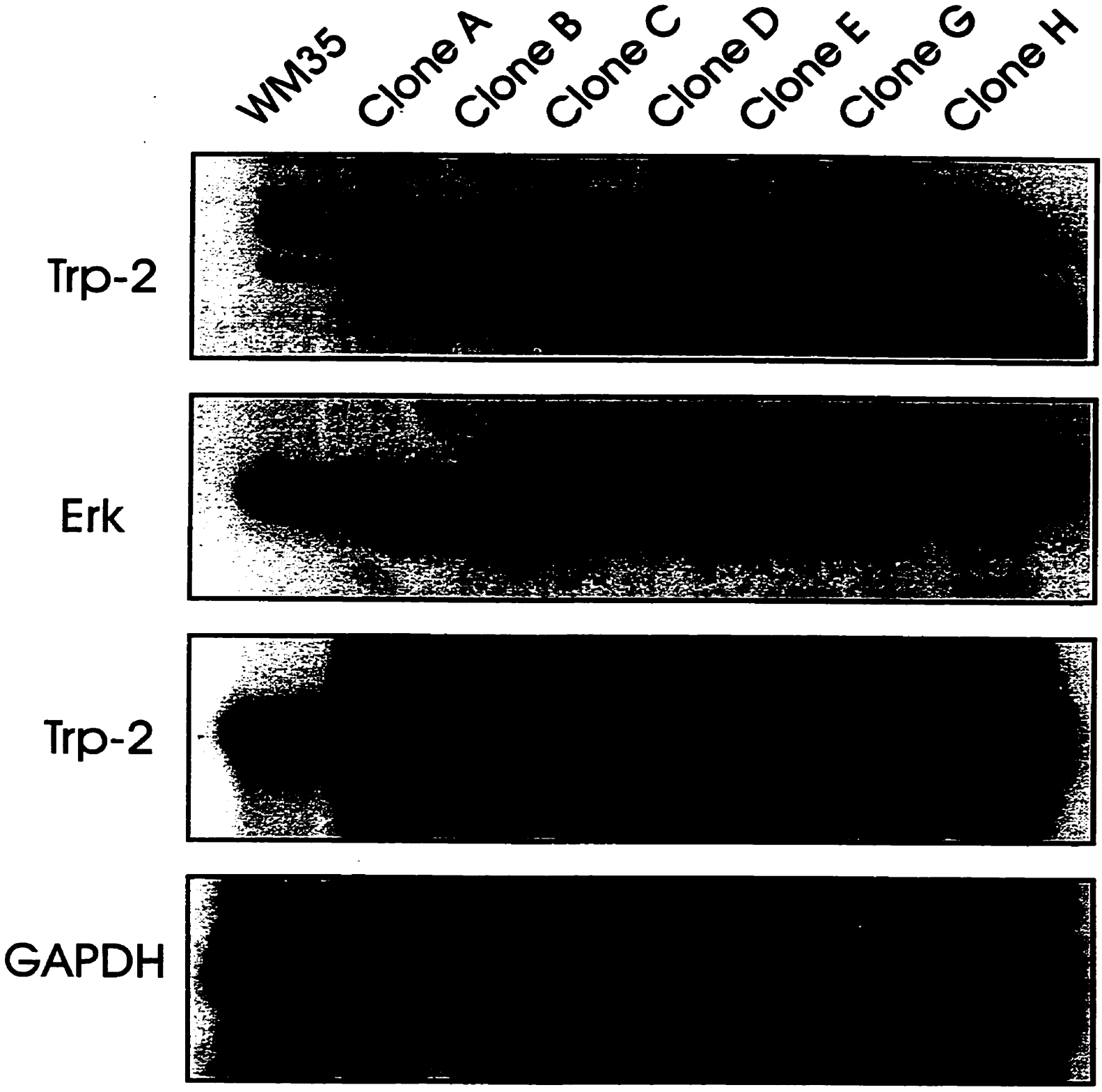
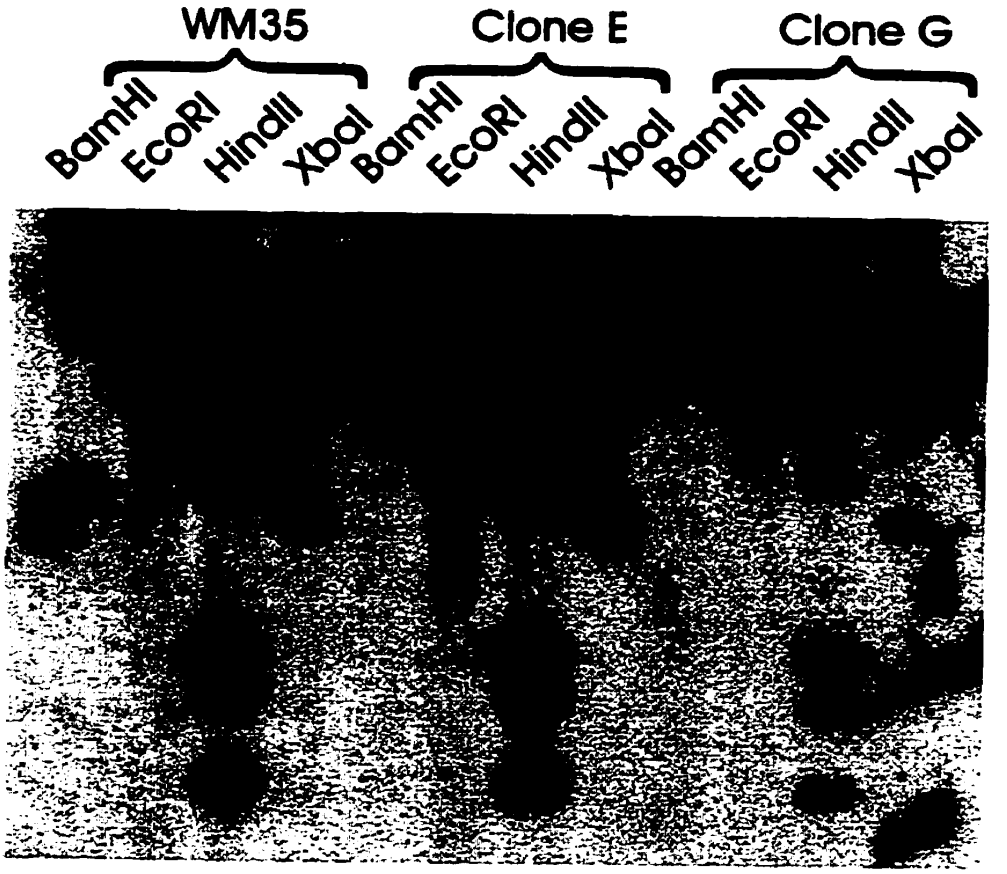

WM35
Clone A
Clone B
Clone C
Clone D
Clone E
Clone G
Clone H
Trp-2
Erk
Trp-2
GAPDH

Figure 2.18 Trp-2 gene was not rearranged in WM35, clones E and G. 20μg of genomic DNA extracted from WM35, clones E and G were completely digested with BamHI, EcoRI, Hind III and Xba I, separated on 0.8% agarose gel and transferred to nitrocellulose. The filter was hybridized with a ^{32}P labelled 1.6Kb BamHI/XbaI Trp-2 fragment as described in Materials and Methods.


WM35
Clone E
Clone G
BamHI
EcoRI
HindII
XbaI
BamHI
EcoRI
HindII
XbaI
BamHI
EcoRI
HindII
XbaI

TRP2

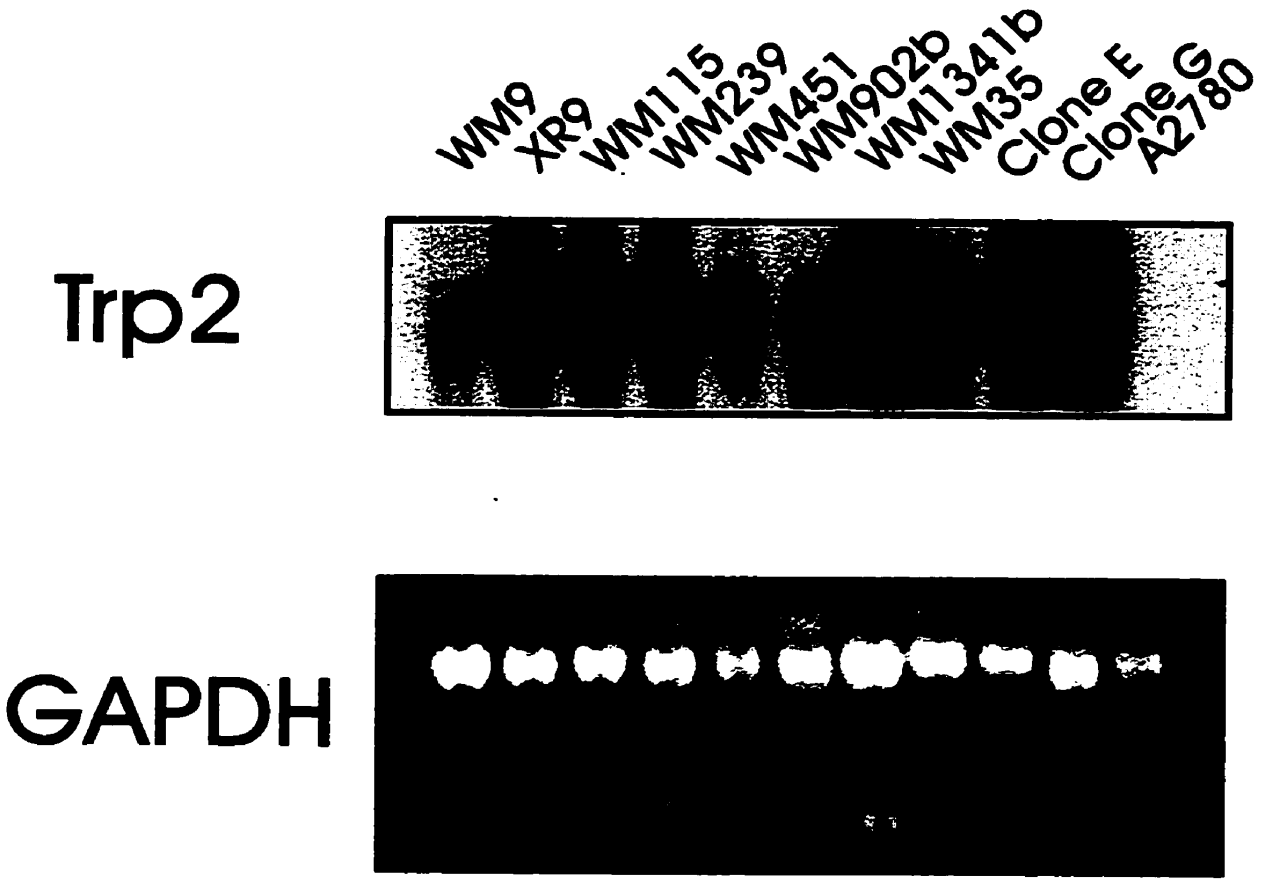
were comparable in the three cell lines examined, suggesting the viral insertion may not be a mechanism of Trp2 gene activation in clones E and G.

2.3.6 Expression of Trp-2 correlates with sensitivity to cisplatin.

In order to provide additional evidence that the over-expression of Trp-2 may correlate with resistance to cisplatin, we examined the expression of Trp-2 in other melanoma cell lines by Northern and Western Blot Analysis. Figure 2.19 shows that the expression of Trp-2 is variable in numerous melanoma cell lines, derived from various stages of melanoma progression (Table 2.3). For example, WM1341b, established from a patient with melanoma lesion at the vertical growth phase expresses very high levels of Trp-2, but WM9, a metastatic melanoma cell line, expresses almost negligible amount of Trp-2 RNA and protein (data not shown). In order to demonstrate that the expression of Trp-2 correlates directly with the degree of sensitivity to cisplatin. The WM9 and WM1341b cell lines were treated with various concentrations of cisplatin for 24 hours. Cell viability was analyzed using the MTS assay. As shown in Figure 2.20, WM1341b and clone E, both cell lines with high Trp-2 expression are more resistant to the cytotoxic effects of cisplatin than WM35. Conversely, the cell line with lowest Trp-2 expression, WM9, is very sensitive to cisplatin treatment; approximately 50% of the cells are nonviable following 6μM of cisplatin treatment for 24 hours. Results obtained from subtraction hybridization and the MTS assay clearly illustrate that the expression of the melanocyte-specific protein, Trp-2, correlates with cisplatin sensitivity in melanoma cells.

As a consequence of our study of the role of Trp-2 in cisplatin resistance, the expression of another melanocytic enzyme involved in the pathway of melanogenesis, Trp-1 (tyrosinase related protein-1) was compared between cisplatin-sensitive and

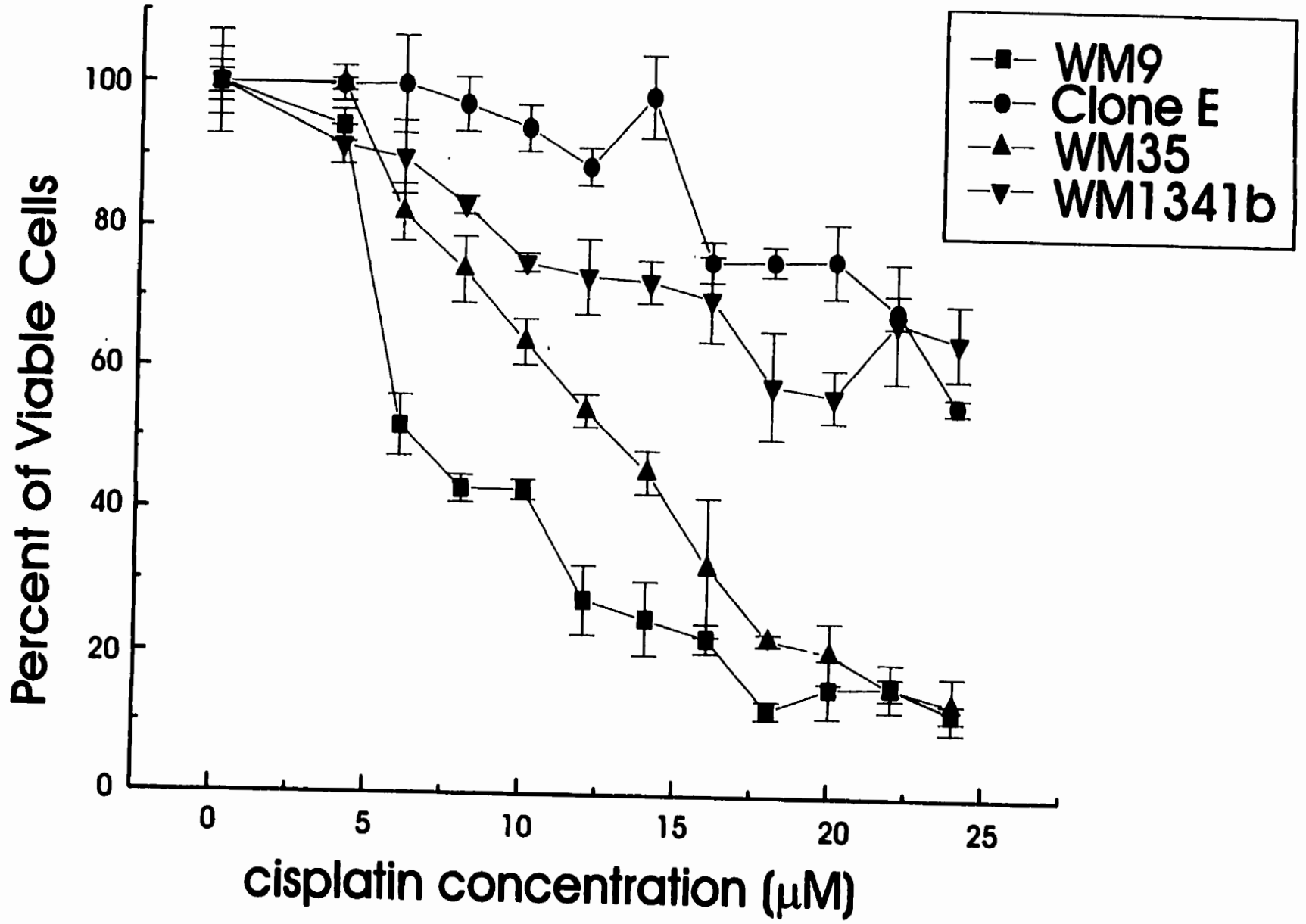
Figure 2.19 Expression of *Trp-2* in melanoma cell lines. 30μg of total RNA was separated on a 1% agarose gel and transferred to nitrocellulose. Trp-2 expression was detected using the 1.6Kb (BamHI/XbaI) Trp-2 fragment. Integrity of RNA was examined using GAPDH.


WM9
XR9
WM115
WM239
WM451
WM902b
WM1341b
WM35
Clone E
Clone G
A2780
Trp2
GAPDH

Human Melanoma Cells	Derivation	TRP1 Expression	TRP2 Expression
WM9	Metastatic	Low	Low
XR9	UVB-resistant variant of WM35	High	High
WM115	VGP	Low	High
WM239	Metastatic variant of WM115	Low	Moderate
WM451	Metastatic	Low	Low
WM902b	VGP	High	Low
WM1341b	VGP	Moderate	High
WM35	RGP	Moderate	Low
Clone E	Cisplatin-resistant variant of WM35	Moderate	High
Clone G	Cisplatin-resistant variant of WM35	Moderate	High

Table 2.3: Expression of Trp-1 and Trp-2 in a panel of melanoma cell lines.

Figure 2.20 Expression of Trp-2 correlates directly with the sensitivity of melanoma cells to cisplatin. $8X10^3$ cells from WM9, clone E, WM35 and WM1341b were cultured in 24-well plates, in quadruplicates for at least 12 hours in a total volume of 200µl of 5% FBS-supplemented media prior to the addition of cisplatin. Cisplatin was added in a total volume of 100µl, and incubated for 24 hours. Four hours prior to cell harvest, 20µl mixture of PMS/MTS (100µl/2ml) was added to the drug-containing media. Cell viability was determined at 490nm, using an ELIZA plate-reader. Mean and standard deviation error bars were determined using Origin 4.1.

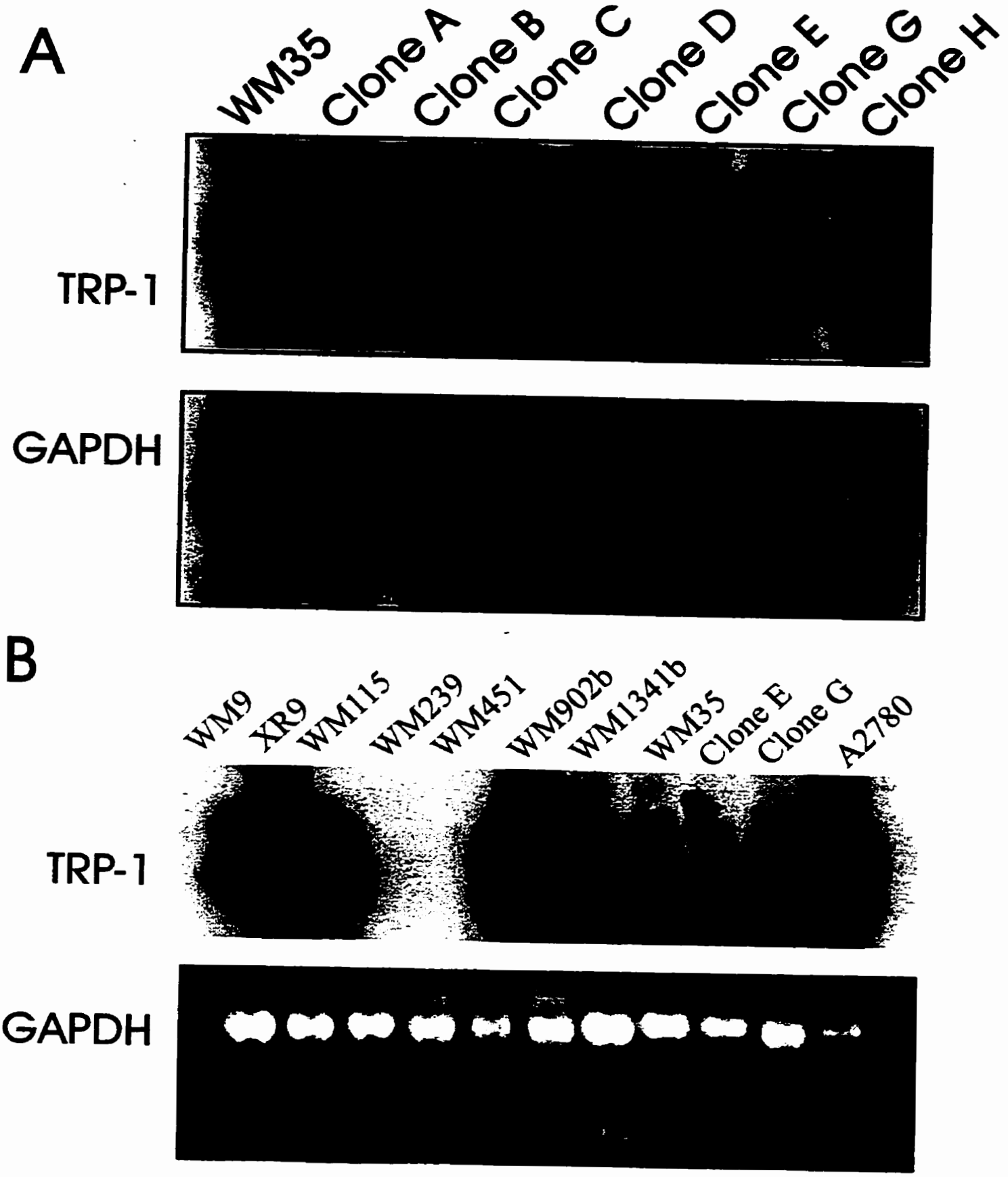

WM9
Clone E
WM35
WM1341b
Percent of Viable Cells
100
80
60
40
20
0
0
5
10
15
20
25
cisplatin concentration (μM)

resistant cell lines. As shown in Figure 2.21A, the expression of Trp-1 does not correlate with the level of sensitivity to cisplatin. Although Trp-1 expression is highest in two cisplatin resistant clones, A and B, the levels of Trp-1 expression in clone G and H are almost negligible, and lower than its expression in WM35 cells. Although the expression of Trp-1 does not appear to correlate with cellular sensitivity to cisplatin, an examination of its expression pattern in numerous melanoma cell lines, established from different stages of melanoma development, revealed that the expression of Trp-1 may correlate inversely with melanoma progression. As shown in Figure 2.21B and Table 2.3 Trp-1 is expressed strongly in cell lines established from patients with radial (RGP) or vertical growth phase (VGP) melanomas, such as WM902b, WM1341b and WM35 cells. However, its expression is low in many metastatic cell lines, including WM451, WM239, and WM9. Interestingly, in a paired cell line isolated from the same patient, the low level of Trp-1 expression is dropped from its earlier VGP derivative as seen in WM115 cells to the metastatic variant WM239 cells, where the expression of Trp-1 is not detectable. Taken together, our data suggests that the expression of Trp-1 is probably not critical to the acquisition of cisplatin resistance in the melanoma cell lines examined in this study. However, the down-regulation of Trp-1 expression may be important for the progression of melanoma cells from its early, non-malignant stage to the malignant and metastatic stage melanoma cells.

2.3.7 Exogenous expression of Trp2 protects against cisplatin-induced apoptosis.

Although the data that we have obtained and described above suggests that the expression of Trp2 correlates with cisplatin sensitivity in cells, WM1341b and WM9 are

Figure 2.21 Expression of Trp-1 does not correlate with cisplatin resistance, but inversely correlates with stage of tumor progression. 30μg of total RNA was extracted from the indicated cell lines, separated on a 1% agarose gel and transferred to nitrocellulose membrane. Trp-1 transcript was detected using the 1.5Kb (EcoRI/BamHI) Trp-1 cDNA fragment. Integrity of RNA was examined using GAPDH probe. **(A)** Trp-1 expression in cisplatin-resistant clones **(B)** Trp-1 expression in panel of melanoma cell lines. A2780 is a cell line established from a patient with ovarian carcinoma.


A
WM35
Clone A
Clone B
Clone C
Clone D
Clone E
Clone G
Clone H
TRP-1
GAPDH
B
WM9
XR9
WM115
WM239
WM451
WM902b
WM1341b
WM35
Clone E
Clone G
A2780
TRP-1
GAPDH

not from isogenic backgrounds and therefore, other factors, apart from their differential expression of Trp2 may account for the difference in their sensitivity to cisplatin.. To determine whether the over-expression of Trp2 may confer a survival advantage to cells determine whether the over-expression of Trp2 may confer a survival advantage to cells following cisplatin treatment, the full length Trp2 cDNA was cloned into the expression vector pcDNA and stablely transfected into the parental, cisplatin sensitive WM35 cells. In addition, the vector alone was transfected into WM35 as a control. After selection with media containing 800μg/ml G418 or Geneticin, 18 transfected clones survived and total protein was extracted in order to examine the expression of Trp2. Western blot analysis with an anti-Trp2 antibody revealed that 6 of these 18 clones over-expressed Trp2 to levels comparable to clone E (Figure 2.22). To determine whether the sensitivity of these transfected clones conferred a survival advantage over WM35 in the presence of cisplatin, WM35, WM35-C4, WM35-C8, WM35-C9, WM35-pCDNA and clone E were treated at various concentration of cisplatin for 24 hours and cell viability was analyzed using the MTS assay. Figure 2.23A shows that all three Trp2-transfected clones of WM35 were more resistant to the cytotoxic effects of cisplatin than WM35, suggesting that the over-expression of Trp2 may indeed be of importance to the acquisition of cisplatin resistance in our system. The LD_{50} cisplatin concentration for WM35-C4, -C8, and –C9 were approximately 2-3 fold higher than WM35, a level that is comparable to the resistant variants of WM35 derived by retroviral insertion.

We have shown previously that in contrast to WM35, clone E cells do not undergo apoptosis following cisplatin treatment. To determine whether the Trp-2-transfected

Figure 2.22 Expression of Trp-2 in WM35-Trp-2 transfected cells. 25μg of total protein extract from the indicated cell lines was subjected to Western blotting as described in Materials and Methods. Trp-2 expression was detected using an anti-Trp-2 antibody (PEP 8). Equal protein loading was demonstrated using anti-Erk antibody.

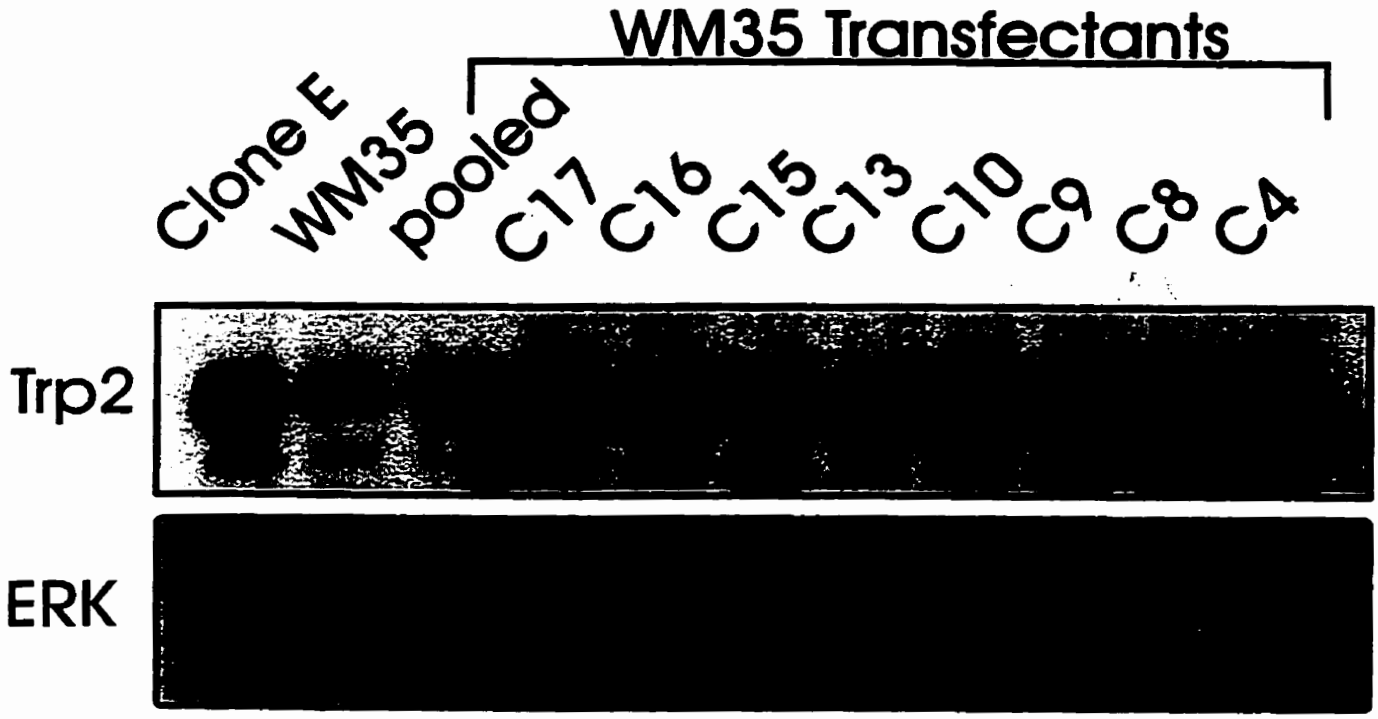
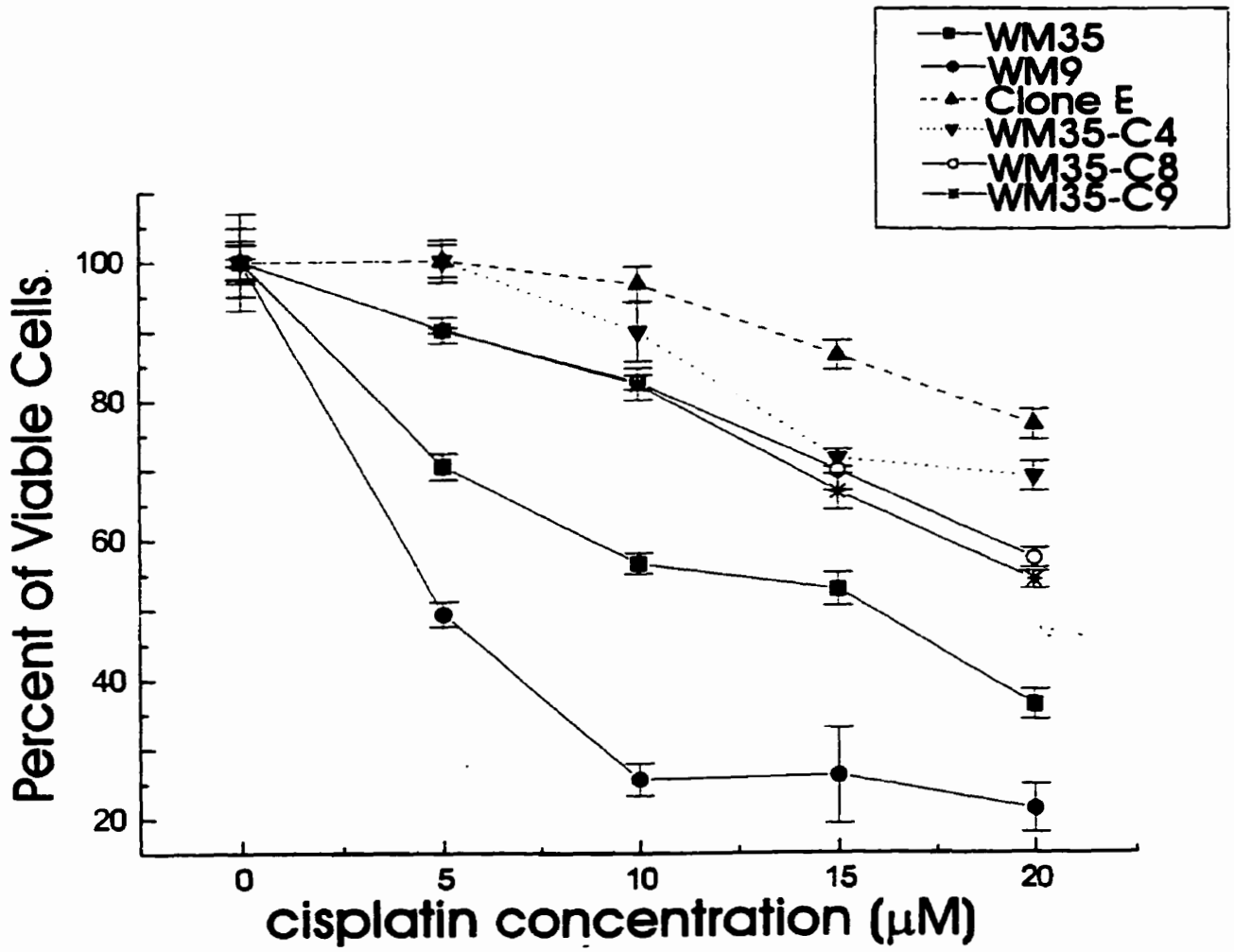
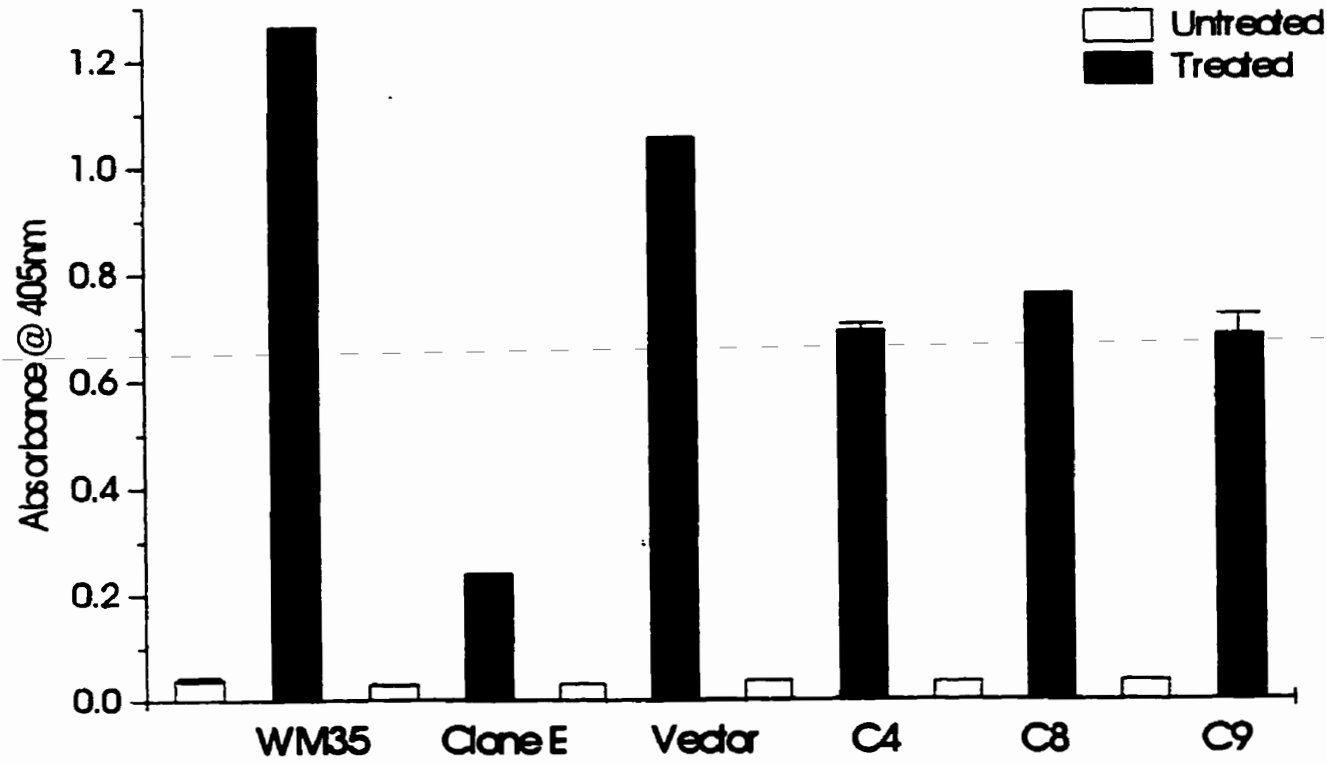

WM35 Transfectants
Clone E
WM35
pooled
C17
C16
C15
C13
C10
C9
C8
C4
Trp2
ERK

Figure 2.23 Expression of Trp-2 confers partial protection to cisplatin-induced apoptosis. **(A)** Sensitivity of WM35-Trp-2 transfected clones to cisplatin were examined using MTS assay. $8X10^3$ cells were cultured in 24-well plates, in quadruplicate, for at least 12 hours in a total volume of 200µl of 5% FBS-supplemented media prior to the addition of cisplatin. Cisplatin was added in a total volume of 100µl, and incubated for 24 hours. Four hours prior to cell harvest, an 20µl mixture of PMS/MTS (100µl/2ml) was added to the drug-containing media. Cell viability was determined at 490nm, using an ELIZA plate-reader. Mean and standard deviation error bars were determined using Origin 4.1. **(B)** Presence of multiples of 180bp oligomers in the cellular cytoplasm examined in WM35-Trp-2 transfected clones. Both adherent and non-adherent cells were harvested in the analysis, using the Cell Death Eliza Detection Kit as described in Materials and Methods.

A


WM35
WM9
Clone E
WM35-C4
WM35-C8
WM35-C9
Percent of Viable Cells
100
80
60
40
20
0
5
10
15
20
cisplatin concentration (μM)

B


Untreated
Treated
Absorbance @ 405nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WM35
Clone E
Vector
C4
C8
C9

clones of WM35 display similar properties upon cisplatin treatment, WM35-C4, -C8, and –C9 were treated with 15μM of cisplatin for 18 hours. Cells were lysed and the amount of mono- and oligonucleosomes produced as a direct effect of cisplatin treatment were quantified using the Cell Death Detection Eliza Kit. Figure 2.23B indicates that apoptosis was significantly suppressed in WM35-C4, C8 and C9 compared with WM35 and WM35-pCDNA cells. However, the level of suppression was not comparable to that evident in clone E. Our data taken from studies using a system to exogenously express Trp2 in WM35 have provided strong evidence that the expression of Trp2 in melanoma cells is at least, in part, significant to the acquisition of cisplatin resistance.

2.4 DISCUSSION

The mechanisms of drug resistance have been vigorously studied and elucidated in both *in vitro* and *in vivo* systems. They include the involvement of membrane-associated drug pumps, increase efficiency of DNA repair mechanisms, up-regulation of cellular detoxifying thiols, and the inhibition of apoptotic pathways. However, these modes of resistance have not been shown to be a major component in the acquisition of clinical resistance in patients. Moreover, strategies employed to overcome drug resistance by targeting these mechanisms have failed to substantially increase tumor cell's sensitivity to chemotherapeutic drugs. Since human malignant melanoma has reportedly shown to be intrinsically resistant to chemo- and radiotherapy, it is reasonable to suggest that cellular events specific and unique to melanoma cells may be involved.

To elucidate the molecular alterations associated with this unique nature of melanoma cells, we have employed a modified subtractive hybridization technique on a pair of cisplatin-sensitive and –resistant melanoma cell lines to identified differentially expressed genes that may contribute to this intrinsic resistance phenotype. We have identified a melanocyte-specific gene, tyroinase-related protein-2 (Trp-2), to be transcriptionally and translationally upregulated in all cisplatin-resistant cell lines established by retroviral insertional mutagenesis strategy. Several factors suggest that the differential expression of Trp-2 in our melanoma cells may be a true phenomenon and probably not the result of artifacts produced by PCR. First, from the nearly one hundred subtractive clones screened, two of the three clones were up-regulated in the cisplatin-resistant clones, of which clone 83 was 1.2kb in length, were homologous to the 3' region of Trp-2. In addition, although the subtractive hybridization method allows for the screening of differentially expressed genes between only two populations of cDNA, Trp-2 has been found to be up-regulated in all seven cisplatin-resistant clones. Thirdly, through our subtractive hybridization procedure, the third differentially expressed clone, clone 65, that is only expressed in the cisplatin-resistant variants, has sequence homology to proviral sequences thus providing us with a positive control for the subtractive hybridization techniques used in this study. Furthermore, we have shown that the expression of Trp-2 in two melanoma cell lines from different stage of disease progression, WM9 and WM1341b correlated with their sensitivity to cisplatin.

The expression of Trp-1 does not appear to correlate with sensitivity toward cisplatin amongst the various melanoma cell lines examined, but correlates with the stage of tumor progression. Previously, Thomson and colleagues have reported an inverse

relationship between the expression of Trp-1 and the stage of melanoma progression (Thomson *et al.* 1988). While nearly 100% of all paraffin sections of primary melanoma stain positively for Trp-1, the expression of the protein was absent and undetectable in greater than 50% of metastatic melanoma frozen sections. However, from the panel of melanoma cell lines examined, we were unable to detect any relationship between the expression of Trp-2 and the stage of melanoma progression from which the cell line was established. Many reports indicate that the inverse relationship between melanoma progression and expression of tyrosinase and Trp-1 co-segregates, without affecting the expression of Trp-2 at all, resulting in lesions that are progressively amelanotic (Orlow *et al.* 1998). Despite the loss of melanin pigment associated with ongoing melanoma progression, the expression of Trp-2 is seemingly unaltered, adding further to our hypothesis that its expression may be important in resistance to chemotherapeutic agents.

Preliminary analysis using Southern blot analysis of genomic DNA digested with various endonucleases suggests that the mechanism of Trp-2 upregulation in the two cisplatin resistant clones, E and G is probably not due to proviral insertion leading to transcriptional activation by the long terminal repeats (LTR) of the retrovirus. The regulation of Trp-2 in the process of melanogenesis is through the transcription factor MITF by binding to the upstream regulatory M boxes which responds to the cellular content of cAMP. Northern blot analysis of MITF demonstrates that MITF is expressed at high levels in all of our cells, including WM35 (data not shown). In addition, WM35 is sensitive to the induction of Trp-2 expression through the addition of forskolin, which has been shown to act through the cAMP pathway, suggesting that other mechanisms of Trp-2 regulation, aside from that mediated through MITF may be involved (data not shown).

Previously, our laboratory has demonstrated that both clones E and G over-express a transcript, termed Cisplatin-Resistant Locus-1 (CRL-1), due to viral insertional activation that is absent in WM35. It is interesting to postulate that perhaps the presence of the encoded protein of CRL-1 may act as a transcription factor and activate the transcription of Trp-2 by binding to the regulatory elements of Trp-2 in the cisplatin-resistant cells. However, CRL-1 is not over-expressed in all cisplatin-resistant clones and hence its expression does not coincide with the over-expression of Trp-2, suggesting that many different mechanisms may be involved.

We have demonstrated that stable transfection of Trp-2 cDNA into WM35 cells conferred resistance to cisplatin, suggesting that the expression of Trp-2 is at least partially responsible for the resistant phenotype. Analysis of cell viability after cisplatin treatment in WM35-Trp-2 transfected clones showed that there is significant protection against the cytotoxicity of cisplatin following treatment in these clones, when compared to WM35. Evidently, results from the Cell Death Eliza assay provide clues to suggest that inhibiting the process of apoptosis following cisplatin treatment may be the mechanism involved when Trp-2 is over-expressed, in either clone E or WM35-C4, -C8, or -C9 cells. Examination of the cellular and biochemical alterations between WM35 and the retrovirally-derived cisplatin-resistant clones has provided evidence to support this theory.

It has been generally accepted that many mammalian cell lines primarily resistant to a single chemotherapeutic agent are often found to be resistant to a variety of different anticancer drugs exerting various mechanisms of action (Hamaguchi *et al.* 1993). Our cisplatin-resistant cells display cross-resistance to methotrexate and carboplatinum, but

not taxol suggesting that the pattern of cross-resistance is not specific to DNA-damaging agents. Their pattern of cross-resistance does not appear to segregate with any one class of chemotherapeutic drugs since resistance to methotrexate arises from DHFR gene amplification (Huennekens 1994). Hamaguchi and colleagues reported that in a panel of human ovarian cancer cell lines, cross-resistance to carboplatin consistently paralleled that of cisplatin (Hamaguchi *et al.* 1993). However, the knowledge gained from studying the pattern of cross-resistance in our cell lines adds further to our hypothesis that an aberrant apoptotic pathway may be involved in the mechanism of cisplatin resistance in our system since chemotherapeutic drugs from various categories with distinct mechanisms of action ultimately tap into the pathways of apoptosis, albeit at different places. Therefore it is likely that cells with defective apoptotic signals may be cross-resistant to chemotherapeutic drugs from various classes. Programmed cell death or apoptosis is a physiological process that plays a critical role in the regulation of tissue homeostasis by ensuring that the rate at which new cells are produced in the body through cell division is offset by a commensurate rate of cell loss. Apoptosis is biochemically and morophogically different from necrosis. Programmed cell death is commonly characterized by cellular membrane blebbing, ruffling of the nuclear membrane, chromosome condensation and the appearance of DNA ladders produced by the degradation of genomic DNA into multiples of 180bp fragments. Defects in the cell death pathway are important not only for the origins of cancer, but also may markedly influence the ability to treat it. In our study of the molecular and cellular mechanism involved in cisplatin resistance in melanoma cells, we have provided evidence through our examination of the biochemical and morphological features of apoptosis, that the

retrovirally-derived cisplatin resistant variants of WM35 have an aberrant ability to undergo apoptosis following cisplatin treatment. We have demonstrated several lines of evidence suggesting an aberrant and delayed apoptosis signalling in clones E and G. Mono- and oligonucleosome fragments produced as DNA undergoes cleavage into multiples of 180bp, characteristic of cells undergoing apoptosis, were detected in the cisplatin-sensitive WM35 cells only, providing direct evidence of apoptosis occurring in WM35 following cisplatin treatment. Furthermore, propidium iodide staining of the floater cell population following cisplatin treatment demonstrated that more than double the number of cells were viable in clone E or G when compared with WM35. In addition, when floater cells were recultured in drug-free media, a large number of these cells adhere and proliferate, in contrast to WM35. Following cisplatin treatment, we have shown that the nuclear protein PARP is cleaved to produce the smaller 85kDa subunit in the sensitive WM35 that is not detectable in clone E, as analyzed by Western Blot Analysis. Since it has been documented in numerous cell lines that the cleavage of PARP from 116 kDa to 85 and 24 kDa to be a hallmark in cells undergoing apoptosis, this and other evidence suggests that a defects leading to the signals required for apoptosis mediated by cisplatin may be involved in our cell lines. In addition, caspase inhibitors, Ac-VAD-cmk (caspases-1 inhibitor), z-DEVD-fmk (caspase-3 inhibitor) and B-D-fmk (BOCDFK, a general inhibitor), which are able to prevent the cleavage of PARP, have been shown to protect more than 80% of auditory sensory cells from cisplatin-induced cell death, suggesting that blocking this cell death pathway at the caspase level effectively rescues these cells (Liu *et al.* 1998, Fuchs *et al.* 1997). The strong induction of stress-activated protein kinase (SAPK) activity in WM35 in contrast to clones E and G supports

evidence of SAPK's function in the activation of signaling pathways that result in apoptosis. Apoptosis was significantly inhibited when SAPK activation was inhibited by the expression of a dominant negative mutant form of SAPK or the upstream protein, SEK, with correlated with the acquisition of cisplatin, UV and heat resistance in a rat fibroblast cell line (Zanke *et al.* 1996, Wang *et al.* 1998). In contrast, the activation of SAPK in several human cisplatin-resistant tumor cell lines was thought to mediate repair of damaged DNA induced by cisplatin and that inhibiting its activity resulted in sensitization to cisplatin (Potapova *et al.* 1997). In the tumor necrosis factor (TNF) receptor system, SAPK activation does not lead to apoptosis induction (Liu *et al.* 1996). The role of SAPK activation in mediating apoptosis or DNA repair is currently unclear. It is possible that under different treatment conditions and/or the presence of various factors promoting apoptosis, the activation of SAPK leads to either DNA repair or apoptosis. Cisplatin stimulates the activity of SAPK in WM35 to more than 5 times its basal level of activity in stark contrast to that observed in clones E or G cells, supporting the observation of apoptosis occurring in WM35. Finally, the observed G_2/M arrest in WM35 supports these observations since it has been previously shown that a G_2 block due to cisplatin-induced DNA damage could in some cells lead to apoptosis (Barry *et al.* 1990, Eastman 1990). Cisplatin may generate DNA adducts in cells, causing G_2/M arrest of the cell cycle and subsequently leading to apoptosis (Chao 1996). Many studies have implicated defects in apoptotic pathways to be important in cisplatin resistance (Segal-Bendirdjian and Jacquemin-Sablon 1996). Resistant cells often exhibit little DNA fragmentation or morphological changes typical of apoptosis when exposed to toxic concentrations of cisplatin (Segal-Bendirdjian and Jacquemin-Sablon 1996).

To suggest that apoptosis induced by cisplatin does not occur in the cisplatin-resistant clones would be misleading. A closer examination of their cellular response to cisplatin provides evidence that apoptosis is delayed and significantly compromised in cisplatin-resistant clones E and G. The cell cycle events that occur as cells are exposed to the cytotoxic effects of cisplatin are different in WM35 and clones E and G cells; interestingly, the execution of cell cycle checkpoints and the up-regulation of cell cycle inhibitors, p21, p53, and p27 appear to occur at the exact same time points in clones E and G following cisplatin treatment. Certainly the induction of p53 followed by p21 expression in WM35 cells occurs at a much earlier time point than that observed in clones E or G cells, suggesting that the signals initiating apoptosis to occur begins by 6 hours post cisplatin treatment in WM35. This is supported by the observation that floater WM35 cells begin to appear by 12 hours following cisplatin treatment (data not shown) in contrast to clone E and G cells in which these populations were only detectable by microscopy at 18 hours. Furthermore, levels of p21 and p53 induction in WM35 is much greater than those detected in clones E and G, even at the later time points. In fact, clone E cells undergo apoptosis to a lesser extent at 18 hours, as shown by analysis of the amount of oligonucleosomes produced. Extrapolating the MTS cell viability data suggests that at higher cisplatin concentrations, the number of viable cisplatin-resistant cells would be significantly decreased, suggesting that a fundamental pathway for apoptosis is preserved in the resistant cells. Our data indicate that the mechanisms which control activation of apoptosis in WM35 cells are different from those which operate in cisplatin-resistant cells. One of the differences probably concerns the expression of Trp-2 in the resistant cells, suggesting that perhaps a scavenger system.

It may be argued that a 2-3 fold difference in sensitivity to cisplatin in our cisplatin-resistant cell lines may not constitute sufficient resistance to cisplatin since a mere increase in drug concentration may render all cells sensitive resulting in completely eradicating any existing resistant cells. However, it is important to bear in mind several important points. Clinically, cocktails of chemotherapeutic agents are given at quite toxic doses to cancer patients to prohibit any likelihood of further augmenting drug concentrations. Often even at these "therapeutic" doses, organ toxicity is prominent as observed in patients treated with cisplatin where the major toxicity is in the kidney. Therefore a 2-3 fold increase in resistance to cisplatin in our resistant cell lines can be considered significant clinically. In addition, not only the adherent cell population of these cell lines are resistant to cisplatin at the studied concentrations, but any floater populations that have detached from the extracellular matrix are still viable.

Our results suggest that the cisplatin-resistant clones have some protective mechanism(s) to endure and tolerate the cytotoxic effects of cisplatin to a greater extent than WM35. Whatever these up-stream cellular modulators may be, the final outcome is a significant delayed and inhibition of apoptosis in the cisplatin-resistant clones. The over-expression of the melanocyte-specific protein, Trp-2, suggests that cellular events regulated by Trp-2, such as the enzymatic synthesis of the melanin polymer may be important in not only protection against ultraviolet radiation but from cisplatin toxicity. This will probably lead to the eventual activation of apoptosis pathway, such as that mediated by the cleavage of PARP protein, or the induction of SAPK activity.

2.5 MATERIALS AND METHODS

2.5.1 Cell Lines and Cell Culture

WM35 was established from a patient with early stage, curable, radial growth phase primary melanoma. It was originally a gift from Dr. M. Herlyn (Wistar Institute, Philadelphia, PA) to Dr. R. S. Kerbel (Sunnybrook Health Sciences Center, Toronto, Canada). The cisplatin-resistant variants of WM35, clones A-I, were established using retroviral insertional mutatgenesis strategies, as previously described (Lu *et al.* 1995). All other melanoma cell lines were obtained from Dr. R. S. Kerbel. All cells were cultured at 37°C in a humidified atmosphere containing 5%CO_2 and was maintained in RPMI 1640 (GIBCO Laboratories, Life Technologies Inc., Grand Island, NY) supplemented with 5% heat-inactivated FBS (Hyclone Laboratories, Logan, UT) and antibiotics. In addition, cultures of cisplatin-resistant variants and transfected cell lines were supplemented with 400μg/ml of Geneticin (G418-sulfate) (GIBCOBRL Laboratories, Life Technologies Inc., Grand Island, NY). Multicellular aggregates (spheroids) were grown by culturing $1X10^5$ cells in 24-well plates previously coated with 1% agarose containing media. The number of viable cells were determined by staining with trypan blue.

2.5.2 Reagents and Antibodies

Cisplatin, carboplatinum, taxol, methotrexate were obtained from the Toronto Sunnybrook Regional Cancer Center (TSRCC) Pharmacy. Propidium iodide was purchased from Sigma Chemical Co. 3-(4, 5-dimethylthiazol-2-yl) -5- (3-carboxy methoxyphenyl) -2 (4-sulfophenyl)-2H-tetrazolium (MTS) and the CellTiter 96 AQ_{ueous} Non-Radioactive MTS Cell Proliferation Assay was purchased from Promega. Cell Death Eliza Assay was purchased from Boehringer Mannehiem.

SAPK antibody was kindly provided by Dr. Brent Zanke of the Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario. p53, p21, p27 antibodies were kindly provided by Dr. Joyce Slingerland (Division of Cancer Biology, Toronto). Bcl-2, Bax and Erk antibodies were obtained from Transduction Research. Anti-PARP antibody was kindly provided by Dr. Jorge Filmus (Division of Cancer Biology, Toronto). Anti-Trp2 antibody (PEP 8) was kindly provided by Vincent Hearing.

2.5.3 Cytotoxicity Assay

Cell viability after drug treatments was assessed using the CellTiter 96 AQ_{ueous} Non-Radioactive MTS Cell Proliferation Assay (Promega). Briefly, 8X103 cells were plated onto 96 well plates, in quadruplicate, in 200µl of media overnight. Next day, various chemotherapeutic agents were added at different concentrations to a final volume of 100µl, and incubated for 24 hours, at 37°C. Four hours prior to harvest, 20µl of the MTS/phenazine methosulfate solutions was added to each well and incubated at 37°C. MTS is bioreduced by cells into a formazan by dehydrogenase enzymes found in metabolically active cells' mitochondria and is soluble in tissue culture medium. The relative cell viability was obtained by measuring the absorbance at 490nm using an ELIZA plate reader.

2.5.4 Cell Death Eliza Assay

The Cell Death Detection Eliza assay was purchased from Boehringer Mannheim and used according to the supplier's protocol. The assay is based on a quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones which allows for the specific determination of mono- and oligonucleosomes, produced as the result of cells undergoing apoptosis in the

cytoplasmatic fraction of cell lysates. Briefly, cells were treated with 15µl of cisplatin for 18 hours and lysed in 200µl of lysis buffer for 30 min. at room temperature. After centrifugation at 200 x g for 10 min., 20µl of the supernatant was transferred into the streptavidin coated microtiter plater for analysis. 80µl of immunoreagent mix (containing anti-histone-Bi, anti-DNA-POD and incubation buffer) were added to each well and incubated for 2 hours at room temperature. After elapsed time, wells were rinsed three times with 250-300µl of incubation buffer, each time followed by gentle suctioning to remove any liquid. For photometric analysis of the amount of mono- and oligonucleosomes produced, 100µl of substrate solution was added to each well and incubated on a plate shaker until the color development is sufficient for a photometric analysis according to the positive control sample (approximately 10-20 min.). Results were obtained by using Eliza Plate Reader set at wavelength 405 nm against substrate solution as a blank.

2.5.5 SAPK/JNK Assay

Cells growing in log phase were treated with 15µM of cisplatin for 1.5 hours. Cells were lysed in 1ml of lysis buffer [50mM Tris (pH 7.4), 50mM NaCl, 2mM $MgCl_2$, 1mM EDTA (pH 8.0), 0.5% IGEPAL CA-630, and proteinase inhibitors, including 0.1mM phenylmethylsulfonyl fluoride, 100µM sodium vanadate, 20µg/ml leupeptin, 50mM NaF_2, 1mM benzamidine] for 45min on rotator, at 4°C. The insoluble material was removed by centrifugation at 14,000 X g for 15 min at 4°C. 200µg of protein was incubated with 15µg GST vector for 4 hours on rotator at 4°C and was subsequently centrifuged at 14,000 X g for 15 min at 4°C. The supernatant was incubated with 5µg of

GST-c-Jun overnight, at 4°C, on rotator. Next day, the complex was washed five times with lysis buffer containing the proteinase inhibitors described above and once with kinase buffer [20mM Hepes (pH 7.0), 20mM MgCl, 20mM benzamidine, 0.1mM sodium vanadate, 2mM DTT, 20μM ATP]. The final complex was resuspended in 30μl of kinase buffer containing 5μCi [γ-^{32}PP] ATP (supplied by Amersham) for 20 min at 30°C. The reaction was terminated with the addition of lysis buffer described above, followed by subsequent boiling for 5 minutes. The phosphorylated proteins were resolved by 10% SDS-PAGE and visualized by autoradiography. Experimental samples were examined for their SAPK content by immunoblotting with SAPK antibody. The antigen-antibody complexes were visualized with Luminol/Enhancer-Peroxide Solution Kit (Pierce, Rockford, Illnois). The level of SAPK activity in each sample was determined from integrated densitometry signals of the phosphorylated GST-c-jun bands using PhosphoImager Scan.

2.5.6 Cell Cycle Analysis

Cells growing at 50% confluency were treated with 15μM of cisplatin. At time 0, 12, 18, 24, 36, 48 hours after treatment, cells were harvested by washing with 2X cold PBS, trypsinized, and resuspended with complete medium and centrifuged at 1000 RPM for 10 min at 4°C. The pellet was resuspended in 0.5ml of media and fix with 5 ml of 70% cold ethanol for 30 min on ice. Ethanol was washed off with cold PBS after centrifugation at 1000 RPM for 10 min at 4C. The pellet was resuspended in 2 ml of 70% cold ethanol and stored in -20°C. In preparing the samples for FACS analysis, each sample was centrifuged at 1000 RPM for 10 min at 4°C and subsequently washed with 5ml of cold 1X PBS. The pellet was resuspended in 0.5 ml of propidium iodide solution

[50µg/ml PI, 0.6% NP-40, 0.5mg RNase A]. Fluorescence activated cell scanning (FACS) analysis was performed using a Becton Dickinson FACScan flow cytometer. The cell cycle profile was analyzed using the Modfit software. Floater cell population was analyzed immediately after harvest and stained with the previously described propidium iodide solution using FACScan analyzer from Becton Dickinson.

2.5.7 Western Blot Analysis

Cells were harvested once with cold PBS and harvested with tyrpsin-EDTA. Cell lysates were prepared using lysis buffer containing 50mM Tris (pH 7.4), 50mM NaCl, 2mM $MgCl_2$, 1mM EDTA (pH 8.0), 0.5% IGEPAL CA-630, and proteinase inhibitors, including 0.1mM phenylmethylsulfonyl fluoride, 100µM sodium vanadate, 20µg/ml leupeptin, 50mM NaF_2, 1mM benzamidine. Lysates were incubated on ice for 40 min and then particulate material removed by centrifugation for 10 min at 14,000 X g. Protein concentration was performed using Bradford assay. 25µg of protein was resolved in SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA) electrophoretically. Equal loading of samples and transfer to nitrocellulose was verified by staining the membrane with Amido Black. Membranes were blocked with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and 5% milk for at least 1 hour, incubated with primary antibody for 1 hour, washed for 3 X 15 min with TBS-T, and developed with horseradish peroxidase-conjugated secondary antibody for 45 min. Blots were washed again for 3 X 15 min with TBS-T, incubated with enhanced chemiluminescence substrate solution (ECL; Amersham) according to the manufacturer's instructions and exposed to Kodak X-Omat film. Biotinylated protein molecular weight markers were also purchased from Amersham.

2.5.8 Isolation of genomic DNA and Southern Blot Analysis

Cells growing in logarimithic phase were harvested with 1X trypsin-EDTA and subsequently centrifuged at 1000 RPM at 4°C. The cell pellet formed was resuspended with 1 volume of Solution A (0.2M NaCl, 0.04M Tris pH 8, 0.02M EDTA), 1 volume of Solution B (0.5% SDS) and 50µg/ml of proteinase K followed by overnight incubation in 57°C waterbath. Next day, 10µg/ml of RNase A was added to the solution and incubated at 37°C for 30 min, followed by 2 phenol/chloroform extractions. Genomic DNA was precipitated with 1/10 volume of 3M NaAc, and 2.2 volume of 95% ethanol, at –70°C for at least 30 min. Genomic DNA as quantified by spectrophotometer and 10µg of DNA was digested with various restriction enzymes overnight and resolved by electrophoresis in 2% agarose. The DNA was acid-depurinated before denaturation and transferred to nitrocellulose. Hybridization with DNA probes was performed as described below.

2.5.9 RNA Extraction and Northern Blot Analysis

Total RNA was isolated from cell lines using TRIZOL (Gibco-BRL), following the manufacturer's protocol. Briefly, cells growing in logimethric phase were harvested, washed and resupsended with approximately 1ml of Trizol. After chloroform extraction, RNA was precipitated with 500µl of isopropanol. After washing the RNA pellet with 70% ethanol, RNA was dissolved in DEPC-treated water. RNA was quantified at 260nm and 280nm using a spectrophometer. 30µg of total RNA was denatured at 65°C for 5 minutes and electrophoresed through a 1% agarose gel containing 0.66M formaldehyde. After transfer to a nitrocellulose membrane filter (Amersham), it was hybridized with DNA probes as described below.

2.5.10 Molecular Hybridization

Radiolabeled DNA probes were synthesized using the Klenow fragment of DNA polymerase I with random hexadeoxyribonucleotides (Boehringer Mannheim) by the method of Feinberg and Vogelstein (Feinberg and Vogelstein, 1983). Briefly, 50ng of DNA was denatured at 100°C for 5 minutes and labelled with 10µl of oligo-mix solution, 5µl of dCTP-P^{32} and 1µl of Klenow fragment of DNA polymerase I. Labelling was performed under the condition of 37°C for at least 30 min. Labelled probes were purified using G50-packed columns. The filters were hybridized with 2 X 10^6 cpm of G_{50}-purified random-primed probe in a hybridization mixture that contained 50% formamide, 10% dextran sulfate, 1.5X SSC (1XSSC = 0.15M NaCl/0.015M sodium citrate, pH 7), 5X Denhardt's solution (1X Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrolidone) at 42°C, over night. The filters were washed initially with two washes of 2X SSC and 0.1% SDS at room temperature for 30 minutes followed by two washes of 0.1X SSC and 0.1% SDS at 65°C for 30 minutes. Filters were exposed to Kodak XRP film at –80C with an intensifying screen.

2.5.11 PCR-Select cDNA Subtraction Hybridization

Subtractive hybridization was performed using the PCR-Select cDNA Subtraction Hybridization kit purchased from CLONTECH (Palo Alto, California) and as described (Diatchenko *et al.* 1996, Gurskaya *et al.* 1996). Briefly, total RNA was harvested from WM35 and clone E cells using TRIZOL (GIBCO-BRL Laboratories) according to the manufacturer's protocol. Polyadenylated RNAs (mRNA) were selected by affinity chromatography on oligo (dT) cellulose (Pharmacia) as described by the supplier. First- and second-strand driver and tester ds cDNA was synthesized from 2µg of WM35 and clone E poly(A)$^+$ RNA respectively using the Great Lengths cDNA Synthesis Kit

(CLONTECH) and 1ng of oligonucleotide Pr16 as a primer with T4 DNA polymerase. The resulting cDNA pellet was dissolved in 10µl of deionized water and digested by *Rsa*I or *Hae*III, followed by phenol extraction and ethanol precipitation. Half of the entire volume of digested tester cDNA (diluted in 5µl of H_2O) was ligated to adapter 1 and adpter 2 in separate ligation reactions at 16°C overnight, using 0.5 units of T4 DNA ligase (Life Technologies) in the buffer supplied from the manufacturer, followed by heat inactivation the next day. Two microliters of driver ds cDNA was added to each of two tubes containing 2µl of adapter 1- and adapter 2-ligated tester cDNA. The samples were mixed, ethanol precipitated and then resuspended in 1.5µl of hybridization buffer [50mM Hepes, pH 8.3/0.5 M NaCl/0.02 mM EDTA, pH 8.0/10% (wt/vol) PEG 8000]. The solution was overlaid with mineral oil, the DNAs were denatured (1.5 min, 98°C), anneal (10 hrs at 68°C). After this first hybridization, fresh heat-denatured driver and hybridization buffer was added and mixture was allowed to hybridized for an additional 10 hours at 68°C). This final hybridization was followed by heat inactivation at 72°C for 7 min and stored at -20°C. Two PCR amplifications were conducted in a total volume of 25µl, containing 1µl of diluted subtracted cDNA, 5µM of PCR primer P1, 5µM of PCR primer P2 and 22µl of PCR master mixture prepared using the Advantage cDNA PCR Core Kit (CLONTECH). PCR was performed with the following parameters: 75°C for 7 min; 30 cycles at (91°C for 30 sec; 68°C for 30 sec; 72°C for 2.5 min); and a final extension at 68°C for 7 min. Following dilution of the PCR product to 10 fold, 1µl was then used as a template in secondary PCR for 10 cycles under the same conditions used for the primary PCR, except PCR primer P1 and P2 were replaced with nested PCR

primer PN1 and PN2, respectively. The PCR products were analyzed by 2% agarose gel electrophoresis, acid-depurinated before denaturation and transferred to nitrocellulose. It was hybridized with the GAPDH (0.5kb) DNA probe. Products from the secondary PCRs were inserted into pCR2.1 vector using a T/A cloning kit (Invitrogen). Cloned cDNAs were amplified for 20 cycles with nested PCR primer PN1 and PN2 primers, using the similar conditions as the secondary PCR described above. 1μl of the PCR product was prepared for molecular hybridization using the Klenow fragment of DNA polymerase I with radom hexadeoxyribonucleotides (Boehringer Mannheim) by the method of Feinberg and Vogelstein (Feinberg and Vogelstein, 1983). Hybridization conditions were similar to the outline above. DNA sequencing was performed by the chain termination reaction manually. Nucleic acid homology searches were performed using the BLAST program through e-mail servers at the National Center for Biotechnology Information (National Institutes of Health, Bethesda).

2.5.12 Establishment of WM35-Trp-2 Transfected Clones

WM35-Trp-2 clones were established through the stable transfection of full length Trp-2 cDNA into WM35, using the Lipofectin (Boehringer Mannehim). The full length Trp-2 cDNA was obtained from Rick Sturm. Transfected cells were selected with 800μg/ml of Geneticin (G418-sulfate) (GIBCOBRL Laboratories, Life Technologies Inc., Grand Island, NY) for more than 2 weeks. Cells resistant to G418 were individually subcloned and expanded in culture.

CHAPTER 3

General Discussion and Future Perspectives

The incidence of malignant melanoma has increased at an alarming rate over the past few decades (Cohen and Falkson 1998). Indications are that it will continue to rise in the forseeable future. Primary prevention of malignant melanoma through education of the general public regarding the hazards of sun exposure is important in an attempt to reduce the incidence of the disease in the future. It can, however, be expected to take many years before a decrease in the number of cases of this disease is seen. Until such time, the medical oncologist will be faced with an increasing number of referrals for both adjuvant therapy and treatment of metastatic disease. About one third of all melanomas detected disseminate, with the metastatic spread occurring either via lymphatic or blood vessels. Metastatic malignant melanoma patients have a median survival of less than 1 year in the most favorable situation. In the treatment of advanced melanoma, the conventional chemotherapy or radiotherapy has not been very successful, although it has shown to exhibit immunologically provocative features (Villikka and Pyrhonen 1996). Human malignant melanoma is a unique tumor system to study drug resistance and particularly intrinsic resistance since it has been reported that chemotherapeutic agents, including cytokines, are largely ineffective in controlling the growth and metastasis of melanoma. Inherent mechanisms of resistance may be specific for the melanocyte in nature. With this in mind, the initial goal of our study was to identify genes that are differentially over-expressed or down-regulated in cisplatin-resistant variants of sensitive WM35 melanoma cells. The strategy of retroviral insertional mutatgenesis allowed advantageous genetic events in the acquisition of cisplatin resistance to be tagged and identified.

Trp-2: essential in acquiring drug resistance?

Although we have determined that over-expression of Trp-2 through transfection studies provides protection from cisplatin-induced apoptosis, however, these cells are significantly more prone to apoptosis when compared with clone E. This suggests that though the over-expression of Trp-2 may be important in the acquisition of resistance, other cellular mechanisms may be at play. In addition, is the expression of Trp-2 essential or required for a resistant phenotype in melanoma cells? To answer this, it is necessary to down-regulate the expression of Trp-2 in cell lines that abundantly over-expression this protein through antisense strategies.

The disappointments from *in vitro* models of drug resistance has stemmed from the inability to translate these findings to *in vivo* systems such as animal models and eventually patient populations. Therefore, it would be essential to compare the sensitivity of the WM35-Trp-2 clones, cisplatin-resistant cell lines, and the parental WM35 cells in immune-compromised mice harboring tumors from these injected cells. Is there significant difference in the sensitivity between these cells to cisplatin *in vivo*? Can resistance be overcome with strategies to down-regulate Trp-2 such as neutralizing antibodies and antisense technology? Certainly since experience has forewarned us against using results from animal models to extrapolate similar conclusions in melanoma patients, consequently efforts to determine its significance in patients would be significantly important.

Trp-2: by what mechanism is resistance mediated?

Melanocytes have three specific enzymes, tyrosinase, Trp-1 (tyrosinase related protein-1), Trp-2 (tyrosinase related protein-2), all of which contribute to the production of the melanin polymer that has been documented to protect against DNA damage from

ultraviolet radiation. Since we have demonstrated that over-expression of Trp-2 confers protection against cisplatin-mediated apoptosis in melanoma cells, it would be important to examine the mechanism by which the over-expression of Trp-2 serves as a cellular protector against the toxic effects of cisplatin. There are several possibilities:

Resistance via direct cisplatin-melanin association

Within the pathway of melanogenesis, there are two regulatory steps, one at the branch point of DOPAquinone, determining whether phaeomelanin or eumelanin will be the major component in melanin, and the other, determining the carboxylated nature of the polymer, by regulating the fate of DOPAchrome. Phaeomelanogenesis is the most favoured pathway under normal conditions in the melanocyte, since addition of cysteine or glutathione to DOPAquinone is approximately 1000 times faster than DOPAquinone cyclization (Ito *et al.* 1980, Prota 1980). The eumelanogenic pathway is reserved for situations of metabolic stress, when the melanocyte is submitted to ultraviolet radiation, or to the prolonged action of hormonal or melanogenic agents. Under these conditions, L-DOPAquinone undergoes rapid cyclization to L-DOPAchrome that leads to the production of melanin with an increased protective capacity. Although we have not examined the effects of cisplatin on either phaeomelanin or eumelanin production, it is reasonable to speculate the most favoured pathway in this condition of cellular stress would be that leading to the synthesis of L-DOPAchrome. The other regulatory event in the melanogenic pathway is at the point of DOPAchrome. It has been well established that Trp-2 catalyzes the tautomerization of L-DOPAchrome to the highly stable carboxylated product DHICA, which contributes to the incorporation of carboxylated units into the final melanin polymer. In the absence of Trp-2, DHI is oxidized either

spontaneously or enzymatically by tyrsoinase. Consequently, while tyrosinase directs the quantity of dopa, from L-tyrosine synthesis, addressed to melanogenesis, Trp-1 and Trp-2 regulate the quality rather than the quantity of pigment formed by governing the amount of carboxylated indole units incorporated into the polymer. Therefore, since only the expression of Trp-2 is altered without any correlation between the expression of Trp-1 and sensitivity to cisplatin in the melanoma cell lines examined, it is reasonable to suggest that the carboxylated nature of melanin, rather then the total amounts of the polymer may be significant in determining sensitivity to cisplatin. The absence of Trp-2 in the melanogenic process may lead to significant alterations in the structure and possibly the function of melanin in its ability to protect cells from free radicals produced by UVB radiation and perhaps from the toxicity of cisplatin as well. The enrichment of melanin in DHICA-derived units, as a result of Trp-2 activity, can lead to spontaneous polymerization of a smaller molecular weight and stable melanin that is brown in color and soluble in aqueous solutions above pH 5 (Arcoa *et al.* 1992, Pawelek 1991). This is in stark contrast to the black, insoluble precipitate that is formed by DHI alone, in absence of Trp-2 or when it is in molar excess to DHICA (Orlow *et al.* 1992). In keeping with this view, melanocytes carrying mutation in Trp-1 gene synthesize a light brown pigment in culture, but a dark-brown to black pigment after phenotypic rescue by a retroviral vector carrying a wild-type Trp-1 gene (Bennett *et al.* 1990). The melanin polymer with a high proportion of DHICA as opposed to DHI has been predicated to have a more ordered structures, since DHICA polymerization is restricted to the positions 4 and 7 of the indole ring (Palumbo *et al.* 1987, D'Ischia *et al.* 1991, Prota 1988). The C2 position of the indole ring of DHICA is blocked by the carboxylic group and C3 is

greatly deactivated by the electrometric effect of the neighboring carboxylic group (Aroca *et al.* 1992). Additionally, it has been shown that DHICA is less cytotoxic to cells than DHI probably due to the higher rate of nonspecific covalent binding of DHI-derived oxidized products to proteins and/or differences in the rates of generation of H_2O_2 (Salinas *et al.* 1994, Urabe *et al.* 1994). Under physiologic conditions of α-MSH (alpha-melanocyte stimulating hormone) stimulation, the levels of Trp-1 increase only moderately, Trp-2 activity remains essentially unaltered while the activity of tyrosinase would strongly increase, leading to an increase in the rate of L-DOPAchrome formation, but with no increase in the ability to convert this compound into DHICA. The accumulation of L-DOPAchrome leads to a rapid spontaneous and tyrosinase-catalyzed oxidation and polymerization of this compound into the melanin polymer thereby increasing the cytotoxicity of the pathway. Therefore the presence of DHICA-enriched melanin may differ from DHI-melanin not in order, size, and color but with inherently altered physiochemical properties, such as chelating capacity compared to the polymer obtained spontaneously via DHI (Aroca *et al.* 1992). Melanin may act as scavengers of metal ions and other agents which could be toxic to the cell by its chelating capacity located at C5 and C6 of DHI and by the indole nitrogen and carboxylic group at C2 of DHICA (Palumbo *et al.* 1987, Stravs-Mombelli and Wyler1987). Owing to the incorporation of the carboxylated units, DHICA-melanins have higher chelating capacity due to the additional center formed by the indole nitrogen and the carboxylic group at C2, as opposed to DHI which has chelating ability at only the C5 and C6 position, and a higher ability to bind drugs by electrostatic interactions (Aroca *et al.* 1992, Palumbo *et al.* 1987). This is important in the case of cisplatin. Although the structure of cisplatin is

stable and relatively unreactive in aqueous environments such as blood plasma, however, at lower chloride concentrations (e.g. intracellularly), cisplatin loses its chlorine atoms and is converted to an aquated, reactive electrophilic species, containing a positive charge, that will bind readily to nucleophilic, negatively charged cellular macromolecules. In the cisplatin-resistant cells that were examined, Trp-2 expression was augmented, probably leading to a greater production of DHICA-melanin within the cell. Carboxylic acids generally contribute to a negative charge environment at physiological pH thereby allowing the melanin polymer to act as a cellular scavengers or molecular sink, depleting the total quantity of active cisplatin available to interact with DNA within the cell. Several investigators have already postulated the possible role of melanin polymers to act as scavenger of cytotoxic agents, such as amines, free radicals and metal ions, thereby preventing undesirable cellular processes (Breathnach 1971, Larsson and Tjalve 1979, Ings 1984). Natural melanins are composed of a large amount of DHICA and small quantities of DHI monomers and are able to absorb light throughout the ultraviolet and visible spectra (Ito 1986, Kollias *et al.* 1991, Nicolaus *et al.* 1964). Moreover, the scavenging of superoxide radicals by melanoma tissue has been explained by reaction of this radical with low-molecular-mass melanins (DHICA-melanins), whereas high-molecular-mass melanins (DHI-melanin) are ineffective (Schwabe *et al.* 1989). Several reports indicate the cisplatin exerts its cytotoxic effects through the production of reactive oxygen species within cells thereby implicating that protection against this particular chemotherapeutic agent may be mediated by a similar mechanism as that of protection against UVB damage (Miyajima *et al.* 1997).

Resistance via unknown function of Trp-2

The expression of Trp-2 in the panel of melanoma cells examined not only correlate directly with their sensitivity to cisplatin but as well to ultraviolet B radiation suggesting that the mechanism which mediates UVB resistance physiologically, may also regulate their sensitivity to cisplatin (personal communication, unpublished). For example, expression of Trp-2 in the UVB-resistant variant of WM35, XR9, is many folds greater than WM35. It can be argued that ultraviolet irradiation produces thymine-thymine dimers (cyclobutylthymine) in the DNA structure and is therefore different from the interstrand or intrastrand dGpG, dApG cross-links produced by cisplatin, however, it has been shown that both types of adducts may be repaired by the endonucleases, polymerases, and ligases functioning in the excision repair pathway (Kraemer and Slor 1985). The importance of DNA repair and the melanoma are exemplified in patients with the rare inherited disorder, xeroderma pigmentosum (XP). Patients with this disease are very sensitive sun exposure and have a risk of developing skin cancer about 100 times that of the general population (Kraemer et al. 1984, Kraemer et al. 1994). Cells from XP patients are hypersensitive to killing by UV and to induction of mutations in their DNA by UV exposure due to abnormalities caused by defects in DNA nucleotide excision repair (Cleaver and Kraemer 1995, Friedberg *et al.* 1995). Work by laboratories throughout the world in the past few years has resulted in cloning of seven different DNA repair genes (XP-A to XP-G) involved in XP (Bootsma *et al.* 1995, Sancar 1995a, Aboussekhra *et al.* 1995). Mice in which the murine homologue of human XP-A and XP-C genes have been inactivated were shown to have a markedly increased susceptibility to UV induction of skin cancer (Nakane *et al.* 1995, De Vries *et al.* 1995, Sands *et al.* 1995). Increased expression of some of the many proteins participating in

the excision repair pathway has been shown to be induced in cisplatin-resistant cells. Perhaps events that govern protection from UV damage may also mediate cisplatin resistance in melanoma. It is possible that Trp-2 may signal downstream events leading to the activation of repair pathways that would efficiently repair the damaged DNA induced by cisplatin or UVB radiation.

It would be essential to understand the precise mechanism by which Trp-2 mediates resistance to cisplatin or to other chemotherapeutic agents so that effective therapy may be used to circumvent this problem. Currently, immunotherapy against different tumors from various stages of melanoma progression is actively being studied. Interest in this are of study concerned with controlling the growth and metastases of melanoma has shifted towards identifying proteins that are specifically and exclusively expression in primary, vertical growth phase and metastatic melanoma. Many studies using cell lines, fresh-frozen sections and animal models have demonstrated the down-regulation of tyrosinase, Trp-1 but not Trp-2 proteins as melanoma progresses to its more aggressive and invasive final stage. Currently, the data suggests that although anti-tyrosinase family of proteins may be effective in relatively early stages of malignancy, anti-Trp-2 therapy may be especially useful for therapeutic interventions at the later stages (Orlow *et al.* 1998). Does targeting of Trp-2 in melanoma elicit more than just immune response in melanoma? Or, as our data suggests, could the down-regulation of Trp-2 protein bring about sensitization to chemo- and radiotherapy, by either inhibiting the contribution of carboxyl nature to the melanin polymer or through the repression of repair mechanisms.

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