IMPORTANCE OF ASC EXPRESSION LEVELS IN THE DEVELOPMENT OF CHEMORESISTANCE IN HUMAN MELANOMA CELL LINES

by

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Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science

Graduate Program in Molecular Biology and Genetics
Boğaziçi University
2008

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DATE OF APPROVAL: 10.June.2008

AKNOWLEDGEMENTS

My special appreciation and thanks go to my thesis supervisor Assoc. Prof. Nesrin Özören for her endless support and belief in me. Without her inexhaustible dynamism the study could not be accomplished. It is a great honor for me to be her first graduating student.

Moreover I would like to express my special thanks to Burcu Sümer. There is no better lab-partner than her. Many thanks also to Şahru Yüksel, Metehan Çifdalöz, Ayaz Najafov, Gamze Küser, Cihan Erkut, Demet Candaş and Tolga Aslan for their friendship, help and support during my experiments.

I am extremely grateful to Erman Alpay, Mehves Cetinkaya and Ilgaz Soykal for being in my life and giving me their friendship at all times.

Last but not least I would like to thank my parents and family for supporting me throughout my education.

This study was supported by Boğaziçi University BAP-B106, TUBITAK-KARIYER 105S350 and EMBO-SDIG 1468 grants to Nesrin Özören ,TUBA-GEBIP (2006) award to Nesrin Özören and TUBITAK-BIDEP 2210 award to Damla Erdoğan.

ABSTRACT

IMPORTANCE OF ASC EXPRESSION LEVELS IN THE DEVELOPMENT OF CHEMORESISTANCE IN HUMAN MELANOMA CELL LINES

ASC (Apoptosis-associated speck like protein containing a CARD) is an adapter protein containing PYRIN and CARD interaction domains. ASC is implicated in apoptosis and innate immune signaling and is required for IL-1β and IL-18 processing via the activation of caspase 1. ASC protein expression is suppressed via methylation of its promoter regions in a high percentage of melanoma and other cancer types, including colorectal, prostate, lung, breast cancers and glioblastomas. ASC protein is absent or downregulated in 62.5 per cent of the melanoma tissue samples and in 58.3 per cent of the investigated melanoma cell lines. Most melanoma cells are highly resistant to widely used chemotherapeutic drugs, thus novel pathways and targets need to be investigated for the development of effective therapies. We aimed to clarify the role of ASC in increasing the chemo-sensitivity of melanoma cell lines, which lack its expression. ASC was re-introduced into two of these cell lines, MeWo and Skmel 19, using a lentiviral infection system, which establishes the stable expression of ASC. Control GFP vector- and ASC vector- infected cells were treated with DNA damaging drugs - doxorubicin, etoposide and dacarbazine and the death receptor ligand TRAIL. In short term viability assays we have found that ASC expression confers slight chemosensitization to doxorubicin (Skmel 19) and etoposide (Skmel 19). A more pronounced effect was observed with TRAIL in Mewo cells. More importantly, we found in long term assays that re-expression of ASC in Mewo and Skmel 19 dramatically decreased their colony forming capacity.

ÖZET

MELANOMA HÜCRE HATLARINDA ASC PROTEİNİ ANLATIM DÜZEYLERİNİN KEMO-DİRENCİN OLUŞUMUNDAKİ ÖNEMİ

ASC, PYRIN ve CARD birimleri bulunan bir adaptör proteindir. ASC'nin programlı hücre ölümünde ve içşel bağışıklık sisteminde görev aldığı belirlenmiştir. Bir yandan IL1β ve IL18 salınımlarını, kaspaz-1 aktivasyonu sayesinde gerçekleştirdiği kanıtlanmıştır. Diğer yandan ASC'nin protein anlatımının melanomalarda, kolon, prostat, göğüs, akciğer gibi kanser çeşitlerinde, promoter bölgesindeki metilasyon sonucu baskılandığı görülmüştür. ASC proteininin melanoma doku örneklerinin yüzde 62.5'inde ve hücre hatlarının yüzde 58.3'ünde baskılandığı bilinmektedir. Çoğu melanoma hücresinin, bilinen kemoterapi ilaçlarına ve radyasyona karşı direnç göstermesi, yeni etkili terapi yöntemlerinin geliştirilmesini gerektirmektedir. Bu çalışmadaki amacımız ASC genini içermeyen melanoma hücrelerine, ASC geni aktarıldığında, kemo-direnç üzerindeki etkisini araştırmaktır. ASC, Mewo ve Skmel 19 melanoma hatlarına lentivirus yardımı ile transfer edilip, protein anlatımında devamlılık sağlanmıştır.

GFP ve ASC enfekte olmuş hücrelere, doxorubisin, etoposit, dakarbazin gibi DNA ya hasar veren ilaçlar ya da TRAIL uygulanmıştır. Kısa vadeli canlılık testlerinde Skmel 19 hücrelerinde, ASC protein anlatımının doxorubisin ve etoposit'e karşı zayıf bir kemo-duyarlılık kazandırdığı tespit edilmiştir. TRAIL ile Mewo hücrelerinde daha güçlü bir etki gözlenmiştir. Daha da önemlisi, ASC içeren Mewo ve Skmel 19 hücrelerinin uzun vadeli testlerde koloni oluşturma özelliklerinin azaldığı görülmektedir.

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LIST OF ABBREVIATIONS

ALL	Acute Lymphocytic Leukemia
APS	Ammonium per sulfate
ASC	Apoptosis-associated speck-like protein containing a CARD
ATG	Autophagy-related Gene
BCC	Basal Cell Carcinoma
BiCNU	Carmustine
bp	Basepair
CARD	Caspase Recruitment Domain
CDK	Cyclin Dependant Kinase
CLL	Chronic Lymphoctic Leukemia
CRADD	CASP2 and RIPK1 Domain Containing Adaptor with Death Domain
DISC	Death-Induced Silencing Complex
DMEM	Dulbecco Modified Eagle's Minimal Essential Medium
DMEM*	DMEM supplemented with Penicillin/Streptomycin, L- Non Essential Amino acids
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR	Death Receptor
DTIC	Dacarbazine
DTT	Dithithreiol
EDTA	Ethylenediaminetetraacetic acid
EGFR	Endothelial Growth Factor Receptor
ER	Endoplasmic Reticulum
FADD	Fas Associated Death Domain
FBS	Fetal Bovine Serum
FG12	Lentiviral vector
FG12-ASC-GFP	Lentiviral vector containing ASC and GFP genes with separate promoters
FG12-GFP	Lentiviral vector containing a GFP gene

FITC	Fluorescein Isothiocyanate
FIV	Feline Immunodeficiency Virus
FMF	Familial Mediterranean Fever
GFP	Green Fluorescent Protein
HBS	HEPES Balanced Salt
HCT116	Human Colon Carcinoma
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency Virus
IAP	Inhibitor of Apoptosis
IFN	Interferon
IL	Interleukin
kb	Kilo Base
kDa	Kilo Dalton
LB	Luria Broth
LTR	Long terminal repeats
mA	Miliamper
MCS	Multiple cloning site
mRNA	Messenger ribonucleic acid
MCS	Multiple Cloning Site
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMSC	Non Melanoma Skin Cancer
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGFR	Platelet-Derived Growth Factor Receptor
pRb	Retinoblastoma protein
PVDF	Polyvinyl difluoride
RA	Retinoic Acid
RGP	Radial Growth Phase
RNA	Ribonucleic Acid

SCC	Squamous Cell Carcinoma
SDS	Sodium dodecylsulfate
SiRNA	Small Interference Ribonucleic Acid
SIV	Simian Immunodeficiency Virus
TAE	Tris-Acetate-EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween 20
TEMED	N,N,N',N',-tetramethylethylenediamine
TMS1	Target of Methylation-Induced Silencing
TMZ	Temotolomide
TRAIL	TNF-Related Apoptosis-Inducing Ligand
UTR	Untranslated region
UV	Ultraviolet
V	Volt
VEGFR	Vascular Endothelial Growth Factor Receptor
VGP	Vertical Growth Phase
Z-VAD-FMK	Benzyloxycarbonyl-Valine-Alanine-Aspartine(OMe) Fluoromethylketone

1. INTRODUCTION

Cancer is a group of diseases characterized by unrestrained cell division and the ability of these cells to invade nearby tissue and spread through the bloodstream and metastasize. People at all ages are prone to this disease since cancer can occur sporadically and in non-familial cases the risk increases with increasing age. In familial cases of cancer, patients already have mutations in cancer critical genes and therefore are predisposed to cancer (Özören & El-Deiry). When there is even a single mutation, genetic instability leads to the accumulation of other mutations rapidly. Once these mutations have accumulated in genes that regulate cell cycle checkpoints (like p53, pRb and others), apoptosis (like Bcl-2 family, caspases, death receptors, Apaf-1 and others), immortalization (telomerase) or angiogenesis, transformation occurs and the invasion of nearby vascular or lymphatic systems by over-proliferation, leads to metastasis into other tissues. Most of the time, patients with tumors can be cured because of early-diagnosis, where the tumor can be excised out, but there is still a chance of relapse. On the other hand, once metastasis has occurred, any type of tissue can be affected, in which case directed therapies are required. Chemotherapy and/or radiation therapy are the most commonly available treatment types.

Cancer is one of the major causes of death, especially in developed countries. Globally there are over 25 million people with cancer; about seven million of which die and 11 million new cases are expected every year. A quarter of the deaths in the United States are of cancer (Jemal et al., 2006) and cancer is just second to heart disease, as a leading cause of death in Turkey (Turkish Association for Cancer Research and Control, 2000). There are about 166 cancer related deaths per 100.000 people in Turkey, according to a study by Jemal et al. 2002, and expected new cases are over 150.000 per year.

Many of the cancer incidences reported are sporadic and environmental factors such as inappropriate diet, smoking tobacco, exposure to asbestos or prolonged UV-exposure, have been show to increase the risk of cancer development. Melanoma of the skin can be of both familial and sporadic occurrence. Over 68.000 new cases were expected in the United States in 2006 and the probability of developing invasive malignant melanoma is

one in 52 males and one in 77 females. With this expectation rate, melanoma is sixth in more than 45 different types of cancers, as a cause of death (Jemal et al., 2006).

1.1. Molecular Biology of Cancer

Cancer is a disease under the influence of two different groups of genes: proto-oncogenes which are activated to become oncogenes, and tumor suppressor genes which are mutated to become inactive. A single mutation is not enough for a normal cell to progress into a cancerous one. Only the stepwise accumulation of mutations in different genes can form a cancerous cell.

Proto-oncogenes become activated by acquiring derangements such as, promoter mutations or gene duplications, increasing the mRNA product, chromosomal translocation, causing gene activation in the wrong tissue type and mutations that increase activity of the protein product. Oncogenes promote the growth, proliferation and immortalization of the cell. Examples are, receptor tyrosine kinases (EGFR, PDGFR, and VEGFR), GTPases (Ras protein), Serine/Threonine kinases (BRAF, CDK's) and transcription factors (Myc). Proto-oncogenes are activated by gain-of-function mutations and therefore a hit in only one of the alleles will have a phenotypic effect (Stehelin et al., 1976).

Unlike proto-oncogenes, tumor suppressors usually need both of the alleles to be mutated. A loss-of-function mutation results in the absence of an important regulatory protein. One major example is the Retinoblastoma gene, mutant forms of which were identified as the cause of retinoblastoma, a tumor of the eye (Friend et al., 1986). The protein product Rb-1 is involved in the G1/S checkpoint of the cell cycle. Once E2F, a transcription factor, is relieved from its inhibitor pRb, it is free to activate S phase genes, constitutively, leading to uncontrolled cell cycle progression. p53, another tumor suppressor protein, which is mutated in 50 per cent of human cancers, is the key regulator of DNA damage repair and cell cycle checkpoints (Finlay et al., 1989). Once the DNA is damaged by double-strand breaks or replication errors, p53 is activated to induce repair mechanisms. In case of severe DNA damage this protein is also able to activate apoptotic

pathways leading to the death of the cell, so that no further progeny of this defective cell is produced. As a result, p53 is named as the ‘guardian of the cell’ and is found mutated in more than half of all cancer types.

Tumor suppressor genes are subject to point mutations, deletions, insertions, inversions, frameshift mutations and other mutations that inactivate the function of the protein product (Sherr, 2004).

Many cancers also originate from viral-oncogenes. Human viruses directly linked to cancer development are papillomavirus, hepatitis B virus, Epstein-Barr virus, and human T-lymphotropic virus. These viruses carry in their genomes oncogenes over-produced inside the cell and lead to, for example, inhibition of the retinoblastoma protein. This would have the same effect as a loss-of function mutation in the gene encoding pRb. In other viral infections, the viral promoter is inserted in front of a proto-oncogene, which starts to be over-expressed and becomes an oncogene (Carrillo-Infante et al., 2007).

1.2. Programmed Cell Death and Its Importance in Cancer

Programmed cell death, apoptosis, is the death of a cell by an intracellular program. The name programmed cell death stems from the fact that, apoptosis occurs within defined steps and additionally requires gene activity. Consequently it is different from simple necrosis, which results mostly in the ruptured destruction of the cell (Özören, 2003).

Apoptosis is an essential process in embryonic development, shaping of the immune system and sustaining tissue homeostasis (Kerr et al., 1994). Disrupted regulation of apoptosis can lead to neurodegenerative disorders, autoimmune diseases and even cancer. In order for the organism to restrain these chaotic situations, the balance between cell death and cell division must be preserved throughout its life for each tissue (Figure 1.1). Therefore it is highly relevant to understand the interactions between players involved at each stage of the apoptotic cell death.

There are two major and several minor pathways that can commence apoptosis: the DNA damage and stress induced intrinsic pathway is mainly coordinated through the mitochondria and the extrinsic pathway through death receptors. Both ultimately result in the permeabilization of the mitochondrial membrane and release of cytochrome c, DNA fragmentation and membrane blebbing (Schuler, 2005).

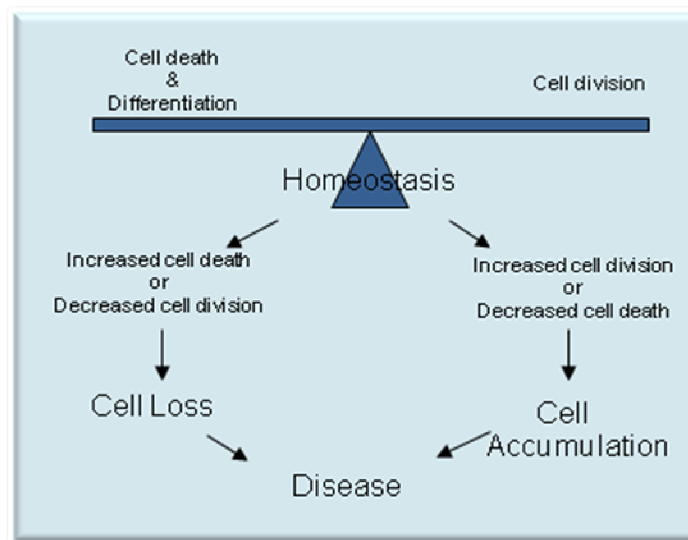


Figure 1.1. Crucial balance between cell division and cell death. When either of the sides of the balance is disturbed it results in disease in the organism.

The intrinsic pathway leads to the activation of Bcl-2 family members that are pro-apoptotic, whereas the anti-apoptotic members of this family are suppressed during apoptosis. Proteins like Bax, Bak and Bad are activated and form pores in the mitochondrial membrane such that cytochrome c is released. At the next phase the formation of the apoptosome together with cytochrome c, Apaf-1 and caspase 9 initiates the caspase cascade and leads to the destruction of the cell which includes inactivation of IAPs, disassembly of cell structures and activation of more apoptotic proteins (Green, 2005).

The afore mentioned crucial players in apoptosis, the members of the Bcl-2 family are divided into two main groups: Anti-apoptotic members, which have similarity to Bcl-2 itself and pro-apoptotic members. The latter is further divided into Bax-like and BH3 domain-only protein groups. Anti-apoptotic members mainly are transmembrane proteins

and are located in the mitochondrial membrane and their role is to maintain mitochondrial integrity. Some are also present in the ER and nuclear membranes. The pro-apoptotic members can be either transmembrane or cytosolic proteins and some are found on the cytoskeleton of normal cell. When an apoptotic signal arrives, they bind to and inhibit anti-apoptotic members, so that cytochrome c is released from mitochondria and the death pathway is initiated (Youle and Strasser, 2008).

All Bcl-2 family members are composed of different combinations of four domains, called the **B**cl-2 **h**omology (BH) domains. There are four such domains (BH1, BH2, BH3 and BH4), each of which gives a defined function to the protein that contains them. Bcl-2 was identified at the Philadelphia translocation t (14:18), placing Bcl-2 after the IgG promoter (Tsujimoto et al., 1984; Clearly et al., 1986) and is the founding member of the family. The protein is regulated by phosphorylation at a serine residue by MAP kinase or Caspase 3 (cpp32). Another anti-apoptotic protein is Bcl-XL which has BH1, BH2 and BH3 domains that form a hydrophobic pocket for interaction with pro-apoptotic proteins, like Bax (Kuwana, 2005). Bax has high homology to Bcl-2 and is a cytosolic protein that translocates to the mitochondria when a death signal arrives. It can form pores and aid the release of cytochrome c. Thereby, Bax oligomerizes with Bak which functions in a very similar way with Bax. Bak also acts together with a BH3 only protein BID upon its cleavage (tBID), to release cytochrome c (Thomadaki, 2006).

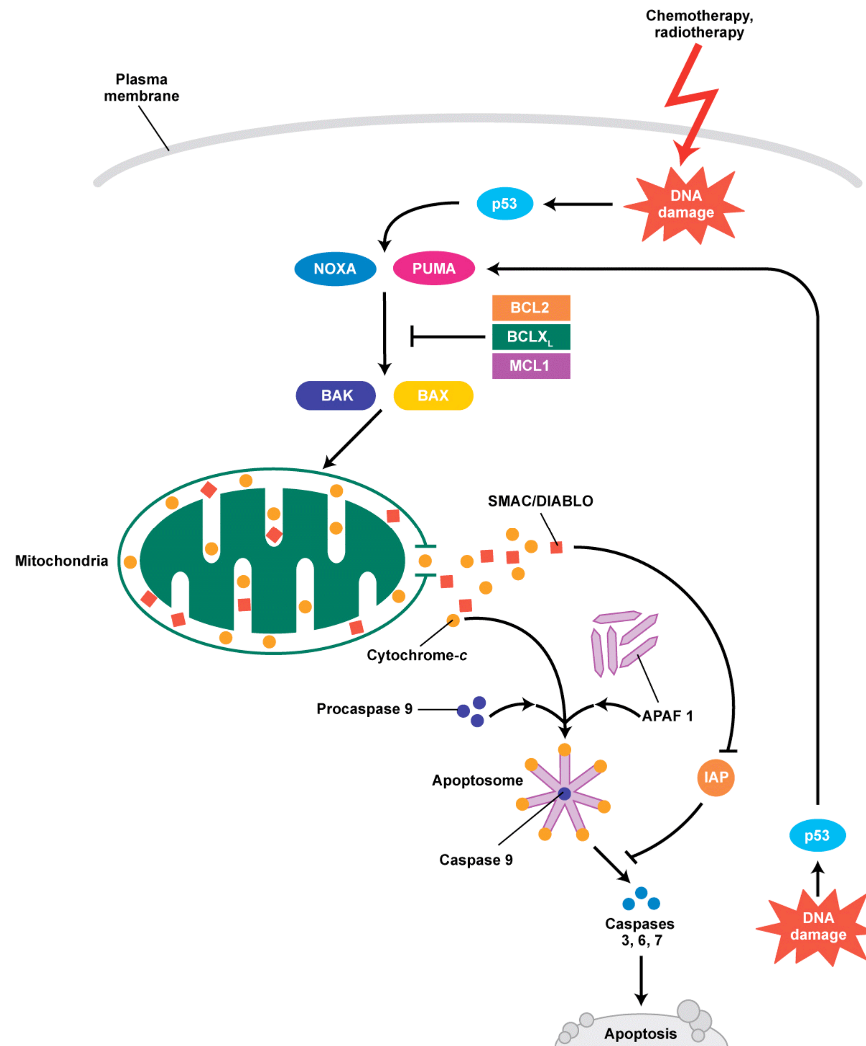


Figure 1.2. Intrinsic pathway of apoptosis. Adopted from Ashkenazi 2002

In the death receptor pathway the signal emerges from neighboring cells bearing death ligands such as FasL, TNFα or TRAIL that bind to death receptors on the surface of the cell to be "killed" (Wang and El-Deiry, 2003). According to the type of adapter protein recruited, TNFα can both provide survival and death signals. If this is FADD the signal is apoptotic if it is RIP or TRAF-2, the cell survives via Nuclear factor-kappa B (NFκB) signaling. The adapter proteins together with Caspase 8 (and Caspase 10) form the death-induced signaling complex (DISC) that transmits the signal downwards (Peter and Krammer, 2003).

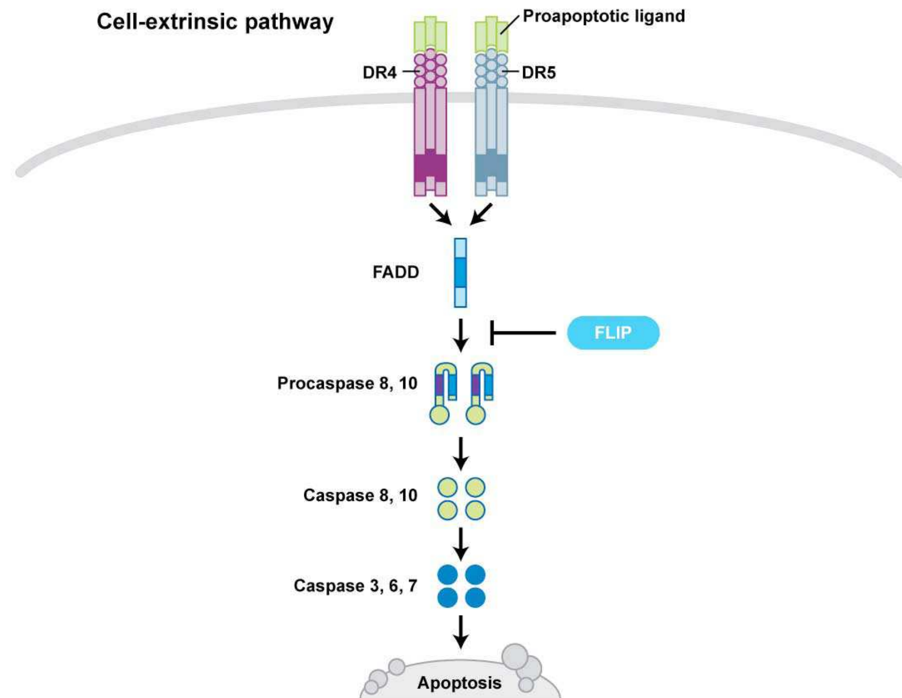


Figure 1.3. Extrinsic pathway of apoptosis (Death receptor pathway). Adopted from Ashkenazi 2002

Another form of death is ER-stress induced apoptosis. ER-stress occurs as a result of accumulation of unfolded proteins, glucose deprivation, aberrant calcium regulation or viral infections (Xu et al., 2005). Normally the cell acts to restore homeostasis, however prolonged stress can lead to cell death. Activation of the transcription factor NFκB, leads to the transcription of proteins in the suicide program.

ER-induced apoptosis has a common path with the intrinsic pathway of apoptosis through the activation of caspase 9. However other caspase are activated upstream of caspase 9 which are, caspase 4 and caspase 12. High concentration of calcium in the cytoplasm or receptor ligand associations at the ER membrane relay down the signal to the mitochondria (Boyce and Yuan, 2006).

A recent advancement in the area of cell death includes autophagy or self-eating of the cell. A basal level of autophagy is always present in a cell and it is required for the turnover of organelles, pathogens, cytoplasm and also proteins. A double membrane autophagosome is assembled one by one with the activation of autophagy-related genes

(Atg), which fuses with the lysosome forming the autolysosome (Vicencio et al., 2008). The major negative regulator of autophagy is mTOR. This protein is inhibited during starvation and leads to the dephosphorylation of Atg-13. Thereby this protein shows a higher affinity for the Atg-1 kinase and their interaction initiates autophagy (Yorimitsu and Klionsky, 2005).

The primary role of autophagy is the adaptation of the cell to nutrient-deprivation, heat or hypoxia but it is also utilized as a backup system for apoptosis when it is inhibited or when cells cannot be removed by macrophages (Levine and Yuan, 2005).

1.3. Melanoma of the Skin

Melanoma, like all other cancer types, arises from the accumulation of mutations in different genes through the lifetime of a person. Predisposing factors are a history of severe sun-burn, fair-haired and/or fair-pigmented phenotype, freckles and intense sun-exposure, rather than cumulative sun-exposures (Hayward, 2003). Besides sporadic occurrences familial cases of the disease are also frequent. These families are ideal for linkage analysis and identifying candidate loci for melanoma development.

Each time a mutation is acquired that changes the growth, survival and death criterion of the cell, different stages of tumor growth and metastasis occur. These mutations may also affect the attachment of the cell to the substratum and their capability of maintaining angiogenesis in the nearby tissue matrix. Identifying changes that lead to more advanced stages in melanoma development, as well as metastasis, will be informative for generating novel treatments for patients (Figure 1.4).

Skin cancer is one of the most common types of malignancy and by far the most dangerous one (Gray-Shopfer, 2007). An early prognosis is important for surgical removal of the tumor and increasing the life expectancy of the patient. However, once the tumor has spread, there is no effective treatment for melanoma and the 5-year survival rate for melanoma is less than 5 per cent. Melanoma progresses through the formation of a benign nevus towards a dysplastic nevus which has an irregular shape and color. At this stage the nevus is able to advance into metastasis (Figure 1.4).

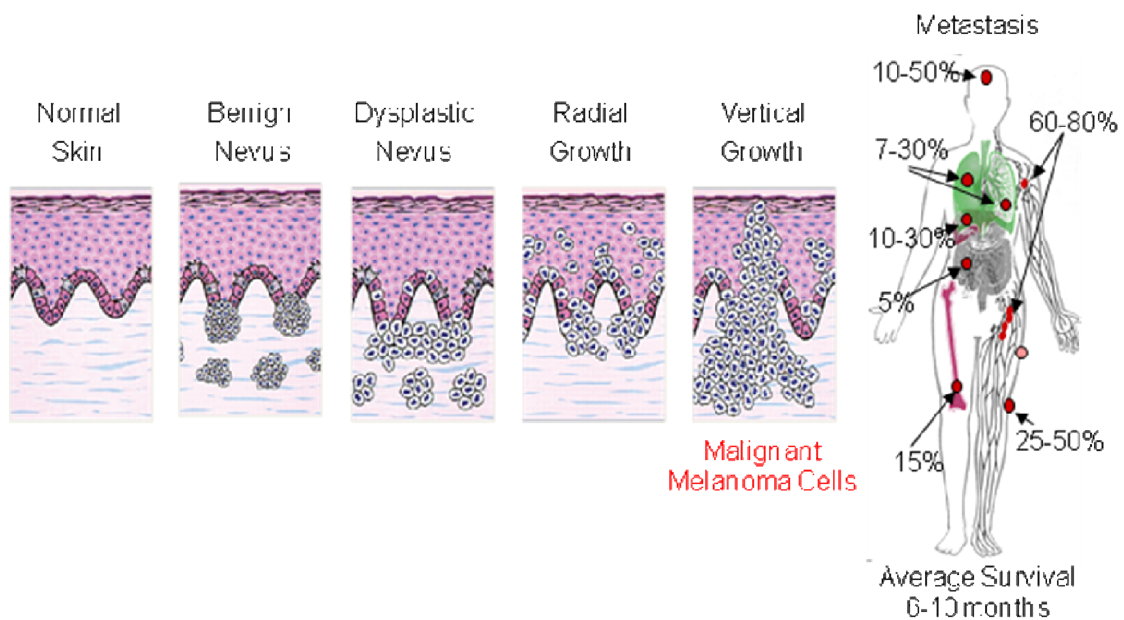


Figure 1.4. Malignant melanoma progression. Adopted from M.S. Soengas.

1.3.1. Skin Cancer and Melanoma Biology

The skin is the largest organ of the body and contains pigmentation provided by the melanocytes in the basal layer of the epidermis. Keratinocytes which are found together with melanocytes are in a larger number and control the growth of the melanocytes. The lower part of the skin, the dermis, is composed of mostly collagen and fibroblasts that produce it. The skin has functions in protection, heat regulation, storage and excretion.

Skin cancer can arise through the transformation of epidermal keratinocytes, which causes a non-melanoma skin cancer (NMSC). Its basis is the cumulative exposure to UV light which is in contrast to the intermittent and intense UV exposure in cutaneous melanoma that occurs by the transformation of melanocytes. Both arise on the sun exposed parts of the body and NMSC are the most common type of skin cancer however less severe than cutaneous melanoma. Types of NMSC are basal cell carcinoma (BCC) occurring in the basal layer of the epidermis and squamous cell carcinoma (SCC) arising in the squamous cells that compose most of the upper layer of the skin (Boukamp, 2005).

Generally melanocytes are tightly regulated by paracrine signaling from the keratinocytes and their proliferation is restricted to the boundary between the epidermis and the dermis (Figure 1.5). However, DNA damage in growth and proliferation regulatory genes, apoptotic genes as well as genes controlling the adhesion of melanocytes to their substratum causes loss of their control by keratinocytes (Chudnovsky, 2005). Once transformed, they are able to invade the epidermis and form naevi. Naevi, although benign, can cause the invasion of nearby epidermal tissue by a radial growth phase (RGP) (Figure 1.4). At this phase the disease is called melanoma. The next step is the vertical growth phase (VGP), in which cells are able to invade the underlying dermis and reach out to the lymphatic and the vascular system and become malignant (Chudnovsky, 2005) (Figure 1.5).

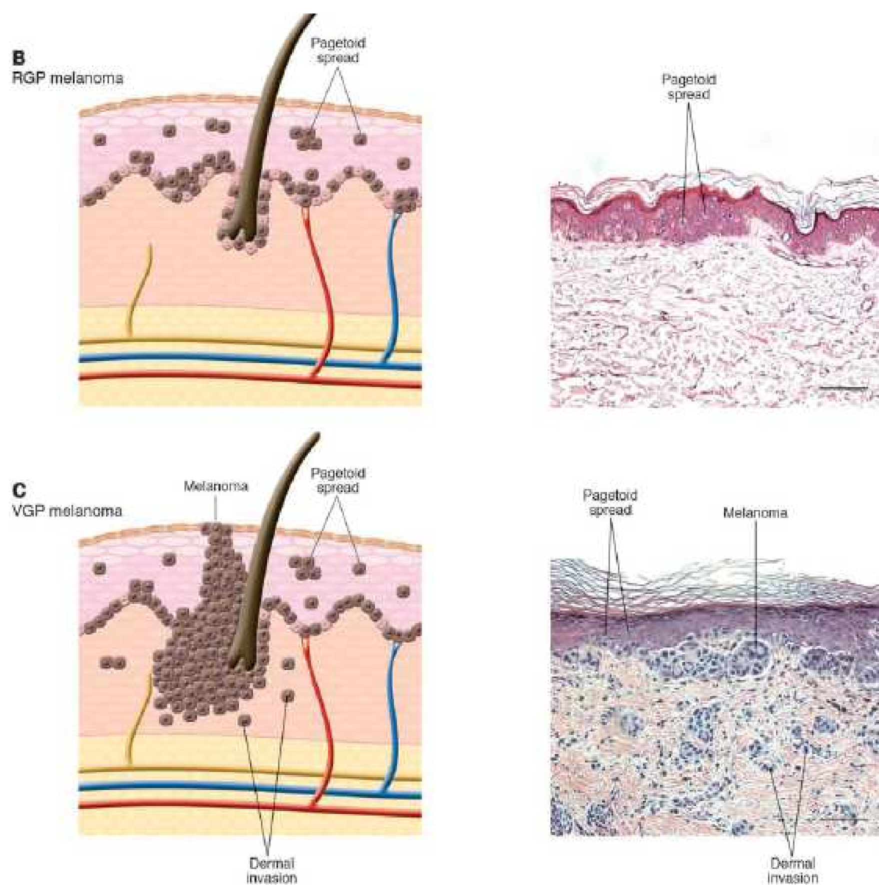


Figure 1.5. Radial and vertical growth phases of transformed melanocytes. Adopted from Chudawski, 2005.

1.3.2. Recent Progress in Melanoma Genetics

A major step toward finding new treatment opportunities for malignant melanoma is the determination of commonly disrupted genes in melanoma patients. The advances in our molecular understanding of melanoma development have come quite slowly. Genes commonly mutated in melanoma are both transcripts of the CDKN2A locus, BRAF, Apaf-1 and PTEN.

Linkage analysis in melanoma patients of familial origin identified 9p21 as a candidate locus for this type of cancer (Cannon-Albright et al. 1992). CDKN2A gene was linked to this locus and it codes for a cell cycle inhibitor protein $p16^{INK4a}$ gene. This protein functions as a cyclin-dependent kinase (CDK) inhibitor and is deactivating the G1 CDK, CDK4/cyclin D1 complex in the G1/S transition of the cell cycle. Together, they make up a checkpoint that tightly controls the Retinoblastoma (pRb) protein that is blocking the action of a major protein in S phase initiation, E2F. The absence of $p16^{INK4a}$ in melanoma patients leads to the release of CDK4/cyclin D1 which is free to phosphorylate pRb and release E2F from its inhibitor. E2F is now free to induce transcription of S phase genes leading to DNA replication and the cell can proliferate further without this crucial checkpoint control mechanism (Bartkova et al., 1996).

The principle types of mutations found in the CDKN2A gene are deletions, but $p16^{INK4a}$ loss also occurs through transcriptional silencing by methylation and also by post-transcriptional events (Morita et al, 1998 and Rubben et al., 2000). CDK4 mutations were also detected in a few of the predisposed melanoma patients. This mutation did not affect the cyclin binding efficiency but lead to decreased binding efficiency of CDK4 with $p16^{INK4a}$.

The fact that certain sporadic cases of melanoma showed linkage to the locus 9p21, but no mutation could be detected in CDKN2A, lead to the conclusion that, other candidate genes might be present in this locus. The gene for the protein $p19^{ARF}$, another cell cycle inhibitor was identified as having overlapping reading frames with $p16^{INK4a}$ (Quelle et al. 1995). $p19^{ARF}$ and $p16^{INK4a}$ have common exon 2 and exon 3 sequences and differ in their first exon.

CDKN2A mutation carriers have a greater than 50 per cent risk of developing melanoma in their 80's (Cannon-Albright 1994; Newton Bishop et al. 2000). Moreover, melanoma patients, found to contain a p16 ^{INK4a} mutation by its own or in combination with BRAF, NRAS or PTEN in isolated lesions, show a survival rate of less than 2 years after lymph node surgery (Daniotti et al., 2004).

BRAF is a Serine/Threonine kinase, activated by binding to Ras which leads to the activation of the MAP kinase pathway via the PI3 kinase and Protein kinase A pathways. In 50 per cent of melanomas the BRAF mutation V599E is present in the early radial growth phases. Mutations of the BRAF gene are seen at a higher percentage in melanoma compared to other cancer types (Davies et al., 2002). They are also maintained in metastatic lesions and can be involved in acquiring a metastatic capacity. BRAF mutations are mostly seen in melanomas arising in body sites susceptible to UV radiation (Shinozaki et al., 2004). The importance of BRAF is underscored by the fact that in one study repression of BRAF V599E mutation by siRNA application was found to abrogate melanoma cell growth (Hingorani et al., 2003). As players of the same signaling pathway, NRAS and BRAF mutations were found to be mutually exclusive in melanomas. NRAS codon 61 mutation is detected in 30 per cent of melanomas. In some melanoma samples, NRAS and BRAF mutations could not be seen but MAPK activation was detected nonetheless.

A study done by Baldi and co-workers on 40 patients with metastatic and non-metastatic melanomas showed weak Apaf-1 expression in 12 patients and absence of Apaf-1 in 22. Furthermore, there is a higher expression of Apaf-1 in thick melanomas with respect to thin melanomas. Changes in Apaf-1 expression also seem to be involved in progression from local invasive to metastatic melanomas (Baldi et al., 2004).

A similar work indicates that the chemoresistance of melanoma cell lines is due to the loss of Apaf-1. In many other cases, for example in colorectal cancer (Bunz et al., 1999), chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL) or lung cancer (Lowe et al., 1994), loss of p53 protein increases chemo-resistance. Melanoma cell lines, which retain p53 and are Apaf-1 negative are also resistant to chemotherapeutic

drugs such as doxorubicin (adriamycin), which normally activates p53 protein to induce apoptosis (Soengas et al., 2001).

PTEN was first identified as a tumor suppressor and cloned in 1997 (Li et al., 1997). In the same year a report about mutations in the PTEN gene in 30-40 per cent of melanoma cell lines (Guldberg et al., 1997) was published, followed by a paper stating ten per cent of primary melanomas also contain mutation in the PTEN gene (Tsao et al., 1998). Ectopic expression of PTEN decreases the tumorigenicity in melanoma cells (Hwang et al., 2001). PTEN is a cytoplasmic phosphatase that is involved in cell cycle regulation and cell death. It downregulates Akt , MAPK and anti-apoptotic proteins such as Bcl-2 and activates pro-apoptotic caspases. PTEN also shows reciprocal inactivation with Ras oncogene, because they are controlling the same protein, Akt (Tsao et al., 2000).

Besides these genes which show mutations in a higher percentage of melanomas, other genes have been implicated, including Noxa, E2F, Trail-R, Bcl-2,Bcl-XL,FLIP, IAP and survivin (Soengas and Lowe, 2003).

1.3.3. Resistance of Melanoma to Chemotherapy and Apoptosis

Melanoma cells are thought to be extraordinarily resistant to chemotherapy because melanocytes are differentiated from motile cells with a particularly high survival capability (Soengas and Lowe, 2003).

Today, melanoma is the least treatable cancer type with a very high resistance to any kind of treatment. Dacarbazine applications, as well as combination strategies fail to give constructive results. The only significant treatment seems to be surgical removal of the tumor and even in this case there is a high chance of relapse. Moreover the survival rate is inversely related to the thickness of the tumor.

Some of the major forms of drug resistance occur due to aberrant transport proteins, enzyme-activation, increased DNA repair and modulation of the apoptotic pathways, the latter being the most important one involved in melanoma chemo-resistance (Helmbach et al., 2001). Many of the drugs widely applied in cancer therapy induce p53, in order to lead

a cell towards cell cycle arrest and death. Therefore p53 gene is target in transformation of cancer cells. However, p53 expression is lost or mutated in only 10 per cent of melanomas (Straume and Akslen, 1997, Lassam et al., 1993 and Albino et al., 1994) and apoptosis cannot be induced in a regular manner. The mutation rate of p53 is not as high as CDKN2A but it is known that downstream targets of p53 are also inactivated. In melanoma, genes involved in the apoptotic pathway are abnormally expressed or are shut down by mutations, deletions or epigenetic silencing. Some of these are anti-apoptotic genes, whose expressions are increased to favor the survival of the cell and others like Apaf-1, various caspase activators, death receptor ligands and pro-apoptotic Bcl-2 family members are decreased in their expression. Chemo-resistance, therefore, found in melanoma samples and cell lines, is probably the result of aberrant functioning of the apoptotic machinery.

Dacarbazine (DTIC) is the only single-agent chemotherapeutic drug that is FDA approved for treatment of advanced melanoma. Other drugs such as carmustine (BiCNU), paclitaxel (Taxol), temozolomide (TMZ) and cisplatin and have also shown single-agent activity in metastatic disease. Furthermore, combination of chemotherapy followed by immunotherapy with Interferon alpha (IFN-α) and Interleukin 2 (IL-2) or simultaneous chemo-immunotherapy have been tested in phase II trials with a response rate of 40-60 per cent (Tarhini and Agarwala, 2006).

Other studies emerging in the field of melanoma therapy point in the direction of inhibiting oncogenes. Drugs developed include, Bcl-2 antisense oligonucleotides, CDK inhibitors, Ras, Raf and Mek inhibitors, Protease inhibitors of the NF-KB survival and angiogenesis pathways and the PI3 pathway involving AKT/PKB kinase (Gray-Schopfer et al., 2007). However, many questions about melanoma development still remain unanswered.

1.4. ASC: A New Target in Melanoma

ASC, (Apoptosis associated speck-like protein containing a CARD) was first described in 1999 by Masumoto et al., as a 22 kDa protein, aggregating during apoptosis into speck-like bodies. ASC is essentially a soluble protein residing in the cytoplasm, however it forms aggregates after the induction of apoptosis with retinoic acid. The gene is mapped to the region 16p11.2-p12 and the protein contains 195 amino acids, including a pyrin-like domain (1-91) and a caspase recruitment domain (CARD) (107-195) Figure 1.6.

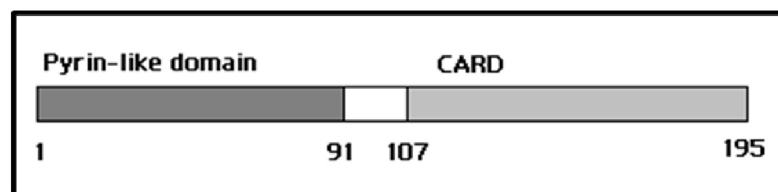


Figure 1.6. Domain structure of ASC, containing a PAAD/PYRIN domain between amino acids one and 91 and a Caspase recruitment domain between amino acids 107 and 195.

The CARD and the PYRIN domains are part of the death fold family and contain six conserved α-helical folds (Chou, et al.,1998). The CARD domain is found in apoptosis related proteins as well proteins involved in innate immunity, i.e., caspases 1, 2, 4, 5, 9, 11, 12, 14, Apaf-1, Ipaf, Nalp-1, Cryopyrin-1 (Inohara and Nunez, 2003). MNDA, IFI16 and AIM 2 are putative tumor suppressors that contain a PYRIN domain and are downregulated in melanoma (DeYoung, 1997). Mutations in the PYRIN domain of certain genes are also associated with a number of autoimmune diseases, such as Familial Mediterranean Fever (FMF).

ASC expression in normal tissues is found in the placenta, anterior horn cells of the spinal cord, renal tubules of the kidney, seminiferous tubules and Leydig cells of the testis, hepatocytes and interlobular bile ducts of the liver, hair follicles, sebaceous and eccrine glands of the skin, epithelial cells in the colon, peripheral blood leukocytes, squamous epithelium of the tonsil and skin. No ASC expression was discovered in the ciliated epithelium of the trachea, glomeruli of the kidney, cardiac muscle, alveolar epithelium of the lung or lymphocytes (Massumoto et al., 2001).

Because of its dual character containing both PYRIN and CARD domains, ASC has a critical role in inflammation and innate immunity as well as in apoptosis and cancer development (Shiohara et al., 2002).

1.5. ASC Connection to Inflammation and Cancer

1.5.1. ASC Connection to Inflammation

Nod-like Receptors (NLRs) are cytosolic proteins activated upon recognition of pathogen stimuli. These proteins also contain a PYRIN or a CARD domain that enables further signaling through the inflammasome (Akira et al., 2006). With its dual character ASC plays a critical role in the innate immune defense. The PYRIN domain enables ASC to interact with NLRs and at the same time the CARD domain provides a docking site for pro-caspase-1, which is activated to induce IL-1β secretion (Martinon et al., 2002). Moreover, ASC knockout data provide evidence about the requirement of ASC for caspase 1 activation and IL-1β and IL-18 secretion in macrophages infected with *Listeria monocytogenes* (Özören et al., 2006).

Another role for ASC was suggested in linking PYRIN domain proteins to NF-κB activation in overexpression studies. NFκB, is a transcription factor that regulates the immune response upon infection and can be induced by stress, cytokines, free radicals, ultraviolet irradiation, and bacterial or viral antigens. NFκB activity is inhibited by the IκB protein. IκB itself is depredated and NFκB is free to enter the nucleus (Stehlik et al., 2002). However, these data could not be confirmed in cells from knockout mice (Özören at al., 2006)

1.5.2. ASC Connection to Cancer

The bipartite structure of ASC suggests a function for this protein not only in the inflammatory response but also in apoptosis. With its CARD, ASC is actively involved in apoptosis when it is over-expressed in different cell lines. On the other hand, the discovery that ASC expression is downregulated or even lost in breast cancer, melanoma, colon, prostate, ovarian cancers and glioblastomas, indicates an important role for it in

tumorigenisis. Considering the fact that cells must override the apoptotic program in order to convert to cancer cells, downregulation of ASC in a methylation-induced fashion might be contributing to this escape.

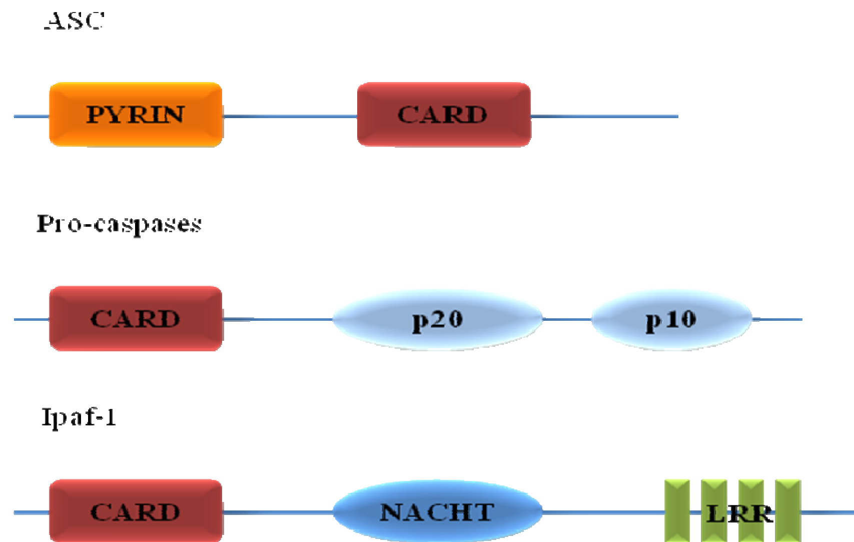


Figure 1.7. Domain structures of ASC, Pro-caspases and Ipaf-1.

1.6. ASC Regulation in Cancer and Apoptosis

ASC was identified in the cytosolic part of HL60 cells after induction of apoptosis with retinoic acid (RA) and the development of monoclonal antibodies against an insoluble fraction obtained from these cells. It was shown to form aggregates speck-like formations during programmed cell death and therefore was given the name ASC (Masumoto et al., 1999) (Figure 1.8.).

A separate group identified the same protein in 2000 while looking for aberrant methylation of genes upon ectopic expression of DNMT1 in breast cancer samples and named it ’target of methylation-induced silencing 1’ (TMS1) (Conway et al., 2000).

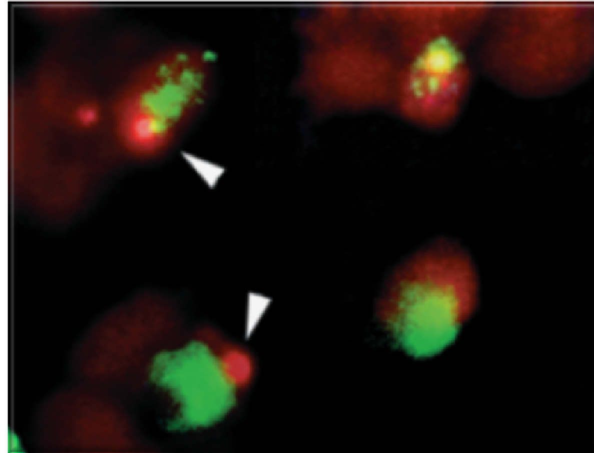


Figure 1.8. ASC protein aggregates in apoptotic cells (Taken from Masumoto, 1999).

1.6.1. Role of Pro-apoptotic ASC Protein In Cell Death

After the identification of ASC as a pro-apoptotic protein different papers about the nature of ASC and its involvement in apoptosis were published. In general, they were stating that apoptosis involving ASC can occur in a caspase-1, caspase-8 or caspase-9 dependent manner.

ASC was shown to be promoting apoptosis in a caspase 9 dependent manner in HEK 293. At the same time, ASC aggregation occurred in the absence of caspases when inhibited with Z-VAD-FMK, which disturbed the processing of general caspases. Therefore the authors speculate a role for ASC upstream of caspases leading to their activation (Mc Connell and Vertino, 2000).

Pyrin, the mutated gene in Familial Mediterranean Fever (FMF) was also found to be colocolized to the speck-like aggregates of ASC and was shown to be favoring apoptosis when present in combination with ASC in these aggregates (Richards et al., 2001).

Another result by Masumoto et al. in 2003 showed a role for active Ipaf-1 and ASC in initiating apoptosis. Ipaf-1 is a CARD containing protein which recruits ASC and caspase-1 to the inflammasome. This complex in turn leads to autoproteolytic activation of caspase-1 and consequent IL-1β and IL-18 maturation (Hiscott et al., 2006). Active Ipaf-1

and ASC are shown to induce apoptosis via caspase-8 dependent pathways which was inhibited by Z-VAD-FMK.

Furthermore, p53 knockdown has been shown to abrogate ASC induction and ectopic expression of ASC increases apoptosis in Saos2 cells. In addition, a p53 induced protein, Bax, was shown to be important in reducing the sensitivity to ASC induced apoptosis, when absent in HCT 116 cells (Ohtshuka et al., 2004). The authors state that the induction of apoptosis with Etoposide shows increased death also in Bax-null cells. Taken together, ASC is involved downstream of p53 in inducing apoptosis in Bax dependent manner. However this is not the only way that leads from p53 through ASC towards apoptosis.

It is known that apoptosis as well cancer can behave differently in different kinds of cell types and there is a redundancy in conditions and pathways that may lead to the death of the cell or its survival.

1.6.2. Epigenetic Methylation in Cancer

In normal cells CpG dinucleotides are methylated by DNA methyltransferases DNMT1, DNMT3a and DNMT3b on the fifth carbon of cytosines (Bestor, 1988; Okano et al., 1998). DNA methylation is a developmentally controlled process that is relayed down to daughter cells by maintenance methyltransferases. Early in the embryo the parental strands are demethylated and then *de novo* methylation takes place between the 8-cell and the blastocyst stages (Howlett and Reik, 1991).

CpG islands that are found in the promoter regions and the first exons of housekeeping genes are hypomethylated, although they are excellent sites of methylation. However, these regions are subject to active transcription and furthermore they are thought to be protected by chromatin structure, replication timing and active demethylation via RNA transcripts from CpG islands themselves.

In cancer cells, on the other hand, there is aberrant methylation of tumor suppressor genes or proto-oncogenes, where, each type of cancer seems to have its own methylated gene profile. Hypomethylation results in activation of proto-oncogenes and latent transposons and promotes chromosomal instability. It is also known that chromosomal instability due to mutations in the genome also results in disrupted methylation in cancer cells. In most of the cases DNMT1 transcript levels are elevated, for example, 2.5-3.7 fold in colon cancer (Lee et al., 1996) or 4.2 in leukemia (Melki et al., 1998).

Hypermethylation of promoter region is known to be present in CDKN2A (p16 and p14), p15, BRCA1, GSTP1, p73, APC (Esteller et al., 2001). Loss of pRb expression by hypermethylation is also a known process of inactivation of a tumor suppressor gene in some cancers (Simpson et al., 2000). Whereas **hyper**methylation is responsible for malfunctioning of tumor suppressors, **hypo**methylations result in activation of proto-oncogenes. A major example is the increase in expression of anti-apoptotic genes like Bcl-2. Once they are activated, anti-apoptotic genes prevent the death of the cancer cell and result in continued proliferation.

1.6.3. ASC Is Subject To Methylation-Induced Silencing In Melanoma and Other Cancer Types

The ASC protein was first shown to be downregulated in 44 per cent of breast cancer cell lines upon induction of DNA (cytosine-5-)-methyltransferase 1 (DNMT1) (Conway et al., 2000). Furthermore in 2003, Guan and coworkers showed ASC promoter methylation in human melanoma. In 62.5 per cent of the melanoma tissue samples and in 58.3 per cent of the investigated melanoma cell lines, ASC expression was reduced or absent. More importantly the reduction of ASC protein was observed more in malignant melanoma samples than in benign melanocytic naevi, designating a possible role of ASC in transforming naevi into metastatic cells. The reduced expression of ASC was found to be methylation-induced, because after treatment with 5-aza-2'-deoxycytidine, which reversed the methylation on Cytosine residues, the expression pattern of ASC returned to normal.

Similar results were obtained in a study with 15 ovarian cancer cell lines, of which 6 had no ASC expression and 2 had significantly lower levels of ASC present (Terasawa et al., 2004). Moreover, a decrease in histone acetylation rate around the ASC promoter was detected and the addition of a histone deacetylase inhibitor together with 5-aza-2'-deoxycytidine increased ASC expression more than when 5-aza-2'-deoxycytidine was added alone.

ASC expression was also found to be decreased or completely absent due to partial or complete methylation of the promoter in prostate cancer cell lines (Das et al., 2006), in colorectal cancer (Ohtsuka et al., 2006) as well as in glioblastoma (Martinez et al., 2005).

ASC being a target of methylation-induced silencing in different types of cancers indicates its important role in transforming normal cells into benign tumors and even malignant cancer cells. Presumably, as an apoptosis inducing protein, ASC has to be shut down or its expression has to be downregulated in order for the cancer cell to survive the apoptotic death-clock and proliferate further.

2. PURPOSE

ASC is a small, cytoplasmic, pro-apoptotic protein that has been identified as the cause of a morphological change in the cell upon induction of apoptosis (Masumoto et al.,1999). It is able to form speck-like aggregates during cell death. Another feature of this protein is the fact that its expression is being suppressed due to methylation induced silencing in various cancer types, such as breast cancer (Conway et al., 2000), ovarian cancer (Terasawa et al., 2004), colorectal cancer (Ohtsuka et al., 2005), glioblastoma (Martinez et al., 2005) or prostate cancer (Das et al., 2006). More interesting is the fact that 62.5 per cent of the melanoma tissue samples and in 58.3 per cent of the investigated melanoma cell lines exhibit reduced ASC expression or the expression is completely lost (Guan et al., 2003).

Furthermore, melanoma is a very resistant form of cancer that presently does not have a successful treatment. Chemotherapy combined with immunotherapy and/or radiotherapy has no or little affect in melanoma patients and their survival rate is as low as 5 per cent (Soengas and Lowe, 2003).

We hypothesized that ASC may be a putative tumor suppressor gene and reintroduction of ASC protein into melanoma cell lines devoid of its expression, will render them more chemosensitive. To test our hypothesis we followed the steps outlined below:

- Re-introduction of ASC protein into melanoma cell lines lacking ASC expression and showing resistance to chemotherapy, using a lentiviral delivery system;

- Test of chemosensitization of *de novo* ASC expressing cells upon drug treatment in short term assays;

- Test of the colony formation potential of *de novo* ASC expressing cells in long term assays.

3. MATERIALS AND METHODS

3.1. Chemicals, Plastic and Glass Ware

All chemicals used in this study were purchased from Sigma-Aldrich (USA) or Merck (Germany) unless otherwise stated in the text. Cell culture media and supplements were obtained from Gibco (Invitrogen, USA). All plastic ware for cell culture purposes were acquired in sterile packages (TPP, Switzerland) and glassware was sterilized by autoclaving at 121°C.

3.2. Cell Lines and Cell Culture

Melanoma cell lines Skmel 19, 28, 103, 147 (kindly provided by M.S. Soengas-University of Michigan, Ann Arbor,USA), Mewo, Malme3M (kindly provided by Mehmet Öztürk- Bilkent University, Ankara,Turkey) and the human embryonic kidney cell lines HEK 293FT (M.S. Soengas) and HEK 293T (Mehmet Öztürk) were grown in DMEM supplemented with 10 per cent FBS, 1mM Sodium Pyruvate, 1X L-Glutamine (Gibco, Invitrogen, USA), 1X Penicillin/Streptomycin and 1X MEM NNA (DMEM*). Cell lines were grown at 37°C and 5 per cent CO_2 and stored in 7 per cent DMSO at -80°C.

3.3. Plasmids

pHCMV-G : Contains VSV-G gene, which provides viral entry into the cell , an HCMV promoter and ampicillin resistance gene.

pRSV rev : Contains the reverse transcriptase gene

pMDLg : Includes gag and pol genes

(Dull et al., 1998, Naldini et al., 1996, Miyoshi et al., 1998)

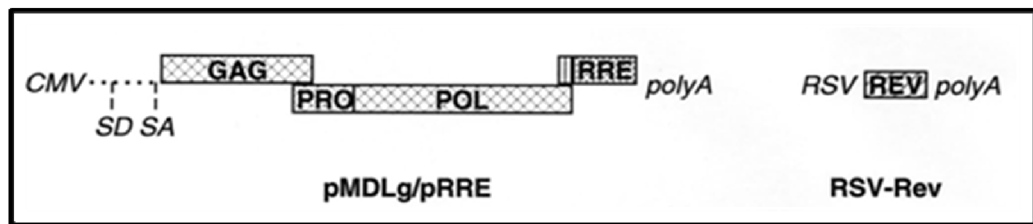


Figure 3.1. pMDLg and pRSV-rev plasmids. Modified from Dull et al., 1998.

The control FG12-GFP vector is depicted in Figure 3.2. and contains the GFP gene with an ubiquitin C promoter. The FG12-ASC vector has the ASC gene cloned into the multiple cloning site (MCS) with a CMV promoter and the GFP gene under the control of its own promoter.

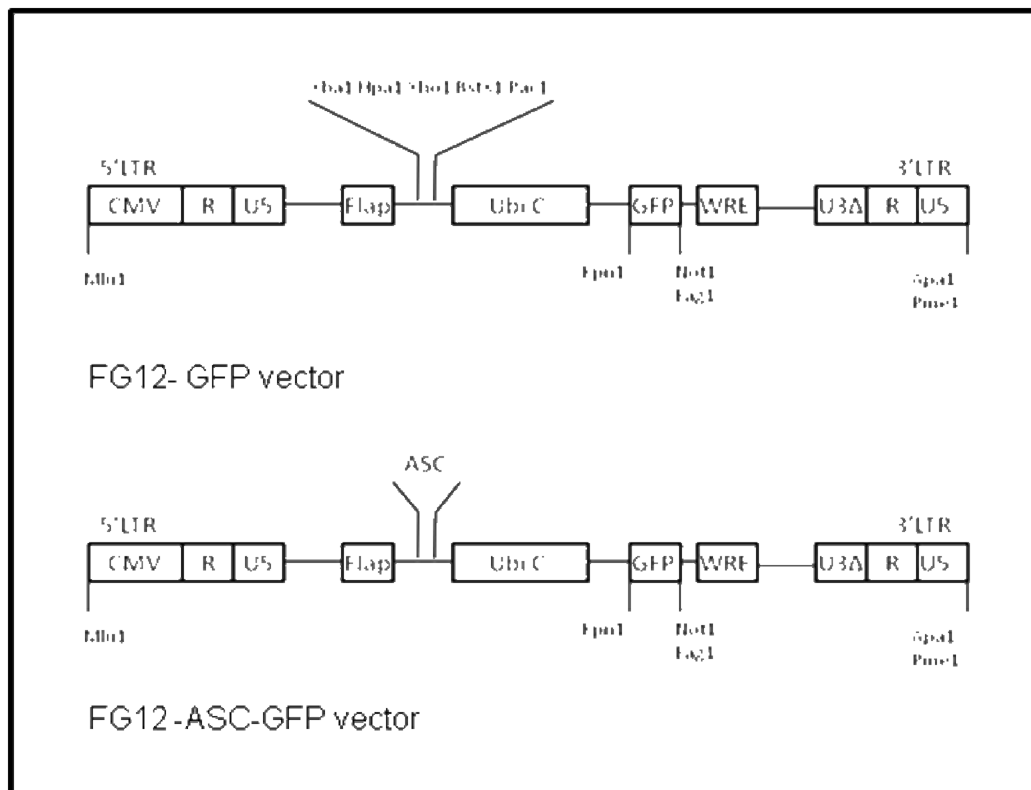


Figure 3.2. FG12 vector constructs.

3.3.1. Competent Cell Preparation

Stock of frozen JM109 E.Coli cells were streaked on LB agar plates (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl and 18 g/l agar) and incubated at 37 ^{o}C. A single, isolated colony was inoculated into 2.5 ml liquid LB medium (10 g/l tryptone, 5 g/l yeast extract and 5 g/l NaCl) for overnight culture at 37 ^{o}C. This culture was used to start a culture of 25 ml which grows until OD_{595} reaches the log phase at around 0.4. Cells were centrifuged at 3000 rpm and 4 ^{o}C for 10 minutes. Pellets were resuspended gently with a pipette in 12.5 ml ice-cold sterile 50 mM $CaCl_2$. The bacteria were incubated on ice for 30 minutes and centrifuged at 3000 rpm and 4 ^{o}C for 10 minutes. Cells were recovered in 2.5 ml of ice-cold sterile 50 mM $CaCl_2$ containing 10 per cent glycerol and stored as 100 µl aliquots at -80 ^{o}C.

3.3.2. Transformation of Bacteria by Heat-shock

The vectors FG12-ASC-GFP, pRSV, pHCMV-G and pMDlg/pRRE were transformed into separately competent JM109 cells. 100 µl of competent cells were used for a single vector transformation.

Competent bacterial cells were thawed on ice for 15 minutes and one µl of plasmid vector (150-200 ng/µl) was added to 100 µl competent bacteria. No DNA was added to a negative control tube. The mixture was incubated for 10 minutes in an ice/water bath and then heat-shocked in a 42 ^{o}C water bath for 40 seconds. The tube was immersed into an ice/water bath for two minutes. One ml of SOC (0.5 per cent yeast extract, two per cent trypton, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) medium was added and the cells were incubated for one hour at 37 ^{o}C with shaking. 10 µL, 100 µL and 250 µL of culture was spread on LB agar plates containing 100 µg/ml ampicilin and grown overnight at 37 ^{o}C.

3.3.3. Plasmid Isolation

Single, well isolated colonies were selected and grown overnight in 5ml liquid LB medium and plasmids were isolated with a QIAprep® Spin MiniPrep Kit (Qiagen, Germany) according to manufacturer's instructions. Their quantity and quality was checked with agarose gel electrophoresis. To obtain higher amounts of plasmid DNA for lentiviral transfection, Endofree® Plasmid Maxi Kit (Qiagen, Germany) was utilized.

3.3.4. Agarose Gel Electrophoresis

DNA samples were mixed in a 1:6 ratio with 6x Loading Dye (Fermentas, Germany) and loaded onto a 1 per cent agarose gel prepared with 1X Tris-Acetate-EDTA (TAE) Buffer (40mM Tris, 20mM acetic acid and 1 mM EDTA) and 0.5 µg/ml ethidium bromide. Gene Ruler 100kb (Fermentas, Germany) was used as a DNA ladder and the gel was run at 120V in 1X TAE. Bands were visualized with UV light and documented with GelDoc imaging system (BioRad,USA)

3.4. Lentiviral Transfection into Packaging Cell Line HEK 293 T

HEK 293T viral packaging cells were plated the day before transfection into 10 cm dishes at $5*10^6$ cells/ml. Prior to transfection medium was replaced with 9 ml of DMEM* containing 25 µM chloroquine. A 500 µl DNA - calcium chloride ($CaCl_2$) solution was prepared with final concentrations of 250 mM calcium chloride, 4 µg of helper plasmids pHCMV-G, pRSV rev, pMDLg and four µg of FG12-ASC. 2X HEPES Buffered Saline (HBS) solution with 280 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4 • $2H_2O$, 12 mM dextrose (D-glucose) and 50 mM HEPES with a pH adjusted to 7 with 0.5 N NaOH, was filter sterilized with a 0.22 µm filter. The DNA-$CaCl_2$ was added drop wise to the HBS Buffer and the solution was applied drop wise onto HEK 293T cells. Cells were incubated at 37°C and 5 per cent CO_2 and the medium was changed after eight hours (Figure 3.3). Biosafety level 2 applies in handling dishes and tubes containing viruses. All trash is bleached first and autoclaved before disposal. Disposable coats and double gloves were used.

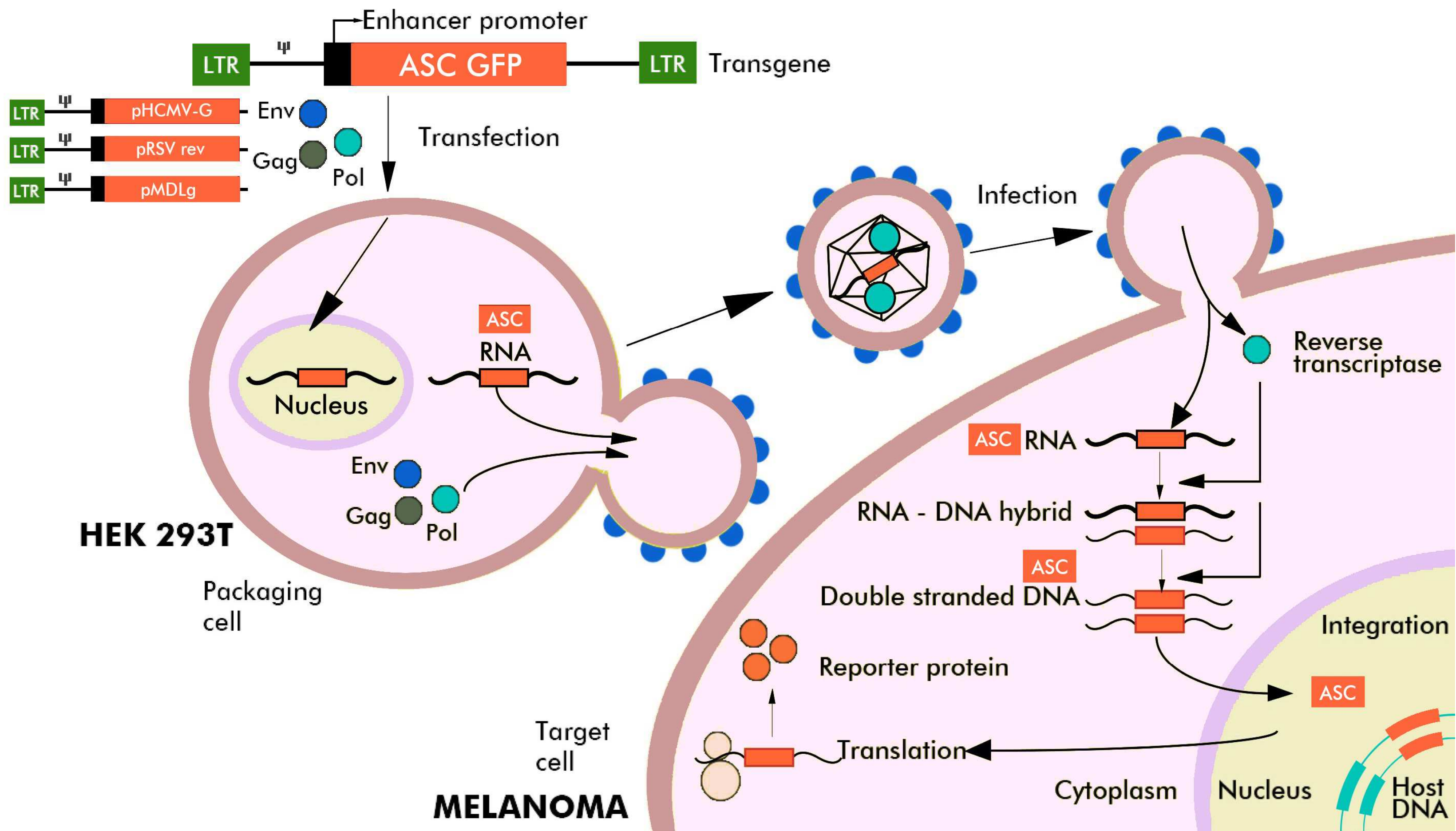


Figure 3.3 Lentiviral transduction of melanoma cells. FG12-ASC, pHCMV-G, pRSVrev and pMDLg vectors are transfected into the packaging cell line HEK293T. There the ASC gene is transcribed first into RNA and then reverse-transcribed and inserted into the cells' genome randomly. The packaging cell line produces viral compartments derived from the other three vectors thereby releasing complete viruses into the supernatant. The supernatant is collected and applied onto the melanopma cells, which integrate ASC into their genome and start to express ASC stably.

3.5. Lentiviral Infection of Melanoma Cell Lines

Melanoma cell lines to be infected were plated on the same day as the transfection into 10 cm dishes at $5*10^6$cells/ml. On the next day, fresh medium was added onto melanoma cells with 4 µg/ml polybreen. The supernatant from transfected HEK 293FT cells was collected with a syringe and filtered through a 45 µm filter onto Mewo and SKMEL 19 melanoma cell lines. HEK 293FT cells were supplied with 6ml of DMEM* medium for a second infection after five hours. Medium was changed after a total of eight hours to eliminate polybreen. GFP production could be monitored after 48 hours (Figure 3.3).

Table 3.1. Summary of derivative cell lines. Nomenclature to be used after lentiviral infection

Cell Line	Vector delivered	New Cell Line 1st Infection	New Cell Line 2nd Infection
HEK 293T	pHCMV-G, pRSV rev, pMDLg' FG12-GFP	-	-
HEK 293T	pHCMV-G, pRSV rev, pMDLg' FG12-ASC	-	-
Skmel 19	FG12-GFP	Skmel 19-FG12-GFP	Skmel 19-FG12-GFP-1
Skmel 19	FG12-ASC	Skmel 19-FG12-ASC	Skmel 19-FG12-ASC-1
Mewo	FG12-GFP	Mewo-FG12-GFP	Mewo -FG12-GFP-1
Mewo	FG12-ASC	Mewo-FG12-ASC	Mewo-FG12-ASC-1

3.6. Drug Treatment and MTS Viability Assay

Cells were plated a day prior to drug treatment into 96-well plates at 4000 cells/well for doxorubicin, etoposide and dacarbazine (DTIC) and 10^4 cells/ml for TRAIL treatment. Treatment concentrations and lengths were as shown in Table 3.2..

Table 3.2. Treatment conditions for doxorubicin, etoposide, DTIC and TRAIL.

	Drug	Cells plated/ml	Concentration of drug	Treatment length (h)
DNA damage drugs	Doxorubicin	4000	1 µg/ml	0, 24,48,72
	Etoposide	4000	25 µg/ml	0, 24,48,72
	Dacarbazine	4000	250 µg/ml	0, 24,48,72
Death Receptor Signaling	TRAIL	10 000	100 ng/ml	0,16

Following treatment with the respective drug concentrations, the viability of the cells was assessed with CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay) (Promega, USA). This assay measures the amount of a tetrazolium compound (yellow), [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, brown) which is reduced by the cells to a soluble formazan product (Cory et al., 1991). The conversion is accomplished by the electron donator NADPH or NADH reductase, therefore requires the presence of live cells. The absorbance of the product is measured at 490 nm with a microplate reader and percent viabilities are determined by taking the OD measurement at hour zero as 100 per cent and calculating the other viabilities respective to this value.

3.7. Bradford Assay

To determine the concentration of proteins in cell lysates Coomassie Plus-The Better Bradford Assay Kit (Pierce,USA) was used according to manufacturer’s manual. Absorbance was measured at 595 nm with Versamax Microplate Reader (Molecular Devices). Protein concentrations were calculated according to the standard curve prepared with BSA, supplied with the kit.

3.8. SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

10^6 cells per well were plated into 6-well plates, one day before treatment. Cells were treated with the drugs and harvested with 200 µl of 4X Laemmli Sample Buffer (250 mM Tris-HCl (pH 6.8), 20 per cent 2-mercaptoethanol, 8 per cent SDS, 40 per cent glycerol and 0.1 per cent bromophenol blue) and kept at -20°C.

Samples were boiled at 95°C for five minutes and run on a 15 per cent polyacrylamide gel (50 per cent Acrylamide:Bis-acrylamide (29:1), 10 per cent 1.875M Tris.HCl (pH 8.8), 0.1 per cent SDS, 1 per cent ammonium per sulfate (APS), 0.1 per cent N,N,N',N'-tetramethyylethylenediamine) with a 5 per cent stacking gel (16 per cent Acrylamide:Bis-acrylamide (29:1), 10 per cent 1.25M Tris-HCl (pH 6.8), 0.1 per cent SDS, 1 per cent APS, 0.1 per cent N,N,N',N'- tetramethylethylenediamine)

The gel was run in 0.3 per cent (w/V) Tris-HCl, 1.44 per cent (w/V) Glycine, 0.1 per cent SDS buffer at 70V until the samples reached the resolving gel and the voltage was increased to 150V.

Blotting papers (Sigma-Aldrich, USA) and PVDF membrane (Milipore, Ireland) were cut to the size of the gel to be transferred and wet in cold transfer buffer (0.293 per cent (w/V) glycine, 0.582 per cent (w/V) Tris-base, 25 per cent Methanol (pH 9)) and methanol, respectively. Semi-dry transfer was done at (area of polyacrylamide gel x 5) mA for 45 minutes.

The PVDF membrane was incubated in 5 per cent fat-free milk powder in 0.9 per cent (w/V) sodium chloride, 10 per cent 1M Tris-HCl (pH 7.5), 1 per cent Tween (TBST) for 1h and in 1° antibody in 1 per cent milk powder in TBST overnight. On the next day the membrane was washed in TBST for 3x2', 1x30', 1x15' and incubated with the 2° antibody conjugated to horse radish peroxidase (HRP) in 1 per cent milk powder in TBST for 1h. The previous washing steps were repeated. Lumi-light Western Blotting Substrate (Roche, Germany) was applied onto the membrane for five minutes and the bands were visualized on the Lumi-light chemiluminescence film (Roche, Germany).

Table 3.3. Antibody concentrations and suppliers.

1° Antibody	kDa	Dilution	Company	2° Antibody	Company
ASC	22	1:10	Supernatant/Masumoto	Anti-Mouse	Pierce
β-tubulin	55	1:1000	Cell Signaling	Anti-Rabbit	Pierce
Caspase-3	17/19/35	1:1000	Cell Signaling	Anti-Rabbit	Pierce

3.9. Caspase 3 Activity Assay

$8x10^6$ cells were plated into 10 cm plates and treated with 100 ng/ml TRAIL, 100 ng/ml TRAIL and Z-VAD-FMK (CaspACE Assay System, Colorimetric, Promega) or left untreated for five hours, unless otherwise stated. Cells were harvested by trypsinization and washed once with ice-cold PBS. Cell Lysis Buffer (CaspACE Assay System, Colorimetric, Promega) was added and tubes were incubated on ice for 10 minutes. With the help of a 0.5 ml syringe, cells were mechanically sheared and the cell debris was centrifuged down at 15000g and 4°C for 20 minutes. 25-100 µg total protein was used for the Caspase Activity Assay (CaspACE Assay System, Colorimetric, Promega) according to manufacturer's manual. The absorbance was measured after 4 hours at 405 nm with a microplate reader (Versamax, Molecular Devices).

3.10. Soft Agar Colony Assay

A 1 per cent noble agar stock solution was prepared from which top and bottom layers of agar were mixed with sodium pyruvate (1 per cent), L-Glutamine (1 per cent), Penicilin/Streptomycin (1 per cent), Non-essential amino acids (1 per cent) and DMEM (1.4 per cent w/V). $2x10^4$ cells per well were plated and incubated at 37°C and 5 per cent CO_2. Every 7 days 200 µl DMEM*/well was supplied to each well. Colonies were defined as populations with ten cells or more and were counted after 3 weeks.

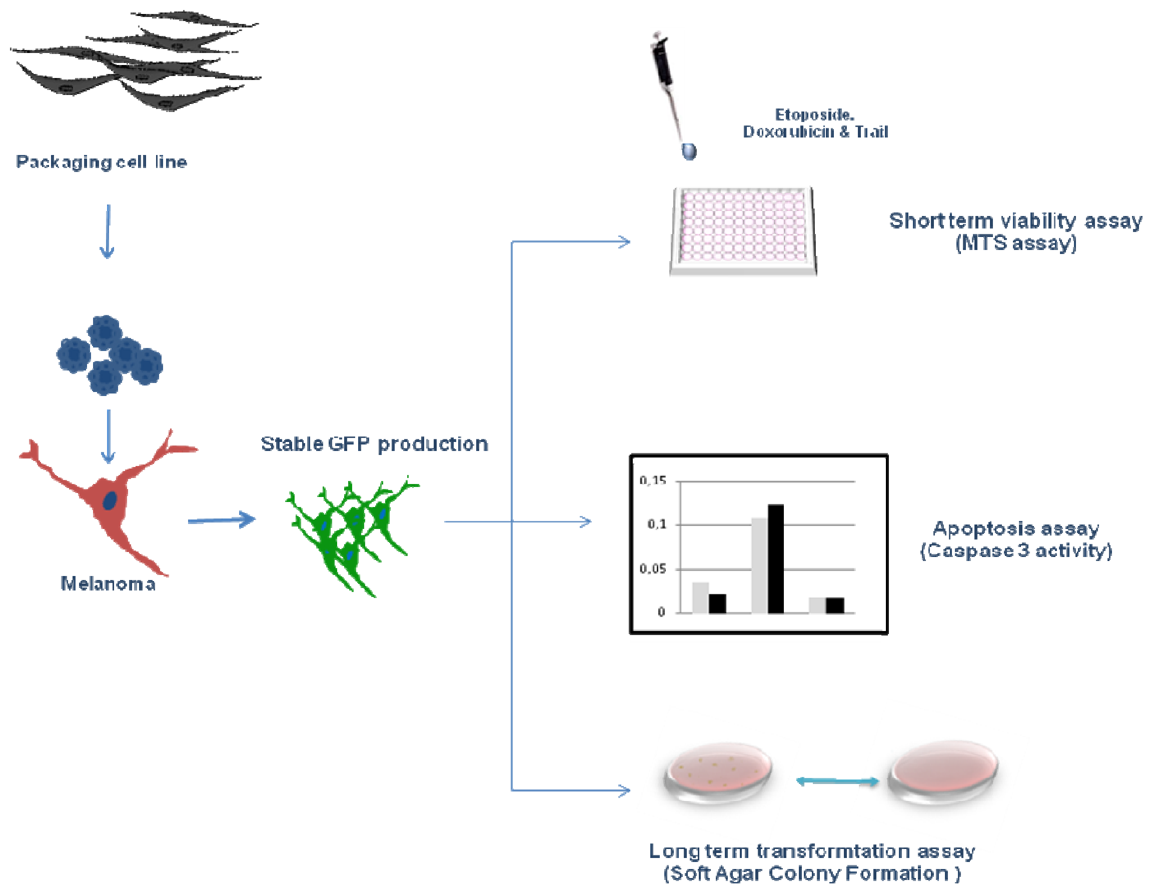


Figure 3.4. Outline of research methodology. Lentiviruses containing the desired gene were generated in packaging cell lines and melanoma cells were infected. These start to produce the desired gene stably after a few days. Transformed cells are subjected to short term viability assays after drug treatments, caspase activity assays and long term soft agar colony formation assays.

4. RESULTS

4.1. Melanoma Cell Lines

Melanoma cell lines Skmel 19, 28,103, 147 and the human embryonic kidney cell lines HEK 293FT were obtained from M.S. Soengas (University of Michigan, Ann Arbor,USA) and MeWo, Malme3M were kindly provided by Mehmet Öztürk (Bilkent University) (Figure 4.1.).

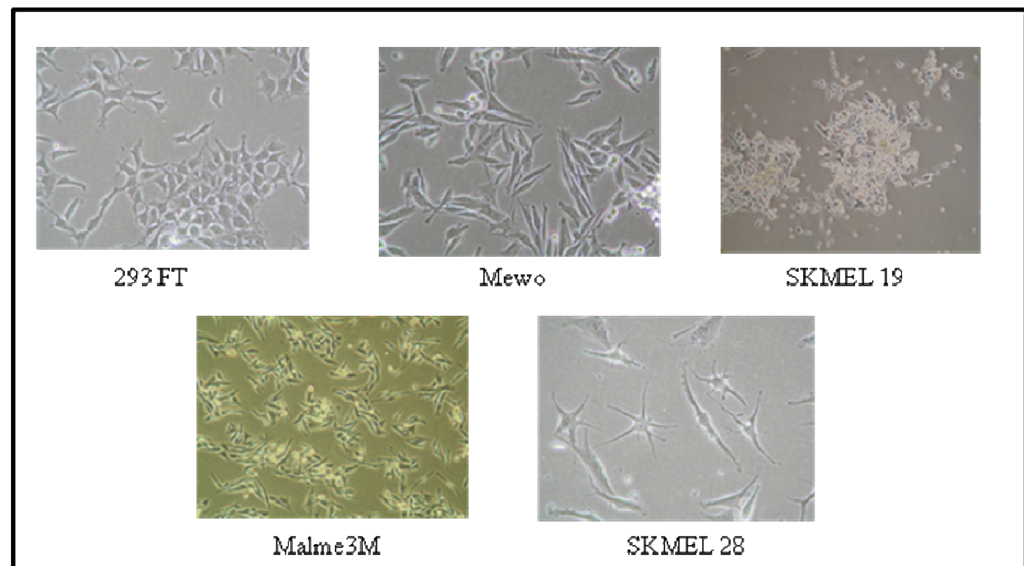


Figure 4.1. Morphology of melanoma cell lines Mewo, Skmel 19, Malme3M, Skmel28 and human embryonic kidney cell line HEK 293 FT. Pictures were taken with a digital camera mounted on a bright light inverted microscope at a magnification of 100X (Nikon Eclipse TS2100).

The cells were successfully cultured and stored in the newly established laboratory conditions.

4.2. Only Two Out of Six Different Cell Lines Show ASC Expression

As previously stated, ASC protein expression is downregulated in about 60 per cent of melanoma cell lines by promoter methylation. In order to assess the ASC expression quality of the cell lines obtained from Bilkent University and University of Michigan, Ann Arbor, a Western blot analysis of these cells was performed.

Lysates were prepared from 10^6 cells per well in 6-well plates and equal amounts of total protein was loaded. It is shown in Figure 4.2 that only two out of six different cell lines are able to express ASC at the protein level.

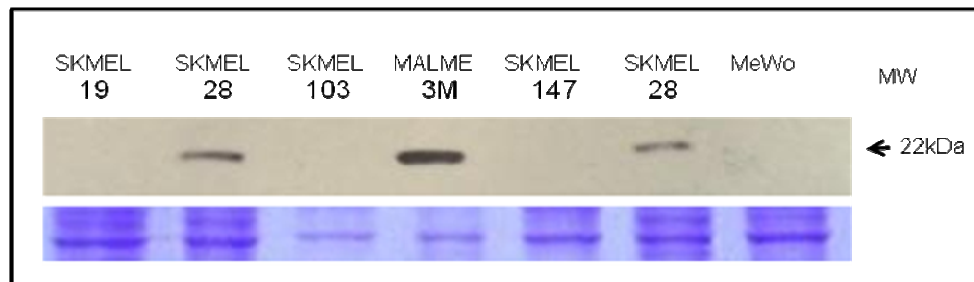


Figure 4.2. Endogenous ASC expression of melanoma cell lines. Of the six cell lines only Skmel 28 and Malme3M show ASC expression. Western blot analysis of melanoma cell lysates was carried out using ASC antibody. (Skmel 103 and Malme3M - 5ng; Skmel 19, 28,147 and Mewo - 15ng)

Next, these cell lines were probed for their sensitivity towards doxorubicin. Cells were treated with 1 µg/ml Doxorubicin and their viability was assessed at 12, 24, 48 and 72 hours by MTS assay (Figure 4.3). It is evident that Mewo and Skmel 19 cells are resistant to doxorubicin induced killing.

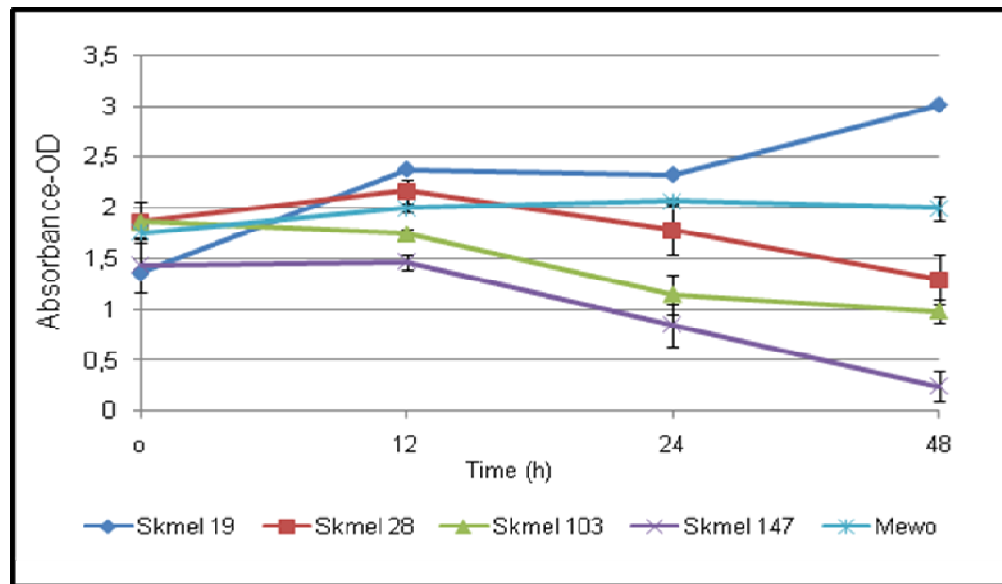


Figure 4.3. Resistance of Mewo and Skmel 19 cell lines to doxorubicin. Mewo and Skmel 19 cells are resistant to doxorubicin treatment. Viabilities were determined using the MTS assay.

For the continuation of experiments, two of the cell lines which do not express ASC and show resistance towards DNA damaging drugs, Mewo and Skmel 19 were chosen. These cells were additionally able to attach and grow fast.

4.3. Establishment of Stable Mewo-FG12-GFP, Mewo-FG12-ASC, Skmel 19-FG12-GFP and Skmel 19-FG12-ASC Cell Lines

HEK 293 T viral packaging cells were transfected with lentiviral helper plasmids pHCMV-G, pRSV rev, pMDLg and the ASC containing vector FG12-ASC or control FG12-GFP vector. Melanoma cell lines, Mewo and Skmel 19 were infected with the viral supernatant of the packaging cells. GFP production could be visualized with an efficiency of 95 per cent after four days (Figure 4.4.). This ratio was followed at two weeks and monthly by counting with a hemocytometer and by casual inspection. The ratio of GFP positive cells remained constant.

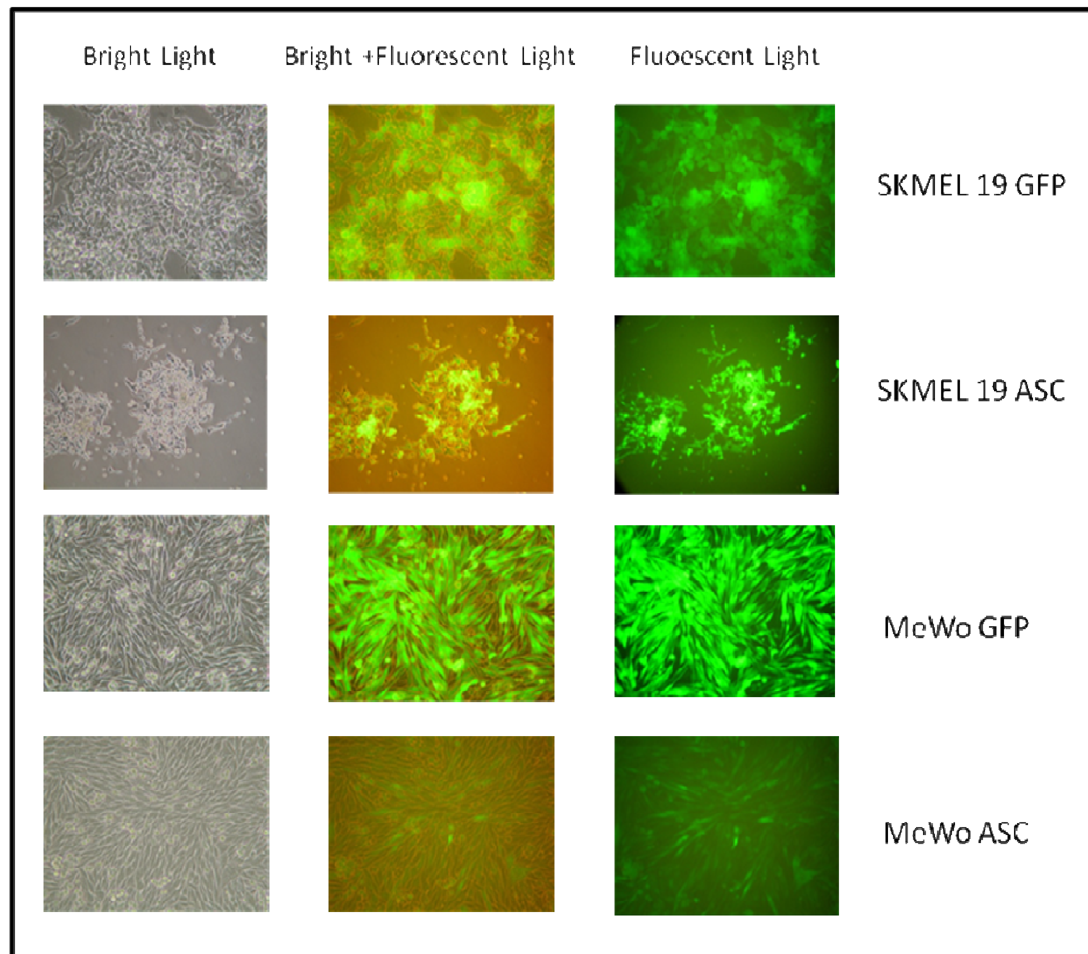


Figure 4.4. Establishment of GFP control and ASC stable lines. Bright Light, Fluorescent Light and merged pictures of infection results (Magnification 100X). ASC carrying cells are also expressing GFP from a separate promoter.

These cell lines, when monitored over a period of 12 months still displayed the same efficiency in expressing the GFP tag.

4.4. Western Blot Analysis of Derivative Cell Lines Show Stable ASC Expression

Following the infection of Mewo and Skmel 19 cell lines, their lysates were prepared in 4x Laemmli Sample Buffer. Equal amounts of total protein, prepared from 10^6 cells, were loaded. SDS PAGE and Western Blotting were done according to Material and Methods 3.8.. Monoclonal ASC antibody was used for the detection of ASC protein in Mewo, Mewo-FG12-GFP, Mewo-FG12-ASC, Skmel 19, Skmel 19-FG12-GFP and Skmel 19-FG12-ASC cell lysates. Figure 4.5. indicates the ASC protein levels in Mewo-FG12-ASC and Skme-FG12-ASC cells and shows the lack of ASC expression in the parental cell lines as well as the control GFP infected cell lines.

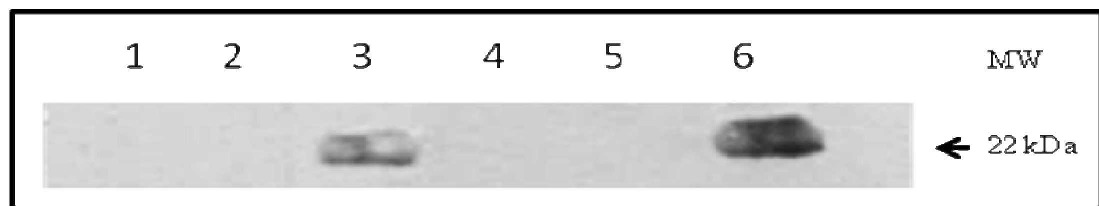


Figure 4.5. ASC expression was reestablished in Mewo and Skmel 19 cell lines. Western analysis of Mewo, Mewo- FG12-GFP, Mewo- FG12-ASC, Skmel 19, Skmel19- FG12-GFP and Skmel 19- FG12-ASC cell lysates (lanes 1-6) using the ASC-Antibody.

4.5. Drug Treatments of ASC Infected Melanoma Cells

It is unfortunate that due to the resistance of melanoma cells, widely used drugs cannot be used to treat patients. After the establishment of *de novo* ASC expressions in two of these lines, Mewo and Skmel 19 we wanted to check the resistance of these cells towards chemotherapeutic drugs such as doxorubicin and etoposide, as well as TRAIL, an inducer of the extrinsic pathway of apoptosis. Suitable doses of drugs were determined according to a literature search and trial experiments.

4.5.1. ASC-infected Mewo and Skmel 19 Cells Show a Slight Sensitization Towards Doxorubicin Treatment

Doxorubicin (Adriamycin) is a widely used chemotherapeutic drug, which is an intercalating agent that disturbs the biosynthesis of macromolecules, such as DNA, by interfering with the action of topoisomerase II. Doxorubicin activates the p53 DNA damage-induced apoptotic pathway (Muller et al., 1998; Zhou et al., 2002).

Mewo- FG12-ASC and Mewo-FG12-GFP control cells were plated at 4000 cells/well in 96-well plates in triplicates and on the next day were treated with 1 µg/ml doxorubicin for 0, 24, 48 and 72 hours. Their viability was measured and percent viabilities were as depicted in Figure 4.6.

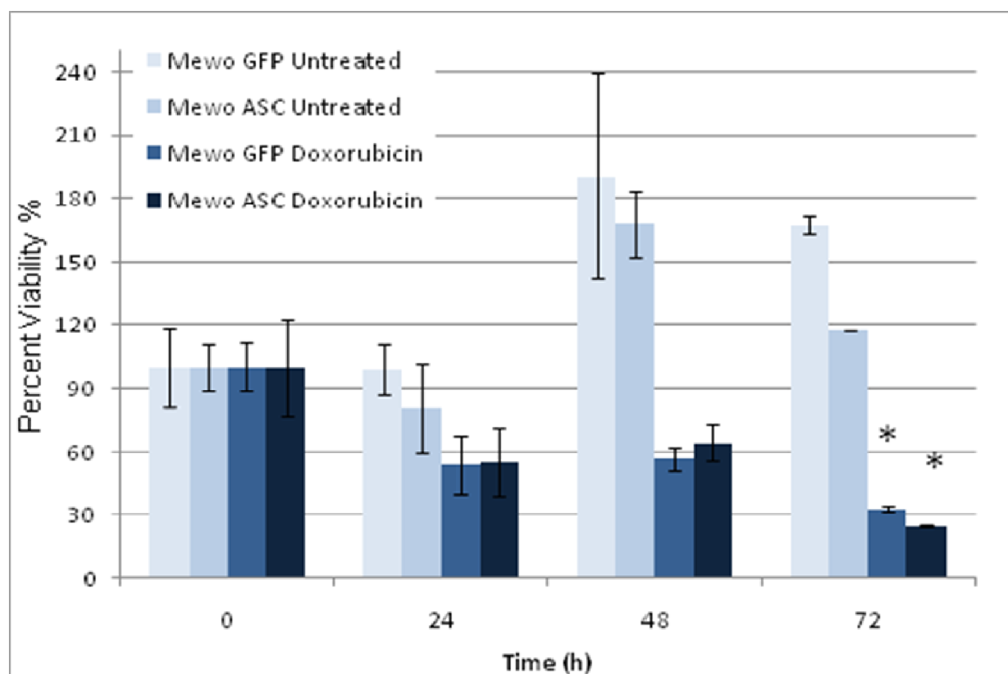


Figure 4.6. Slight sensitization of Mewo-FG-12-ASC after treatment with 1 µg/ml doxorubicin. Changes in percent viabilities of Mewo-FG12-GFP and Mewo-FG12-ASC were determined after treatment for 0, 24, 48 and 72 hours. The viability of cells on day 0 after the treatment was set at 100 per cent. No statistical significance (*).

When compared to treated control cells (Mewo-FG12-GFP), ASC expressing Mewo cells (Mewo-FG12-ASC) show 8 per cent higher loss of viability at 72 hour time point. Similar results were obtained in repeated experiments.

Treatment of Skmel 19-FG12-ASC cells with 1 μg/ml doxorubicin exhibits around 10 per cent higher loss of viability than control GFP cells at 48 hour time point (Figure 4.7).

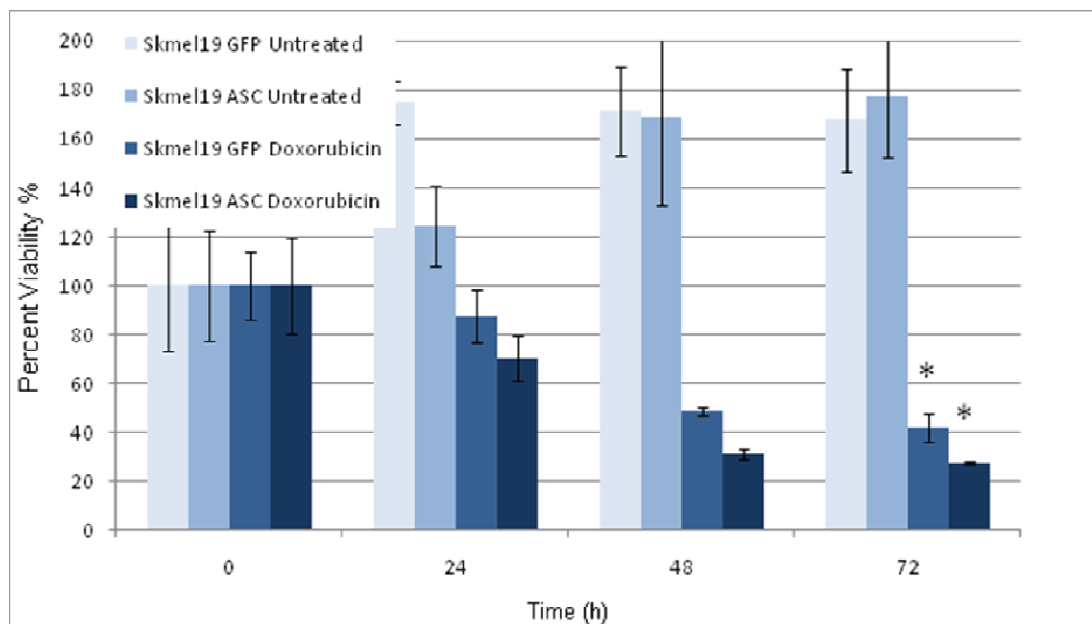


Figure 4.7. Slight sensitization of Skmel 19-FG12-ASC cells after treatment with 1 μg/ml doxorubicin. Percent viabilities of Skmel-FG12-GFP and Skmel-FG12-ASC were determined after treatment for 0, 24, 48 and 72 hours. The viability of cells on day 0 after the treatment was set at 100 per cent. Results are statistically significant at $p < 0.05$.

These data show that ASC expression alone is capable of rendering melanoma cells significantly more susceptible but cannot completely overcome their doxorubicin chemoresistance.

4.5.2. Etoposide Treated Skmel 19-FG12-ASC Cells Show Growth Inhibition

Etoposide mode of action is similar to doxorubicin and kills the cells by interfering with biosynthetic pathways. Mewo-FG12-ASC, Skmel 19-FG12-ASC and control cells were treated with 25 µg/ml etoposide or left untreated for 0, 24, 48 and 72 hours. Mewo-FG12-ASC cells when subjected to etoposide show a loss of viability that is 11 per cent higher than control GFP infected cells at 72 hour time point (Figure 4.8).

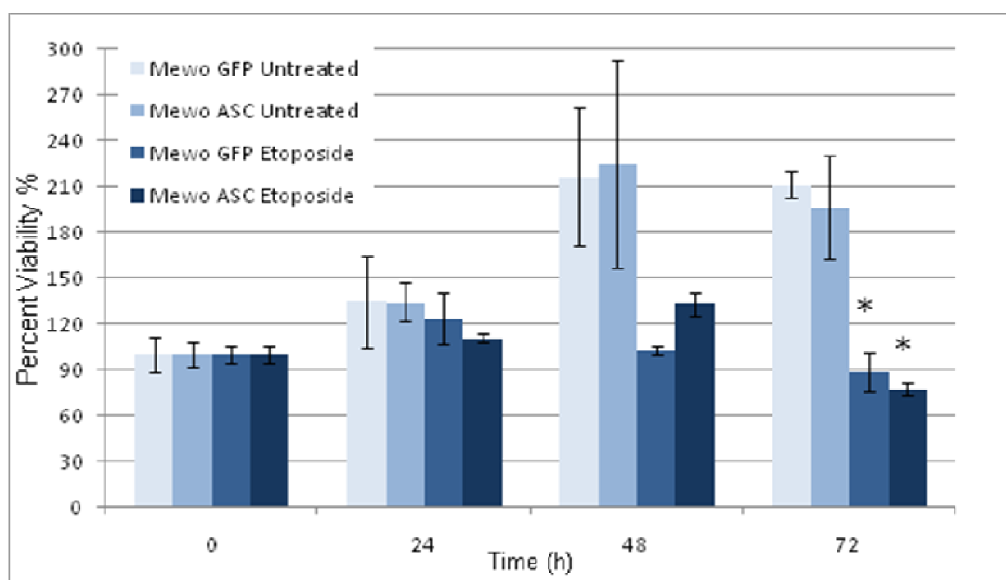


Figure 4.8. Slight sensitization of Mewo 19-FG12-ASC cells after treatment with 25 µg/ml etoposide. Percent viabilities of Mewo-FG12-GFP and Mewo-FG12-ASC were determined after treatment for 0, 24, 48 and 72 hours. The viability of cells on day 0 hour after treatment was set at 100 per cent. No statistical significance.

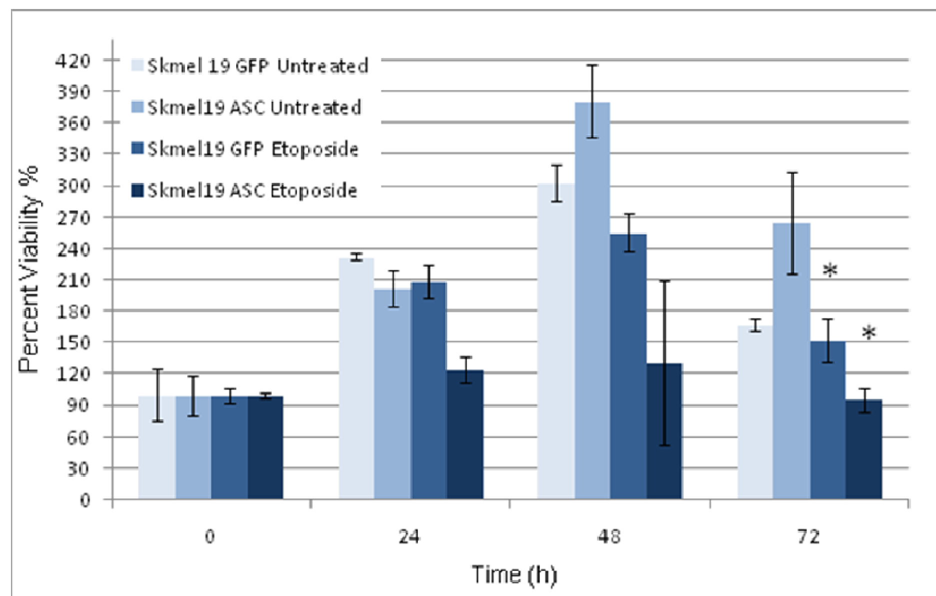


Figure 4.9. Growth arrest of Skmel 19-FG12-ASC after treatment with 25 µg/ml etoposide. Percent viabilities of Skmel 19-FG12-GFP and Skmel 19-FG12-ASC were determined after treatment for 0, 24, 48 and 72 hours. The viability of cells on day 0 after the treatment was set at 100 per cent. Statistically significant at $p<0.05$.

Skmel 19-FG12-GFP cells, both in the treated and untreated, columns show an in-phase growth curve. On the other hand Skmel 19-FG12-ASC cells are displaying a profound inhibition in growth (Figure 4.9) which is statistically significant.

Thus, it is clear that ASC expression alone cannot overcome the etoposide resistance of Mewo and Skmel 19 cells but may have a slight sensitization effect.

4.5.3. Mewo-FG12-ASC and Skmel 19-FG12-ASC Cells Are Not Sensitized Towards Dacarbazine (DTIC) Treatment

Dacarbazine (DTIC) is a cytotoxic drug belonging to the family of alkylating agents that has been FDA approved in 1975 for melanoma treatment. It is being used in treatment of malignant melanoma and Hodgkin lymphoma since 2006, however with a success rate of 5 per cent. Consequently, we wanted to test whether the addition of ASC expression to the cells has an effect on their sensitivity towards DTIC.

4000 cells/well were plated into 96-well plates in triplicates and were treated with 250 μg/ml of DTIC over a period of 72 hours. Both Mewo and Skmel 19 cells that were infected with ASC do not show any sensitization towards DTIC (Figure 4.10 and Figure 4.11).

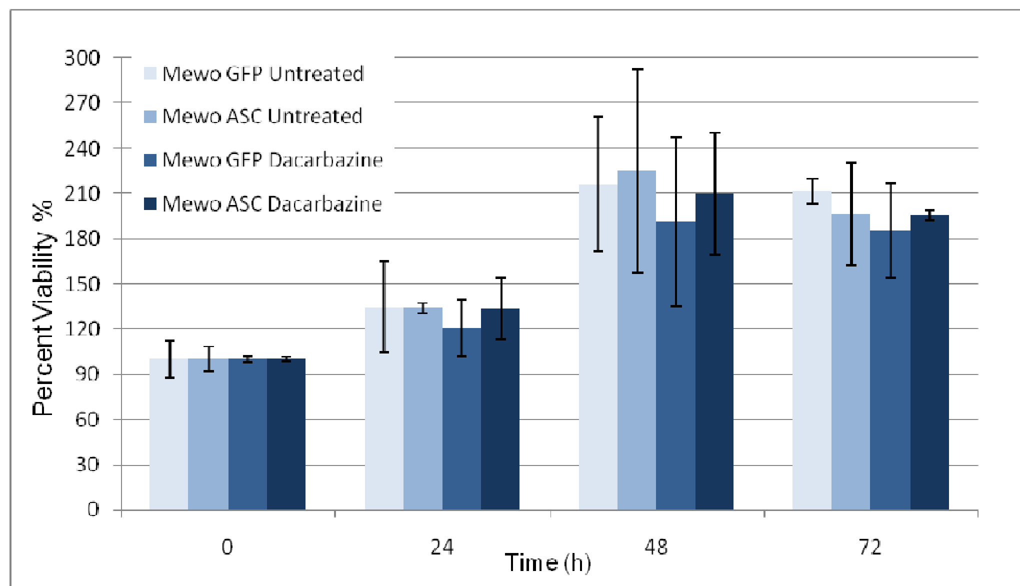


Figure 4.10. Lack of sensitization to dacarbazine treatment in Mewo cells. Percent viabilities of Mewo-FG12-GFP and Mewo-FG12-ASC were determined after treatment for 0, 24, 48 and 72 hours. The viability of cells on day 0 after the treatment was set at 100 per cent.

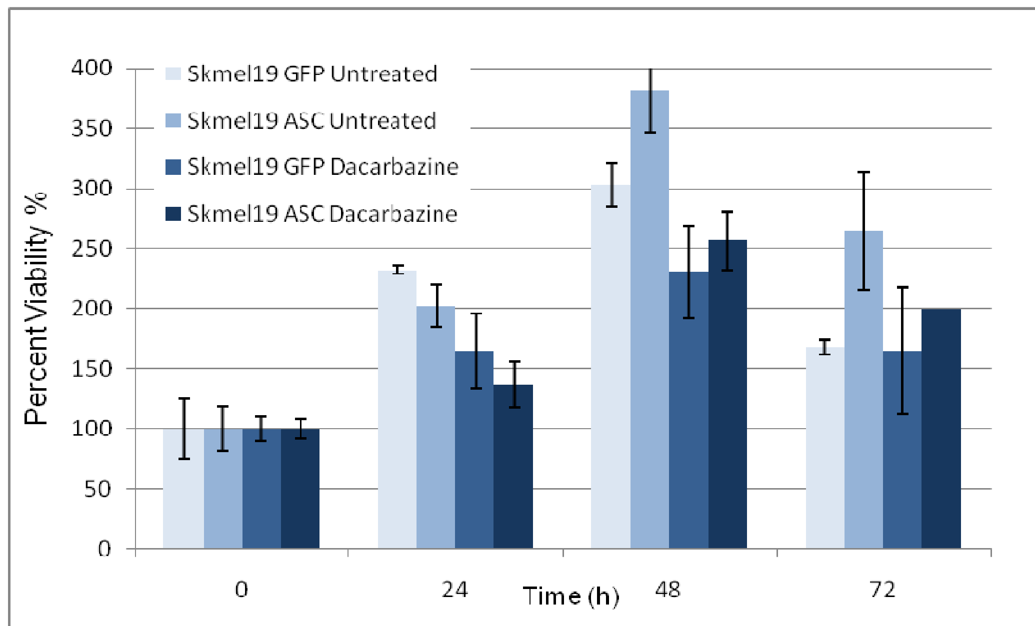


Figure 4.11. Lack of sensitization to dacarbazine treatment in Skmel 19 cells. Percent viabilities of Skmel-FG12-GFP and Skmel-FG12-ASC determined after 0, 24, 48 and 72 hour treatments. The viability of cells on day 0 after the treatment was set at 100 per cent.

From the data above it becomes clear that *de novo* ASC expression alone cannot overcome the resistance of Mewo and Skmel 19 cells to killing induced by DNA damaging and cytotoxic drugs.

4.5.4. TRAIL Kills ASC-infected Mewo Cells More Efficiently

TNF-related apoptosis inducing ligand (TRAIL) is a type II transmembrane protein, which activates the extrinsic pathway of apoptosis (death receptor pathway) by binding and activating death receptor 4 and/or 5 (DR4; DR5). Thereby, it induces DISC formation and the concomitant recruitment and activation of caspase 8. TRAIL is also a promising cancer treatment agent and studies are under way to initiate clinical trials with TRAIL in humans.

We wanted to test whether ASC induction in melanoma cells increases the sensitivity of these towards TRAIL. Therefore, Mewo-FG12-GFP and ASC and Skmel 19-FG12-GFP and ASC cells were plated in triplicates at a density of 10^4 cells/well into a 96-well plate and were treated with 100 ng/ml TRAIL or were left untreated for 16 hours. Measurements were taken at 0 hour and 16 hour points.

Interestingly, Mewo-FG12-ASC cells display higher loss of viability by 30 per cent compared to Mewo-FG12-GFP cells treated with TRAIL which does not have an effect on their viability.

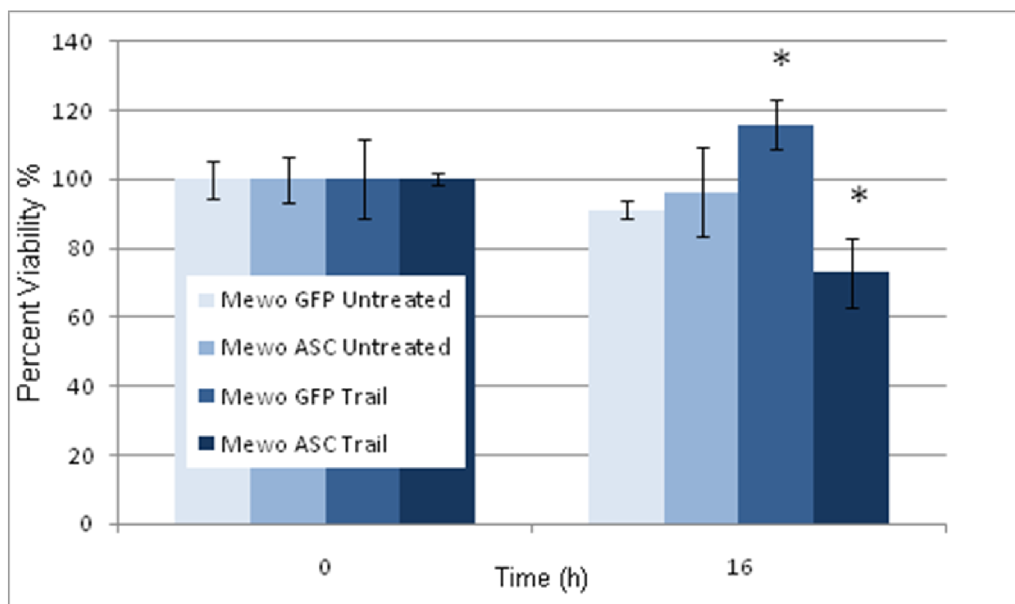


Figure 4.12. ASC expression sensitizes Mewo cells to TRAIL treatment. Mewo-FG12-ASC displays a higher loss of viability when treated with 100 ng/ml TRAIL. Results are statistically significant at $p<0.05$ (*).

TRAIL is a very potent death inducer and is able to kill cells within 5 hours. However experiments in which viability determination is done via the MTS assay require a treatment period of 16 hours, as done in other publications (Garnett et al., 2006, Martinez-Lorenzo et al., 2006). Since TRAIL treatment resulted in a significant viability loss in the *de novo* ASC expressing cells we wanted to confirm these data by more direct apoptotic assays.

4.6. Caspase 3 Activity of TRAIL Treated Mewo-ASC Cells

Caspase 3 is a major effector caspase that is activated by initiator caspases 8, 9 or 10 upon induction of the respective pathways. Its activity is a measure of apoptotic activity going on in a cell population. CaspACE™ Assay System, Colorimetric (Promega), constitutes of a calorimetric substrate that is cleaved by the DEVDase activity of caspase 3.

Cells were plated into 10 cm plates at a density of $8x10^6$ cells/plate. Cells were left untreated or treated with TRAIL and irreversible, cell-permeable pan-caspase inhibitor Z-VAD-FMK or the drug alone. Equal amount of protein was used for the assay in a 96-well plate. After four hours of incubation, measurements were taken at 405 nm with a microplate reader (Versamax, Molecular Devices).

The pan-caspase inhibitor Z-VAD-FMK inhibits apoptosis induction and serves as a specific negative control for the experiment (Figure 4.13). TRAIL treated Mewo-FG12-ASC cells show a slightly higher activity in caspase 3 than is seen in control cells, which is in accordance with previous findings shown in Figure 4.12. They do not however show a significant difference.

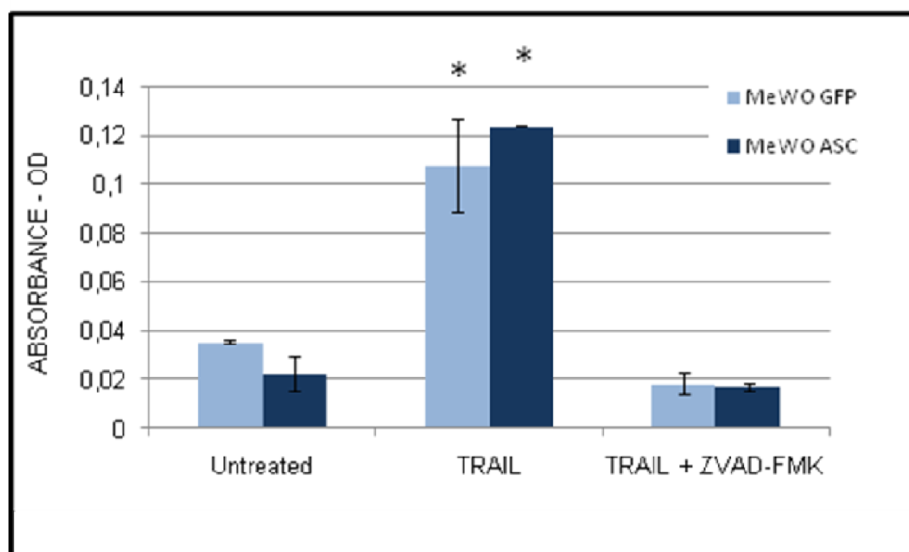


Figure 4.13. Higher caspase 3 activity in ASC expressing Mewo cells. Mewo-GFP and ASC were left untreated, treated with 100 ng/ml TRAIL or TRAIL and 50 µM Z-VAD-FMK. No statistical significance.

One unexpected result is the detection of caspase 3 activity in Mewo-FG12-GFP cells, which showed no loss in viability by the MTS assay in Figure 4.12. We do not have an explanation for this discrepancy.

In order to check the pro-caspase 3 protein status of these cells, western blot analysis was carried out on control GFP infected and ASC infected Mewo cell lines. 10^6 cells were plated into 6-well plates and were left untreated or treated with 100 ng/ml TRAIL. Caspase 3 antibody was able to detect the pro-caspase 3 form and its loss indicates the cleavage, thus activation of this protein.

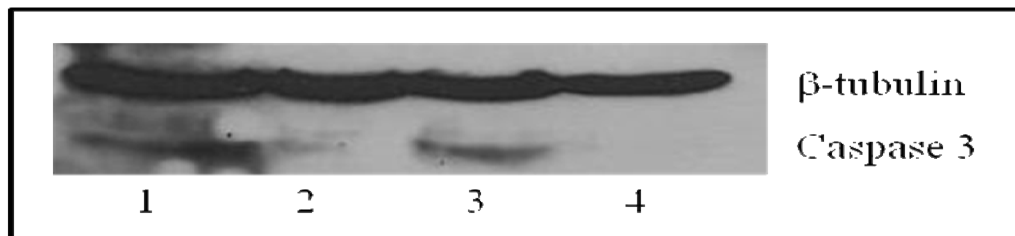


Figure 4.14. Pro-caspase 3 is lost upon treatment with TRAIL in Mewo cells. Lanes 1-4: Mewo-FG12-GFP untreated, Mewo-FG12-GFP +100ng/ml TRAIL, Mewo-FG12-ASC untreated, Mewo-FG12-ASC +100ng/ml TRAIL.

Again it was surprising to find pro-caspase 3 disappear in lane 2 which is in accordance with the caspase 3 activity assay but is contrasting the results of the MTS assay. The data above shows that ASC expression may sensitize Mewo cells to killing by TRAIL through the death receptor pathway.

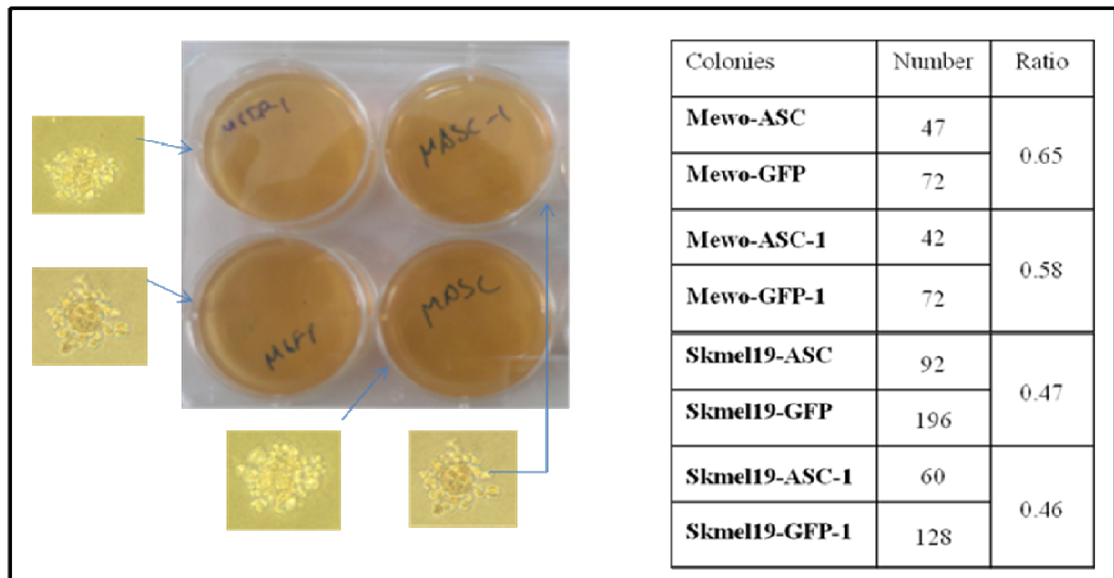
4.7. ASC Infected Mewo and Skmel 19 Cells Display Dramatically Reduced Anchorage-Independent Growth In Long Term Assays

The short term viability assay results described above provided us with weak clues about the tumor suppressive abilities of ASC in melanoma. We reasoned that a better test for the possible tumor inhibitory role of ASC may be through long term colony formation assays.

Normal cells grow and divide only after forming attachments with their substratum, as is provided by plastic or glass surfaces of tissue culture plates. However, transformed cells such as with melanoma cell lines are able to form colonies without the need for attachments. The semi-solid media that is made of soft agar and DMEM*, allows individual cells to develop into single colonies. When cells lose their transformed character they are not able to form colonies in this attachment-free substratum.

Equal numbers of Mewo-FG12-GFP, Mewo-FG12-ASC, Skmel 19-FG12-GFP and Skmel 19-FG12-ASC cells obtained from two different infections were plated into soft agar culture medium as described in Materials and Methods. Cells were allowed to grow and were supplemented with new culture medium weekly. Colonies were counted after three weeks with a bright light microscope. The average colony was observed to contain more than 40 cells however; we counted all colonies having more than ten cells. Both Mewo and Skmel 19 cells formed compact colonies. The experiments were repeated at least twice for each cell type.

Interestingly, in all of the experiments *de novo* ASC expressing cells showed dramatically fewer numbers of colonies compared to controls (Figure 4.15). In the first trial using cells from the first lentiviral infection we found that Mewo-FG12-ASC cells formed 47 colonies whereas Mewo-FG12-GFP cells formed 72 colonies. In the second trial using the cells from a second lentiviral infection, these numbers were 42 and 72 respectively. As for the Skmel 19 cells, the first trial results were even more promising such that only 92 colonies were observed in ASC expressing cells compared to 196 in the control condition. For the second infection of Skmel 19 cells these results were 60 colonies of Skmel 19-FG12-ASC versus 128 colonies of Skmel 19-FG12-GFP.



Colonies	Number	Ratio
Mewo-ASC	47	0.65
Mewo-GFP	72	
Mewo-ASC-1	42	0.58
Mewo-GFP-1	72	
Skmel19-ASC	92	0.47
Skmel19-GFP	196	
Skmel19-ASC-1	60	0.46
Skmel19-GFP-1	128	

Figure 4.15. ASC expression suppresses the anchorage-independent growth of melanoma cells. ASC expressing cells have decreased number of colonies formed in the long term soft agar colony formation assay. Number of colonies counted of ASC infected to control GFP infected cells and their ratios. Data represents two trials using cell populations from two separate lentiviral transductions.

When we compared the ratios of the colony numbers of ASC infected cells over controls (approx. 0.6 for Mewo and 0.46 for Skmel 19), we concluded that *de novo* ASC expression has an inhibitory effect on the anchorage-independent growth of melanoma cell lines. Furthermore, we detected that the Skmel 19 cells which were slightly more responsive to DNA damaging drugs, also seem to be affected to a higher degree in the long term assay.

5. DISCUSSION

ASC is a novel adapter protein with critical involvement in two pathways, namely the innate immune signaling and apoptosis signaling cascades, both of which play significant roles in cancer development. The apoptosis arm of ASC related functions is the focus of this thesis.

The ASC protein has already been assigned a pro-apoptotic role in apoptosis (Masumoto et al., 1999). Furthermore, its expression is lost via promoter hypermethylation in various types of cancer and about 58.3 per cent of melanoma cell lines do not show ASC expression (Guan et al., 2003).

These findings make ASC a promising candidate tumor suppressor gene. Thus, we hypothesized that reintroduction of ASC expression into the chemoresistant melanoma cell lines devoid of this protein will give us a suitable system to test the importance of ASC in apoptosis signaling and chemosensitization.

Initially, we carried out a screen for ASC protein expression in the panel of melanoma cell lines that we could obtain and found that only two out of six show ASC expression (Figure 4.2), which correlates with previous findings (Guan et al., 2003).

Next, we investigated the chemsensitivity of this panel of cell lines to the commonly used chemotherapy drug doxorubicin and found that most are resistant (Figure 4.3). Actually, the presence or absence of ASC protein expression does not directly correlate with the chemoresistance status of the cell lines. This finding is not surprising when we take into account the widely accepted notion that cancer cells carry mutations in multiple genes (generally more than four) and/or aberrations in several different pathways. As such, the introduction of a single pro-apoptotic protein into completely chemoresistant cells would be expected to have only a partial effect.

Among the melanoma cell lines lacking ASC expression and showing complete chemoresistance to doxorubicin we chose Mewo and Skmel 19 to continue our studies.

After the introduction of control FG12-GFP and FG12-ASC lentiviral vectors into these cells and verification of *de novo* ASC expression only in Mewo-FG12-ASC and Skmel 19-FG12-ASC (Figure 4.4 and Figure 4.5), we carried out the short term chemosensitization assays using drugs targeting the DNA damage-induced - and death receptor-induced - apoptotic pathways. In the category of DNA damage-inducing drugs such as doxorubicin and etoposide, we found a very minimal sensitization of about 10 per cent in Skmel 19 and Mewo cells (Figure 4.6, Figure 4.7, Figure 4.8 and Figure 4.9). Chemosensitization was measured as loss of viability in ASC expressing cells compared to GFP expressing controls using the MTS assay. When we tested the effect of dacarbazine, the FDA (USA) approved drug for melanoma treatment, we did not observe any difference between ASC positive and negative cells (Figure 4.10 and Figure 4.11).

We obtained more promising results with TRAIL, a strong apoptosis inducing death receptor ligand. Mewo-FG12-ASC cells showed 30 per cent higher loss of viability compared to GFP control cells (Figure 4.12). When we tried to measure apoptosis rates more directly using a colorimetric substrate-cleavage assay, we found that caspase 3 activities were present in both GFP control and ASC expressing Mewo cells, with slightly higher activity in ASC containing cells (Figure 4.13). Besides, western analysis of pro-caspase 3 levels in TRAIL treated Mewo control and ASC expressing cells show equal levels of loss of protein (Figure 4.14). Upon apoptosis induction, pro-caspases are generally cleaved to their active subunits and loss of the pro-form verifies ongoing apoptotic signaling.

The colorimetric caspase 3 activity and the western blot results are in correlation with each other, however, they do not explain the 30 per cent difference in viability observed in the MTS assay. Most probably this is due to the nature and timing of the different tests. For example, MTS assays measure mitochondrial activity and it is known that even dying cells do not lose all their mitochondria and the initial steps of apoptotic signaling require energy generation. As a strong apoptosis inducer, TRAIL induced killing becomes evident even at five hours in some sensitive lines as examined by microscopy. To

detect loss of viability using MTS the treatment is usually extended to 16 or 24 hours. On the other hand, caspase activity is an earlier event in apoptosis and can be measured even after five hours. We conclude that it is practically challenging to explain the observed partial TRAIL chemosensitization effects with Mewo-FG12-GFP and Mewo-FG12-ASC melanoma cell lines, each composed of a random mixture of cells carrying lentivirally mutated genomes. We suspect that more consistent results could be obtained from single cell derivative cell lines and the TRAIL experiments will be repeated to reach a final decision.

Even though the short term chemosensitization assays detailed above failed to provide clear evidence about the importance of ASC expression in apoptosis induction in two of the chemoresistant melanoma cell lines, they do not disqualify ASC from being a putative tumor suppressor gene. Another widely accepted test of tumorigenicity is the anchorage-independent growth of cells in a semi-solid medium such as agar. Colonies are formed from a single cell without being able to attach to their substratum. Normal cells require this attachment via integrin proteins in order to start to grow and divide. However, cancer cells are not anchorage-dependent and form colonies nonetheless. This type of assay provides long term insight about the tumorigenic potential of the cells.

Most strikingly, when we applied the soft agar colony formation assay to Mewo-FG12-ASC, Skmel 19-FG12-ASC and corresponding control cells we observed a significant decrease in the number of colonies in ASC expressing cells in two different trials using cells from two different infection experiments. While in one of the trials, Mewo-FG12-ASC form 47 colonies, GFP control cells form 72 colonies. Similarly, Skmel 19-FG12-ASC cells formed only 92 colonies whereas; GFP expressing Skmel 19 cells formed 196 (Figure 4.15). The data was consistent in the two trials.

Interestingly, the ratio of number of colonies formed by Skmel 19-FG12-ASC cells over control GFP cells is lower than the same ratio for Mewo cells. This finding correlates with the 10 per cent chemosensitization observed in the short term doxorubicin and etoposide treatment assays.

The dramatic loss of colony forming capacity observed after ASC expression, in the absence of any chemical treatment, provides strong evidence about the tumor suppressive capacity of ASC in melanoma. It is imminent at this stage, to test the combination of ASC expression with chemotherapy (doxorubicin, etoposide and TRAIL) in long term soft agar colony formation assays and *in vivo* tumor formation experiments in immune-compromised mouse hosts. These studies initiated with melanoma cell lines should be carried out in parallel with other cancer types where ASC expression is frequently lost. It will be also very interesting to investigate whether the involvement of ASC in innate immune signaling could be harnessed to kill chemoresistant cancer cells more efficiently.

REFERENCES

Akira, S., S. Uematsu and O. Takeuchi, 2006, "Pathogen recognition and innate immunity" *Cell*, Vol. 124, pp. 783-801.

Albino, A. P., M. J. Vidal, N.S. McNutt, C. R. Shea, V.G. Prieto, D. M. Nanus, J. M. Palmer and N. K. Hayward, 1994, "Mutation and expression of the p53 gene in human malignant melanoma", *Melanoma Research*, Vol. 4, pp. 35-45.

Ashkenazi, A. , 2002, " Targeting death and decoy receptors of the tumour-necrosis factor superfamily", *Nature Review Cancer*, Vol. 2, pp. 420-430.

Baldi, A., D. Santini, P. Russo, C. Catricala, A. Amantea, M. Picardo, F. Tatangelo, G. Botti, E. Dragonetti and R. Murace, 2004, "Analysis of APAF-1 expression in human cutaneous melanoma progression", *Experimental Dermatology*, Vol. 13, pp. 93-97.

Bartkova, J., J. Lukas, P. Guldberg, J. Alsner, A.F. Kirkin, J. Zeuthen and J. Bartek, 1996, "The p16-cyclin D/Cdk4-pRb pathway as a functional unit frequently altered in melanoma pathogenesis", *Cancer Research*, Vol. 56, pp. 5475-5483.

Bestor, T. H., 1988, "Cloning of a mammalian DNA methyltransferase", *Gene,* Vol. 74, pp. 9-12.

Bishop, J. A., R.C. Wachsmuth, M. Harland, V. Bataille, E. Pinney, K. P. Mac, L. Baglietto, J. Cuzick and D. T. Bishop, 2000, "Genotype/phenotype and penetrance studies in melanoma families with germline CDKN2A mutations", *Journal of Investigative Dermatology*, Vol.114, pp. 28-33.

Boukamp, P., 2005, "Non-melanoma skin cancer: what drives tumor development and progression?", *Carcinogenesis*, Vol. 26, pp. 1657-1667.

Boyce, M., and J. Yuan, 2006, “Cellular response to endoplasmic reticulum stress: a matter of life or death”, *Cell Death and Differentiation*, Vol. 13, pp. 363-373.

Bunz, F., P. M. Hwang, C. Torrance, T. Waldman, Y. Zhang, L. Dillehay, J. Williams, C. Lengauer, K.W. Kinzler and B. Vogelstein, 1999, “Disruption of p53 in human cancer cells alters the responses to therapeutic agents”, *Journal of Clinical Investigation*, Vol. 104, pp. 263-269.

Cannon-Albright, L. A., D. E. Goldgar, L. J. Meyer, C. M. Lewis, D. E. Anderson, J. W. Fountain, M. E. Hegi, R. W. Wiseman, E. M. Petty and A. E. Bale, 1992, “Assignment of a locus for familial melanoma, MLM, to chromosome 9p13-p22”, *Science*, Vol. 258, pp. 1148-1152.

Cannon-Albright, L. A., L.J. Meyer, D. E. Goldgar, C. M. Lewis, W. P. McWhorter, M. Jost, D. Harrison, D. E. Anderson, J. J. Zone and M. H. Skolnick, 1994, “Penetrance and expressivity of the chromosome 9p melanoma susceptibility locus (MLM)”, *Cancer Research*, Vol. 54, pp. 6041-6044.

Carrillo-Infante, C., G. Abbadessa, L. Bagella and A. Giordano, 2007, “Viral infections as a cause of cancer (review)”, *International Journal of Oncology*, Vol. 30, pp. 1521-1528.

Chou, J. J., H. Matsuo, H. Duan and G. Wagner, 1998, “Solution structure of the RAIDD CARD and model for CARD/CARD interaction in caspase-2 and caspase-9 recruitment”, *Cell*, Vol. 94, pp. 171-180.

Chudnovsky, Y., P. A. Khavari and A. E. Adams, 2005, “Melanoma genetics and the development of rational therapeutics”, *Journal of Clinical Investigation*, Vol. 115, pp. 813-824.

Clark, S. J. and J. Melki, 2002, “DNA methylation and gene silencing in cancer: which is the guilty party?”, *Oncogene*, Vol. 21, pp. 5380-5387.

Conway, K. E., B. B. McConnell, C. E. Bowring, C. D. Donald, S. T. Warren and P. M. Vertino, 2000, "TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers", *Cancer Research*, Vol. 60, pp. 6236-6242.

Cory, A. H., T. C. Owen, J. A. Barltrop and J. G. Cory, 1991, "Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture", *Cancer Communications*, Vol. 3, pp. 207-212.

Daniotti, M., M. Oggionni, T. Ranzani, V. Vallacchi, V. Campi, D. Di Stasi, G. D. Torre, F. Perrone, C. Luoni and S. Suardi, 2004, "BRAF alterations are associated with complex mutational profiles in malignant melanoma", *Oncogene*, Vol. 23, pp. 5968-5977.

Das, P. M., K. Ramachandran, J. Vanwert, L. Ferdinand, G. Gopisetty, I. M. Reis and R. Singal, 2006, "Methylation mediated silencing of TMS1/ASC gene in prostate cancer", *Molecular Cancer*, Vol. 5, p. 28.

Davies, H., G. R. Bignell, C. Cox, P. Stephens, S. Edkins, S. Clegg, J. Teague, H. Woffendin, M. J. Garnett and W. Bottomley, 2002, "Mutations of the BRAF gene in human cancer", *Nature*, Vol. 417, pp. 949-954.

DeYoung, K. L., M. E. Ray, Y. A. Su, S. L. Anzick, R. W. Johnstone, J. A. Trapani, P. S. Meltzer and J. M. Trent, 1997, "Cloning a novel member of the human interferon-inducible gene family associated with control of tumorigenicity in a model of human melanoma", *Oncogene*, Vol. 15, pp. 453-457.

Dowds, T. A., J. Masumoto, L. Zhu, N. Inohara and G. Nunez, 2004, "Cryopyrin-induced interleukin 1beta secretion in monocytic cells: enhanced activity of disease-associated mutants and requirement for ASC", *Journal of Biological Chemistry*, Vol. 279, pp. 21924-21928.

Dull, T., R. Zufferey, M. Kelly, R. J. Mandel, M. Nguyen, D. Trono and L. Naldini, 1998, “A third-generation lentivirus vector with a conditional packaging system”, *Journal of Virology,* Vol. 72, pp. 8463-8471.

Esteller, M., P. G. Corn, S. B. Baylin and J. G. Herman, 2001, “A gene hypermethylation profile of human cancer”, *Cancer Research*, Vol. 61, pp. 3225-3229.

Finlay, C. A., P. W. Hinds and A. J. Levine, 1989, “The p53 proto-oncogene can act as a suppressor of transformation”, *Cell*, Vol. 57, pp. 1083-1093.

Friend, S. H., R. Bernards, S. Rogelj, R. A. Weinberg, J. M. Rapaport, D. M. Albert and T. P. Dryja, 1986, “A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma”, *Nature*, Vol. 323, pp. 643-646.

Gray-Schopfer, V., C. Wellbrock and R. Marais, 2007, “Melanoma biology and new targeted therapy”, *Nature*, Vol. 445, pp. 851-857.

Green, D. R., 2005, “Apoptotic pathways: ten minutes to dead”, *Cell*, Vol. 121, pp. 671-674.

Guan, X., J. Sagara, T. Yokoyama, Y. Koganehira, M. Oguchi, T. Saida and S. Taniguchi, 2003, “ASC/TMS1, a caspase-1 activating adaptor, is downregulated by aberrant methylation in human melanoma”, *International Journal of Cancer,* Vol. 107, pp. 202-208.

Guldberg, P., P. Straten, A. Birck, V. Ahrenkiel, A. F. Kirkin and J. Zeuthen, 1997, “Disruption of the MMAC1/PTEN gene by deletion or mutation is a frequent event in malignant melanoma”, *Cancer Research,* Vol. 57, pp. 3660-3663.

Hanada, M., D. Delia, A. Aiello, E. Stadtmauer and J. C. Reed, 1993, “Bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia”, *Blood*, Vol. 82, pp. 1820-1828.

Hayward, N. K., 2003, “Genetics of melanoma predisposition”, *Oncogene*, Vol. 22, pp. 3053-3062.

Helmbach, H., E. Rossmann, M. A. Kern and D. Schadendorf, 2001, “Drug-resistance in human melanoma”, *International Journal of Cancer*, Vol. 93, pp. 617-622.

Hingorani, S. R., M. A. Jacobetz, G. P. Robertson, M. Herlyn and D. A. Tuveson, 2003, Suppression of BRAF(V599E) in human melanoma abrogates transformation”, *Cancer Research*, Vol. 63, pp. 5198-5202.

Hiscott, J., R. Lin, P. Nakhaei and S. Paz, 2006, “MasterCARD: a priceless link to innate immunity”, *Trends in Molecular Medicine*, Vol. 12, pp. 53-56.

Howlett, S. K., and Reik, W. (1991). Methylation levels of maternal and paternal genomes during preimplantation development. *Development* 113, 119-127.

Hwang, P. H., H. K. Yi, D. S. Kim, S. Y. Nam, J. S. Kim and D. Y. Lee, 2001, Suppression of tumorigenicity and metastasis in B16F10 cells by PTEN/MMAC1/TEP1 gene”, *Cancer Letters*, Vol. 172, pp. 83-91.

Inohara, N. and G. Nunez, 2003, “NODs: intracellular proteins involved in inflammation and apoptosis”, *Nat Rev Immunology*, Vol. 3, pp. 371-382.

Jemal, A., R. Siegel, E. Ward, T. Murray, J. Xu, C. Smigal and M. J. Thun, 2006, “Cancer statistics” , 2006, *Cancer J Clin,* Vol.56, pp. 106-130.

Kerr, J. F., C. M. Winterford and B. V. Harmon, 1994, “Apoptosis. Its significance in cancer and cancer therapy”, *Cancer*, Vol. 73, pp. 2013-2026.

Kuwana, T., L. Bouchier-Hayes, J. E. Chipuk, C. Bonzon, B. A. Sullivan, D. R. Green and D. D. Newmeyer, 2005, “BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly”, *Molecular Cell, Vol.* 17, pp. 525-535.

Lassam, N. J., L. From and H. J. Kahn, 1993, “Overexpression of p53 is a late event in the development of malignant melanoma”, *Cancer Research*, Vol. 53, pp. 2235-2238.

Lee, P. J., L. L. Washer, D. J. Law, C. R. Boland, I. L. Horon and A. P. Feinberg, 1996, Limited up-regulation of DNA methyltransferase in human colon cancer reflecting increased cell proliferation”, *Proceedings National Academy of Science U S A*, Vol. 93, pp. 10366-10370.

Levine, B. and J. Yuan, 2005, “Autophagy in cell death: an innocent convict?”, *Journal of Clinical Investigation*, Vol. 115, pp. 2679-2688.

Li, J., C. Yen, D. Liaw, K. Podsypanina, S. Bose, S. I. Wang, J. Puc, C. Miliaresis, L. Rodgers and R. McCombie, 1997, “PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer” *Science*, Vol. 275, pp. 1943-1947.

Lowe, S. W., S. Bodis, A. McClatchey, L. Remington, H. E. Ruley, D. E. Fisher, D. E. Housman and T. Jacks, 1994, “ p53 status and the efficacy of cancer therapy in vivo”, *Science*, Vol. 266, pp. 807-810.

Martinez, R., G. Schackert and M. Esteller, 2007, “Hypermethylation of the proapoptotic gene TMS1/ASC: prognostic importance in glioblastoma multiforme”, *Journal of Neurooncology*, Vol. 82, pp. 133-139.

Martinez, R., G. Schackert and M. Esteller, 2007, “Hypermethylation of the proapoptotic gene TMS1/ASC: prognostic importance in glioblastoma multiforme”, *Journal of Neurooncology*, Vol. 82, pp. 133-139.

Martinon, F., K. Burns and J. Tschopp, 2002, “The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta”, *Mol Cell*, Vol. 10, pp. 417-426.

Masumoto, J., S. Taniguchi, K. Ayukawa, H. Sarvotham, T. Kishino, N. Niikawa, E. Hidaka, T. Katsuyama, T. Higuchi and J. Sagara, 1999, “ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells” *Journal of Biological Chemistry*, Vol. 274, pp. 33835-33838.

Masumoto, J., S. Taniguchi, J. Nakayama, M. Shiohara, E. Hidaka, E., T. Katsuyama, S. Murase and J. Sagara, 2001, “Expression of apoptosis-associated speck-like protein containing a caspase recruitment domain, a pyrin N-terminal homology domain-containing protein, in normal human tissues”, *Journal of Histochemistry and Cytochemistry*, Vol. 49, pp. 1269-1275.

McConnell, B. B. and P. M. Vertino, 2004, “TMS1/ASC: the cancer connection”, *Apoptosis*, Vol. 9, pp. 5-18.

Melki, J. R., P. Warnecke, P. C. Vincent and S. J. Clark, 1998, “Increased DNA methyltransferase expression in leukaemia”, *Leukemia*, Vol. 12, pp. 311-316.

Miyoshi, H., U. Blomer, M. Takahashi, F. H. Gage and I. M. Verma, 1998, “Development of a self-inactivating lentivirus vector”, *Journal of Virology*, Vol. 72, pp. 8150-8157.

Morita, R., A. Fujimoto, N. Hatta, K. Takehara and M. Takata, 1998, “Comparison of genetic profiles between primary melanomas and their metastases reveals genetic alterations and clonal evolution during progression”, *Journal of Invesigaivet Dermatology*, Vol. 111, pp. 919-924.

Muller, M., C. A. Scaffidi, P. R. Galle, W. Stremmel and P. H. Krammer, 1998, “The role of p53 and the CD95 (APO-1/Fas) death system in chemotherapy-induced apoptosis”, *European Cytokine Network,* Vol. 9, pp. 685-686.

Naldini, L., U. Blomer, P. Gallay, D. Ory, R. Mulligan, F. H. Gage, I. M. Verma and D. Trono, 1996, "In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector", *Science*, Vol. 272, pp. 263-267.

Ohtsuka, T., X. F. Liu, Y. Koga, Y. Kitajima, Y. Nakafusa, C. W. Ha, S. W. Lee and K. Miyazaki, 2006,." Methylation-induced silencing of ASC and the effect of expressed ASC on p53-mediated chemosensitivity in colorectal cancer", *Oncogene*, Vol. 25, pp. 1807-1811.

Ohtsuka, T., H. Ryu, Y. A. Minamishima, S. Macip, J. Sagara, K. I. Nakayama, S. A. Aaronson and S. W. Lee, 2004, "ASC is a Bax adaptor and regulates the p53-Bax mitochondrial apoptosis pathway", *Nature Cell Biology,* Vol. 6, pp. 121-128.

Okano, M., S. Xi and E. Li, 1998, "Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells", *Nucleic Acids Research*, Vol. 26, pp. 2536-2540.

Ozoren, N. and W. S. El-Deiry, 2003, "Cell surface Death Receptor signaling in normal and cancer cells", *Seminars in Cancer Biology*, Vol. 13, pp. 135-147.

Ozoren, N., J. Masumoto, L. Franchi, T. D. Kanneganti, M. Body-Malapel, I. Erturk, R. Jagirdar, L. Zhu, N. Inohara and J. Bertin, 2006, "Distinct roles of TLR2 and the adaptor ASC in IL-1beta/IL-18 secretion in response to Listeria monocytogenes", *Journal of Immunology*, Vol. 176, pp. 4337-4342.

Peter, M. E. and P. H. Krammer, 2003, "The CD95(APO-1/Fas) DISC and beyond", *Cell Death and Differentiation*, Vol. 10, pp. 26-35.

Quelle, D. E., F. Zindy, R. A. Ashmun and C. J. Sherr, 1995, "Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest", *Cell*, Vol. 83, pp. 993-1000.

Richards, N., P. Schaner, A. Diaz, J. Stuckey, E. Shelden, A. Wadhwa and D. L. Gumucio, 2001, “Interaction between pyrin and the apoptotic speck protein (ASC) modulates ASC-induced apoptosis”, *Journal of Biological Chemistry*, Vol. 276, pp. 39320-39329.

Rubben, A., P. Babilas, J.M. Baron, A. Hofheinz, M. Neis, F. Sels and M. Sporkert, 2000, “Analysis of tumor cell evolution in a melanoma: evidence of mutational and selective pressure for loss of p16ink4 and for microsatellite instability”, *Journal of Investigative Dermatology*, Vol. 114, pp. 14-20.

Schuler, M. and D. R. Green, 2005, “Transcription, apoptosis and p53: catch-22”, *Trends in Geneicst*, Vol. 21, pp. 182-187.

Sherr, C. J., 2004, “Principles of tumor suppression”, *Cell*, Vol. 116, pp. 235-246.

Shinozaki, M., A. Fujimoto, D. L. Morton and D. S. Hoon, 2004, “Incidence of BRAF oncogene mutation and clinical relevance for primary cutaneous melanomas”, *Clin Cancer Research*, Vol. 10, pp. 1753-1757.

Shiohara, M., S. Taniguchi, J. Masumoto, K. Yasui, K. Koike, A. Komiyama and J. Sagara, 2002, “ASC, which is composed of a PYD and a CARD, is up-regulated by inflammation and apoptosis in human neutrophils”, *Biochemistry and Biophysics Research Communication*, Vol. 293, pp. 1314-1318.

Simpson, D. J., N. A. Hibberts, A. M. McNicol, R. N. Clayton and W. E. Farrell, 2000, “Loss of pRb expression in pituitary adenomas is associated with methylation of the RB1 CpG island”, *Cancer Research*, Vol. 60, pp. 1211-1216.

Soengas, M. S., P. Capodieci, D. Polsky, J. Mora, M. Esteller, X. Opitz-Araya, R. McCombie, J. G. Herman, W. L. Gerald and Y. A. Lazebnik, 2001, “Inactivation of the apoptosis effector Apaf-1 in malignant melanoma”, *Nature*, Vol. 409, pp. 207-211.

Soengas, M. S. and S. W. Lowe, 2003, “Apoptosis and melanoma chemoresistance”, *Oncogene*, Vol. 22, pp. 3138-3151.

Srinivasula, S. M., J. L. Poyet, M. Razmara, P. Datta, Z. Zhang and E. S. Alnemri, 2002, “The PYRIN-CARD protein ASC is an activating adaptor for caspase-1”, *Journal of Biological Chemistry*, Vol. 277, pp. 21119-21122.

Stehelin, D., 1976, “The transforming gene of avian tumor viruses”, *Pathological Biology (Paris)*,Vol. 24, pp. 513-515.

Stehlik, C., L. Fiorentino, A. Dorfleutner, J. M. Bruey, E. M. Ariza, J. Sagara and J. C. Reed, 2002, “The PAAD/PYRIN-family protein ASC is a dual regulator of a conserved step in nuclear factor kappaB activation pathways”, *Journal of Experimental Medicine*, Vol. 196, pp. 1605-1615.

Straume, O. and L. A. Akslen, 1997, “Alterations and prognostic significance of p16 and p53 protein expression in subgroups of cutaneous melanoma”, *International Journal of Cancer*, Vol. 74, pp. 535-539.

Tarhini, A. A. and S. S. Agarwala, 2006, “Cutaneous melanoma: available therapy for metastatic disease”, *Dermatological Therapy*, Vol.19, pp. 19-25.

Terasawa, K., S. Sagae, M. Toyota, K. Tsukada, K. Ogi, A. Satoh, H. Mita, K. Imai, T. Tokino and R. Kudo, 2004, “Epigenetic inactivation of TMS1/ASC in ovarian cancer”, *Clinical Cancer Research*, Vol. 10, pp. 2000-2006.

Thomadaki, H. and A. Scorilas, 2006, “BCL2 family of apoptosis-related genes: functions and clinical implications in cancer”, *Critical Review of Clinical Laboratory Science*, Vol. 43, pp. 1-67.

Tsao, H., X. Zhang, E. Benoit and F. G. Haluska, 1998, “Identification of PTEN/MMAC1 alterations in uncultured melanomas and melanoma cell lines” *Oncogene*, Vol. 16, pp. 3397-3402.

Tsao, H., X. Zhang, K. Fowlkes and F. G. Haluska, 2000, “Relative reciprocity of NRAS and PTEN/MMAC1 alterations in cutaneous melanoma cell lines”, *Cancer Research*, Vol. 60, pp. 1800-1804.

Tsujimoto, Y., L. R. Finger, J. Yunis, P. C. Nowell and C. M. Croce, 1984, “Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation”, *Science,* Vol. 226, pp. 1097-1099.

Vicencio, J. M., L. Galluzzi, N. Tajeddine, C. Ortiz, A. Criollo, E. Tasdemir, E. Morselli, A. Ben Younes, M. C. Maiuri, S. Lavandero and G. Kroemer, 2008, “Senescence, apoptosis or autophagy? When a damaged cell must decide its path--a mini-review”, *Gerontology*, Vol. 54, pp. 92-99.

Wang, S. and W. S. El-Deiry, 2003, “TRAIL and apoptosis induction by TNF-family death receptors”, *Oncogene*, Vol. 22, pp. 8628-8633.

Xu, C., B. Bailly-Maitre and J. C. Reed, 2005, “Endoplasmic reticulum stress: cell life and death decisions”, *Journal of Clinical Investigation*, Vol. 115, pp. 2656-2664.

Yorimitsu, T. and D. J. Klionsky, 2005, “Autophagy: molecular machinery for self-eating”, *Cell Death and Differentiation*, Vol. 12 Suppl 2, pp. 1542-1552.

Youle, R. J. and A. Strasser, 2008, “The BCL-2 protein family: opposing activities that mediate cell death”, *Nature Review Molecular Cell Biology*, Vol. 9, pp. 47-59.

Zhou, M., L. Gu, F. Li, Y. Zhu, W. G. Woods and H. W. Findley, 2002, “DNA damage induces a novel p53-survivin signaling pathway regulating cell cycle and apoptosis in acute lymphoblastic leukemia cells”, *J Pharmacol Exp Ther*, Vol. 303, pp. 124-131.