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THE ROLE OF P53 IN DEATH RECEPTOR-MEDIATED APOPTOSIS OF TESTICULAR GERM CELLS IN RESPONSE TO MONO-(2-ETHYLHEXYL) PHTHALATE TREATMENT

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by

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Dedication

To my parents Bhavani and Chandrasekaran

and to Srinath and Anand

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Mono-(2-ethylhexyl) phthalate (MEHP) is the toxic metabolite of the common plasticizer di-(2-ethylhexyl) phthalate (DEHP). Exposure to DEHP or MEHP typically leads to testicular atrophy in laboratory animals. The prevalence of DEHP as an environmental contaminant is therefore cause for concern due to its potential to affect human testicular function and fertility. In the testis, MEHP induces the injury of the supportive cells called Sertoli cells, leading to Fas death receptor-dependent apoptotic elimination of germ cells. This dissertation examines the molecular mechanisms that lead to this loss of germ cells resulting from testicular exposure to MEHP. The p53 tumor suppressor protein is proposed as the cellular stress sensor that sensitizes specific germ cells to Fas-dependent cell death in response to the MEHP-induced Sertoli cell injury. This is based upon recent reports that demonstrate p53's ability to initiate the localization

of Fas to the plasma membrane of cells in a transcription-independent manner, in addition to its ability to transcribe various pro-apoptotic proteins including Fas.

In experiments involving the exposure of pre-pubertal p53 wild-type and mutant mice (C57 x 129S/v) to a single oral dose of 1g MEHP /kg body weight, we were able to demonstrate that mice lacking the p53 gene were partially protected from the testicular toxicity induced by MEHP. These studies enabled us to establish that a lack of p53 expression affected the activation of the Fas pathway in germ cells after MEHP exposure, partly by affecting Fas membrane localization and also by influencing the cellular retention of an inhibitor of this pathway, the c-FLIP (L) protein. To further clarify these results, we examined the effect of Fas activation in GC-2*spd* (ts) cells, which are germ cells that demonstrate variable activation of p53 depending on the temperature that they are maintained at. The activation of the p53 protein increased the sensitivity of GC-2 cells to undergo Fas-mediated apoptosis by modulating Fas expression on the germ cell membrane. Additionally, activation of Fas caused an increased tagging of c-FLIP (L) with ubiquitin, indicating its targeting for degradation. Thus the p53 status of GC-2 cells influenced the membrane expression of Fas, subsequently influencing the degradation of the anti-apoptotic protein c-FLIP (L). In summary, the results indicate that p53 promotes Fas activated germ cell apoptosis in response to MEHP-induced Sertoli cell injury, by instigating the degradation of c-FLIP (L) protein *via* its ubiquitinylation.

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List of Abbreviations

ABP	Androgen binding protein
AIDS	Acquired immuno deficiency syndrome
AIF	Apoptosis inducing factor
Apaf-1	Apoptotic protease activating factor-1
ANT	Adenine nucleotide transporter
ATM	Ataxia telangiectasia mutated protein
ATP	Adenosine triphosphate
BCG	Bacillus calmette-guerin
BIR	Baculoviral IAP repeat
CAD	Caspase activated deoxyribonuclease
CARD	Caspase recruitment domain
c-FLIP	Cellular-FLICE inhibitory protein
DAB	Diaminobenzidine
DcR	Decoy receptor
DD	Death domain
DED	Death effector domain
DEHP	Di- (2-ethylehexyl) phthalate
DISC	Death inducing signaling complex
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA-PK	DNA dependent protein kinase
DR	Death receptor
DTT	Dithio threitol
EDTA	Ethylenediamine tetra acetic acid
ERK	Extracellular signal regulated kinase
FADD	Fas associated death domain protein

FAK	Focal adhesion kinase
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
GC-2 *spd* (ts)	Germ cell spermatid (temperature sensitive)
Gld	Generalized lymphoproliferative disease
GSH	Glutathione
HCl	Hydrochloric acid
HSF	Heat shock factor
IAP	Inhibitors of apoptosis
ICAD	Inhibitory caspase activated deoxyribonuclease
IGF-I	Insulin-like growth factor- I
JNK	c-Jun N terminal kinase
Lpr	Lymphoproliferation
MBCD	Methyl β cyclo dextrin
MEHP	Mono- (2-ethylhexyl) phthalate
MEKK1	Mitogen activated protein kinase kinase 1
MOMP	Mitochondrial outer membrane permeabilization
NGF	Nerve growth factor
NFκB	Nuclear factor κB
NOAEL	No observed adverse effect level
PAK2	p21 activated kinase
PARP	Poly ADP ribose polymerase
PI	Propidium iodide
PKC	Protein kinase C
PLAD	Pre-ligand assembly domain
PMSF	Phenyl methyl sulphonyl fluoride
PPAR	Peroxisome proliferator activated receptor
PS	Phosphotidyl serine
PT	Permeability transition

PVC	Poly vinyl chloride
PVDF	Poly vinylidene fluoride
RAR	Retinoic acid receptor
RIP	Receptor interacting kinase
ROS	Reactive oxygen species
RZF	Ring zinc finger
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAIL	TNF related apoptosis inducing ligand
TRAF	TNFR associated factor
TUNEL	Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end labeling
VDAC	Voltage dependent anion channel

Chapter 1. Introduction

The plasticizer di-(2-ethylhexyl) phthalate (DEHP) is a common environmental contaminant that induces testicular atrophy in rodents and higher mammals (Kavlock *et al.* 2002). Of particular concern is the ability of DEHP to affect testicular functions in younger animals. Human fetuses, infants and children are at an elevated risk of exposure to DEHP from placental transfer, lactating milk, rattles, pacifiers, bottles, and toys. Therefore, in the last several decades, extensive studies have been conducted to determine the cellular and molecular determinants of DEHP toxicity in order to assess its effects on human reproductive health.

DEHP toxicity mediated by its major toxic metabolite mono- (2-ethylhexyl) phthalate (MEHP) primarily targets the support cells of the testis or Sertoli cells (Gray and Gangolli 1986). The injured Sertoli cells are unable to perform various secretory and regulatory functions and also undergo specific ultra-structural changes (Creasy *et al.* 1988). Hence, they cannot adequately execute their support role toward germ cells. Consequently, this leads to increases in germ cell death by a process called apoptosis and in case of longer exposures to DEHP or MEHP, to the appearance of testicular lesions and atrophy (Thomas *et al.* 1978; Richburg and Boekelheide 1996). The work in our laboratory has been geared toward identifying the cellular stress sensors and activators that sensitize specific germ cell populations to die in response to the MEHP-induced

Sertoli cell injury. However, before a description of these studies is given, a description of the anatomy and physiology of the testis is provided in the following section.

1.1. Overview of the Testis:

The paired mammalian testes reside in the scrotal sac below the main trunk of the organism (Russell 1990). Each testis in the scrotum is individually covered by an inelastic fibrous capsule called the tunica albuginea that imparts shape to the testis and maintains its contents. The testis is itself composed of two compartments: the seminiferous tubules and the interstitial compartment. The interstitial compartment is the less structurally organized of the two compartments and contains the blood and lymph vessels. The prominent cell types in the interstitial space are Leydig cells, which primarily secrete the hormone testosterone, and vascular cells including macrophages. The lymphatic space is not very well defined across species, but is commonly separated from the seminiferous tubules by endothelial cells.

The seminiferous tubules are coils of convoluted tubules. All the tubules begin and end at an excurrent duct system called the rété testis. Each tubule has multiple straight sections that follow the long axis of the testis and upon transverse cross-sectioning of the testis appear almost circular or oval. The seminiferous tubule wall is formed by one or more layers of myoid cells that separate the interstitium from the tubule's contents. Myoid cells being of contractile nature are responsible for the tubule's

movements to expel mature germ cells called spermatozoa. A basement membrane is found between the lymphatic endothelial cells and the myoid cells and also between the myoid cells and the cells of the tubule. The seminiferous tubule is composed of both Sertoli cells and germ cells in a distinctive spatial arrangement. The following sections will describe some of the physical and functional features of the interactions between Sertoli cells and germ cells. They will also emphasize how the unique relationship between the two cell types influences the balance between life and death for germ cells.

1.1.1. The Sertoli cell and its Functions:

The somatic cells of the seminiferous epithelium are the Sertoli cells. They were termed the 'mother' cells of the testis by Enrico Sertoli, who first described them in a paper published in 1865 (Sertoli 1865). Sertoli cells vary in size from 75-100 μm and appear columnar (Russell 1993; Skinner 2005). These cells are attached to the basement membrane and extend into the lumen of the seminiferous tubule. They have a number of cytoplasmic processes that go around and between various germ cells. They also possess a large polymorphic nucleus with distinct tripartite nucleoli (see **Fig. 1.1** for representation of Sertoli cell-germ cell associations in the testis). The Sertoli cells function in a number of capacities within the testis. They perform roles that are as diverse as physical and nutritional support for germ cells, to active phagocytosis of germ cell cytoplasmic bodies and debris. Hence they influence the microenvironment within the tubules of the testis by affecting the structural, endocrine and nutritional components

essential for the survival and functioning of germ cells therein. Sertoli cells perform their structural role by serving as physical scaffolds that maintain in place several germ cells at varying stages of division or differentiation. Sertoli cells also form a 'blood testis barrier' when they establish tight junctions with adjacent Sertoli cells. These tight junctions separate the seminiferous tubule into two compartments, a basal and an adluminal compartment. Tight junctions do not allow the movement of most macromolecules through them. The basal compartment abutting the basement membrane has access to resources from the vascular system, while the germ cells in the adluminal compartment are limited in the resources received due to the presence of the tight junctions. Hence these cells have to receive most substrates from Sertoli cell secretions. Sertoli cells therefore perform nutritive functions by delivering sugars, amino acids, energy substrates, lipids, metals, and vitamins; and secretory functions by secreting a variety of proteins, ions, hormones, proteases, metal carrier proteins, growth and paracrine factors to germ cells in the adluminal compartment and to other cells within the tubule as well.

Regulatory factors secreted by Sertoli cells are typically glycoproteins. These include androgen binding protein that binds and transports testosterone; transferrin a ferric ion shuttling protein that moves these ions from the basal side of Sertoli cells to germ cells; other metal ion transport proteins; proteases that are involved in the tissue remodeling as specific germ cells called spermatids differentiate; several growth and paracrine factors such as insulin like growth factor I, transforming growth factors α and β, müllerian inhibiting substance, inhibin, activin and many more. These factors influence

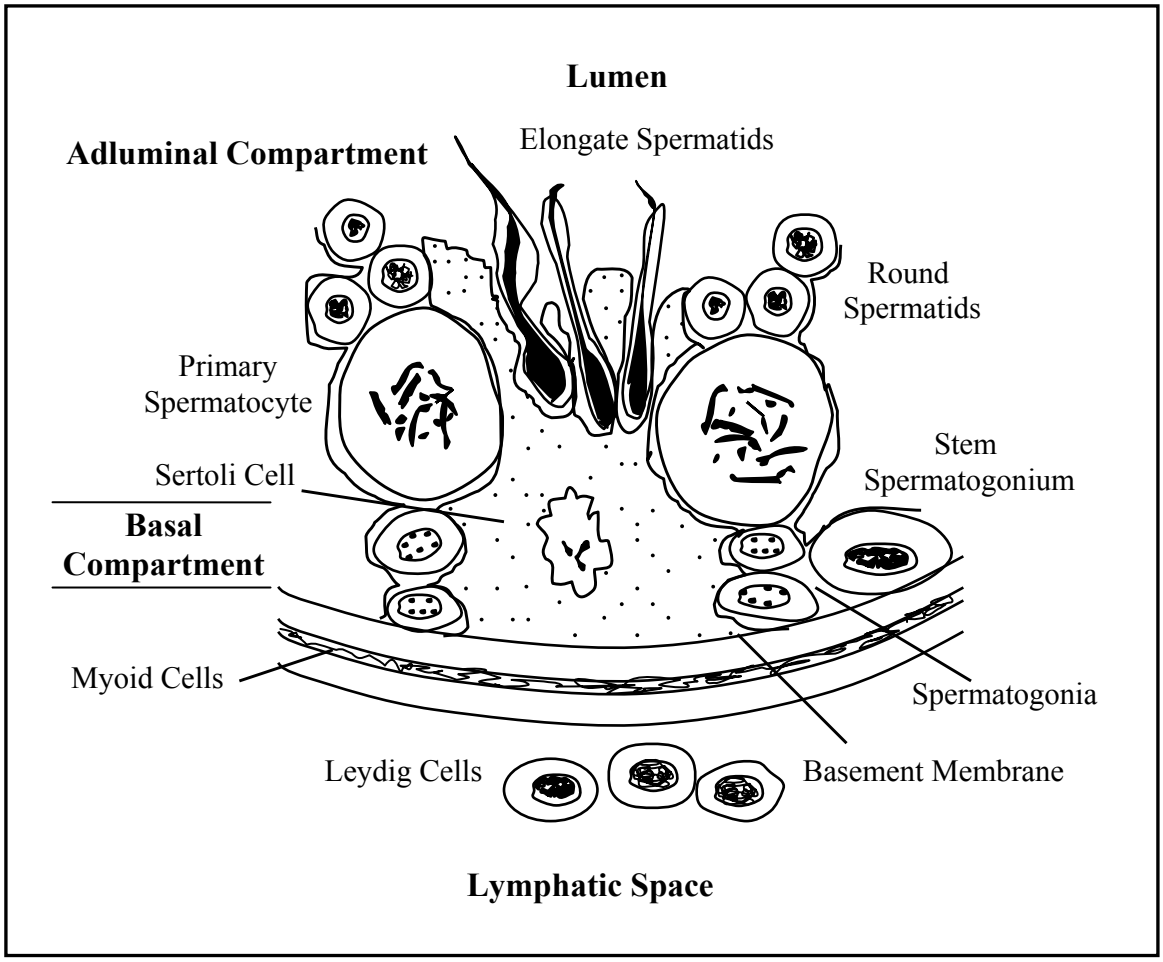

Fig. 1.1. Schematic representation of Sertoli cell-germ cell interactions within seminiferous tubules. Sertoli cells extend from the basement membrane of the tubule to the lumen. Their cytoplasmic processes surround and support the various germ cell types. Stem germ cells divide to form spermatogonia (in basal compartment of tubule); spematogonia divide and differentiate to become spermatocytes, then round and elongate spermatids and eventually spermatozoa (all present in the adluminal compartment).

the growth and proliferation of germ cells and also that of Sertoli cells. They also affect differentiation and other functions that affect the normal testicular function and fertility of the organism.

1.1.2. Germ cells and Spermatogenesis:

The process by which stem germ cells eventually form mature haploid cells called spermatozoa (the male gamete), is called spermatogenesis (Russell 1990). Sertoli cells play a very important role in maintaining this process of spermatogenesis. Sertoli cells ensure the transition of dividing and differentiating germ cells closer to the lumen of the tubule, so that germ cells may be released when they become spermatozoa. Additionally, the various regulatory proteins secreted by Sertoli cells as well as the hormones testosterone and FSH (follicle stimulating hormone), are critical for ensuring the growth, proliferation and differentiation of germ cells and hence the timing of spermatogenesis.

Germ cells originate as stem germ cells that can be found in the basal compartment of the seminiferous tubule adjoining the basement membrane and Sertoli cell membranes (Russell 1990). These cells undergo mitotic cell division to either regenerate themselves or become the proliferative spermatogonia, A_p (p-paired). A_p spermatogonia then undergo several mitotic divisions to generate A_{al} (al-aligned) cells with as many as 4-32 A_{al} cells found connected to each other *via* cytoplasmic bridges called intercellular bridges. These A_{al} cells undergo differentiation to become A_1

spermatogonia. Successive mitotic cell divisions give rise to 5 more types of differentiated spermatogonia, A_2-A_4, In (intermediate) and B type cells. Thus, a thousand cells may arise from one stem cell spermatogonium. Interestingly, the total numbers of Sertoli cells in the testis has been found to establish the upper limit of sperm cell production (Huckins 1978). Huckins (1978) demonstrated in his paper that only 25% of the theoretically possible numbers of pre-leptotene spermatocyte cells were produced from A_1 spermatogonia.

Spematocytes are the germ cells that arise from the cell division of B type spermatogonia. These are the preleptotene spermatocytes and they are the last germ cell type to undergo DNA synthesis. All the germ cells until the preleptotene stage are confined to the basal compartment of the seminiferous tubule. The preleptotene spermatocytes then transition to form leptotene spermatocytes and start to move away from the basal compartment. These cells move toward the adluminal compartment, passing through intermediate compartments that are formed by the breaking and reforming of tight junctions between two Sertoli cells. These primary spermatocytes then initiate meiotic cell division once they reach the adluminal compartment. Meiosis I which initiates the separation of homologous chromosomes is prolonged and prophase I (leptotene to pachytene to zygotene to diplotene phases) can take up to 3 weeks to complete. Secondary spermatocytes formed after meiosis I then undergo a shorter meiosis II and the genetic material is halved.

The haploid cells formed after meiotic cell divisions are called round spermatids. These cells then undergo the elaborate process of cell transformation without cell division called spermiation. The round spermatids progressively elongate, form flagella and refine their acrosomal structure while losing their extra cytoplasmic baggage. The formation of the motile flagellum and the acrosomal structure which carries lytic enzymes, along with the modification of cytosolic and nuclear material clearly signals preparations for fertilization. Elongate spermatids called spermatozoa are eventually released into the lumen of the seminiferous tubules. These spermatozoa pass through the rété testis and reach the epididymis.

1.1.3. Sertoli Cell Injury and its Importance to Germ Cell Viability:

Sertoli cell involvement in the maintenance of an appropriate extracellular environment for germ cells and in spermatogenesis, have emphasized the crucial role played by Sertoli cells in ensuring the well-being of germ cells within the testis. Studies on the effects of various chemical toxicants such as phthalates, 2,5-hexanedione, nitroaromatics like 1,3-dinitrobenzene, and the chemotherapeutic drug cisplatin in the testis (Strandgaard and Miller 1998; Lee *et al.* 1999; Sawhney *et al.* 2005) go a step further by demonstrating that causing Sertoli cell dysfunction eventually affects germ cell viability.

In response to these chemical toxicants, several Sertoli cell functions were found to be affected. These included the alteration of the ultrastructure of Sertoli cells with the

modification of the microtubule filament and intermediate filament structures (Richburg and Boekelheide 1996; Boekelheide *et al.* 2003) and the formation of Sertoli cell vacuoles. The alterations in Sertoli cell structure may be responsible for observations of germ cell detachment in response to the Sertoli cell toxicants. These chemical exposures also caused disruption of Sertoli cells' metabolic functions and disrupted hormone/regulatory protein secretions (Heindel and Chapin 1989; Williams and Foster 1989; Thysen *et al.* 1990; Reeve *et al.* 2002). These were followed by observations of germ cell death by a process called apoptosis or programmed cell death leading to testicular atrophy. Thus the original definition of 'mother cell' for Sertoli cells was a very apt description of Sertoli cell functions. The loss of normal Sertoli cell functions appears to ultimately affect the ability of the testis to maintain their germ cell populations.

The study of chemical toxicants affecting only Sertoli cells engenders an appreciation for the paracrine nature of these toxicity models. Germ cell death by the process of apoptosis turns out to be one of the primary consequences of Sertoli cell injury. When the ability to perform their normal support functions are compromised, Sertoli cells do not themselves undergo death in most cases, but cause the germ cells that are dependent on them for survival to die. Cell death by apoptosis is typically mediated in response to direct injury to the cells from a variety of cues including growth factor withdrawal, DNA damage, oxidative stress, loss of extracellular matrix support and others. The Sertoli cell injury model offers us the unique opportunity to study the molecular and cellular changes associated with cell death due to indirect injury instead. In

our laboratory, we utilize the testicular exposure to phthalates to study germ cell apoptosis. However, before a description of this injury model can be provided, one must have a fundamental understanding of the process of apoptosis which is described in the following section.

1.2. Apoptosis:

The word 'apoptosis' is derived from the Greek language and translates to mean 'falling of leaves'. Apoptosis is a form of physiological cell death that was first observed in the development process of various multi-cellular organisms. This observation of cell death in different cellular systems was demonstrated several times over in the last 150 years, but the term "programmed cell death" to describe cell death in tadpole and insect metamorphosis was first coined by Lockshin and Williams in 1965 (Lockshin and Williams 1965). In 1972, Kerr, Wyllie and Currie (Kerr *et al.* 1972) were the first to conceive of the now commonly used term 'apoptosis' to describe the process of controlled cell deletion. They believed that this term aptly described cell death based on the Greek meaning of the word. They were able to demonstrate that apoptosis was a basic biological phenomenon with distinct morphological features that could be initiated or inhibited by a variety of stimuli, both physiological and pathological and that it was initiated in all kinds of cells.

Kerr *et al.* (Kerr *et al.* 1972) were the first to collectively describe all the morphological features associated with apoptosis; nuclear and cytoplasmic condensation,

membrane blebbing, the break up of the cells' components into membrane bound fragments or apoptotic bodies, the uptake of the apoptotic bodies by other cells by phagocytosis, and degradation of the remnants of these cells. They very clearly demonstrated that apoptosis took place in a wide variety of tissues, adult, young or embryonic, neoplastic or normal tissue, in response to noxious agents, or as a physiological phenomenon in development or otherwise. They were also able to show the absence of inflammation after apoptosis.

After this seminal paper, the field would get its next impetus from the genetic characterization of the nematode, *Caenorhabditis elegans* that was carried out by the groups of Horvitz and Sulston in the 1970's. They made the discovery that 131 cells of the 1090 somatic cells of the worm always died during development. Horvitz's group at the Massachusett's Institute of Technology then went on to identify the key components/genes involved in the cell death program of the worm and their studies would provide some of the most basic insights into the initiation and propagation of the apoptotic machinery in cells. They identified the genes Ced-3and 4 as being essential for apoptosis and Ced-9 as being anti-apoptotic (Ellis and Horvitz 1986); (Hengartner *et al.* 1992). Ced-3 and 4 are the worm homologs of the proteolytic enzymes called caspases and the adaptor protein APAF-1 (apoptotic protease activating factor-1), while Ced-9 is the homolog of Bcl-2, an anti-apoptotic protein family member. Meanwhile, in 1975 a serum factor induced by endotoxin in BCG (Bacillus Calmette-Guerin) vaccine infected mice was found to kill transplanted tumors (Carswell *et al.* 1975). The discovery of this

factor called the Tumor necrosis factor or TNF, would in turn lead to the discovery of other cytokines and death receptors that could initiate apoptosis in cells.

Apoptosis is considered an important regulator of cellular homeostasis within tissues and organs. Apoptotic cell death does not merely sculpt organs during development, but also enables the removal of old and damaged components of the adult body in the absence of inflammation. It therefore helps to maintain cell numbers and tissue size. Apoptosis can thus be deemed an essential and very fundamental physiological function for an organism and as necessary as cell proliferation and differentiation (Vaux and Korsmeyer 1999). The process of apoptosis also helps to defend the body by promoting cell death of the invading microorganism. However, if the process goes awry, it has been found to contribute to the pathogenesis of several diseases and disorders (Thompson 1995). Excessive cell death has been found to lead to autoimmune disorders such as AIDS (Acquired immunodeficiency syndrome), several neurodegenerative diseases such as Parkinson's and Alzheimer's, ischemic injury related myocardial infarction and stroke, amongst others. On the other hand, diseases associated with prolonged cell survival such as cancer, viral infections and immune disorders such as systemic lupus erythematosus arise in part due to a failure of apoptotic processes within the body. Apoptosis is also initiated when the cell is exposed to stress inducing conditions such as DNA damage, oxidative stress, growth factor withdrawal, radiation injury, chemical toxicants, and physical injury such as heat shock (Held 1997; Chandra *et al.* 2000; Rich *et al.* 2000; Robertson and Orrenius 2000; Beere 2001). A cell will

typically try to repair the damage inflicted upon it and all attempts will be made to avert cell death. However, when the damage is irreparable, cells will turn on the cell death machinery. Understanding the nuances to this process will therefore remain one of the enduring questions for science.

1.2.1. The Biochemistry of Apoptosis:

The process of apoptosis is possible due to the concerted action of a family of cysteine proteases called caspases (for reviews see Hengartner 2000; Degterev *et al.* 2003). A majority of the caspases are activated only after the initiation of apoptosis and are responsible for the basic cellular changes that eventually lead to cell death. These caspases are proteases with an active site cysteine moiety and they cleave all their protein substrates after an aspartate residue in the P1 position. Caspases typically recognize a tetrapeptide sequence with the aspartate residue being the C-terminal residue on all their substrates and usually cleave their substrates only at one or very few sites. Their action leads to protein activation or inactivation, or disassembly of a multiprotein complex, or the release of proteins from such a complex. Over 100 caspase substrates have been identified to date (Fischer *et al.* 2003).

Some of the substrates cleaved by caspases are responsible for the characteristic morphological changes in cells undergoing apoptosis (Hengartner 2000; Fischer *et al.* 2003). For instance, the cleavage of ICAD or inhibitory caspase activated DNase releases

the active DNase CAD which was in complex with ICAD. CAD is an endonuclease that cuts DNA at the linker regions between nucleosomes. This results in the formation of DNA fragments of lengths corresponding to multiples of 180 base pairs. These fragments of DNA can be visualized on an agarose gel as a ladder and is used typically as a marker of apoptosis in that cell population. Caspases also inactivate the DNA repair protein PARP (poly ADP-ribose polymerase) by cleavage and thus prevent repair of fragmented DNA. Caspases cleave a number of structural proteins such as lamins that lead to the collapse of nuclear lamina and hence the shrinkage and budding of the nucleus. They also cleave cytoskeletal proteins such as actin, tubulin, and vimentin, in addition to proteins involved in cell adhesion and anchorage such as FAK (focal adhesion kinase) and catenins. The cleavage of PAK2 (p21 activated kinase) and the structural proteins fodrin and gelsolin, are believed to contribute to the formation of membrane blebs.

Other caspase substrates are several signaling molecules which when activated are involved in the signaling reactions necessary to advance the apoptotic state. Caspases themselves are activated by cleavage by other caspases. Proteins such as Bid, MEKK1 (MAP kinase kinase kinase 1), some forms of PKC (protein kinase C), calcineurin, etc. which are involved in stress signaling are activated by caspase cleavage. On the other hand, proteins involved in DNA repair and survival signaling, such as ATM (ataxia telangiectasia mutated protein), DNA-PK (DNA-dependent protein kinase), Akt, HSF (heat shock factor) are typically inactivated. Thus caspases are the basic work-horses of

the apoptotic machinery that are responsible for most of the reactions leading up to cell death.

Apoptotic cells are cleared from the extracellular milieu by phagocytes or by the phagocytic action of neighboring cells. However, for apoptotic cells to be recognized for clearance, a signaling system has evolved which involves the amino phospholipid, phosphotidyl serine (PS) (Savill and Fadok 2000; Schlegel and Williamson 2001). PS is typically present in the inner leaflet of the plasma membrane lipid bilayer. In apoptotic cells, the PS is externalized and serves as a signal for macrophages and other phagocytic cells. The asymmetric distribution of phospholipids in the plasma membrane is disrupted during apoptosis by the inactivation of an ATPase or translocase that keeps PS internalized and also by the activation of a scramblase protein that externalizes PS. The signals that activate this event are not well defined, but seem to be largely caspase-independent. Macrophages in turn express lectin-like receptors, integrin receptors, scavenger receptors, and other molecules which may or may not act in concert to recognize PS. Once apoptotic bodies are cleared from the field and their components recycled, the process is essentially completed.

1.2.2. Activation of Caspases, the Effectors of Apoptosis:

There are 11 known human caspases, 11 murine caspases, 7 Drosophila caspases and 4 in *C. elegans* (for review see Degterev *et al.* 2003). Caspases possess an active site cysteine- which is part of a conserved pentapeptide 'QACXG' sequence. Caspase-1 or

interleukin 1-β processing enzyme (ICE) was the first human caspase discovered. Like all proteases, caspases are expressed as zymogens or inactive forms of themselves. The most important question that then arises is, how do these 'effectors' of apoptosis get activated?

Caspase zymogens are composed of three domains- an N-terminal prodomain, a p20 and a p10 domain (Degterev *et al.* 2003; Hengartner 2000). These zymogens are processed by cleavage to separate the p20 and p10 domains first and then the p20 domain from the prodomain. The active enzyme is a heterotetramer consisting of two p20 and two p10 subunits with two active sites at the opposite ends. Interestingly, the processed sites on caspases are also the tetrapeptide motifs with aspartate at the P1 position common to all caspase substrates, indicating that caspases proteolytically process themselves. Indeed, the group of caspases called executioner caspases comprising the short prodomain caspases, 3, 6 and 7, are cleaved by active initiator caspases. The activated executioner caspases then become involved in most of the reactions previously described that initiate the biochemical and morphological changes during apoptosis. This process of caspase activation however does not account for the activation of the very first caspases in this cascade, namely the initiator caspases.

Initiator caspases include caspases 1, 2, 4, 5, 8-12, and 14 and have longer prodomains than the executioner caspases. Caspases 4, 5, 10 and 14 are strictly human caspases, while caspases 11 and 12 can only be found in mice. Caspase 1, 5 and 11 are involved in mediating inflammation, while the remaining initiator caspases are involved

in mediating apoptosis. The two initiator caspases that are most commonly activated in response to various stress stimuli are the caspases 8 and 9. Caspase-8 is the key initiator caspase in death receptor activated apoptotic pathways, while caspase-9 is the primary caspase that initiates cell death *via* the mitochondrial stress pathway. In response to apoptosis inducing stimuli, these caspases are typically recruited to multi-protein complexes, where their increased concentration and proximity to other zymogen molecules is thought to enable autocatalytic processing of the caspases. In fact, a model called the 'induced proximity model' was proposed in 1999 by Salvesen and Dixit (Salvesen and Dixit 1999) that predicted that the clustering of initiator caspases would allow for their self-processing and activation, as the zymogens possessed some latent proteolytic activity themselves. Interestingly, caspase-9 possessed high catalytic activity when in the presence of its cytosolic co-factors and did not seem to require cleavage to be activated (Stennicke *et al.* 1999). Recent studies by Salvesen's group (Boatright *et al.* 2003) have led to the proposal of a unified model for apical caspase activation. They were able to demonstrate that both caspases 8 and 9 were activated by dimerization and that internal proteolysis of these caspases was a secondary event after dimerization of the caspases. The model for apical caspase activation may still undergo further refinement, but that for executioner caspases remains straightforward. Executioner caspases are incapable of processing substrates unless they are themselves activated, and this adds a very important level of checks and balances against accidental cell death.

1.2.3. The Adaptors in the Apoptotic Pathway:

To enable death signals to be transmitted to the caspases, multiprotein complexes are formed in the cell. In case of caspase-8, death receptors activated at the cell membrane by their ligands bind to an adaptor protein called FADD (Fas associated death domain protein) *via* interactions between death domains (DD) at their C-terminal ends (Chinnaiyan *et al.* 1995; Boldin *et al.* 1996). FADD, in turn through its N-terminal death effector domain (DED), binds to one of the two DED's expressed on the prodomain of a caspase-8 zymogen molecule (Muzio *et al.* 1996; Medema *et al.* 1997). The complex formed by activated death receptors, FADD and caspase-8 is called the death-inducing signaling complex or DISC. Caspase-8 monomers brought in proximity to each other at the DISC are then activated by autocatalytic processing.

Signaling through the mitochondrial pathway involves the release of an inner membrane mitochondrial protein called cytochrome c. Caspase 9 is activated when it is bound to its adaptor protein APAF-1, the mammalian homologue of Ced-4, *via* interactions between their caspase recruitment domains (CARD) (Hofmann *et al.* 1997). CARD domain may be found as part of the prodomain of caspase-9. The two proteins along with cytochrome c and ATP/dATP form a very large protein complex called the apoptosome (~700-1400 kDa), which enables caspase-9 to achieve the right conformation for activation (Cain *et al.* 2002).

Thus the processing and activation of the initiator caspases 8 and 9 is achieved by the formation of large complexes, the DISC and the apoptosome respectively. The formation of these complexes is achieved by protein-protein interactions *via* conserved domains, namely DD's, DED's and CARD's which are composed of 6 α-helices and are conserved across species (Hofmann *et al.* 1997).

1.2.4. Regulators of Apoptosis:

Regulators of apoptosis are those proteins that either directly bind to adaptors that recruit initiator caspases and affect caspase activation, or bind directly to caspases themselves, or alter the function of key pro-/anti-apoptotic proteins, and therefore alter cell fate. Thus these proteins can either be inhibitors or pro-apoptotic proteins themselves.

c-FLIP (cellular-FLICE inhibiting protein) is an inhibitor of the death receptor activated pathway that binds the adaptor protein FADD, when FADD is present in the DISC (Irmler *et al.* 1997). The c-FLIP protein was discovered at about the same period of time by 7 independent groups (Goltsev *et al.* 1997; Han *et al.* 1997; Hu *et al.* 1997; Inohara *et al.* 1997; Irmler *et al.* 1997; Shu *et al.* 1997; Srinivasula *et al.* 1997). The protein has been shown to exist as three alternately spliced forms, (L), (S) and (R) (Irmler *et al.* 1997; Golks *et al.* 2005). All three forms have considerable homology to the caspase-8 molecule. The (L) form structurally resembles the caspase-8 protein the most, with two N-terminal DED's and a caspase catalytic domain without the active site

cysteine. The (S) and (R) forms of c-FLIP contain only the prodomain with its two DED's, followed by a short stretch of amino acids. c-FLIP (S) and (R) completely inhibit caspase-8 cleavage, while the (L) form will allow partial processing of caspase-8 at the DISC (Krueger *et al.* 2001; Golks *et al.* 2005). The role of c-FLIP (L) in caspase-8 inhibition remains controversial as it has been alternatively described as pro- or anti-apoptotic by different groups; however it appears that the level of expression of the c-FLIP (L) predisposes its ability to act as a pro- or anti-apoptotic protein (higher the expression, more anti-apoptotic its effect) (Chang D.W 2002; Micheau *et al.* 2002; Boatright *et al.* 2004). In addition, their results (Chang D.W 2002; Micheau *et al.* 2002; Boatright *et al.* 2004) indicate that c-FLIP (L) forms a heterodimer with the caspase-8 molecule more readily than caspase-8 homodimer formation at the DISC. The formation of the heterodimer does not prevent activation of the caspase-8 zymogen, but perhaps restricts the heterodimer to the DISC. Thus, the caspase-8 homodimer may mediate cytotoxicity by its ability to move to the cytosol, while the caspase-8-c-FLIP (L) heterodimer acts locally at the cell membrane and is restricted in its choice of substrates.

Another key regulator at the DISC is the receptor interacting kinase or RIP. RIP is a serine threonine kinase that can induce apoptosis when overexpressed (Nagata 1997). It has been shown to bind the Fas death receptor *via* DD's (Peter and Krammer 2003) but preferably binds the adaptor protein TRADD at the DISC of activated TNF death receptors. RIP bound to TRADD recruits other proteins that mediate the activation of the pro-survival NFκB (NF- nuclear factor) pathway (Nagata 1997). The adaptor protein

TNF receptor associated protein 2 (TRAF2) is also recruited to the DISC, where it is implicated in activating the stress kinase c-Jun N-terminal kinase (JNK).

The Bcl-2 family of proteins are amongst the most important regulators of apoptosis expressed by cells. This family has both pro- and anti- apoptotic members and about 20 mammalian members are known to exist (for review see Cory *et al.* 2003). The Bcl-2 proteins respond to intracellular stress cues such as DNA damage and cytokine deprivation and the interaction between opposing family members ultimately decides the fate of the cell. The pro-survival members of the family include Bcl-2, Bcl-x_L, and Bcl-w, while the pro-apoptotic proteins include Bax, Bak, Bid, Bim, Noxa, Puma, etc. This family of proteins can also be subdivided based on the presence of certain domains in their protein structure. The anti-apoptotic proteins and the Bax sub-family of pro-apoptotic proteins (Bax, Bak and Bok) carry three conserved BH domains, BH1, 2 and 3 while the BH3 only sub-family of pro-apoptotic proteins expresses only the BH3 domain. The BH3 only proteins when activated typically block the activity of the anti-apoptotic Bcl-2 proteins by docking in the hydrophobic groove formed by their BH1, 2 and 3 domains. Bid is the only BH3 only protein that interacts directly (albeit transiently) with the Bax proteins. Bax and Bak form oligomeric pores on the mitochondrial membranes in response to apoptotic cues and enable the release of cytochrome c. Thus the Bcl-2 proteins play a very important role in initiating the mitochondrial stress pathway, leading to the activation of caspase-9 and other downstream effectors of apoptosis. Bcl-2, originally discovered as an oncogene in human follicular lymphoma, is also a very

critical survival molecule that is responsible for providing another source of checks to the apoptotic process.

The inhibitors of apoptosis or IAP family of proteins directly bind to both initiator (caspase-9) and executioner caspases (caspase-3 and -7) and block their activity (for review see Liston *et al.* 2003). This family of proteins which include c-IAP 1 and 2, XIAP and Survivin are cytosolic proteins with 70-80 amino acid domains called baculoviral IAP repeat (BIR) domains with which they bind to caspases. IAP's with multiple BIR domains bind caspase-9 through their BIR3 domain and caspases 3 and 7 through their BIR2 domains. These proteins also carry a Ring zinc finger domain (RZF) at their C-terminus that possesses E3 ubiquitin ligase activity. These RZF's enable the IAP's to target the caspases bound to them for degradation. Negative regulators of IAP's are also expressed by cells. These include the mitochondrial proteins Smac/Diablo and Omi/HtrA2. Smac and Omi are released from the mitochondrial inter-membrane space when apoptotic signals cause cytochrome c release. Both proteins once released usually bind IAP's at their BIR domains and prevent their activity, thereby enabling apoptotic pathways to proceed.

1.2.5. Apoptotic Pathways:

Initiation of apoptotic signaling pathways in a cell occurs typically by the activation of death receptors on the cell membrane, or when the mitochondria release

mediators of apoptosis in response to stress stimuli. These two pathways are not the only transduction pathways, but are certainly the two main ones that eventually lead to activation of executioner caspases and the reactions mediated by them. Schematic representations of these two pathways are presented in **Figs. 1.2 and 1.3**.

Death receptors are members of the TNF receptor family/nerve growth factor (NGF) receptor family (for reviews, see Nagata 1997; Ashkenazi and Dixit 1999). The members of the family that mediate cell death include the TNF receptors 1 and 2, DR3 (DR- death receptor, Apo-3), Fas (Apo-1, CD95), DR4 (TRAIL-R1), DR5 (TRAIL-R2, Killer), DR6, DcR1 and 2 (DcR-decoy receptor) and osteoprotegrin (OPG). The receptors contain 2-6 repeats of a cysteine rich subdomain in their extracellular domain, a transmembrane domain and a cytosolic domain that carries the death domain. Decoy receptors and OPG are incapable of transmitting death signals due to the lack of extracellular or cytosolic domains or the presence of truncated domains. The other death receptors transmit signals *via* their DD interactions as mentioned in section 1.2.3. These receptors are only activated when they are bound to their cognate ligands as trimers.

Interestingly, both TNF receptors and Fas pre-associate as trimers *via* pre-ligand binding association domains (PLAD) before they are activated and do not trimerize only when bound to their ligands as originally predicted (Chan *et al.* 2000; Siegel *et al.* 2000). The death ligands are members of the TNF subfamily of cytokines and are TNF, FasL, Apo-3L, and TRAIL (TNF related apoptosis inducing ligand). These ligands have a

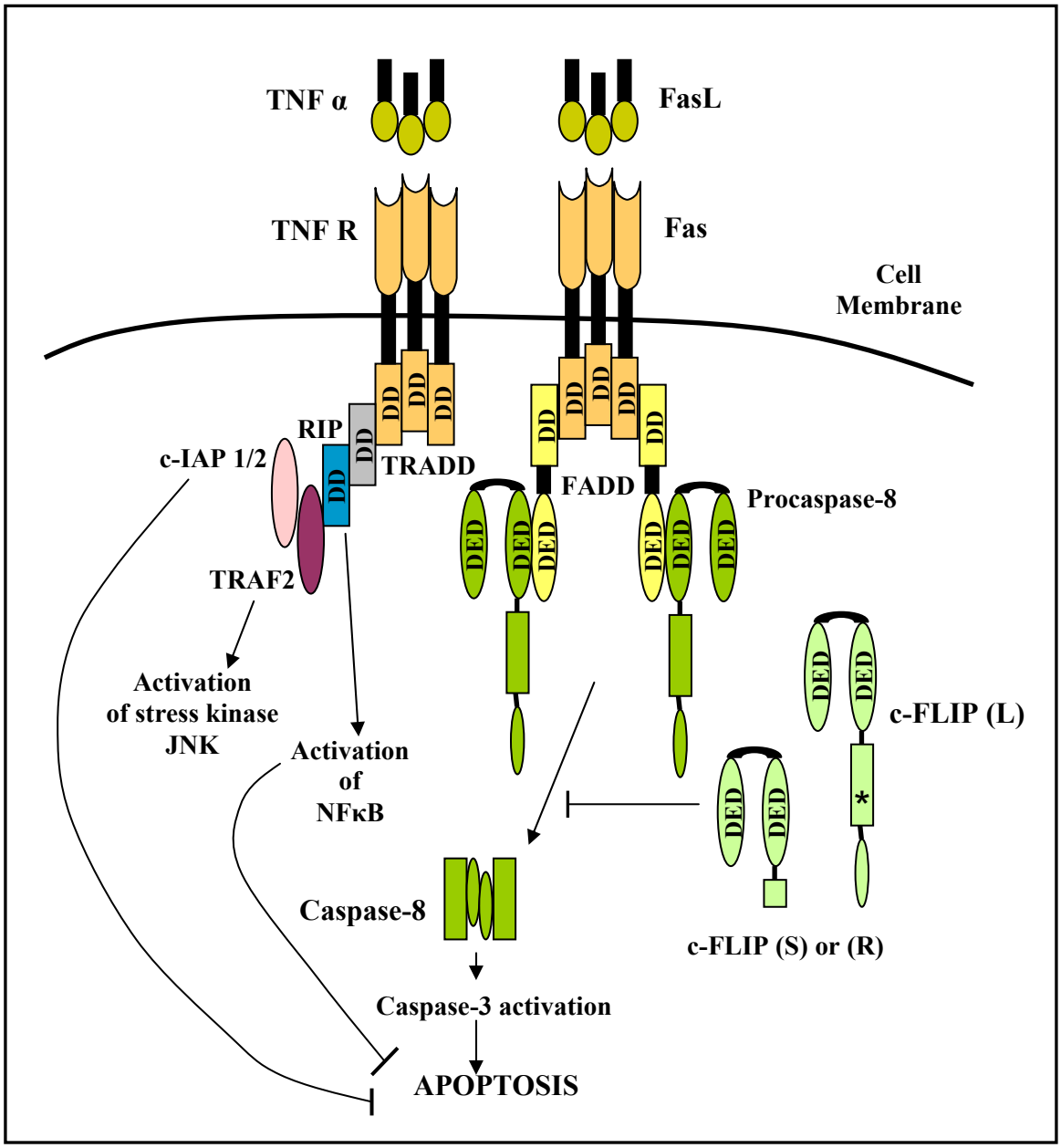

Fig. 1.2. Representation of the apoptosis pathway initiated by the activation of death receptors. ('*' represents the active site mutation of c-FLIP (L)).

cytoplasmic N-terminus and an extracellular C-terminal tail and are type II membrane proteins. The binding of the death ligands to death receptors put into motion a signal transduction cascade which involves the formation of the DISC, activation of proximal caspase-8 enzymes and the downstream proteolysis and activation of executioner caspases.

The second apoptotic pathway which involves the mitochondria proceeds due to the initiation of mitochondrial outer membrane permeabilization (MOMP) (for review see Green and Kroemer 2004). MOMP is accompanied by the dissipation of the mitochondrial inner transmembrane potential or $\Delta\Psi_M$. This causes the release of proteins found in the space between the inner and outer mitochondrial membranes such as cytochrome c, AIF (apoptosis inducing factor), Smac, etc. MOMP is usually caused by the formation of a pore in the inner and outer membranes together, called the permeability transition pore or PT pore, or by the formation of a pore only on the outer membrane created by the oligomerization of Bax and/or Bak. The PT pore is formed by the adenine nucleotide transporter (ANT) and the voltage dependent anion channel (VDAC). Some reports indicate that Bax and Bak can bind to the VDAC as well. MOMP is triggered by a variety of stimuli, including viral proteins, increased intracellular Ca^{2+}, reactive oxygen species, metabolic perturbations, toxicants like ethanol, heavy metals and salicylates, etc. MOMP also eventually leads to loss of mitochondrial functions which are critical for the cell to survive. Cytochrome c once released binds to APAF-1 in the presence of ATP/dATP and together they can bind caspase-9 to form the apoptosome.

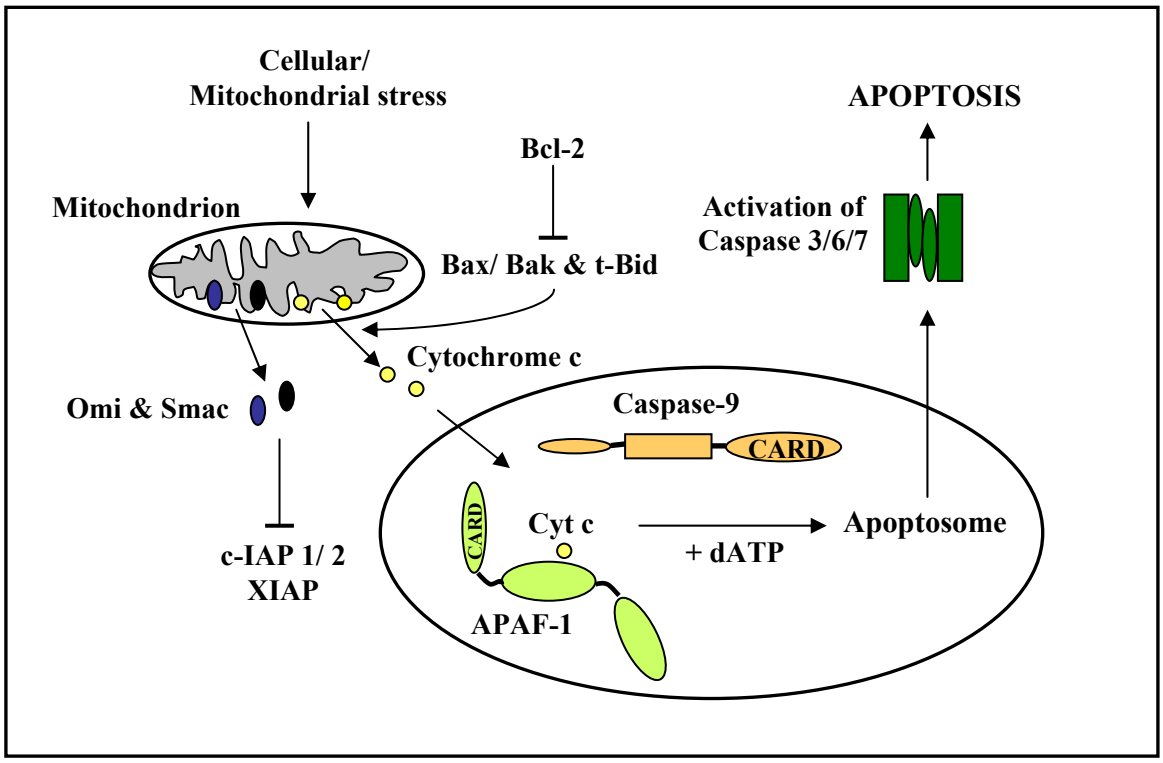

Fig. 1.3. Representation of the apoptosis pathway initiated by mitochondrial stress. The formation of the apoptosome by caspase-9, APAF-1, dATP and cytochrome c released from the mitochondria, leads to the activation of executioner caspases and hence apoptosis.

The activation of caspase-9 in turn leads to activation of caspases 3, 6 and 7. Executioner caspases eventually cleave subunits in the respiratory complexes I and II of the mitochondria, thereby completely destroying mitochondrial energy production and hence putting the cell well on its way to cell death.

The two apoptotic pathways described can also be categorized as type I and II pathways (Scaffidi *et al.* 1998). Cells that are described as type I cells typically activate caspase-3 due to robust formation of the DISC complex after FasL exposure, leading to the activation of large amounts of caspase-8 which cleave procaspase-3. Type II cells alternatively do not mediate the formation of such a strong DISC complex when exposed to FasL. Instead, minimal caspase-8 activation leading to the cleavage of the Bid protein, the binding of t-Bid to Bax and/or Bak and the subsequent MOMP occurs, and caspase-3 is activated due to the activation of caspase-9. Thus type I cells such as thymocytes and peripheral T cells may initiate cytochrome c release in response to FasL, but will still undergo cell death in spite of Bcl-2 over-expression (Scaffidi *et al.* 1998; Huang *et al.* 1999). Hepatocytes on the other hand, which are type II cells, fail to undergo apoptosis in the presence of excess Bcl-2.

1.3. The Phthalate Injury Model and Germ Cell Apoptosis

In our laboratory we study the effects of different environmental and therapeutic chemicals including phthalates and cisplatin on testicular function. This section will

provide a brief introduction to di-(2-ethylhexyl) phthalate or DEHP, which is a commonly used plasticizer in polyvinyl chloride (PVC) products and other plastics. A discussion of the general characteristics of DEHP, its toxicity profile and how this relates to testicular changes will also be provided.

DEHP imparts flexibility to plastics and is utilized widely in a number of commercial products (this information is based on the National Toxicology Program-Center for Evaluation of Risks to Human Reproduction or the NTP-CERHR panel report on DEHP, published in October 2000; available online at http://cerhr.niehs.nih.gov/news/phthalates/DEHP-final.pdf or as published in the journal Reproductive Toxicology in 2002, Kavlock *et al.* 2002). It is used in the manufacture of building products (wallpaper, cable and wire insulation), car products (car seats, vinyl upholstery), clothing (footwear, raincoats), food packaging, children's products (toys), and in medical devices (IV bags, blood storage bags, etc.). Due to its lack of covalent bonding to the PVC, DEHP leaches out from these various objects. Hence DEHP gets widely dispersed in the environment from its release during manufacture, from consumer goods, and during their waste incineration. DEHP bio-accumulates but does not bio-magnify in the food chain, as it can be metabolized and is biodegradable. The typical routes of exposure to DEHP are *via* ingestion and inhalation. The physical properties of very low vapor pressure and poor water solubility limit DEHP's concentration in the air and water to 10^{-3} to 10^{1} ppb (parts per billion). Thus, the chief route of exposure to DEHP is though ingestion. The range of human exposure from all sources excluding non-

dietary ingestion and medical and occupational exposure are an estimated 3-30 μg/ kg of body weight/ day.

Anxiety about the use of phthalates and especially DEHP in consumer products has arisen from the observation of liver, kidney, testicular and developmental toxicity in various organisms (for reviews see Thomas *et al.* 1978; Kavlock *et al.* 2002). In fact, the observation of testicular atrophy and anti-androgenic effects in very young rodents has led to the ban of DEHP's use in children's products such as pacifiers, rattles and teethers in the US and Canada. DEHP when orally ingested by different organisms cause the following liver effects: peroxisomal proliferation, increased liver weights, hepatocellular hypertrophy and hyperplasia sometimes leading to formation of adenomas and carcinomas (in rodents), and increases in enzyme markers relevant to peroxisomal proliferation. However, DEHP is not classified by the US regulatory agencies as a carcinogen as the studies that produced the carcinomas in rodents used extremely high daily doses of DEHP, beyond normal exposure levels. Developmental toxicity in rodent pups exposed in-utero to DEHP typically manifested itself as morphological abnormalities of the axial and appendicular skeleton, cardiovascular system, and the eye and neural tube, while kidney effects of DEHP included decreased kidney weights in males and increased weights in females.

The NOAEL or 'no observed adverse effect level" for DEHP toxicity from DEHP exposure studies in rodents have been highly variable on account of the duration, species

and age differences in each study. One study on the general toxicity of DEHP reported a NOAEL as low as 3 mg/kg body weight/day. Based on these results and considering that the typical daily exposure to DEHP does not exceed 3-30 µg/ kg of body weight/ day for human adults, the ambient exposure to DEHP cannot be considered toxic. Therefore, one needs to justify studying reproductive toxicity as caused by DEHP and its main metabolite mono-(2-ethylhexyl) phthalate.

1.3.1. Mono-(2-ethylhexyl) Phthalate and Sertoli Cell Injury:

DEHP is a testicular toxicant and causes changes such as decreased testicular weights, testicular lesions and atrophy, and changes in androgen hormone secretion when different organisms are exposed to the toxicant (Kavlock *et al.* 2002). However, it is a product of DEHP metabolism, called mono-(2-ethylhexyl) phthalate or MEHP that produces the effects attributed to DEHP for testicular toxicity in rodents (Gray and Gangolli 1986; Sjoberg *et al.* 1986). MEHP is a monoester phthalate produced by the hydrolysis of DEHP by stomach, pancreatic and intestinal esterases (for review see Albro and Lavenhar 1989) (Also see **Fig. 1.4** for representation of the hydrolysis reaction involved in the metabolism of DEHP to MEHP). Sensitivity to the testicular effects of DEHP is species specific with rodents being the most sensitive, and dogs, marmosets and monkeys being less sensitive. This variation in sensitivity may be attributed to the differences in the metabolism of DEHP. Humans can typically cause phase II biotransformation of MEHP by causing its glucuronidation

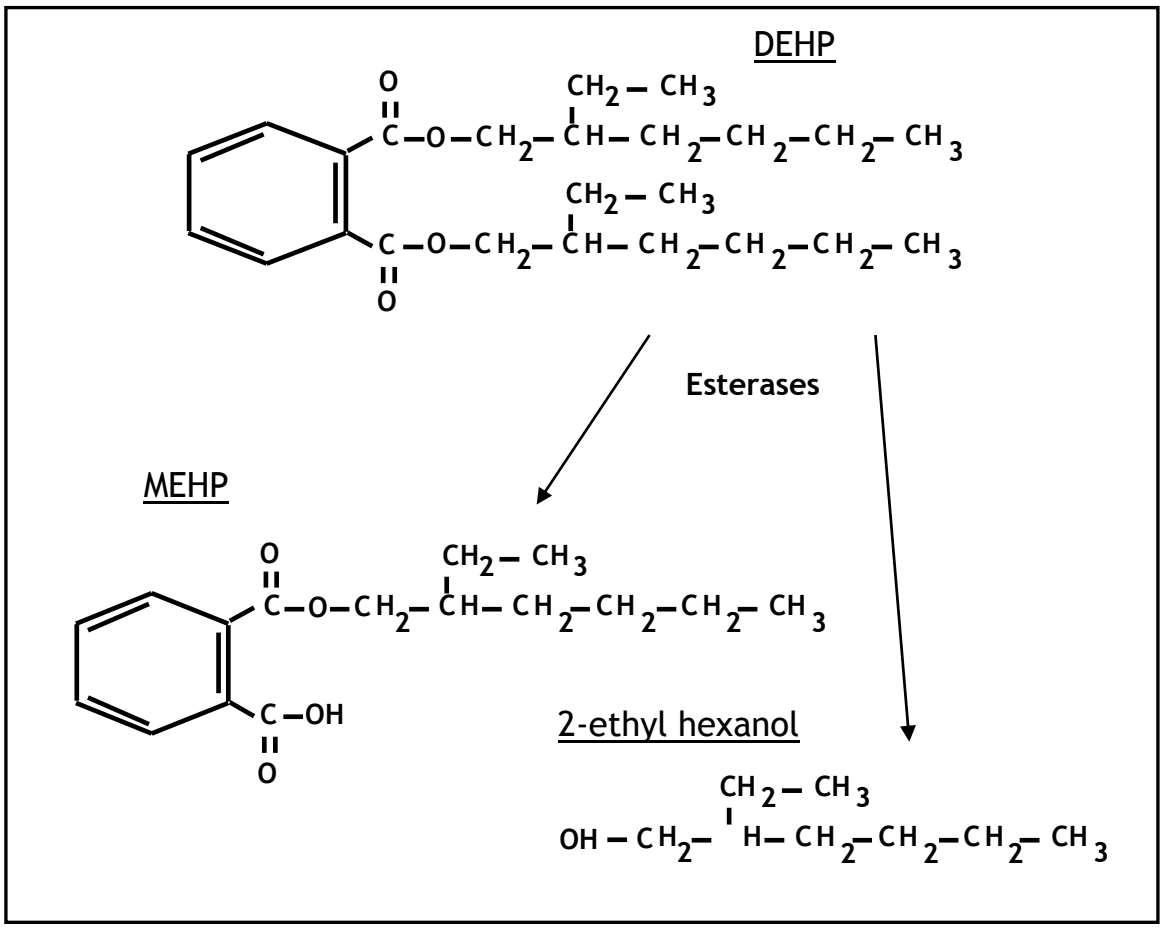

Fig 1.4. Schematic representation of the hydrolysis reaction involved in the metabolism of DEHP to MEHP

and subsequently excrete it, while rats and mice further hydrolyze MEHP (Schmid and Schlatter 1985). Interestingly, human children up to 3 months of age do not possess the ability to glucuronidate and they may also receive DEHP and MEHP *via* milk from their mothers or through placental transfer (Dostal *et al.* 1987; Cresteil 1998).

With exposure to phthalates from toys, placental transfer, lactating milk, pacifiers, bottles and other objects, human fetuses, infants and younger children are exposed to DEHP at much higher levels than adults. Recent studies also revealed that when rats were exposed to DEHP in utero during their period of sexual differentiation (from gestational day 14 to post natal day 3), this led to reduced testosterone synthesis (Gray *et al.* 2000; Parks *et al.* 2000). This in turn caused the demasculinization of the male pups as they displayed decreased anogenital distances, retention of nipples, a vaginal pounch, cleft phallus with hypospadias, undescended testes in some cases, and other changed features that were retained into adulthood. In addition, the testicular effects of DEHP and MEHP are age-dependent, with fetal, neonatal and young pre-pubertal rats being the most sensitive to their effects and the adult animals being insensitive (Gray and Gangolli 1986; Dostal *et al.* 1987; Teirlynck *et al.* 1988; Li *et al.* 2000). Thus, with slightly deficient xenobiotic-metabolism capabilities and at the appropriate age for toxicant effects to be more intense, concern has been raised about the effects of phthalates on the future fertility of human children. This justifies continuing research on the testicular effects of phthalate esters.

The other interesting aspect to MEHP toxicity is its cell specificity. MEHP specifically targets Sertoli cells of the testis. Research by several labs demonstrate that Sertoli cells are the primary targets as evidenced by reduced androgen binding protein and seminiferous tubule secretions only in immature rats (Gray and Gangolli 1986); germ cell detachment from Sertoli cell-germ cell co-cultures only after MEHP exposure and not with DEHP (Gray and Gangolli 1986); reduced proliferation of Sertoli cells in neonatal rats (Dostal *et al.* 1988; Li *et al.* 2000); alterations in Sertoli cell vimentin filaments and cell ultrastructure (Creasy *et al.* 1988; Richburg and Boekelheide 1996); reduced pyruvate secretion, transferrin secretion, cellular ATP levels and mitochondrial succinate dehydrogenase activity in cultured Sertoli cells (from pre-pubertal rats) exposed to MEHP; increased lactate production (Chapin *et al.* 1988; Moss *et al.* 1988; Thysen *et al.* 1990); and inhibition of FSH stimulated cyclic AMP accumulation in Sertoli cell cultures also from immature rats (Heindel and Chapin 1989).

In addition, in their 1986 study, Gray and Gangolli (Gray and Gangolli 1986) demonstrated that MEHP did not cross the blood testis barrier in young rats and hence was not directly in contact with germ cells of the adluminal compartment. Further studies demonstrated that spermatocytes and spermatids were the primary germ cell types that underwent apoptosis in response to MEHP in pre-pubertal rodents (Dostal *et al.* 1988; Richburg and Boekelheide 1996; Giammona *et al.* 2002). Hence, the phthalate injury model is ideal to study the mechanisms governing indirect injury to germ cells as previously discussed in section 1.1.3. The use of pre-pubertal (or immature) animals in

our study ensures a paracrine model of toxicity, without the inherent death of the injured cells themselves to confuse the issue. In our laboratory, we study the molecular and cellular changes that lead to germ cell apoptosis after exposure of rodents to MEHP. These studies will ultimately help us in understanding how fertility may be maintained or how testicular injury may be alleviated in males despite exposures to various environmental insults.

1.3.2. Physiological vs Pathological Control of Germ Cell Apoptosis: Participation of the Fas-FasL Signaling Pathway in Germ Cell Apoptosis in Response to Cellular Stress:

Testicular germ cell apoptosis has been observed under both physiological and pathological conditions. As mentioned in section 1.1.2, the number of Sertoli cells in a seminiferous tubule limits the numbers of germ cells they can support (Huckins 1978). Thus, germ cell proliferation is balanced in the testis by their cell death and this process is critical for the continued fertility of the organism. Indeed, dysregulation of the physiological germ cell apoptotic processes can lead to either the formation of testicular cancers or conversely to the decreased fertility of the organism.

To control the clonal expansion of germ cells, the apoptosis of type A_2, A_3 and A_4 spermatogonia is routinely observed in the testis (Allan *et al.* 1987). Various cues including paracrine signals (stem cell factor (SCF), leukemia inhibitory factor, desert hedgehog) and endocrine signals (testosterone and pituitary gonadotrophins) regulate

germ cell numbers in the normal testis (for review see Print and Loveland 2000). The loss of signals from the survival factor SCF, or androgen withdrawal or the use of a gonadotrophin antagonist leads to increased germ cell apoptosis (Billig *et al.* 1995; Woolveridge *et al.* 1999; Guerif *et al.* 2002). In addition, for the development of functional spermatogenesis, the first wave of spermatogenesis is accompanied by a massive wave of germ cell apoptosis which occurs during the 3rd week of life in rodents (Rodriguez *et al.* 1997). Interestingly, both the pro- and anti-apoptotic members of the Bcl-2 family are required for this process to occur normally. Bax-deficient mice and Bcl-2 transgenic mice have both been found to skip this first wave of apoptosis, followed by a complete break-down of spermatogenesis and end up being sterile (Knudson *et al.* 1995; Rodriguez *et al.* 1997; Russell *et al.* 2002). Another member of the Bcl-2 family, the Bcl-w protein, is also critical for the efficient functioning of spermatogenesis. Bcl-w is found expressed in Sertoli cells and in other cell types within seminiferous tubules, except in spermatids. The lack of Bcl-w expression leads to the progressive loss of both germ cells and Sertoli cells in these mice (Russell *et al.* 2001).

Other cellular regulators such as the tumor suppressor protein p53 have also been examined for their role in germ cell apoptosis. In mice expressing reduced amounts of p53, a testicular giant cell degenerative syndrome has been observed (Rotter *et al.* 1993). p53 which is a protein that regulates the processes of apoptosis, cell cycle arrest and DNA repair (details discussed in the next section) is normally detectable only in pachytene spermatocytes (Schwartz *et al.* 1993). Thus the reduced expression of p53 in

these meiotic cells may account for the formation of giant cells that are genetically defective. However, the appearance of giant cells is mouse strain specific and depending on the background strain, p53 null mice can be fertile (Rotter *et al.* 1993). Interestingly, the death receptor Fas and its ligand FasL may not be involved in physiological germ cell death. One report (Boekelheide *et al.* 1998) suggested that Fas and FasL expression was increased in rats between the ages of 16-35 days. However, male mice lacking the functional FasL protein (*gld* mice) and/ or p53 did not seem to have any overt reproductive or developmental defects, while their female counterparts had developmental abnormalities and were sub-fertile (Embree-Ku and Boekelheide 2002). Therefore, based on studies of the various knockout and transgenic mice, it would appear that physiological germ cell apoptosis in the testis is primarily mediated by the Bcl-2 family members and that this may be influenced by either paracrine or endocrine factors.

In contrast, depending on the type of physical injury or chemical toxicant to which rodents have been exposed, the Fas-FasL system plays a partial or primary role in the initiation of germ cell apoptosis. Thus, the death receptor-ligand system may predominantly function in a situation where stress-induced germ cell death occurs instead of being involved in apoptosis initiated under normal conditions in the testis. Interestingly, the Fas receptor is predominantly expressed in germ cells, while FasL is expressed in Sertoli cells (Lee *et al.* 1997; Richburg *et al.* 1999; Koji *et al.* 2001). The expression of FasL in Sertoli cells may explain the immune privilege of the testis

(Bellgrau *et al.* 1995). The cellular localization of these two proteins is therefore important for the paracrine activation of cell death in germ cells.

The following examples will help in the appreciation of the role played by Fas during testicular injury. When testicular torsion of spermatic cords which impedes blood flow to the testis and causes ischemic conditions is alleviated by reperfusion of the tubules, this leads to an increase in germ cell apoptosis (Lysiak *et al.* 2000; Koji *et al.* 2001). The release of cytochrome c and the increased expression of Fas in the spermatocytes and spermatids undergoing cell death in response to this injury, implicate both the mitochondrial and the death receptor pathways in germ cell apoptosis caused by ischemia-reperfusion. Testicular exposure to radiation on the other hand, indicates a role for p53 and Fas in spermatogonial apoptosis (Beumer *et al.* 1998; Lee *et al.* 1999; Embree-Ku *et al.* 2002). Spematogonia of p53 null mice are radio-resistant as are germ cells expressed by mice carrying a dysfunctional Fas gene, or the lpr^{cg} mice. In yet another physical injury model to the testis, that of cryptorchidism involving the retraction of the testis into main trunk of the organism and thereby the elevation of its ambient temperature, both p53 and Fas may have a role to play in germ cell death. Using p53 null mice and *lpr* mice (also dysfunctional for Fas receptor), researchers demonstrated a sequential mechanism of p53- and Fas-mediated germ cell apoptosis in cryptorchid testes (Ogi *et al.* 1998; Yin *et al.* 2002). However, not all responses to testicular injury utilize the Fas-FasL pathway. Exposure to mild testicular hyperthermia involves the activation

of Bax-mediated germ cell apoptosis without activation of the death receptor pathway (Yamamoto *et al.* 2000; Vera *et al.* 2004).

When rodents are exposed to chemical toxicants such as 2,5 hexanedione and MEHP, Fas and FasL proteins prove to be the critical regulators of germ cell apoptosis (Lee *et al.* 1997; Lee *et al.* 1999). In fact, when *gld* mice (lacking functional FasL) were exposed to MEHP, they were significantly protected against its toxicity (Richburg *et al.* 2000; Giammona *et al.* 2002). The specific injury to Sertoli cells by both 2,5 hexanedione and MEHP is a very critical aspect to these injury models. Both toxicants caused an increase in FasL expression in Sertoli cells and the concomitant increase in Fas on germ cell membranes, followed by enhanced germ cell apoptosis (Lee *et al.* 1997; Lee *et al.* 1999; Richburg *et al.* 1999). Based on the above research, it was postulated that injury by these toxicants to the main 'support' cell of the testis abrogated the ability of these Sertoli cells to maintain normal functions and led to the paracrine elimination of Fas expressing germ cells.

As discussed in section 1.1.3, the injury to Sertoli cells provides us with a unique opportunity to study germ cell apoptosis initiated by indirect injury. Thus, the observation of Fas-mediated germ cell apoptosis in response to phthalate induced injury to Sertoli cells forms the basis for the commencement of this thesis. An interesting question that arose from these observations was the question of how Fas was 'regulated' or 'directed' or 'activated' in germ cells after MEHP exposure. MEHP does not directly injure germ

cells and no biochemical changes in germ cells except for the Fas response and the subsequent apoptosis of germ cells had been reported. It was therefore intriguing to determine how germ cells sensed stress from the injured Sertoli cells and how this elicited a Fas mediated apoptotic response. To answer these questions, we proposed a role for the p53 protein in the germ cell apoptosis caused by testicular exposure to MEHP and the reasons are explained in the subsequent section.

1.4. p53 and its Potential Role in MEHP Mediated Germ Cell Apoptosis:

DNA double strand breaks and lesions, chromosomal aberrations, activation of oncogenes, hypoxia, ribonucleoside triphosphate depletion, cold and hot shock, excess nitric oxide and growth factor withdrawal are examples of conditions of cellular stress that can cause the activation of a very unique molecule called p53 (for reviews see Levine 1997; Harris and Levine 2005). Over 50% of human cancers have been reported to contain mutations in the p53 gene. In fact, a report in 1994 by Hollstein M *et al.* (Hollstein *et al.* 1994) compiled over 2500 somatic mutations of the p53 gene in various human tumors and tumor cell lines. This list albeit long, was actually incomplete and did not include germ-line mutations that can cause individuals to inherit faulty alleles of the p53 gene, like those suffering from the Li-Fraumeni syndrome. p53 is thus the most critical tumor suppressor protein in the cell and this is borne out by the sheer number of functions that have been attributed to this protein. In response to different kinds of cellular stress, p53 can mediate cell cycle arrest, senescence, DNA repair, differentiation

or apoptosis. It is able to carry out such diverse functions because of its ability to transactivate a large number of genes in a sequence specific manner (for reviews see Stewart and Pietenpol 2001; Vousden and Lu 2002). Thus, genes encoding proteins involved in a variety of functions such as p21, 14-3-3-σ and GADD45 that are involved in cell cycle arrest, Fas, DR5, PUMA, Noxa and Bax in apoptosis, and the xeroderma pigmentosum p48 protein in DNA repair are all target genes transactivated by p53.

The human p53 protein is 393 amino acids long and can be divided into three functional domains, an acidic amino-terminal transactivation domain, a central DNA binding domain and a basic carboxy-terminal oligomerization domain (for reviews see Levine 1997; Stewart and Pietenpol 2001). p53's ability to function as a transcription factor is based on the binding of co-factors to, and modifications of the N-terminal transactivation domain. For example, Mdm-2 which is the chief regulator of p53's functions, keeps the p53 protein inactive in normal cells by binding to it at the N-terminus and targeting it to the proteosome for degradation. The phosphorylation of various serine and threonine residues in the transactivation domain by stress-response kinases in the cell affects the interaction between Mdm-2 and p53, allowing the release of p53 from Mdm-2. This allows p53 to localize to the nucleus where it binds to the promoter regions of various genes *via* its DNA binding domain. The DNA binding domain recognizes four repeats of a specific consensus sequence, namely 5' PuPuPuC(A/T) 3', arranged as pairs of inverted repeats. The transactivation domain meanwhile completes its functions by interacting with various co-factors and proteins of

the transcription machinery to enable the initiation of transcription. The C-terminal domain on the other hand, contains both the nuclear localization signal and the nuclear export signal, along with an oligomerization domain. The oligomerization domain plays a part in the transcriptional process as well. It enables the homo-tetramerization of p53, which is required to initiate transcription. The nuclear export signal gets masked in the process and p53 is retained in the nucleus to complete its work. In addition, the C-terminus is also phosphorylated and acetylated and these modifications are also important for the activation of p53. Hence, depending on the source of cellular stress, p53 is modified in a number of different ways by phosphorylation, acetylation, methylation, sumoylation and ubiquitination to mediate the appropriate response of inhibition of cellular progression to enable repairs, or apoptosis or senescence to terminate the life cycle of a cell (for review see Woods and Vousden 2001).

1.4.1. The Role of p53 in Apoptosis:

The first response to cellular stress by p53, does not always lead to the initiation of apoptosis. Unless the cellular damage is severe and hence irreparable, cells prefer not to initiate the cell death machinery which is quite irrevocable. In fact, DNA repair was found to be initiated in the early stages of p53-induced apoptosis in the mouse mammary carcinoma cell line MOD and the apoptotic response was reversed if the apoptotic stimulus was removed (Geske *et al.* 2000). In addition, most of the apoptotic proteins that are transactivated by p53 (exception being PUMA) have lower affinity binding sites for

p53 at their promoter sites, than do proteins such as p21 and Mdm-2 (Vousden and Lu 2002). Therefore, it is suggested that p53 may activate several apoptotic mediators simultaneously depending on the nature of the cellular stress and the cell type exposed to it. For example, in human leukemia cells, Bax and Fas were only up-regulated in cells containing functional p53 (Kobayashi *et al.* 1998) or in the BV173 cells, but not in p53 null HL60 cells. However, depending on the source of stress, exposure to radiation or treatment with the chemotherapeutic drug ara-C, the specific regulators involved in initiating apoptosis were different. Fas was activated by p53 in the BV173 leukemic cells only after exposure to ara-C and not after radiation exposure, while Bax was up-regulated by both treatments.

p53 mediated apoptosis can proceed through the death receptor activated pathway or through activation of the mitochondrial pathway. In a variety of cell types, the expression of Fas and/or DR5 can be induced to initiate apoptosis in a p53-dependent manner when cells are exposed to either ionizing radiation or chemotherapeutic drugs (Wu *et al.* 1997; Kobayashi *et al.* 1998; Muller *et al.* 1998; Wu *et al.* 1999; Petak *et al.* 2000; Burns *et al.* 2001). DR5 null mice were radioresistant in an organ specific way (Finnberg *et al.* 2005) while *lpr* mice (having a functional mutation of the Fas protein) displayed thymocyte apoptosis in response to the radiation exposure (Strasser *et al.* 1995). Additionally, p53 also triggered the apoptosis of thymocytes and spleenocytes *in vitro*, in response to radiation damage in a Fas-independent manner (Fuchs *et al.* 1997). The other dysfunctional Fas mutant mice, lpr^{cg} mice, were found to have significantly reduced germ

cell apoptosis after exposure to ionizing radiation (Embree-Ku *et al.* 2002). Thus, as mentioned in the previous paragraph, p53 can activate mediators of apoptosis based on the context of the cells' surroundings and the source of stress.

Alternatively, p53 mediates the release of cytochrome c leading to the induction of apoptosis *via* the mitochondrial pathway. This release of cytochrome c is based upon the movement of Bax to the mitochondrial membrane (Schuler *et al.* 2000; Gao *et al.* 2001). Additionally, mice or cells deficient for Apaf-1, Bid, caspase-9, Noxa or PUMA were all found to protect cells (from different organelles/sources) from p53-induced apoptotic responses (Soengas *et al.* 1999; Sax *et al.* 2002; Villunger *et al.* 2003).

A transcription-independent role for p53 during apoptosis has also been demonstrated. In 1998, Bennett M *et al*. (Bennett *et al.* 1998) showed that in human vascular smooth muscle cells, activation of p53 caused the trafficking of Fas receptors from the storage vesicles of the Golgi to the cell membrane in a transcription-independent manner. This sensitized the cells to apoptotic cell death. p53 also directly acts at the mitochondria to release cytochrome c and initiate apoptosis in response to DNA damage or hypoxia, amongst other things (Marchenko *et al.* 2000; Sansome *et al.* 2001; Chipuk *et al.* 2004; Erster *et al.* 2004). In these studies, a fraction of cytosolic p53 localized to the mitochondrial membrane or inter-membrane space. p53 was described to act in a similar fashion to the BH3 only proteins like Bid, and caused the oligomerization of Bax at the mitochondrial membrane while releasing all pro-apoptotic Bcl-2 proteins from their

interaction with Bcl-x_L. Thus p53 may augment its transcriptional activation of apoptotic proteins with the non-transcriptional activation of either death receptors or the mitochondrial stress pathway.

1.4.2. The role of p53 in MEHP-mediated germ cell apoptosis : Hypothesis and Specific Aims:

Given the extremely important role played by p53 in cellular apoptosis, it would be reasonable to assume that p53 may be involved in mediating germ cell apoptosis in response to MEHP toxicity, as a 'universal stress sensor'. Moreover, germ cell apoptosis is an extremely important physiological process that ensures the correct transmission of information to progeny. Therefore, the involvement of a critical tumor suppressor protein in mediating this process after toxicant induced injury is likely. As mentioned in section 1.3.2, response to MEHP toxicity to Sertoli cells manifests itself as Fas-mediated germ cell apoptosis. Fas is a p53-transcribed gene and appears to also be modulated in a transcription-independent manner by p53. Additionally, spermatocytes which always express detectable levels of p53 (Schwartz *et al.* 1993)are the predominant germ cell type to undergo apoptosis after testicular exposure to MEHP in pre-pubertal rodents (Richburg and Boekelheide 1996). This raises the possibility that these cells may regulate Fas expression in response to MEHP in a p53-dependent manner.

Exposure to MEHP may not cause direct injury to the germ cells, but leads to the loss of germ cell attachment to Sertoli cells and decreased nutritional support to germ

cells by the injured Sertoli cells. p53 is typically described to transactivate death receptors in response to DNA damage or other specific cellular injuries. However, in the absence of overt cellular damage, we postulate that p53 mediates changes in germ cell Fas expression by a transcription-independent mechanism in response to MEHP mediated stress. In pre-pubertal rats exposed to MEHP, previous work by this laboratory has demonstrated that an increased localization of Fas receptors on germ cell membranes is critical for ensuring germ cell apoptosis (Richburg *et al.* 1999). These observations therefore lead up to the working hypothesis of this dissertation: "p53 sensitizes germ cells to Fas mediated apoptosis by modifying the membrane localization of Fas, after exposure to MEHP".

In order to determine if p53 plays a role in germ cell apoptosis in response to testicular toxicity caused by MEHP, our first specific aim involved testing the response of pre-pubertal mice lacking p53 protein expression (p53 -/-) and their wild type littermates (p53+/+) to an acute oral dose of MEHP. These mice were also utilized to characterize the differences in Fas response to MEHP, i.e. its localization to germ cell membranes, based on the p53 status of the mice. Analyses of DR5 receptor membrane localization was also carried out in testicular cells, as a means to compare the effect of p53 activation on one of its alternate transcriptional targets. To further clarify the functional significance of our *in vivo* results, in our second aim, we analyzed the relationships between these proteins in a transformed germ cell line called GC-2*spd* (ts) cells. The activation of p53 in these cells could be modified by altering the temperature at which they were grown as

the cells express a temperature sensitive mutation of the p53 protein. This enabled us to model the cells as p53+/+and p53 -/- cell types as well. The results from our experiments are presented in subsequent chapters.

Chapter 2: Materials and Methods

2.1. Animals

Approximately three week old, male C57BL/6J x 129S/v chimeric mice were obtained from The University of Texas M.D. Anderson Cancer Center, Science Park Research Division, Smithville, TX. These mice were either deficient for p53 (p53-/-) or were wild- type littermates (p53+/+) and were originally created at UT MDACC, Houston by Dr. Lawrence Donehower's research group (Donehower *et al.* 1992). All animals were acclimatized for 1 week prior to experiments. The animal room climate was kept at a constant temperature (74 $\pm$ 2°F) at 35-70% humidity with a 12h alternating light-dark cycle. Animals were given standard lab chow and water *ad libitum*. All procedures involving animals were performed in accordance with the guidelines of the University of Texas at Austin's Institutional Animal Care and Use Committee in compliance with guidelines established by the National Institutes of Health.

2.2. Cell Culture and Treatment Protocols

Germ cell-2 spermatid (temperature sensitive) cells or GC-2*spd* (ts) cells were obtained from the American Type Culture Collection (Rockville, MD). They were cultured at 37 ºC in a 5% CO_2 atmosphere, in Dulbecco's modified Eagles's media (DMEM, Life Technologies, Carlsbad, CA) containing 4.5g/L glucose, L-glutamine, 110 mg/L sodium pyruvate, pyridoxine hydrochloride, and supplemented with 10% fetal

bovine serum, and 100 units/ml penicillin-streptomycin. These cells express a temperature sensitive p53 mutant protein ($p53^{Val\text{-}135}$), which completely localizes in the nucleus only at the lower temperature of 32 ºC, and not at 37 ºC (Hofmann *et al.* 1994). In all experiments, p53 was activated by transferring cells cultured for 24 h at 37 ºC, to an incubator maintained at 32 ºC for a further 24 h.

GC-2 cells that had been in culture for 48 h (grown at either temperature) were exposed to various treatments including JO2 (anti mouse-Fas antibody, 554254, Pharmingen, San Diego, CA, at 5µg/ml), Hamster IgG (Isotype control for JO2, 553961, Pharmingen, at 5µg/ml), mono-(2-ethylhexyl) phthalate (MEHP, TCI America, Portland, OR, at 200 µM), DMSO (Sigma Aldrich, San Diego, CA, at equivalent volume as MEHP), MG132 (Alexis Biochemicals, 260-092-M001, San Diego, CA, at 0.5, 1, 5 and 10 µM) and human TRAIL (TRAIL was a kind gift from Dr. Shawn B. Bratton; used at 1µg/ml) for time periods indicated in the results. For experiments involving pretreatment of GC-2 cells with the cholesterol depletor, methyl β-cyclodextrin (MBCD, C4555, Sigma Aldrich, at 1, 5 and 10 mM), cells were exposed to MBCD in serum free media for 30 min at 37 ºC. The cells were then washed to remove any trace of MBCD and then exposed to JO2. Only cells maintained at the p53 permissive temperature were subjected to MBCD pretreatment and were returned to 32 ºC for the JO2 treatments thereafter, for the indicated periods of time shown in the results. The concentrations used for JO2 and MEHP treatments were based on previous studies in primary cell cultures from testicular cells (Lee *et al.* 1997; Lee *et al.* 1999).

2.3. MEHP Exposure Protocol

To evaluate the consequences of MEHP-induced Sertoli cell injury on testicular germ cell apoptosis, 26-30 day old male mice (p53+/+ and p53-/-) were given a single dose of MEHP (1 g/kg) by oral gavage, a standard procedure for the investigation of MEHP-induced testicular toxicity (Thomas and Thomas 1984). Both the dose and the time points used were selected based on our previous work (Lee *et al.* 1997; Lee *et al.* 1999; Richburg *et al.* 1999; Giammona *et al.* 2002). MEHP (>97% purity) was purchased from TCI America. Animals received MEHP in corn oil at a volume equal to 4 ml/kg. Control animals received a similar volume of vehicle (corn oil). Vehicle and MEHP-treated animals were killed by CO_2 inhalation at the time points indicated. Both testes were rapidly removed, one testis was quickly frozen in liquid N_2 and stored at -80°C and the other testis was immersion fixed overnight in Bouin's solution (Polysciences, Inc., Warrington, PA), washed in 70% EtOH-Li_2CO_3 (saturated solution, Mallinkrodt, Paris, KY) and embedded in paraffin.

2.4. Analysis of Apoptotic Index in Testicular Cross-Sections by TUNEL Assay

Apoptotic fragmentation of DNA in mouse testis cross sections was evaluated using terminal deoxy-nucleotidyl transferase-mediated digoxigenin-dUTP nick end labeling (TUNEL) using the ApopTag^{TM} kit (Intergen, Purchase, NY) and standard protocols for paraffin sections as previously reported (Seaman *et al.* 2003). 4 μm paraffin sections from Bouin's fixed and paraffin embedded testes were obtained. Following warming at

60 ºC for 1 h, the sections were placed in Citru Solv^{TM} (Fisher Scientific, Pittsburgh, PA), rehydrated in a graded alcohol series and boiled in 0.01 M sodium citrate solution to unmask antigens. The sections were then soaked in 2% H_2O_2 solution to quench endogenous peroxidase activity. Digoxigenin-dUTP end-labeled DNA was detected with anti-digoxigenin-peroxidase antibody followed by peroxidase detection with 0.05% diaminobenzidine (DAB; Peroxidase substrate kit, Vector Labs, Burlinggame, CA) as the substrate. After antibody and substrate development, the tissue sections were counterstained with a 1:14 dilution of hematoxylin in water. TUNEL-positive germ cells were quantified in each tissue section by counting the number of brown TUNEL-positive cells in each round seminiferous tubule. The apoptotic index reflects the percentage of seminiferous tubule cross sections with >3 TUNEL-positive germ cells. Two testis cross-sections per mouse, with a minimum of at least 100 tubule cross-sections, were analyzed and the data are presented as averages ± SEM from 3-4 mice. In the control mouse testis, the percentage of seminiferous tubules with more than three TUNEL-positive cells is approximately 4%, so an increase in apoptosis is easily determined using this method of analysis.

2.5. Apoptosis Measurement in GC-2 Cells by Flow Cytometry

The Annexin V-Propidium Iodide (PI) assay was used to identify apoptotic cells *via* Annexin-V's ability to bind to externalized phosphotidylserine molecules (van Engeland *et al.* 1998). PI is expelled from live and early-apoptotic cells which maintain

their cell membrane integrity, while being retained by dead cells or cells undergoing secondary necrosis/late apoptosis. These cells are then identified based on their "apoptotic" characteristics (Annexin positive-stain or Annexin and PI positive-stain) using a flow cytometer. Cells in culture after appropriate treatment(s) were collected by trypsinization at 400g for 6 min. They were re-suspended in fresh media for about 30 min to neutralize the effects of trypsin. The cells were pelleted by centrifugation at 400g and the cell pellets were suspended in 1X binding buffer (from the ApoAlert Annexin-V FITC apoptosis kit, Clonetech, San Diego, CA) to wash them. They were collected once again by centrifugation and the cell pellet was re-suspended in 1X binding buffer. Approximately 10^5-10^6 cells/ sample were incubated with 5 µl of Annexin-V conjugated with fluorescein isothiocyanate (FITC) available from the kit, for 5-10 min. The cells were then incubated with 10 µl of PI for 2-5 min. Flow cytometric analysis of these cells was then carried out on a Coulter Epics XL cytometer.

2.6. Immunocytochemistry (ICC)

Localization of Fas or p53 was examined in acetone-methanol (1:1)-fixed, Triton X-100 permeabilized, GC2 cells grown on chamber slides (Lab Tek, 154526, Hatfield, PA) with or without a 3 h JO2 treatment, using rabbit (anti-Fas, sc-716) or goat (anti-p53, sc-1312) polyclonal antibodies purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The primary antibodies were detected using Alexa-fluor® 488 conjugated secondary antibodies from Molecular Probes, Eugene, OR. Images were viewed using a Nikon E800

microscope, captured with the Nikon Cool-SNAP digital camera, and processed using MetaMorph Imaging System software (v 6). Assay controls included incubation of the cells without primary antibody or by substituting the primary antibody with rabbit/goat IgG at the same dilution as primary antibody.

2.7. Immunoprecipitation (IP)

GC-2 cells were treated with JO2 for a period of 3 or 6 h before being collected for analysis. Cells were lysed using a RIPA lysis buffer containing 150 mM NaCl, 1% IPEGAL, 0.5 % sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), and 1 mM PMSF. Lysates were pre-cleared with 50 µl (50 % slurry) of protein-G sepharose beads (Amersham Pharmacia Biosciences, Piscataway, NJ) in 500 µl of the lysis buffer for 1 h. The supernatant was retained following clearing, and was incubated with 2.5 µg of a rat monoclonal anti-c-FLIP antibody (ab16078, AbCam, Cambridge, MA) for an hour at 4 ºC. Following this incubation, 50 µl of sepharose was added to the supernatant and the incubation continued for a further two hours. The captured antibody-protein-G complexes were then washed thrice with lysis buffer and once with PBS. After the addition of SDS running buffer, protein G was removed from the protein complex by boiling for 5 minutes, after which the complexes were loaded onto a 10-12% Bis-Tris gel and separated by electrophoresis. The proteins were transferred to a nitrocellulose membrane and subsequently detected with the c-FLIP rat monoclonal antibody or a rabbit polyclonal antibody against ubiquitin (SPA-200, Stressgen, Victoria, BC).

2.8. Semi-Quantitative RT-PCR Analysis and Real Time-PCR Analysis

Total RNA was isolated from testicular tissue or GC-2 cells using the QIAGEN (Valencia, CA) RNeasy RNA isolation kit. First strand complementary cDNA was made using 1 μg (for GC-2 cells) or 2 μg (for testicular tissue) total RNA in the presence of Superscript II reverse transcriptase and oligo-dT primer (both from Life Technologies, Carlsbad, CA). PCR reaction was performed using 2 μl of the cDNA product and Taq DNA polymerase (Roche Applied Science, Indianapolis, IN). PCR products of 469bp for Fas, 297bp for c-FLIP (L) and 389bp for β-actin were amplified using the following primers for Fas, 5' catgccaacctggtaaaaaaaaagttgagg 3' and 5' attggtatggtttcacgactggaggttcta 3'; c-FLIP (L), 5' aatgtggactctaagcccctgcaacc 3'and 5' cgtaggagccaggatgagtttcttcc 3'; and β-actin, 5' aggcatcctgaccctgaagtac 3' and 5' tcttcatgaggtagtctgtacg 3'. β-actin mRNA was used as an internal control for the semi-quantitative analysis. Conditions for the co-amplification of Fas and β-actin were 92ºC for 1 min, 59ºC for 1 min, and 72ºC for 35 sec for 38 cycles in 1.5 mM $MgCl_2$, while that for the co-amplification of c-FLIP (L) and β-actin were 92ºC for 1 min, 58ºC for 1 min, and 72ºC for 40 sec for 32 cycles in 1.5 mM $MgCl_2$.

The primers for the real time-PCR reaction for c-FLIP (L) were 5' aaccccagaccgttggtgt 3' and 5' cgccaagctctgctcca 3'; and for β-actin were 5' ccagcagatgtggatcagca 3' and 5' cttgcggtgcacgatgg 3'. Primers were designed with the aid of Primer Express, generating a 64 bp product for both β-actin and c-FLIP. Real time was performed on a Stratagene Mx3000P thermocycler using the Brilliant SYBR green PCR

master mix (600548, Stratagene, La Jolla, CA). The PCR conditions included a 10 min, 95 °C activating cycle, followed by 45 amplification cycles of 95 °C for 30 sec, 60 °C for 1 min, and 72° C for 30 sec. To control against mRNA variation between samples, actin controls were used for each sample. A baseline calibrator was run for each cDNA set used and individual samples were run in duplicate. As the expression levels of each sample were normalized to a single calibrator sample, the results from the samples at both temperatures can also be compared with each other.

2.9. siRNA Transfection Protocol

Small interfering RNA or siRNA technology is used to cause sequence specific degradation of mRNA (of a desired gene), thereby resulting in gene silencing. We obtained siRNA against mouse c-FLIP (S/L) (sc-35389) from Santa Cruz Biotechnology. It was utilized to knock down the protein expression levels of c-FLIP (L) in GC-2 cells. GC-2 cells were plated in 6 well culture plates in DMEM media containing serum, but lacking antibiotics. The cells were allowed to grow to 40-50% confluence before being transfected with siRNA against c-FLIP or with a control siRNA that was fluorescein tagged. The siRNA was incubated in transfection medium along with the transfection reagent (both provided by Santa Cruz for use in siRNA transfection protocols) at room temperature for 25 min before being added to culture wells. The appropriate quantities of each reagent used, was based on the recommendations of the company. The cells were

incubated with the siRNA mixture for a period of 24-48 hours and then subjected to mRNA or protein analysis, as discussed in the results.

2.10. Sucrose Density Gradient Ultra-centrifugation for the Isolation of Lipid Raft Domains within Cell Membranes

The protocol for sucrose density gradient ultracentrifugation was adapted from the protocol by Gajate and Mollinedo (Gajate and Mollinedo 2001), with modifications. Frozen mouse testes or GC2 cells were homogenized in 1 ml of lysis buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM PMSF and 1 % Triton X-100. The nuclear and other cellular debris were removed by centrifugation at 1000g for 10 min. The supernatant was then mixed with an equal volume of an 80 % sucrose solution (made in the lysis buffer without PMSF and Triton X-100) and added to the bottom of an ultra-centrifuge tube. The mixture was further over-layed with 4 ml of a 30 % sucrose solution, followed by 4 ml of a 5 % sucrose solution (both sucrose solutions also made in lysis buffer lacking PMSF and Triton X-100). The entire sucrose density gradient column was then subjected to ultracentrifugation using a swinging bucket rotor in an Optima XL-100 centrifuge at 140,000g for 16 h at 4 ºC. Fractions were collected from the top of the ultra-centrifuge tube, with the top most fraction being designated fraction 1 and the bottom-most, being fraction 10. Lipid raft domains should be the fractions with the lowest buoyant density

and are detergent resistant. Thus the top fractions in the column, collected in the 5-30 % sucrose gradient region are likely to be the lipid raft domains or fractions.

2.11. Western Blot Analysis

Frozen mouse testes/GC2 cell pellets (collected by trysinization and centrifugation at 400g for 6 min) were placed in homogenization buffer (either in phosphate buffered saline containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μM sodium orthovanadate, 10 μM E64, and 1X Complete Mini protease cocktail (Roche Applied Science, Indianapolis, IN) for total lysate preparation, or in buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 25 mM NaF, 1 mM sodium orthovanadate, 1 mM dithio threitol, 1 μg/ml Aprotinin, and 1 μg/ml leupeptin for samples undergoing membrane fractionation) and homogenized with a Dounce homogenizer. For total lysate preparation, homogenized samples were incubated on ice for 30 min, centrifuged at 14,000g for 20 min at 4 ºC, and supernatants collected. Homogenized samples for membrane fractionation were immediately centrifuged at 100,000g for 1h at 4 ºC. The pellet was lysed in fractionation buffer containing 0.8% Triton X-100. After 20 min of incubation, the pellet suspension was centrifuged at 12,000g for 15 min and the supernatant collected. The concentration of proteins in the samples was determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA).

Equal concentrations of proteins from samples were applied to NuPage 10% Bis-Tris gels (Invitrogen, Carlsbad, CA) and electrophoresed. Seperated proteins were transferred onto a PVDF membrane (Invitrogen) and incubated with primary antibodies against caspase-8 (AAP-118, Stressgen, Victoria, BC, Columbia), c-FLIP (06-697, Upstate Cell Signaling Systems, Charlottesville, VA), DR5 (AF837, R&D Systems Inc., Minneapolis, MN), or Fas (sc-716, SantaCruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies (SantaCruz Biotechnology) were used to detect primary antibodies. Signals were detected by the use of an ECL kit (Amersham Pharmacia Biosciences). Images were collected by a Kodak DC-290 digital camera and densitometric analysis carried out using Kodak 1D Image Analysis Software (Kodak Digital Sciences, Rochester, NY). Each protein was analyzed in a minimum of 3-4 independent groups of mice or GC-2 sample sets, and representative blots have been presented. Equal loading was verified by either comparing expression levels of actin or, when analyzing testicular or GC-2 plasma membrane protein levels, by staining the blots with amido-black.

Western blot analyses of Fas and Flotillin-2 expression were performed for fractions separated by sucrose density gradient ultracentrifugation (procedure discussed in section 2.10) from testicular or GC-2 samples. Equal volumes of each fraction for each sample were loaded onto a 10 % Bis-Tris gel and electrophoresed. All the fractions from each sample were analyzed together. Additionally, for testicular samples from each MEHP treatment set, only fraction 5 for each sample was also analyzed on a blot together.

The procedure for western transfer, protein detection with primary and secondary antibodies, signal detection with ECL and image collection and analysis with the KODAK 1D software were carried out as described in the previous paragraph. The primary antibody for Flotillin-2 was obtained from BD Transduction Labs (610383, San Jose, CA).

2.12. Statistics

Significance between groups was evaluated using parametric single factor analysis of variance (ANOVA) with Fisher's protected least significance differences (PLSD) test comparison with a significance value of $p<0.1$ using Statview^{TM} software (SAS Institute Inc., Cary, NC). The significance value of $p<0.1$ was considered based on the high degree of variability in the results from *in vivo* experiments. The range in ages of the animals used in these experiments from 26-30 days of age, could perhaps account for the variation.

Chapter 3: The p53 protein influences the sensitivity of testicular germ cells to MEHP-induced apoptosis by increasing the membrane levels of Fas and DR5 and decreasing the intracellular amount of c-FLIP

3.1. Introduction and Rationale

In the rodent testis, the participation of the Fas signaling system has been observed in response to different forms of testicular injury including chemical injury, cryptorchidism, radiation injury, and ischemia-reperfusion (Lee *et al.* 1997; Ogi *et al.* 1998; Lee *et al.* 1999; Koji *et al.* 2001; Embree-Ku *et al.* 2002). Our group has previously reported that Sertoli cells express FasL and initiate the apoptotic elimination of Fas-expressing germ cells in a paracrine manner, after challenge with MEHP (Lee *et al.* 1997; Lee *et al.* 1999). In contrast, other investigators have reported that FasL is only expressed by mature spermatozoa and play a role in their survival in the female genital tract (D'Alessio *et al.* 2001). As discussed previously in section 1.3.2 of Chapter 1, evidence for a functional role of FasL/Fas signaling within the testis after injury comes from studies utilizing *gld* mice. Although *gld* mice display apparently normal spermatogenesis, after exposure to MEHP these mice exhibit a significant reduction in the incidence of germ cell apoptosis as compared to their wild-type (C57BL/6J) counterparts (Richburg *et al.* 2000; Giammona *et al.* 2002). Therefore, the significant attenuation of testicular germ cell apoptosis in *gld* mice indicates the functional participation of the Fas-signaling system in MEHP-induced germ cell apoptosis.

MEHP is a well characterized Sertoli cell toxicant (Thomas *et al.* 1978; Gray and Gangolli 1986; Creasy *et al.* 1988). It disrupts Sertoli cell function and causes an increase in germ cell apoptosis, in both young rats and mice, leading to testicular atrophy (Lee *et al.* 1997; Lee *et al.* 1999; Giammona *et al.* 2002). After MEHP-induced Sertoli cell injury, only a distinct subclass of germ cells, the primary spermatocytes and early round spermatids, undergo apoptosis. Previous work by our laboratory demonstrated that increased germ cell apoptosis in response to MEHP in the rat testis was preceded by increases in membrane localization of Fas (Richburg *et al.* 1999). However, the mechanisms by which these specific subclasses of germ cells elicit this membrane expression of Fas are currently unknown.

The p53 tumor suppressor protein is widely known to influence the transcription of the gene for Fas and DR5 in many cell types under conditions of cellular stress (Owen-Schaub *et al.* 1995; Muller *et al.* 1998; Wu *et al.* 1999; 2000; Takimoto and El-Deiry 2000). Recently, several investigators have also suggested that the p53 protein may be instrumental in the trafficking of Fas to the cell membrane from stores in the Golgi complex in a transcription-independent manner (Bennett *et al.* 1998; Beltinger *et al.* 1999). The impetus of the present work was to therefore test the involvement of p53 in modulating the sensitivity/membrane localization of Fas in germ cells. MEHP has not been implicated in direct injury to germ cells, but instead, through injury to Sertoli cells, creates an inhospitable external environment for them (Gray and Gangolli 1986; Chapin *et al.* 1988). We therefore hypothesize that MEHP-mediated Sertoli cell injury may lead

to a secondary p53 response by germ cells, resulting in the increased sensitivity of germ cells to undergo apoptosis.

Here we demonstrate, using p53 knockout (p53-/-) mice, that the localization of Fas and DR5 on the germ cell membrane and the ability of the death receptors to initiate apoptosis is, in part, dependent on the expression of p53. Importantly, the changes in Fas membrane expression appear to be mediated by p53 in a transcription-independent manner. In addition, we also report the novel finding in the testis that p53 influences the cellular levels of c-FLIP, a key inhibitory protein of the Fas intracellular signaling pathway. These data for the first time suggest an interaction between the death-receptor and the internal "stress-sensor" p53 protein in the regulation of testicular germ cell apoptosis after MEHP-induced Sertoli cell injury.

3.2. Results

3.2.1. P53-/- mice have attenuated rates of MEHP-induced germ cell apoptosis:

Analysis of testicular histology of p53+/+ and p53-/- mice revealed similar minor aberrations in the seminiferous epithelium including Sertoli cell vacuoles and germ cell sloughing (**Fig. 3.1 A-D**) that are likely due to the background strain of these mice. TUNEL analysis (**Fig. 3.1E**) showed a biphasic apoptotic response after MEHP exposure in both the p53+/+ and p53-/- mice with an early apoptotic peak incidence being reached

at 1.5 h and a late peak incidence occurring at 24 h after the acute dose of MEHP. The observed second peak of apoptosis matches the results of our earlier work in the C57 mice (Giammona *et al.* 2002). However, tissue had not been collected previously at the 1.5h time point. The p53+/+ animals show a ~4-fold increase in peak apoptosis at both time points, while p53 -/- animals display a ~3-fold increase with respect to their control at the same points. A direct comparison of the apoptotic index between the p53+/+ and p53-/- mice revealed a significant reduction (~40%) in the incidence of germ cell apoptosis in the p53-/- mice at 1h, 1.5h and 24h. In both mouse strains the specific sub-types of germ cells undergoing apoptosis are the same at all time points (**Fig. 3.1 A-D**).

3.2.2. Membrane death receptor expression is not up regulated after MEHP in p53-/- mice:

The levels of Fas in testicular membrane preparations reached significantly elevated levels by 12h in the p53+/+ animals after MEHP exposure (**Fig. 3.2 A,C**) and remained elevated 0.5 fold above control levels at 24h. In these mice, membrane DR5 levels were also found to increase within 1.5 h and remain at an elevated levels up to 12 h after MEHP exposure (**Fig. 3.2 B,D**). Increases in membrane Fas and DR5 levels were not detected above untreated controls in p53-/- mice (**Fig. 3.2 A-D**). Basal levels of Fas in p53-/- mice were not significantly different from those in p53+/+ mice (**Fig. 3.2 A**). These results were obtained from 4 independent groups of animals.

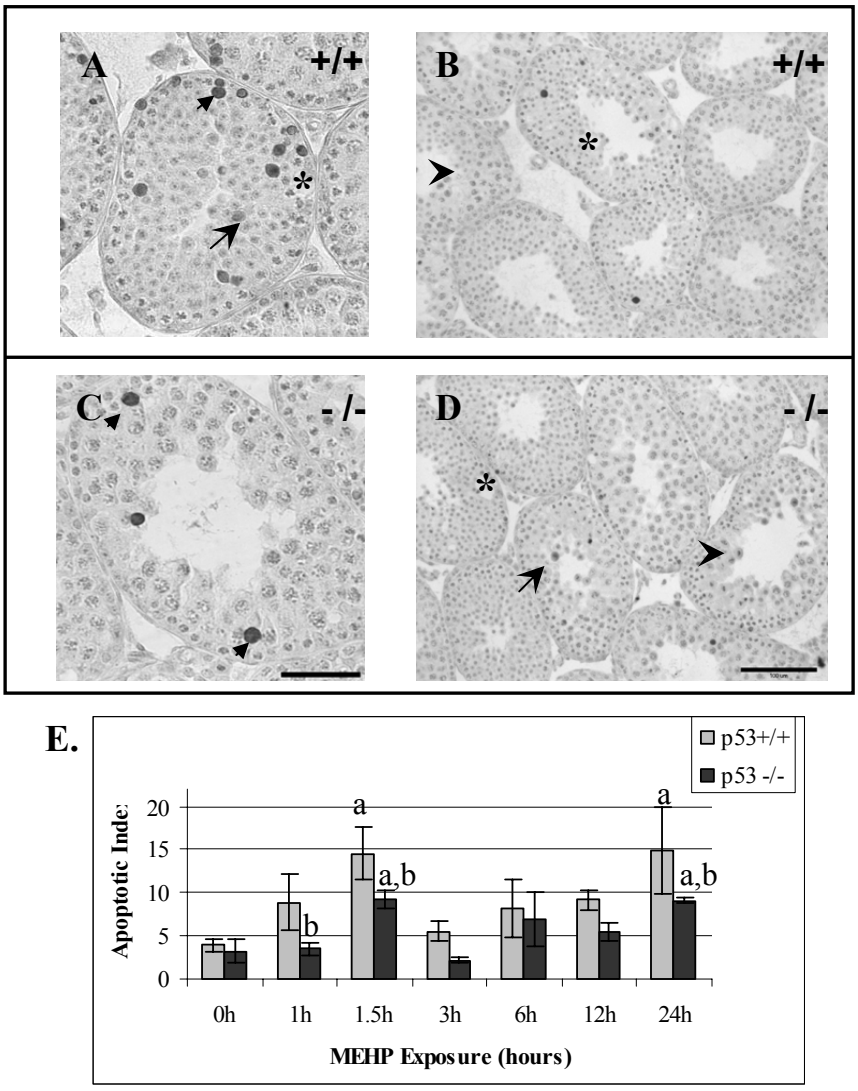

Fig. 3.1. Attenuation of germ cell apoptosis in p53-/- mice after MEHP exposure. (A-D) Cross sections of testes collected from p53+/+ (A,B) and p53-/- (C,D) mice counterstained with hematoxylin for TUNEL analysis. Scale bars for A,C =50μm, and for B,D =100μm. Sertoli cell vacuoles (asterisks) and sloughed germ cells (arrow heads) observed in the seminiferous tubules of both p53+/+ and p53-/- mice. TUNEL positive (brown cells) round spermatids (larger arrows) and primary spermatocytes (smaller arrows) are indicated. (E) Apoptotic index was calculated as described in the *Materials and Methods*. Values represent the mean ± SEM. 'a' denotes significant differences ($p<0.1$) in apoptotic index between untreated and MEHP-treated mice of the same strain, 'b' denotes significant differences ($p<0.1$) between p53-/- and p53+/+ mice at matched time points. The data is representative of results obtained from 3 or 4 mice per time point.

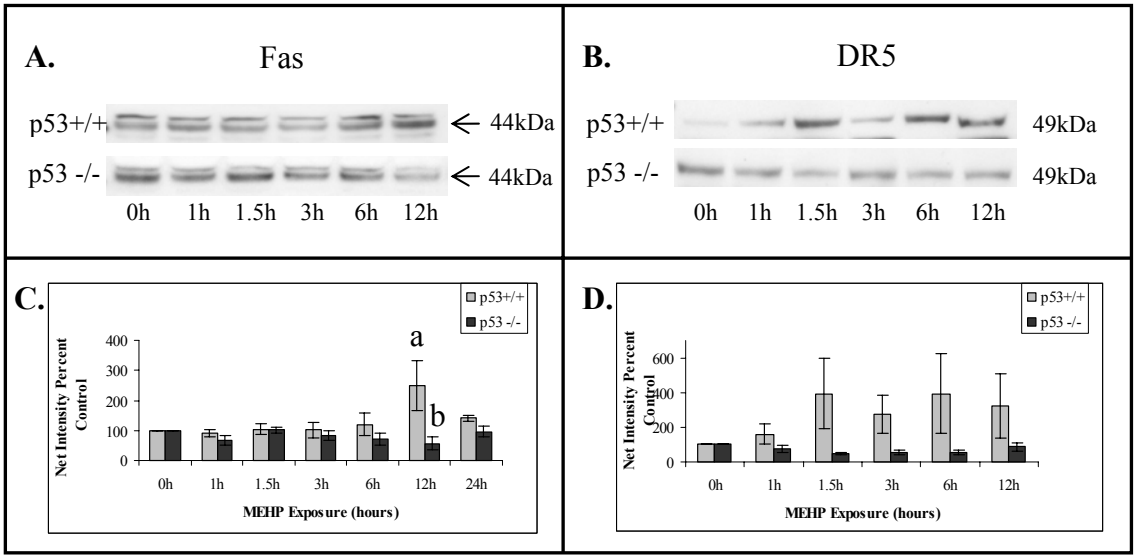

Fig. 3.2. Western blot analysis of Fas and DR5 in membrane fractions of testicular homogenates. Representative blots from 4 testicular samples for each time point are provided. (A) Fas (~44 kDa) band intensity and (C) graphical representation of densitometric analysis in the p53+/+ and p53-/- mice exposed to MEHP. (B) DR5 (~49 kDa) band intensity and (D) graphical quantitation in the p53+/+ and p53-/- mice exposed to MEHP. Values represent the mean ± SEM. 'a' denotes significant differences ($p<0.1$) in membrane death receptor levels between untreated and MEHP-treated mice of the same strain, 'b' denotes significant differences ($p<0.1$) between p53-/- and p53+/+ mice at matched time points.

3.2.3. The presence of p53 does not cause transcriptional up-regulation of death receptors in response to MEHP:

Fas mRNA levels were evaluated in testicular samples of animals at various time points after MEHP exposure using semi-quantitative RT-PCR. After toxicant exposure, no significant changes are observed in the Fas mRNA expression in both p53+/+ and p53-/- animals (**Fig 3.3 A,B**). RT-PCR results for Fas for each time point is normalized with β-actin. Similar analyses of DR5 mRNA levels revealed an absence of transcription after MEHP exposure in both p53+/+ and p53-/- mice (**Fig. 3.3 C**).

3.2.4. MEHP exposure does not lead to caspase-8 cleavage in p53-/- animals:

Procaspase-8 cleavage is a hallmark indicator of death receptor activation. Murine procaspase-8 is known to be efficiently cleaved to fragments of ~44 kDa, ~31 kDa (dimer of the 18kDa and 10kDa subunits), ~20 kDa and ~10 kDa sizes (Van de Craen *et al.* 1998). Here we measured the formation of both the 44 kDa (**Fig. 3.4 A,B**) and 31 kDa (data not shown) fragments as detected by western blot analysis of total testis homogenates of p53+/+ and p53-/- mice exposed to MEHP. In both mouse strains, untreated control animals express measurable amounts of the processed forms. A significant increase in the 44 kDa processed form of caspase-8 is measured in the p53+/+ mice at 1h, 1.5h, 3h, and 24h after MEHP treatment (**Fig. 3.4 A,B**). However, no

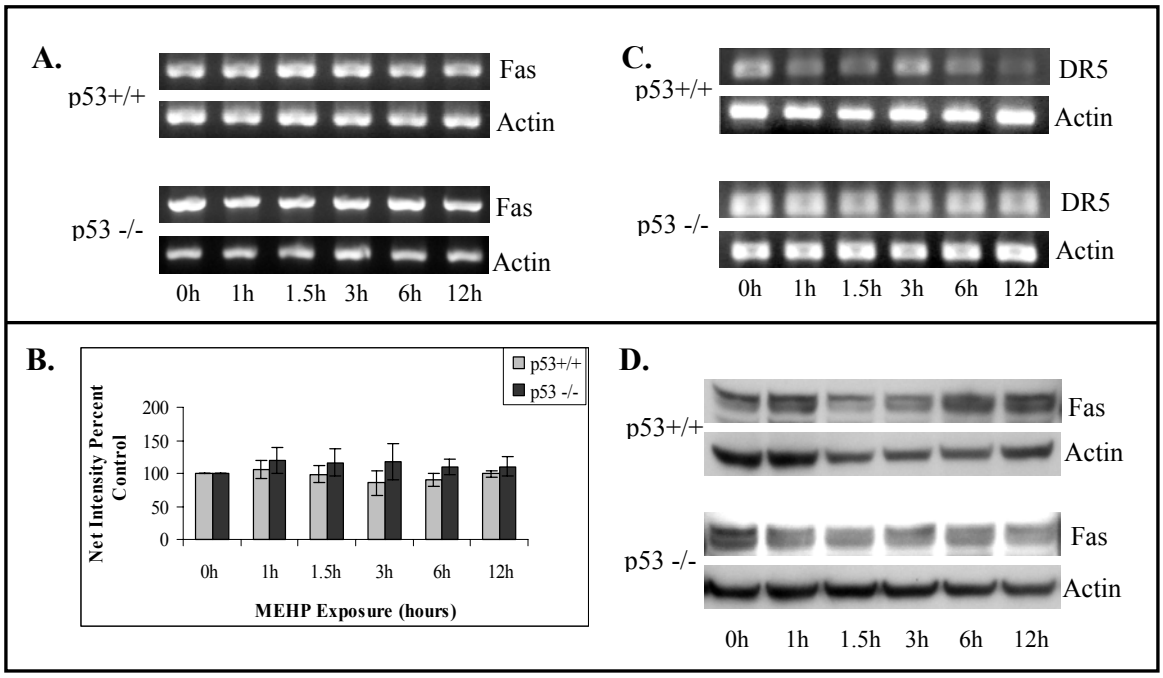

Fig. 3.3. Semi-quantitative RT-PCR analysis of testicular Fas and DR5 mRNA levels after MEHP exposure and Western analysis of total cellular Fas protein. Representative gels for Fas mRNA (A) and DR5 (C) in p53+/+ and p53-/- mice, and graphical representation of densitometric analysis (B), represent the results of 3 experiments. β-actin is used as an internal control. Values are mean ± SEM. No significant changes are observed. (D) Representative blots of total cellular samples from p53+/+ and p53 -/- mice exposed to MEHP and analyzed for Fas and Actin at each time point.

significant differences with respect to untreated controls are observed in the p53-/- mice (**Fig. 3.4 A,B**).

3.2.5. c-FLIP protein levels are increased only in p53-/- mice, while mRNA levels change similarly in both p53+/+ and p53-/- mice:

Western blot analysis of total testis homogenates was carried out to analyze changes in c-FLIP (L) and (S) forms after MEHP treatment. While no significant changes in c-FLIP (L) and (S) protein levels were observed in the p53+/+ mice (**Fig. 3.5 A,C**), increased expression of both forms was measured in the p53-/- animals; c-FLIP (L) (**Fig. 3.5 B,C**) and (S) levels (**Fig. 3.5 B**) were enhanced (~2-5 fold) in p53-/- mice as early as an hour after toxicant exposure. In the p53-/- mice, c-FLIP (L) levels were significantly higher than treatment controls at 1.5h, 3h and 6h after MEHP exposure and a significant difference between corresponding c-FLIP (L) levels in p53+/+ and p53-/- mice were observed at these time points as well. Evaluation of mRNA levels of c-FLIP (L) shows similar basal levels and no significant changes in the transcriptional regulation of c-FLIP (L) in both p53+/+ and p53-/- mice following MEHP exposure (**Fig. 3.6 A-C**).

3.3. Discussion

Previous work from our laboratory established the participation of the Fas/FasL signaling system in the initiation of testicular germ cell apoptosis after exposure of

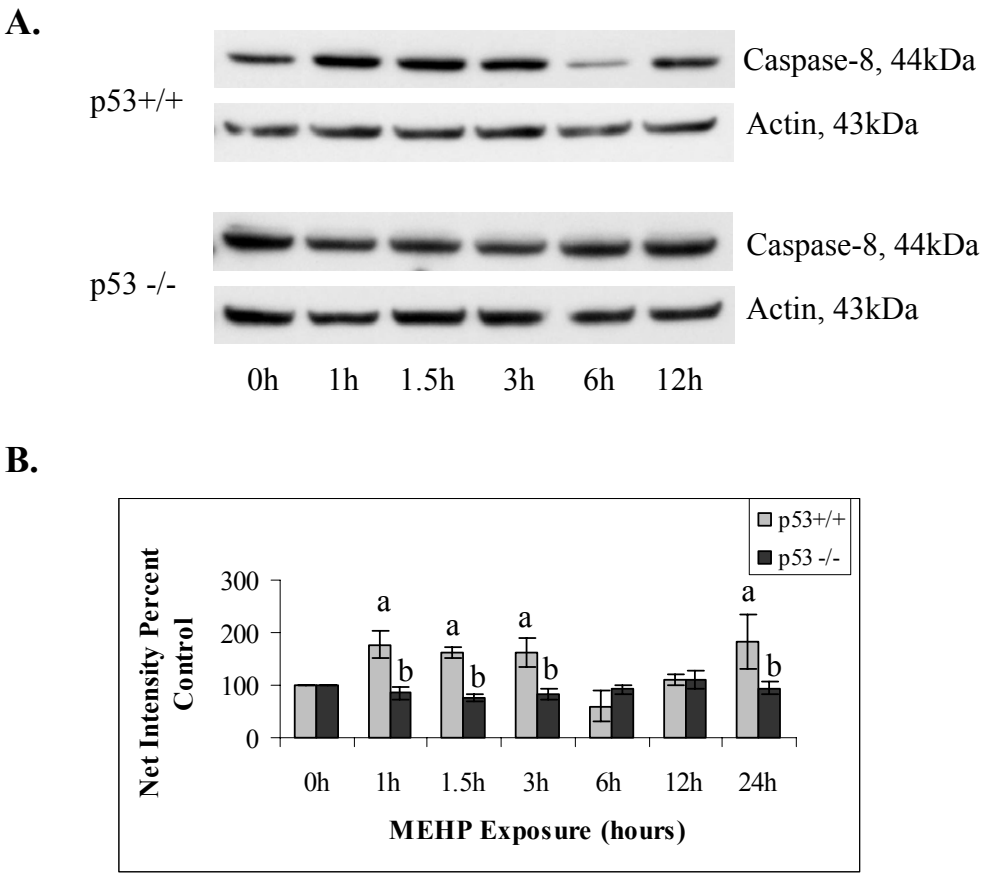

Fig. 3.4. Procaspase-8 processing in response to MEHP exposure. Representative blots (A) for the ~44 kDa processed form of caspase-8 in p53+/+ and p53-/- mice after exposure to MEHP. Graphical representation (B) of the densitometric analysis for the 44 kDA form of caspase-8, normalized for actin, from 4 testicular samples per time point. Values are mean ± SEM. 'a' denotes significant differences ($p<0.1$) in processed caspase-8 levels between untreated and MEHP-treated mice of the same strain, 'b' denotes significant differences ($p<0.1$) between p53-/- and p53+/+ mice at matched time points.

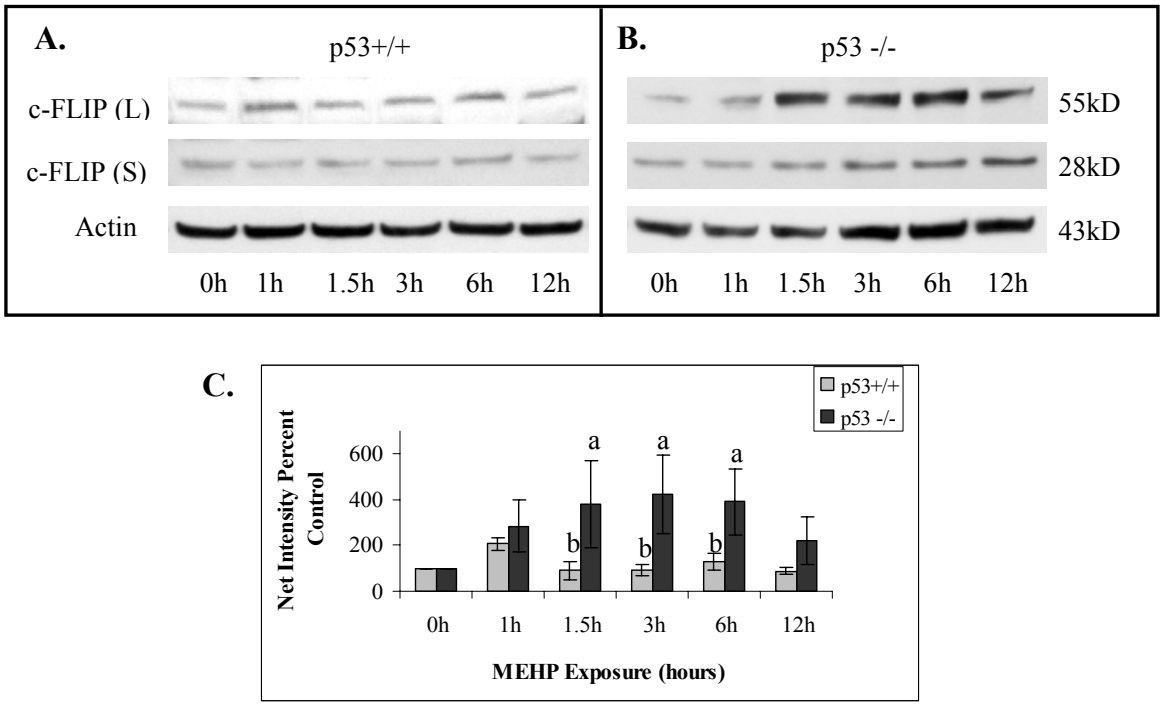

Fig. 3.5. **Western blot analysis of c-FLIP protein levels in response to MEHP exposure.** Representative blots for c-FLIP (L) (55 kDa) and c-FLIP (S) (28 kDa) from p53+/+ (A) and p53-/- (B) mice exposed to MEHP. (C) A graphical representation of densitometric analysis of c-FLIP (L) levels from 3 experiments, normalized for actin. Values are mean ± SEM. 'a' denotes significant differences ($p<0.1$) in c-FLIP (L) levels between untreated and MEHP-treated mice of the same strain, 'b' denotes significant differences ($p<0.1$) between p53-/- and p53+/+ mice at matched time points.

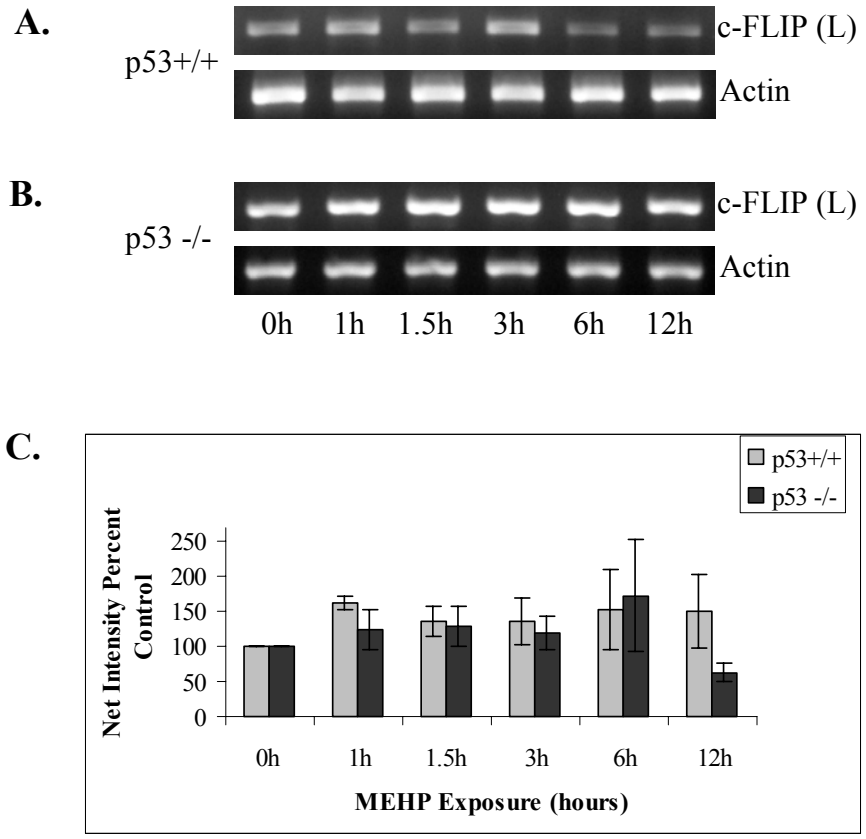

Fig. 3.6. Semi-quantitative analysis of c-FLIP (L) mRNA levels by RT-PCR after MEHP exposure. Representative gels for c-FLIP (L) mRNA from p53+/+ (A) and p53-/- (B) mice exposed to MEHP, and graphical representation of densitometric analysis (C), represent the results of 3 experiments. β-actin is used as an internal control. Values are mean ± SEM. No significant changes are observed.

rodents to the Sertoli cell toxicant MEHP (Lee *et al.* 1997; Lee *et al.* 1999). FasL expressed by Sertoli cells appears to interact with Fas expressed by a specific subset of germ cells to trigger their apoptotic demise. In our previous investigations it was clear that the expression of Fas on the germ cell membrane was a prerequisite for their sensitivity to apoptosis (Richburg *et al.* 1999). However, the mechanisms by which germ cells increase their surface expression of Fas have not been established. A novel observation of the dependence of the p53 protein in the trafficking of Fas to the cell membrane in human vascular smooth muscle cells has been described (Bennett *et al.* 1998). The participation of the p53 tumor suppressor protein in the modulation of apoptosis is widely described to occur by several well-known mechanisms involving the cell cycle protein p21, and the modulation of the transcriptional expression of various pro-apoptotic genes including PUMA, NOXA, Bax, DR5, PIG3 and even Fas in some cells (el-Deiry *et al.* 1993; Miyashita and Reed 1995; Polyak *et al.* 1997; Munsch 2000; Takimoto and El-Deiry 2000; Villunger *et al.* 2003). In the present study we aimed to investigate whether p53-deficient mice displayed alterations in their surface membrane expression of Fas and whether these mice would be less sensitive to the MEHP-stimulated loss of testicular germ cells.

The p53-/- mice used in this study showed only minor alterations in testicular histology (**Fig. 3.1 C,D**). However, these minor changes in the seminiferous epithelium appeared to be due to the background strain of these mice (C57/129S/v) since the paired wild-type mice displayed similar changes in the seminiferous epithelium (**Fig. 3.1 A,B**).

Comparison of wild-type p53+/+ versus p53-/- mice testes after MEHP exposure revealed a trend of lowered germ cell apoptosis after MEHP exposure in p53-/- mice (Fig. 1E). However significant decreases (~ 40%) in apoptosis in the p53-/- mice were only measured at 1h, 1.5h and 24h. Because these time points corresponded to the two peaks of apoptosis seen in p53+/+ mice, the difference in the sensitivity of these mice was perhaps easier to resolve. Interestingly, the germ cell subtypes undergoing apoptosis in response to MEHP exposure were the same for both the p53+/+ and p53-/- mice (**Fig. 3.1 A-D**). In our previous studies examining the sensitivity of *gld* mice to MEHP-induced germ cell apoptosis, we demonstrated a significant protection from apoptosis beginning as early as 6 h after exposure with maximal decreases (~50%) in apoptosis observed 12 and 24 h after MEHP exposure (Richburg *et al.* 2000; Giammona *et al.* 2002). Although our present findings in the p53-/- mice do not reveal a similar robust protection as that seen in *gld* mice, it does reveal that p53 plays at least a partial role in modulating the MEHP-stimulated germ cell apoptosis.

Based on the findings of Bennett (Bennett *et al.* 1998) and Beltinger (Beltinger *et al.* 1999), we predicted that p53-/- mice would display reduced membrane levels of Fas and would not show large increases after MEHP exposure. We show that p53-/- mice display high basal membrane expression levels of Fas (but not statistically different from levels in p53+/+ mice) in the testis (**Fig. 3.2A**). In accordance with our predictions, after MEHP exposure, increases in membrane Fas were only observed in p53+/+ mice (**Fig. 3.2 A,C**). These findings may explain, in part, the observed attenuation of MEHP-

induced germ cell apoptosis in p53-/- mice. However, the abundant basal levels of Fas expression that are observed may account for the germ cell apoptosis that does occur.

In the present work we show that DR5, another death receptor whose expression is modulated by p53 activation, is abundantly expressed in p53-/- mice. Similar to the observations of Fas expression in p53-/- mice after MEHP exposure, DR5 membrane levels did not increase in p53-/- mice (**Fig. 3.2 B,D**). However, DR5 membrane expression was abundantly up-regulated in p53+/+ mice as early as 1.5 hours after MEHP exposure and was maintained at high expression levels through 12 h. Although the expression profile of DR5 correlated very well with the first phase of caspase-8 activation as well as germ cell apoptosis in p53+/+ mice after MEHP exposure, the ability of the DR5/ TRAIL system to initiate cell death in normal tissues is unresolved (Jo *et al.* 2000; Nesterov *et al.* 2002; Almasan and Ashkenazi 2003). Future investigations will therefore be aimed at functional studies to test TRAIL's ability to instigate germ cell apoptosis in the testis. However, our data do indicate that Fas and DR5 are expressed in p53-/- mice, although their ability to be increased on the membrane after MEHP exposure and increase germ cell sensitivity is severely limited.

Our initial interest at assessing the p53 protein as a likely modulator of Fas was based upon p53's reported ability to cause membrane trafficking of Fas by a transcription-independent mechanism (Bennett *et al.* 1998; Beltinger *et al.* 1999). In support of this idea, we found that Fas mRNA levels in the testis of p53+/+ mice did not

significantly increase after MEHP exposure (**Fig. 3.3 A,B**). We were also unable to detect significant changes in the total Fas protein levels in the wild type p53+/+ mice after MEHP exposure (**Fig 3.3 D**). Therefore, the increased levels of Fas receptor in the membrane preparations of p53+/+ mice at 6h and 12h after MEHP are not due to the increased expression of the protein *via* transcription. Although the testicular membrane preparations used in our experiments are comprised of membranes of sub-cellular organelles as well as the plasma membrane of these cells, changes in Fas protein levels from Golgi and ER do not likely account for the observed changes in total membrane levels since any increases in Fas protein in these organelle membranes reflects the production of new protein; a process which is not increased as evidenced by the absence of significant increases in both Fas mRNA and total protein levels. Our observations that Fas levels are not increased in the testicular cell membrane preparations of p53-/- mice after MEHP exposure supports the hypothesis that functional p53 is required for Fas protein transport from Golgi or other intracellular storage sites to the membrane. Another intriguing result is the absence of increased DR5 transcription (**Fig. 3.3 C**) in both p53+/+ and p53-/- mice after MEHP exposure. These observations indicate the absence of active transcription by p53 in the testis, even of a robustly transcribed p53 gene DR5, after MEHP treatment.

To further investigate whether death receptors are activated in p53-/- mice, we evaluated procaspase-8 processing. Caspase-8 is autocatalytically processed from its pro-form to active form at the DISC (Muzio *et al.* 1996), which is created only when Fas is

activated. We observed the formation of the 44 kDa (**Fig. 3.4 A**) and ~31 kDa (data not shown) processed forms of caspase 8. The baseline levels of these processed forms are similar between the p53+/+ and p53-/- mice. However, following MEHP exposure, statistically significant increases in the level of the 44 kDa form of caspase 8 (**Fig. 3.4 B**) and in the 31kDa form (data not shown) were observed only in the p53+/+ mice, demonstrating a more robust activation of Fas in animals expressing p53. Moreover, the formation of enhanced levels of active caspase-8 that preceded the two major apoptotic peaks in our wild type mouse exposed to MEHP supports our earlier reports implicating Fas activation in MEHP-mediated germ cell apoptosis. The outcome of these experiments supports our hypothesis; death receptor activation in mice exposed to MEHP is p53-dependent.

The absence of p53 did not drastically reduce the basal membrane expression levels of Fas, though it may be responsible for the inability of Fas levels to increase on the membrane in response to MEHP exposure. However, these basal membrane levels of Fas could be sufficient for triggering germ cell apoptosis that we do observe. Therefore, the attenuation of apoptosis in p53-/- mice may be due to other modulators of the death receptors' activity. The Fas activated pathway can be inhibited by the cellular FLICE inhibitory protein (c-FLIP), which contains two death effector domains and can bind FADD (Irmler *et al.* 1997). Mammalian cells express two splice variants of c-FLIP, c-FLIP long (L) and c-FLIP short (S), which can function to inhibit death receptor mediated apoptosis (Krueger *et al.* 2001) by inhibiting caspase-8 activation. c-FLIP (L)

lacks a cysteine at the active site of its protease domain and inhibits apoptotic signaling by only allowing partial processing of procaspase-8, while c-FLIP (S) lacking the protease domain, merely binds at the DISC to completely prevent procaspase-8 processing (Irmler *et al.* 1997; Krueger *et al.* 2001). It has been reported that c-FLIP protein can be degraded in cancer cells in a p53-dependent manner (Fukazawa *et al.* 2001). In addition, c-FLIP expression has been reported in mouse testis and has been implicated in protecting the immortalized germ cell line, GC-1spg, from Fas-mediated apoptosis (Giampietri *et al.* 2003). Therefore, we evaluated if changes in c-FLIP protein levels in the testes occurred in both the p53+/+ and p53-/- mice following MEHP exposure and correlated with the sensitivity of germ cells to undergo apoptosis. In the p53+/+ mice, the levels of c-FLIP (L and S) protein were similar and unchanged after MEHP-treatment while c-FLIP (L and S) levels significantly increased in p53-/- mice after MEHP exposure (**Fig. 3.5 A-C**). Analysis of c-FLIP (L) mRNA levels revealed similar transcriptional profiles of c-FLIP (L) in response to MEHP toxicity, regardless of p53 status (**Fig. 3.6 A-C**). The absence of c-FLIP protein up regulation in response to MEHP in p53+/+ mice, despite comparable transcription in both mouse models suggests that c-FLIP may be degraded much more rapidly in the p53+/+ mice as has been suggested before (41). This is also supported by the enhanced Fas/c-FLIP ratio in the p53-/- mice (data not shown). It is plausible that 2-5 fold higher levels of c-FLIP in the p53-/- mice may be responsible, in part, for the reduced caspase 8 processing observed despite their having high Fas membrane levels.

In the present study we have made the following novel observations: 1) The absence of p53 expression correlates with an attenuation of germ cell apoptosis after MEHP exposure, 2) MEHP-induced increases in the membrane levels of Fas and DR5 occurs only in p53+/+ mice, 3) p53 mediates increased membrane levels of Fas on germ cell membranes in response to MEHP in a transcription-independent manner, 3) MEHP-induced caspase-8 processing is enhanced in the testis of p53+/+ mice but not in p53-/- mice and, 5) Attenuation of MEHP-triggered germ cell apoptosis in p53-/- mice correlates with increased testicular c-FLIP protein levels. Taken together, these findings indicate that p53 may play a multi-tiered role in MEHP-induced germ cell apoptosis. The p53 protein not only serves to promote death receptor membrane localization, but by bringing about the degradation of c-FLIP, p53 may act to ensure that Fas can be maximally activated in germ cells in response to MEHP-induced testicular injury.

Chapter 4: The influence of p53 status on Fas membrane localization, c-FLIP ubiquitinylation and sensitivity of testicular germ cells to undergo Fas-mediated apoptosis

4.1. Introduction and Rationale

We demonstrated a potential mechanism by which the activation of Fas and/or DR5 in germ cells *in vivo* is modulated in response to MEHP. The p53 protein mediates death receptor membrane localization and also alters the protein levels of c-FLIP (L) in this toxicant injury model. Interestingly, only after exposure to MEHP do the p53 -/- mice express very high levels of c-FLIP (L). The p53 protein influences the degradation of the c-FLIP protein *via* the process of ubiquitinylation in colon cancer cells (Fukazawa *et al.* 2001). Ubiquitinylation is the process by which intracellular proteins in eukaryotes are targeted for degradation (Ciechanover *et al.* 2000). Proteins conjugated with four or more ubiquitin molecules, enables their recognition and subsequent cleavage by the proteasome (Jan-Michael Peters and Robin Harris 1998). We demonstrated that the p53 status of the mice affected only the protein levels of c-FLIP and not their mRNA levels. Therefore we hypothesized that death receptor activated apoptotic pathway in germ cells could be affected by the modification of protein levels of c-FLIP (L) perhaps by ubiquitinylation, and in a p53-dependant manner. To further characterize the involvement of p53 in the sensitivity of germ cells to Fas- and/or DR5 mediated apoptosis, we utilize

the transformed germ cell line called GC-2*spd* (ts) that has a temperature sensitive expression of p53.

Germ cell-2 spermatid (temperature sensitive) cells or GC-2*spd* (ts) cells were originally created by Hofmann (Hofmann *et al.* 1994) to study the process of germ cell differentiation. These cells were created by transforming preleptotene spermatocytes from 6 week old BALB/c mice, using the SV40 virus' large T antigen and co-transfecting them with a plasmid carrying a p53 mutant gene ($p53^{Val\text{-}135}$). The mutant p53 protein is fully active at 32 ºC but completely inactive at 39 ºC and only partially active at 37 ºC due to improper folding of the protein that inhibits its nuclear localization. Therefore, by modulating the temperature at which GC-2 cells grow, we could functionally create p53+/+ or p53 -/- germ cells. Furthermore, this cell line is an appropriate *in vitro* model since these cells were derived from spermatocytes; which is the germ cell type most sensitive to apoptosis after toxicant-induced Sertoli cell injury.

Since germ cells are not the direct targets of MEHP (Gray and Gangolli 1986), in the present work we use the anti-Fas antibodies (JO2) or human TRAIL ligand to mimic the contribution of Sertoli cell FasL/TRAIL and elicit germ cell apoptosis. Here we report a p53- dependent increase in membrane Fas expression in GC-2 cells maintained at 32 ºC and increased sensitivity to JO2 treatment only at this p53 permissive temperature. However, no significant differences in DR5 membrane levels are reported between the cells grown at the p53 permissive and non-permissive temperatures. Additionally,

increased sensitivity to TRAIL was only observed in the presence of non-toxic concentrations of the protein synthesis inhibitor, cycloheximide in cells at the p53 permissive temperature.

At the non-permissive p53 temperature, GC-2 cells expressed higher levels of c-FLIP (L) protein in response to JO2 and decreasing the amounts of c-FLIP *via* an siRNA approach re-sensitized these cells to JO2-induced apoptosis. The siRNA transfected cells were however, not sensitized to TRAIL treatment. We also demonstrate a robust increase in the ubiquitinylation of c-FLIP (L) at the p53 permissive temperature implying an active degradation mechanism for c-FLIP within these cells. These findings demonstrate that the GC-2 system can be used to successfully model death receptor-activated germ cell apoptosis and is useful for the study of the dynamics between the death receptors and their regulatory proteins.

4.2. Results

4.2.1. Expression profile of various apoptotic proteins in GC-2 cells based on p53 activation status:

Immunocytochemistry was performed to determine the cellular localization of p53 at different temperatures. As shown in **Fig. 4.1 A (i-ii)**, p53 localized entirely in the nucleus at the permissive temperature of 32 ºC **(Fig. 4.1 A, ii)**, while being expressed in

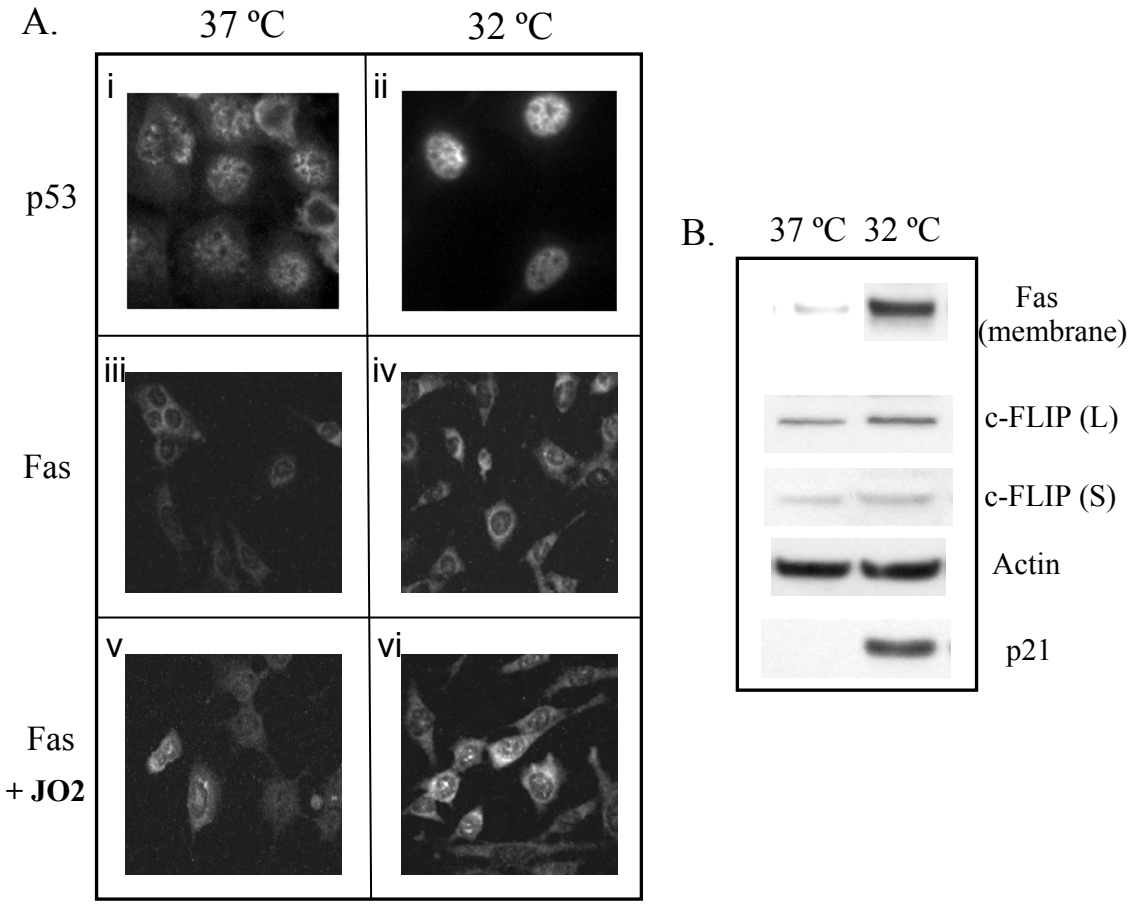

Fig. 4.1. Expression profile of apoptotic proteins in GC-2 cells. (A) Representative images of p53 (i, ii) and Fas (iii-vi) stained GC-2 cells at both the non-permissive (37 ºC) (i, iii, v) and permissive (32 ºC) (ii, iv, vi) p53 temperatures. GC-2 cells were either treated with JO2 (v, vi) or untreated (iii,iv) before being immunostained for Fas. Cells were immunostained with primary antibodies against p53 or Fas and secondary antibodies conjugated to FITC. (B) (C) Representative western blots for Fas (in membrane fraction), c-FLIP(L) and (S), and p21 are shown here from cells grown at both the non-permissive and permissive p53 temperatures. The data represent results from 3 independent experiments.

both cytosolic and nuclear compartments when grown at 37 ºC **(i)**. These results are similar to those demonstrated previously (Hofmann *et al.* 1994). Fas expression in GC-2 cells was also analyzed by ICC and by western blot analysis of membrane fractions of these cells. Fas was detected by ICC in cells grown at both temperatures, with cells at 32 ºC expressing abundant amounts of Fas (**Fig. 4.1 A, iii-iv**). Cells grown at both temperatures showed punctuate Fas staining in response to exposure to JO2 (**Fig. 4.1 A, v-vi**). The Fas membrane expression profile as determined by western blots, was equally different between the two cell types, with a 8-10 fold increase in the membrane Fas levels in cells at 32 ºC over those grown at 37 ºC (**Fig. 4.1 B**). However, the levels of DR5 in membrane fractions of the two cell types were found to be similar (data not shown). The activation of p53 at 32 °C, and not at 37 ºC is demonstrated by the levels of the p53 transcription product p21, as cells grown at 32 °C show abundant expression of the p21 protein while those at 37 ºC do not (**Fig. 4.1 B**). c-FLIP (L) and (S) were also found to be expressed in GC-2 cells at both temperatures and at comparable levels (**Fig. 4.1 B**).

4.2.2. GC-2 cells are insensitive to MEHP treatment, while sensitivity of GC-2 cells to anti-Fas (JO2) and TRAIL treatment is dependent on p53 activation

GC-2 cells grown at 37 ºC and 32 ºC were exposed to 200 μM MEHP for up to 24 hours in order to confirm that MEHP does not act directly on germ cells and instigate their demise. Analysis of incidence of cellular apoptosis in these cells by flow cytometry using the Annexin V-PI assay, revealed no increases in cell death in GC-2 cells after

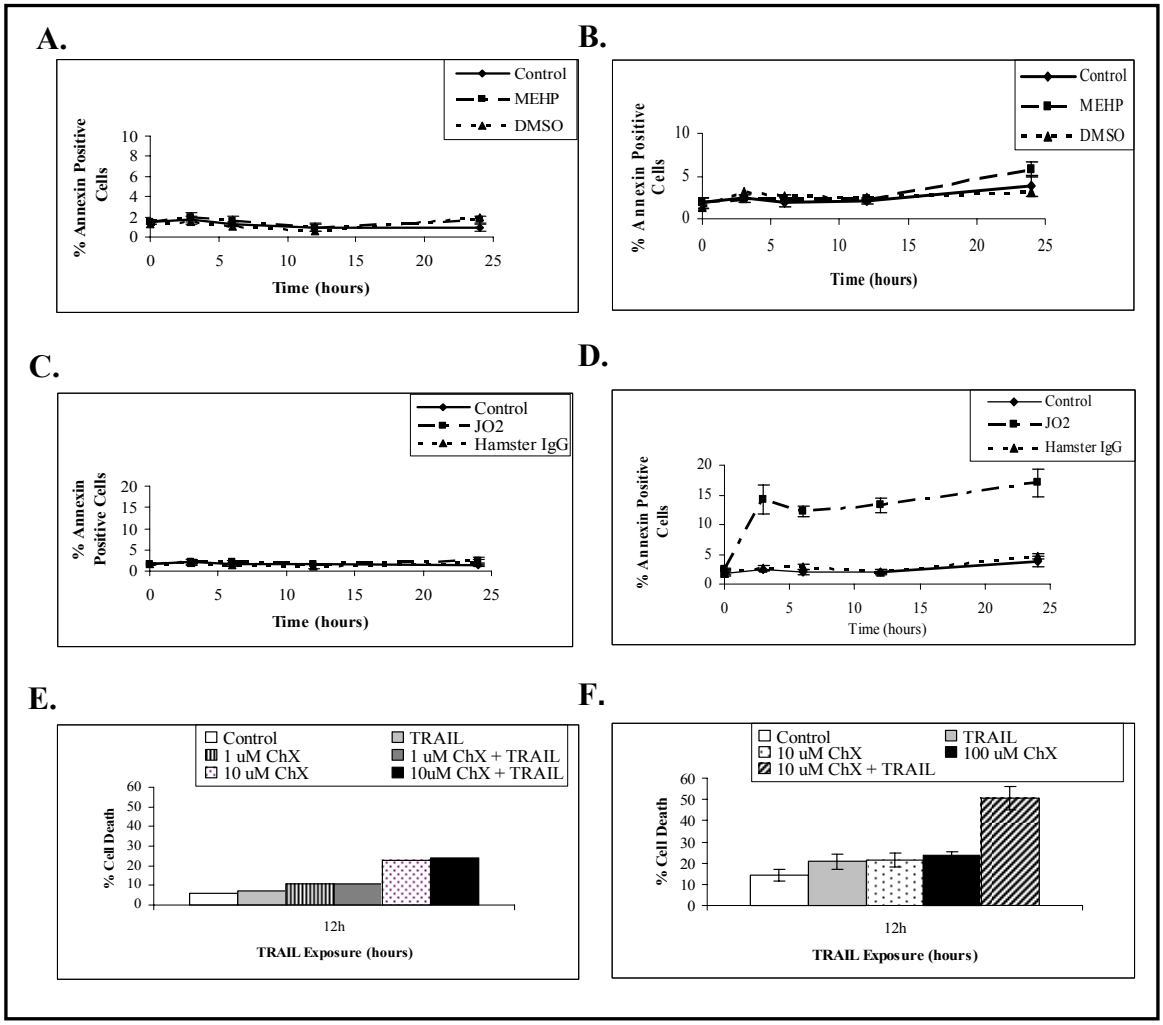

Fig. 4.2. Analysis of apoptosis levels in GC-2 cells by Annexin-PI assay after exposure to MEHP or JO2 or TRAIL. (A) A graphical representation of annexin positive GC-2 cells at the non-permissive p53 temperature and (B) at the permissive p53 temperature, after exposure to MEHP, DMSO or matched time point controls. (C). A graphical representation of annexin positive GC-2 cells at the non-permissive p53 temperature and (D) at the permissive p53 temperature, after exposure to JO2, Hamster IgG or matched time point controls. (E). A graphical representation of annexin positive + annexin and PI positive GC-2 cells at the non-permissive p53 temperature and (D) at the permissive p53 temperature, after exposure to human TRAIL, cycloheximide (ChX), cycloheximide + TRAIL or matched time point controls. The data represent results from 3 independent experiments (except results on panel E- they represent data from 2 experiments).Values are mean ± SEM.

exposure to MEHP or DMSO vehicle control (**Fig. 4.2 A,B**). These results were obtained from three independent groups of experiments.

Earlier results indicated that transferring GC-2 cells to a lower temperature not only activated p53 but also caused a substantial increase in membrane Fas levels in these cells. Flow cytometric analysis of annexin V-PI stained cells indicates that GC-2 cells at the p53 permissive temperature display a near 5-fold increase in cell death (15-20% annexin positive cells compared to 2-4 % positive stain for control cells) by 24 h after exposure to JO2 (**Fig. 4.2 C,D**). Cells grown at the non-permissive p53 temperature were not killed upon exposure to JO2. Cells grown at either temperature were also not responsive to the presence of hamster IgG, an isotype control for the JO2 antibody.

Cells were exposed to human TRAIL ligand in the presence or absence of a protein synthesis inhibitor, cycloheximide. The cytotoxicity of cycloheximide is evident even at 10 μM in cells at 37 ºC, while a higher dose of 100 μM is the cytotoxic dose in cells at 32 ºC. Only cells at the p53 permissive temperature however, demonstrate increased levels of apoptosis (as demonstrated by flow cytometric analysis of annexin and annexin-V + PI stained cells, **Fig. 4.2 E,F**) in the presence of TRAIL and the non-toxic dose of cycloheximide. These cells do not undergo enhanced cell death in response to the TRAIL ligand alone.

4.2.3. p53 status of GC-2 cells determines the protein expression levels of c-FLIP (L) in response to anti-Fas treatment, but not mRNA levels:

Western blot analysis of c-FLIP (L) levels in GC2 cells treated with JO2 show that c-FLIP (L) levels are elevated by 3-fold in GC2 cells grown at 37 ºC at both 3 and 6 h after exposure, while they remained below control levels in cells at 32 ºC (**Fig. 4.3 A**). Real-time PCR assessment of c-FLIP (L) transcription in response to JO2 addition revealed that cells grown at both temperatures do not demonstrate any significant changes in their c-FLIP(L) mRNA levels at any time point after JO2 treatment (**Fig. 4.3 B, i, ii**). Direct comparison of c-FLIP (L) mRNA levels of control samples at 32°C and 37°C indicate that at 32°C GC-2 cells express approximately 0.6 fold more c-FLIP (L) mRNA as compared to cells at 37°C.

4.2.4. Transfection of GC-2 cells with siRNA against c-FLIP (S/L) leads to reduced c-FLIP (L) mRNA levels, decreased protein expression and increased sensitivity to anti-Fas treatment, but not to TRAIL exposure:

Levels of c-FLIP (L) mRNA were analyzed using semi-quantitative RT-PCR at 24 h, 30 h and 48 h after c-FLIP siRNA transfection in cells grown at 37 ºC. A reduction in mRNA levels by about 50-60 % (normalized to β-actin) was observed at the 24 h time point (**Fig. 4.4 A**). However, the reduction was not maintained beyond this time point, with mRNA levels returning to baseline levels by 30 h. Comparisons of c-FLIP (L)

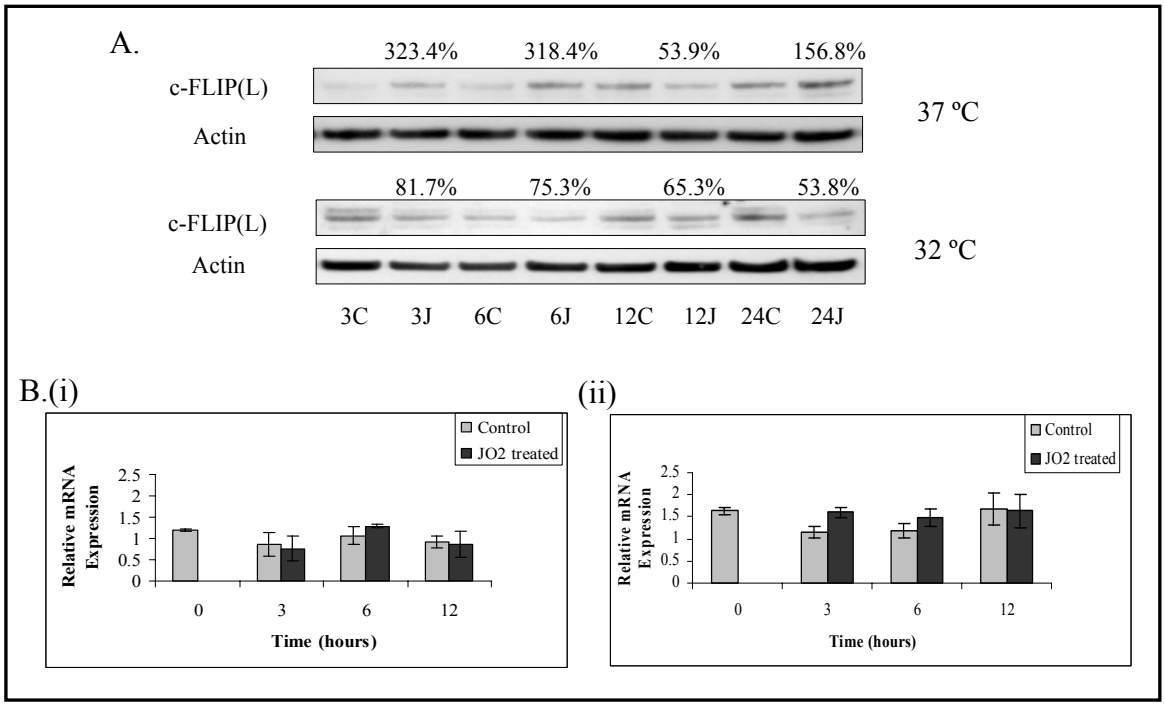

Fig. 4.3. Western blot analysis of c-FLIP(L) protein levels, and real-time PCR analysis of mRNA levels in response to JO2 treatment (A) Representative blots from 3 samples for each time point are provided; c-FLIP(L) (~55 kDa) bands and actin (~43 kDa) are shown here for cells grown at both 37 ºC and 32 ºC temperatures after JO2 treatment, with their matched time controls. 'C' represents controls, 'J' represents JO2 treated cells. The net intensity of c-FLIP (L) expression as a percent of untreated matched time control and normalized to actin are indicated above each treated sample. (B- i and ii) A graphical representation of relative expression levels (relative to the same calibrator) of c-FLIP (L) mRNA (from 3 samples for each time point) with or without JO2 treatment at non-permissive (i) and permissive (ii) p53 temperatures. Values are mean ± SEM.

mRNA levels were made against control siRNA transfected cells, as well as with cells that had not undergone transfection . At 48 h after transfection, we were able to demonstrate a significant reduction in c-FLIP (L) protein levels by western blot analysis (60-80 % reduction) in siRNA transfected cells, compared to corresponding controls (**Fig. 4.4 B**).

GC-2 cells at the non-permissive temperature of 37 ºC and transfected with siRNA for c-FLIP, were exposed to anti-Fas or TRAIL treatment. Cells exposed to JO2 underwent a 4-fold increase in apoptosis levels compared to matched time controls at both 6 and 12 h after exposure, as determined by flow cytometric analysis of annexin-positive cells (**Fig. 4.4 C**). However cells exposed to TRAIL remained insensitive to its effects, despite the reduction in c-FLIP (L) levels (data not shown).

4.2.5. Ubiquitinylation of c-FLIP in GC-2 cells is increased when p53 is active and after exposure to JO2:

To determine if c-FLIP undergoes post-translational modification by ubiquitin, c-FLIP was immunoprecipitated from lysates of GC-2 cells following exposure to JO2. The complexes collected through this procedure showed significant differences by western blot analysis in levels of the ubiquitin-tagged c-FLIP between cells at the p53 permissive or non-permissive temperatures (**Fig. 4.5 A**). Polyubiquitinylated forms of c-FLIP were

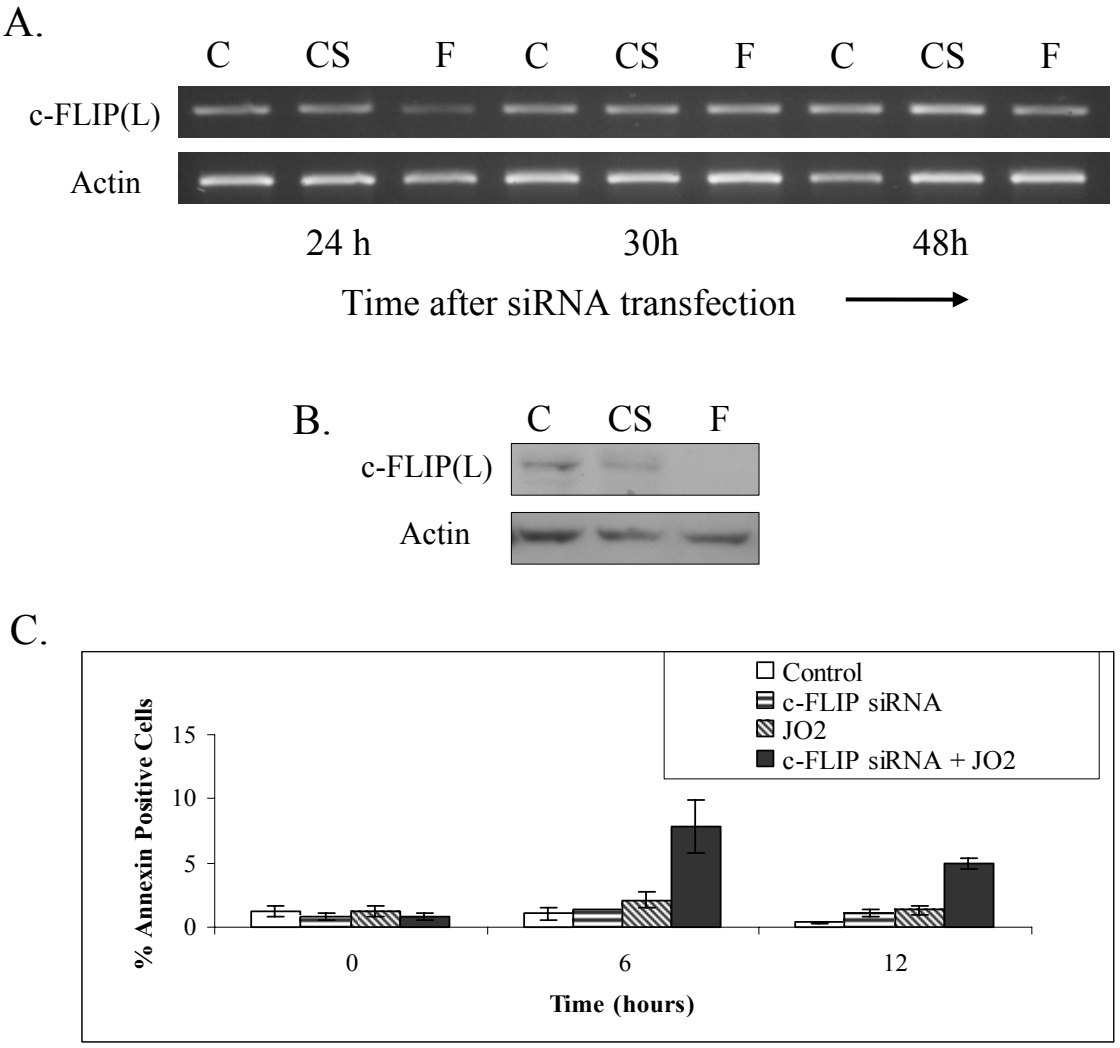

Fig. 4.4. **Analysis of c-FLIP(L) mRNA and protein levels, and apoptotic levels in response to JO2 after knockdown of c-FLIP(L) by siRNA.** (A) Representative gels for c-FLIP (L) mRNA from GC-2 cells at the non-permissive p53 temperature exposed to c-FLIP siRNA for 24h, 30h and 48h. β-actin is used as an internal control. 'C' represents controls, 'CS' represents control siRNA treated cells and 'F' represents c-FLIP siRNA treated cells. (B) Representative western blots are provided; bands for c-FLIP(L) (~55 kDa) and actin (~43 kDa) are shown here for cells grown at the non-permissive p53 temperature, transfected with control siRNA (CS), c-FLIP siRNA (F) or matched time controls (C) at 48 h post transfection. (C) A graphical representation of annexin positive GC-2 cells +/- c-FLIP siRNA transfection and exposed to JO2, or their matched time point controls. Values are mean ± SEM. The data represent results from 3 independent experiments.

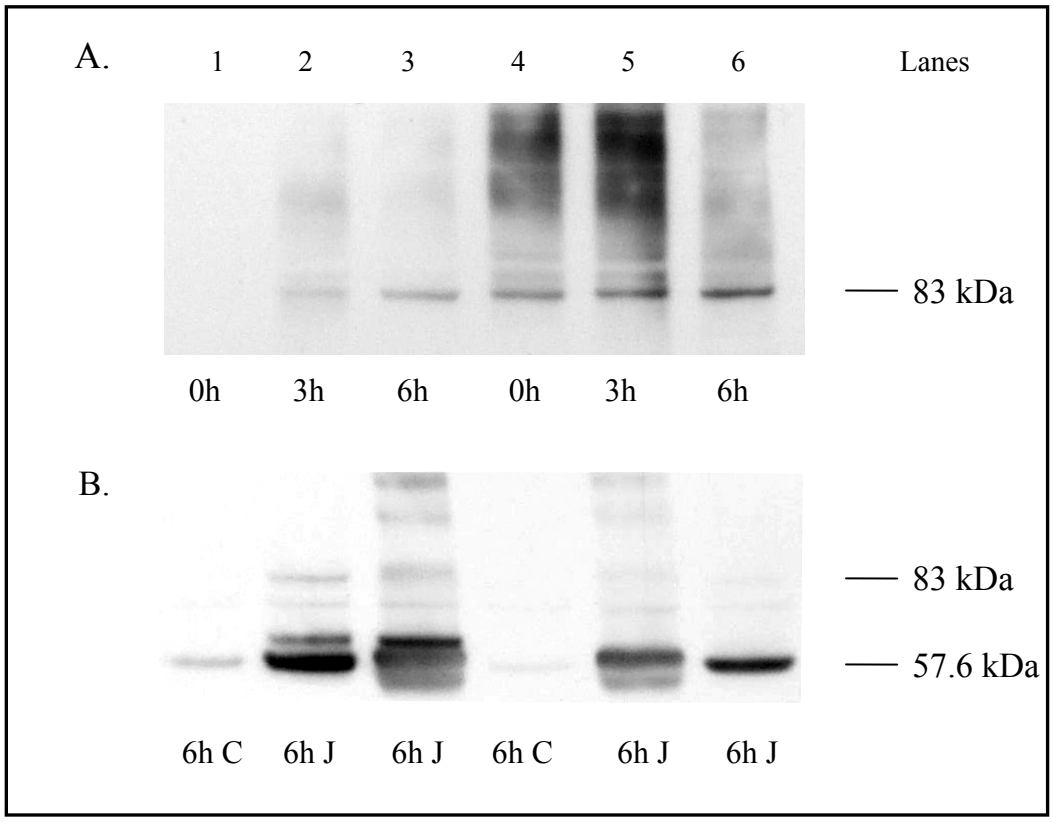

Fig. 4.5. Immunoprecipitation analysis of ubiquitinylation of c-FLIP(L) in GC-2 cells exposed to JO2. (A)) Representative blots from 3 samples for each time point are provided; protein complexes bound to c-FLIP were probed for ubiquitin tag in cells grown at both 37 ºC and 32 ºC and exposed to JO2. Lanes 1-3 represent samples at the non-permissive temperature treated with JO2, while lanes 4-6 represent samples from cells at the permissive p53 temperature also exposed to JO2 for indicated time periods. (B) Representative blots from 3 samples for each sample are provided; Lysates from the pull-down assay were probed for c-FLIP(L) at 6h after JO2 exposure. 'C' represents controls and 'J' represents JO2 treated samples.

revealed as a collection of bands (molecular weight of 83 kDa to 98 kD) on the western blot that corresponded to the predicted mass of c-FLIP (L) plus ubiquitin. Polyubiquitinylated levels of c-FLIP were low in GC-2 cells grown at the p53 non-permissive temperature but were at readily detectable levels after JO2 addition (**Fig. 4.5 A, lanes 1-3**). Cells cultured at the p53 permissive temperature exhibited a higher basal level of ubiquitinylation which also increased 3 h after JO2 treatment (**Fig. 4.5 A, lanes 4-6**). Interestingly, at 6 h after JO2 exposure, the levels of the polyubiquitin labeled c-FLIP complex had already begun to decline in these cells. Immunoprecipitated complexes were also probed using the anti-c-FLIP rat monoclonal antibody. c-FLIP (L) was similarly expressed in both cell types in the absence of JO2 treatment. With the addition of JO2, three or more protein bands approximately 8.5 kDa apart in addition to the naïve c-FLIP protein band were generated, corresponding to the sizes expected for ubiquitin tagged c-FLIP protein (**Fig. 4.5 B**). The data show a reduction in ubiquitinylated c-FLIP by 6 h after JO2 exposure at the p53 permissive temperature, likely through post-transcriptional targeting to the proteasome.

4.3. Discussion

In general, depending upon the physical injury or chemical toxicant that the testis is exposed to, germ cell apoptosis is initiated by either the activation of the death receptor pathway or *via* activation of the stress response pathway involving the mitochondrial release of cytochrome c (Lee *et al.* 1997; Lee *et al.* 1999; Embree-Ku *et al.* 2002; Vera *et*

al. 2004). Sertoli cell injury mediated by the active phthalate monoester, MEHP, results in the death of germ cells through the paracrine interaction of Sertoli cell produced FasL with Fas expressed on germ cells (Richburg *et al.* 1999). Our previous work *in vivo* demonstrated a role for p53 in Fas receptor membrane localization as a late event in response to MEHP, and in DR5 membrane localization as well (Chandrasekaran and Richburg 2005). In addition, we have shown that the activation of the death receptor pathway (as indicated by procaspase-8 processing) in testicular germ cells was abrogated in mice lacking the expression of p53, a condition that may be directly linked to the increased expression of c-FLIP (L). These findings *in vivo* led us to hypothesize that the c-FLIP (L) protein is modified in a p53-dependent manners in germ cells after death receptor activation. The p53 protein is a transcription factor that in many cell types causes the up-regulation in levels of pro-apoptotic proteins, such as Bax, Puma, Noxa, Fas, DR5, etc (Miyashita and Reed 1995; Munsch 2000; Takimoto and El-Deiry 2000; Villunger *et al.* 2003). Therefore, the aim of the present *in vitro* work with GC-2 cells was to functionally characterize the relationships between Fas, DR5, p53 and c-FLIP in germ cell apoptosis.

GC-2 cells were originally created to provide an in vitro model of germ cell differentiation (Hofmann *et al.* 1994). GC-2 cells were created from spermatocytes that had been immortalized with the SV40 virus's large T antigen and co-transformed with a mutant p53 protein. The p53 protein is activated by proper folding and nuclear localization at the lower temperatures in GC-2 cells, where it binds the large T antigen

and suppresses its proliferative ability. This enables the cells to undergo differentiation. However, two years after establishing the GC-2 cell line, the authors were unable to detect markers for the various differentiated germ cell types, in cells grown at the p53 permissive temperature (32 ºC) (Wolkowicz 1996). The cells were therefore deemed unsuitable for further studies. However, here we show that these cells serve as a suitable model to study apoptosis, due to their differential p53 activity based on the temperature they are maintained at. Moreover, GC-2 cells were found to express Fas, DR5 and c-FLIP (L) as shown by western blot analyses, or ICC (**Fig. 4.1 A,B**).

GC-2 cells were created from spermatocytes, the germ cell sub-type most sensitive to MEHP induced apoptosis (Richburg *et al.* 1999). However, MEHP does not act directly on germ cells to mediate their apoptotic demise, rather, MEHP-induced germ cell apoptosis occurs indirectly *via* Sertoli cell expressed FasL activating germ cell Fas receptors. Therefore, as expected, we were unable to observe increases in the incidence of apoptotic GC-2 cells following exposure to MEHP at either the p53 permissive or non-permissive temperatures (**Fig. 4.2 A,B**). In the present study, we used the Fas agonistic antibody, JO2, to mimic *in vitro* the elucidation of FasL by Sertoli cells and the activation of Fas-dependent germ cell apoptosis. The JO2 antibody has been shown to efficiently trigger apoptosis in murine cells expressing the Fas receptor (Ogasawara *et al.* 1993). GC-2 cells expressed nearly 10-fold more Fas protein on the cell membrane at the p53 permissive temperature compared to the cells at the non-permissive temperature (**Fig. 4.1**

B) and could easily account for the sensitivity of GC-2 cells maintained at 32 ºC to JO2-induced apoptosis (**Fig. 4.2 D**).

On the other hand, we were unable to demonstrate that GC-2 cells were sensitive to TRAIL treatment (**Fig. 4.2 E,F**). The membrane DR5 levels in both cell types remained unchanged (data not shown) despite the differences in p53 status. This could perhaps account for the similar responses to TRAIL by cells at both temperatures. Despite TRAIL's ability to kill cancer cells, the sensitivity of normal cells to TRAIL treatment has been disputed over the years, although the susceptibility of normal hepatocytes (Jo *et al.* 2000), normal prostate epithelial cells (Nesterov *et al.* 2002) and thymocytes (Simon *et al.* 2001) to TRAIL treatment have been demonstrated. TRAIL has also been implicated in mediating cell survival of primary vascular endothelial cells by activating the ERK pathway (Secchiero *et al.* 2003). Thus, the absence of an apoptotic response to TRAIL by GC-2 cells demonstrates that TRAIL may not promote germ cell death and hence DR5 may not have a role in MEHP induced germ cell apoptosis. Additionally, we have used the human form of the TRAIL ligand for our experiments. Human TRAIL is nearly 70 % homologous to its mouse form and could potentially not be useful to kill murine cells thereby explaining our results. However, cells at the p53 permissive temperature could be sensitized to TRAIL treatment in the presence of the protein synthesis inhibitor cycloheximide (**Fig. 4.2 F**). Further studies are necessary to explore the sensitivity of GC-2 cells to the murine form of TRAIL and to clarify the issue beyond doubt.

Cycloheximide can sensitize cells to TRAIL mediated apoptosis by causing a reduction in c-FLIP protein levels (Kreuz *et al.* 2001; Brooks and Sayers 2005). Additionally, the over-expression of c-FLIP (L) is responsible for inhibiting TRAIL-induced apoptosis in prostate cancer and malignant mesothelial cells (Rippo *et al.* 2004; Zhang *et al.* 2004). Thus c-FLIP siRNA transfected GC-2 cells at the non-permissive p53 temperature were also exposed to TRAIL to determine if the lack of TRAIL sensitivity in these cells was due to inhibition of DR5 activation by c-FLIP (L). The transfected cells could not be killed by TRAIL (data not shown). The sensitivity of GC-2 cells to TRAIL in the presence of cycoloheximide and in a p53-dependent manner cannot be explained by the suppression of c-FLIP (L) expression. Further work needs to be done to identify the factors responsible for this phenomenon, which could include abrogated NFκB or ERK signaling since these cell survival inducing pathways can be activated when some cell types are exposed to TRAIL (Secchiero *et al.* 2003).

In our *in vivo* studies, we observed an increase in c-FLIP (L) protein levels after MEHP exposure without any changes in the rates of its transcription and only in the p53 knockout mice. Thus, activation of Fas appeared to be a requisite for the retention or stabilization of c-FLIP (L) in these germ cells, (Chandrasekaran and Richburg 2005). Similar to the *in vivo* data, we found that GC-2 cells maintained at the p53 non-permissive temperature had increased levels of the c-FLIP (L) protein only after exposure to the JO2 antibody (**Fig. 4.3 A**). Upon examination of c-FLIP (L) protein expression in GC-2 cells treated with JO2, we detected ~3 fold increases in c-FLIP (L) after 3 and 6 h

of JO2 exposure (**Fig. 4.3 A**) only in cells lacking active p53. On the other hand, in cells at the permissive p53 temperature, c-FLIP (L) protein levels were decreased by nearly 25-50 % of the control values after JO2 treatment (**Fig. 4.3 A**). The observed increase of c-FLIP in cells maintained at 37 ºC cells could not be accounted for by changes in its transcription as demonstrated by real-time PCR analysis (**Fig. 4.3 B, i, ii**). Taken together these observations suggest that *in vitro*, increases in c-FLIP levels in the absence of p53 are due to an alteration in the stability and/or degradation of the protein as well.

p53 influences c-FLIP ubiquitinylation in colon cancer cells (Fukazawa *et al.* 2001). To examine if c-FLIP (L) was being degraded post-transcriptionally in a p53-dependent manner we performed IP experiments to detect ubiquitinylation of c-FLIP (L) in GC-2 cells after JO2 exposure. In cells at the p53 non-permissive temperature, ubiquitin bound protein complexes pulled down by the anti-FLIP antibody and identified by a ubiquitin antibody could only be detected after JO2 treatment (**Fig. 4.5 A, lanes 1-3**), indicating that c-FLIP was being tagged for degradation in these cells only after Fas activation. Interestingly, in p53 permissive cells, very high expression levels of ubiquitinylated complexes could be detected in untreated control cells; with further increases in ubiquitinylation occuring 3 h after JO2 exposure (**Fig. 4.5 A, lanes 4-6**). A comparison of the levels of these polyubiquitin complexes revealed that the c-FLIP protein in the cells at the p53 permissive temperature were more robustly ubiquitinylated than those at the non-permissive temperature. The c-FLIP protein was therefore being more actively targeted to the proteasome for degradation in cells maintained at the p53

permissive 32 ºC. However, when the immunoprecipitated complexes were probed with an antibody against c-FLIP, the intensity of the bands of c-FLIP (L) protein complexed to ubiquitin from both cell types appeared similar (**Fig. 4.5 B**). The apparent similarity in c-FLIP (+/- ubiquitin) levels between the samples can be accounted for by the differences in cell density of GC-2 cells grown at the two temperatures (more dense at 37 ºC) and hence the relative number of c-FLIP molecules pulled down during the IP assay. Experiments were also performed with the proteasome inhibitor MG-132 to demonstrate that inhibiting c-FLIP protein degradation would decrease the sensitivity of GC-2 cells at the p53 permissive temperature to JO2 (data not shown). However, the use of MG-132 and another proteasome inhibitor lactacystin proved to be extremely cytotoxic to the GC-2 cells even at concentrations less than 1 μM.

To test if the retention of c-FLIP (L) was responsible for the insensitivity of GC-2 cells maintained at 37 ºC to JO2, we transiently reduced c-FLIP protein expression by transfecting these cells with siRNA against c-FLIP. siRNA transfected cells displayed a 60-80 % reduction in the protein levels of c-FLIP (L) (**Fig. 4.4 B**) and this resulted in sensitizing the cells to JO2 induced Fas-activation. A near 4-fold increase in GC-2 apoptosis was observed as a result of the siRNA c-FLIP protein reduction (**Fig. 4.4 C**). These results affirm a role for c-FLIP (L) in attenuating Fas activated apoptosis in germ cells. The increases in apoptotic levels from 1-2 % in control c-FLIP siRNA transfected cells to about 8 % in siRNA transfected cells exposed to JO2 are minimal though significantly different. These results can perhaps be explained by the measured low Fas

membrane expression levels (**Fig. 4.1 B**) in cells grown at the non-permissive p53 temperature. Therefore, the combination of reduced membrane Fas levels and increased c-FLIP (L) expression in GC-2 cells after JO2 exposure likely account for the decreased sensitivity of these cells to apoptosis.

An important insight gained by these experiments is the indication that the p53 status of the cell may have a strong influence over the extent of c-FLIP (L) protein degradation within distinct germ cell subtypes. However, it should be noted that the ubiquitinylation of c-FLIP (L), as evident from the results in **Fig. 4.5 B**, occurred in both the cell types irrespective of their p53 status and only when Fas was activated (i.e. after JO2 treatment). Thus, the degradation of c-FLIP (L) is coupled to its recruitment to the DISC. We also show that the p53 status of GC-2 cells directly corresponds to the expression levels of Fas on the germ cell membrane. The binding of FasL to Fas on the membrane of germ cells determines the amount of DISC formed and therefore, DISC formation is likely more robust in p53 permissive GC-2 cells with the greater concentrations of Fas. The protein c-FLIP (L) is typically recruited to the formed DISC complex. To negate c-FLIP's inhibitory influence and ensure that the cell undergoes apoptosis, the cell may mediate degradation of c-FLIP at this point of its recruitment. In our *in vivo* model, the expression levels of membrane Fas in the p53 -/- versus p53+/+ mice were not significantly different except at the 12 h time point after MEHP exposure. Thus *in vivo* the increased expression of c-FLIP (L) protein in the p53-/- mice between 1-12 h after MEHP exposure correlated to an absence of their degradation as influenced by

the p53 status of the cell and perhaps not due to relative differences in the ability of these cells to form a DISC complex. An analysis of DISC formation in germ cells of p53+/+ and p53 -/- mice exposed to MEHP would therefore be important to clarify whether p53 status influences the relative amount of DISC formed, and hence c-FLIP (L) degradation. In GC-2 cells however, the p53 protein does influence the expression levels of membrane Fas and hence the extent of c-FLIP retention.

In our *in vivo* model we weren't entirely able to correlate the differences in apoptosis between p53+/+ and p53 -/- mice to p53 dependent alterations of membrane Fas levels, perhaps due to the use of total testicular lysates for analysis. Nevertheless we were able to demonstrate that p53 influenced the stability/retention of the c-FLIP (L) protein after MEHP exposure, which was confirmed by the ubiquitinylation studies carried out in GC-2 cells. Our results from both studies, when taken together, indicate that the activation of p53 in germ cells due to cellular stress leads to Fas activated apoptosis by ensuring the degradation of c-FLIP (L) protein.

Chapter 5: The influence of p53 status on the localization of Fas to lipid raft domains of germ cell plasma membranes: Correlation to increased sensitization of germ cells to MEHP-induced apoptosis

5.1. Introduction and Rationale:

In Chapter 3, an *in vivo* animal model was used to demonstrate that p53 plays a role in the increased cell membrane localization of Fas and DR5 in germ cells after exposure to MEHP (**Fig. 3.2 A,B**). Our *in vitro* GC-2 cell model demonstrated that germ cells could only be sensitized to apoptotic cell death in the presence of anti-Fas antibodies and not with TRAIL treatment (**Fig. 4.2 C-F**). Thus, in response to MEHP exposure germ cell apoptosis is most likely not mediated by a DR5-activated pathway. Interestingly, the induction of increased Fas expression at the germ cell membranes is a late event that only coincides with a second wave of germ cell apoptosis, while increased membrane DR5 was observed as early as an hour after MEHP exposure and maintained at high levels thereafter. MEHP induced toxicity yielded both an early and a late increase in germ cell apoptosis that peaked at 1.5 h and 24 h respectively post-exposure (**Fig. 3.1 E**). Both waves were preceded by increased processing and hence activation of caspase-8 (**Fig. 3.4 A-C**). These results indicate an activation of death receptors during both the early and late apoptotic events. However, in the absence of increased membrane Fas expression for the first 6 h after MEHP exposure (**Fig. 3.2 A**, **C**), and perhaps a lack of contribution from the augmented DR5 membrane receptors toward germ cell apoptosis,

an alternate explanation is required to clarify how increases in caspase-8 processing occur coincident with the first wave of apoptosis.

The increases in c-FLIP (L) levels in the p53 -/- mice as early as 1 h after exposure to MEHP could indicate that c-FLIP degradation in the p53+/+ mice may sustain caspase-8 activation with only the basal levels of the Fas receptor being present (**Fig. 3.5 B,C**). However, as shown in **Fig. 3.5 A**, the levels of c-FLIP (L) in the p53+/+ mice are not significantly decreased after MEHP treatment and may not completely account for the enhanced caspase-8 activation. Therefore, we hypothesize that in the absence of their increased expression, Fas receptors are targeted to a specific microdomain within the cell membrane called lipid rafts in a p53-dependent manner, enabling the formation of the DISC and hence the processing of caspase-8 in response to MEHP. Lipid rafts are specialized microdomains in cell membranes that are cholesterol and sphingomyelin enriched (for review see Zajchowski and Robbins 2002). A large number of signaling molecules that mediate different signaling pathways such as epidermal growth factor receptor, ceramide, diacylglycerol, Ras, Shc, Src-family of kinases and many more can be found localized in lipid rafts. In fact, the DISC is formed in the lipid rafts of $CD4^+$ T cells in the event of Fas-induced apoptosis (Scheel-Toellner *et al.* 2002). The movement of Fas receptors within the membrane to mediate signaling could explain enhanced processing of caspase-8 in the absence of changes to Fas membrane levels after toxicant exposure.

Lipid rafts are detergent resistant microdomains that are typically associated with integral membrane proteins called caveolins and flotillins and form a liquid ordered phase in membranes due to their lipid composition (Brown and London 1997; Zajchowski and Robbins 2002). These domains not only contain cholesterol and sphingomyelin, but also glycosphingolipids and proteins anchored to the plasma membrane by glycophosphotidylinositol. The ordering of the domains by the presence of a high concentration of saturated fatty acid chains with intercalated cholesterol causes the resistance of these domains to detergents. Lipid rafts can be separated thus based on both a detergent insolubility and low buoyant density in sucrose density gradients. Caveolins are the principal protein component of lipid rafts and may be found associated with flotillins in many cell types. Flotillin-2 or epidermal surface antigen (ESA) is more ubiquitously expressed than Flotillin-1 and is expressed in the testis (Volonte *et al.* 1999).

Antitumor agents such as the ether lipid, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine, cisplatin and the natural phytoalexin, resveratrol (3,5,4'-trihydroxystilbene), cause the redistribution of Fas to membrane lipid rafts to initiate apoptosis of either leukemic or colon cancer cells (Gajate and Mollinedo 2001; Delmas *et al.* 2004; Lacour *et al.* 2004). Muppidi and Siegel (Muppidi and Siegel 2004) demonstrated that restimulation of activated T cells lead to their death due to the translocation of Fas to lipid raft domains. The same authors also demonstrated that type I and type II cells differ in their ability to respond to Fas activation due to the differences in Fas localization at lipid rafts. These different studies demonstrated that the disruption

of lipid rafts by cholesterol depleting agents such as methyl β-cyclodextrin (MBCD) or filipin, also disrupted the localization of Fas to lipid rafts and significantly reduced Fas-mediated apoptosis. Hence, based on the above studies that demonstrate that relocation of Fas within the cell membrane can sensitize cells to Fas-mediated cell death, we decided to test for differences in membrane Fas localization in germ cells from p53 -/- and p53 +/+ mice and in GC-2 cells at both the p53 permissive and non-permissive temperatures.

5.2. Results

5.2.1. Expression Profile of Fas in Lipid Raft Fractions of GC-2 Cell Membranes:

GC-2 cells maintained at both the p53 permissive and non-permissive temperatures were subjected to sucrose density gradient ultracentrifugation to separate lipid raft domains in their cell membranes from the non-lipid raft domains. As shown in **Fig. 5.1**, the fractions obtained by ultracentrifugation were analyzed for both Fas and Flotillin-2 expression by western blot analysis. As expected, Flotillin-2 expression was abundant in the fractions obtained from the top layers of the sucrose density gradient, i.e. the fractions representing the least dense and detergent resistant layers of the gradient (Fractions 3 and 4 in this case, **Fig. 5.1 A,B**). Therefore, these fractions deemed the lipid raft fractions were analyzed further for Fas expression. Cells at the p53 permissive temperature had abundant expression of Fas in almost all fractions unlike cells at the non-permissive temperature (**Fig. 5.1 A,B**). Fas levels in lipid raft fractions 3 and 4 were only

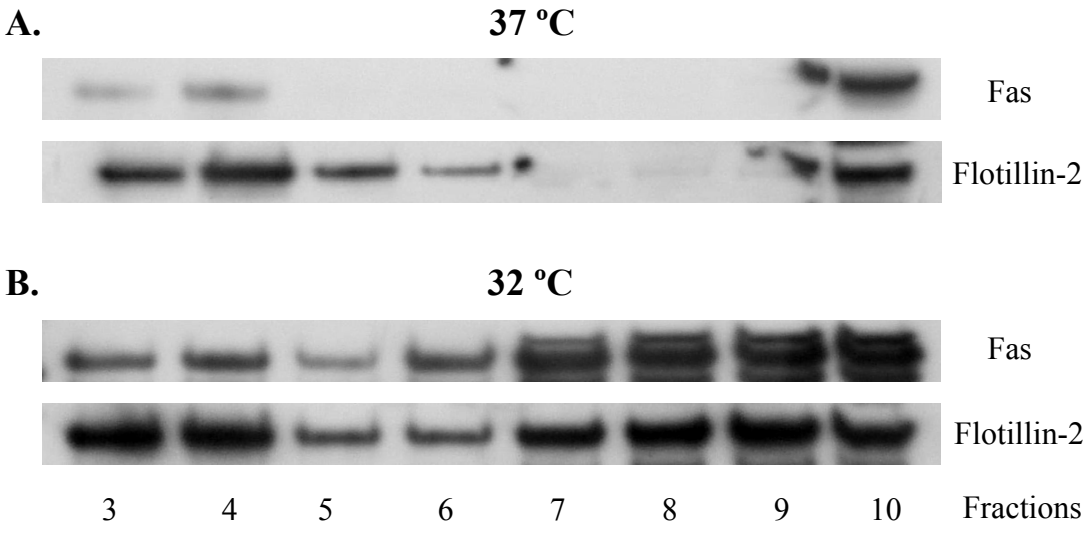

Fig. 5.1. Expression profile of Fas in lipid raft fractions of GC-2 cells. Representative blots (from 2 sample sets) for Fas and Flotillin-2 expression in cell membrane fractions of GC-2 cells at 37 ºC (A) and 32 ºC (B), obtained by sucrose density gradient ultracentrifugation. Fractions 3 and 4 represent lipid raft fractions, while fractions 8-10 donate non-lipid raft fractions.

slightly higher in cells at the p53 permissive temperature than those at the non-permissive temperature when normalized to corresponding Flotillin-2 levels in each fraction (data not shown).

5.2.2. Disruption of Lipid Rafts in GC-2 Cells at the p53 Permissive Temperature Does Not Significantly Reduce Sensitivity of these Cells to JO2:

GC-2 cells maintained at 32 ºC were pretreated with 1 mM, 5 mM or 10 mM of the cholesterol depletor MBCD for 30 min in serum free media at 37 ºC, washed and exposed to JO2. MBCD caused a collapse of cell membrane microdomains as evident from the clustering of Flotillin-2 protein in the non-raft fractions (Fractions 8-10) only (0 h sample, **Fig. 5.2 A**) compared to untreated cells as determined by western blot analysis. Importantly, 6 h after the MBCD had been washed out, GC-2 cells still displayed the collapsed membrane architecture with Flotillin-2 remaining concentrated in the non-raft fractions (**Fig. 5.2 B)**. The higher doses of MBCD (5 and 10 mM) were protective against JO2 mediated apoptosis at the early time points of 3 and 6 h after exposure as demonstrated by flow cytometric analysis of annexin-V positive cells, but not at the later time point of 12 h (**Fig 5.2 C**). However, this protection was significant only at the 3 h time point, wherein cells exposed to JO2 after pretreatment with MBCD experienced a nearly 40-50 % reduction in overall levels of apoptosis.

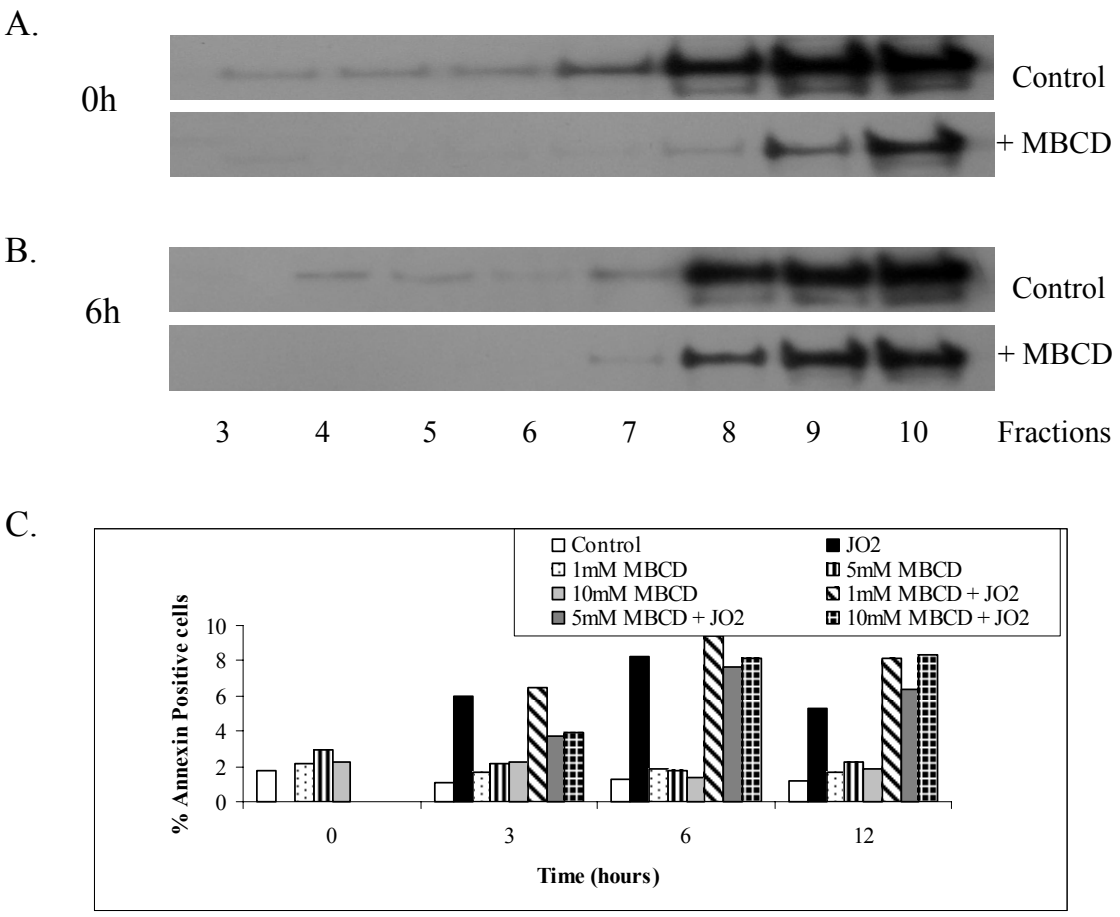

Fig. 5.2. Analysis of sensitivity of GC-2 cells at the p53 permissive temperature to JO2 after disruption of lipid raft domains. (A) and (B) are representative blots for Flotillin-2 (~46 kDa) in control or MBCD treated GC-2 cells at the p53 permissive temperature. (A) represents samples from cells collected 0h after MBCD pre-treatment, while (B) represents samples from cells collected 6h after the pre-treatment. (C) A graphical representation of annexin-positive GC-2 cells, pre-treated with MBCD and exposed to JO2, as well as matched time and pre-treatment only controls. The results represent data from 2 independent experiments.

5.2.3. Absence of Fas Expression Changes in Lipid Raft Domains of Testicular Germ Cells after MEHP Exposure Regardless of p53 Status:

Testicular samples from p53+/+ and p53 -/- mice were also subjected to sucrose density gradient ultracentrifugation to separate lipid raft membrane fractions. For testicular samples, Flotillin-2 expression was most significant in fraction 5 (**Fig 5.3 A**) and all further analyses of Fas expression were carried out in the fraction 5 samples for each MEHP treated sample. Western blot analyses of Fas expression in testicular samples showed no significant changes to Fas expression (or Flotillin-2) after exposure to MEHP, in lipid raft fraction 5 (Fig 5.3 C). Moreover, the ability to detect Fas expression in these fractions was quite poor.

5.3. Discussion

Sensitivity to MEHP in the testis is manifested in the form of increased germ cell apoptosis. We hypothesized that p53 plays an important role in sensitizing specific germ cells subtypes to apoptosis caused by withdrawal of support from MEHP injured Sertoli cells, *via* their increased expression of membrane Fas. However, despite some evidence of increased membrane localization of Fas in our *in vivo* model (Chapter 3), we were unable to completely correlate the activation of the Fas pathway (i.e. increased processing of procaspase-8) to increased expression of Fas on germ cell membranes in response to MEHP. Therefore, we have tried to determine whether in the absence of additional Fas

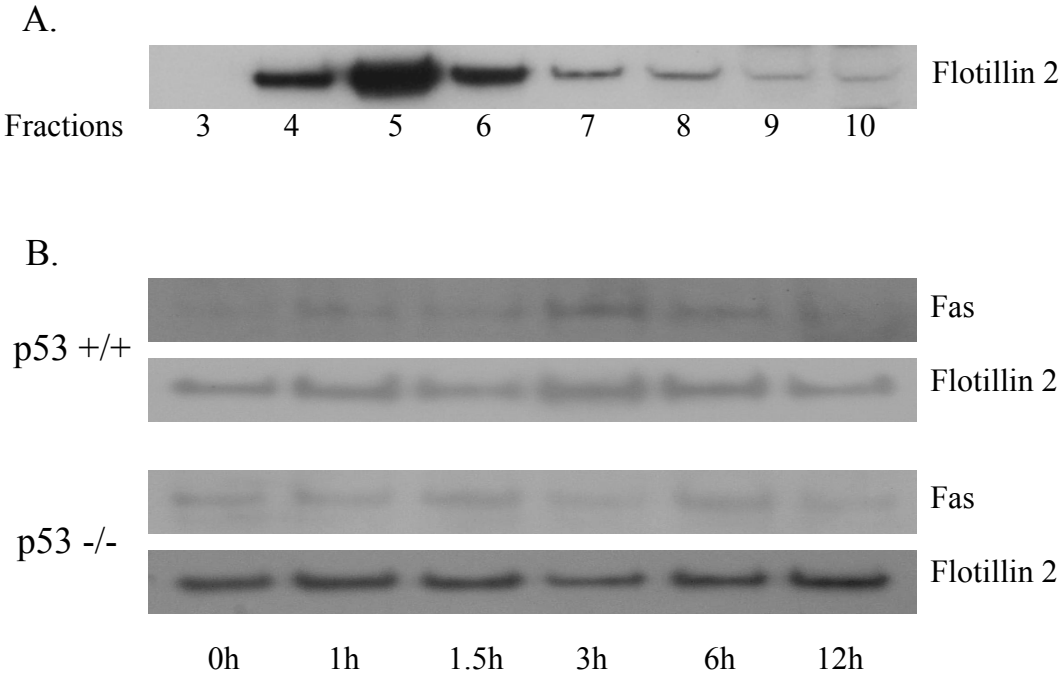

Fig 5.3. Analysis of changes in Fas localization to lipid raft domains. (A) is a representative blot for Flotillin-2 expression in testicular membrane fractions obtained by sucrose density ultracentrifugation. (B) Representative blots for Fas expression in lipid raft fraction 5 from testes of p53+/+ and p53 -/- mice in response to MEHP exposure. Flotillin-2 is used an internal control. The data represents the results from 2 independent experiments.

movement to the germ cell membrane, its movement within the cell membrane into a specific functional microdomain could account for activation of the Fas pathway after MEHP mediated injury. The relocation of Fas into lipid raft domains within the cell membrane is responsible for the sensitization of a number of cell types including T cells, colon cancer cells, and malignant mesothelial cells to apoptosis (Gajate and Mollinedo 2001; Delmas *et al.* 2004; Lacour *et al.* 2004; Muppidi and Siegel 2004). Hence, we evaluated if testicular samples from p53+/+ and p53 -/- mice exposed to MEHP and GC-2 cells maintained at both the p53 permissive and non-permissive temperature, were capable of modifying Fas levels in their membrane lipid raft domains in response to cellular stress and in a p53-dependent manner to mediate apoptosis.

GC-2 cells were analyzed for differences in lipid raft expression of Fas receptors at both 37 ºC and at 32 ºC. Although the cells at the p53 permissive temperature expressed extremely abundant levels of Fas protein in almost all the fractions obtained from the sucrose density gradient fractionation procedure, the levels of Fas in the fractions corresponding to the lipid raft domains (Fractions 3 and 4) were only modestly higher than in fractions from the 37 ºC cells (when normalized to Flotillin-2 levels, **Fig. 5.1 A,B**). GC-2 cells at 37 ºC had increased retention of c-FLIP (L) compared to cells at 32 ºC as discussed in Chapter 4, and could be sensitized to anti-Fas induced apoptosis when c-FLIP levels were knocked down. Thus it appears that GC-2 cells are capable of mediating Fas localization in lipid rafts irrespective of their p53 status and that raft

localization may not be the primary factor responsible for different sensitivities of these cells to anti-Fas treatment.

Cholesterol depletion from cell membranes disrupts lipid raft domains as they are cholesterol enriched domains. Thus, all proteins located within lipid raft domains would cluster in non-raft domains when these rafts are disrupted or dismantled. If Fas is localized in lipid rafts, a cholesterol depletor such as MBCD should cause its relocation as well as that of Flotillin-2, a raft associated protein from that domain. GC-2 cells at the p53 permissive temperature were exposed to MBCD pretreatment. As expected, this pretreatment caused Flotillin-2 relocation from raft domains in the cell membranes of GC-2 cells (**Fig 5.2 A,B**). However, despite the disruption of lipid rafts, the GC-2 cells were not completely protected against JO2 treatment, with significant protection being achieved only for a brief period of time (up to 3 h) after exposure to JO2 (**Fig 5.2 C**). This lack of protection against JO2 could not be explained by any inherent cytotoxic effects of MBCD. The appropriate sample control cells exposed to MBCD but not JO2, displayed slightly higher levels of apoptosis compared to untreated controls, but these levels were not significantly higher. Interestingly, a recent report (Gniadecki 2004) showed that cholesterol depletion leading to Fas clustering in non-raft domains in the plasma membranes of keratinocytes improved the ability of these cells to undergo Fas-induced apoptosis. Again, activation of Fas mediated apoptosis in GC-2 cells may not depend on the localization of Fas to lipid raft microdomains.

Testicular samples from p53+/+ and p53 -/- mice exposed to MEHP were also analyzed for changes in Fas expression in lipid raft domains by western blots. When Fas expression in a specific fraction 5, enriched in Flotillin-2 was examined, no significant changes could be observed in response to MEHP exposure (**Fig. 5.3 B**). No movement of Fas into these lipid raft domains above control expression levels could be observed. Thus, we were unable to determine if MEHP induced-Sertoli cell injury which mediates the paracrine activation of Fas on germ cells requires their localization to lipid raft domains. We cannot rule out the possibility that our analysis was not very sensitive as we had a difficult time in visualizing the bands for Fas protein. On the other hand, a recent report (Henkler *et al.* 2005) demonstrated that membrane associated FasL molecules could induce the clustering of Fas receptors without needing Fas to localize at lipid rafts. Perhaps, the increases in Sertoli cell FasL expression observed in response to MEHP (Richburg *et al.* 1999) is sufficient to trigger Fas activation corresponding to the first wave of germ cell apoptosis in the p53+/+ mice without requiring movement of additional receptors to the germ cell membrane. Furthermore, a more cell specific analysis of membrane Fas levels in the MEHP-sensitive spermatocytes and spermatids, could resolve some of these questions. The *in vivo* data looked at the membrane levels of Fas receptor from a whole testis preparation. Thus, contributions from other cell types within the testis would have obscured changes in Fas expression, specific to the cell types most sensitive to the MEHP toxicity. Taken together, we could not determine whether Fas activation in germ cells in response to MEHP induced Sertoli cell injury, requires the localization of Fas to lipid rafts in a p53-dependent manner.

Chapter 6: Concluding Remarks

DEHP is a commonly used plasticizer found widely dispersed in the natural environment due to its constant leaching out from plastic products. The study of the effects of DEHP and its active toxic metabolite MEHP on the reproductive system arise from the observation of testicular atrophy in rodents and other higher mammals exposed to DEHP or MEHP. Testicular atrophy is preceded by increases in germ cell apoptosis caused by the injury to Sertoli cells by MEHP, thereby compromising their ability to function properly. Various studies have been carried out to understand the mechanism by which both Sertoli cells and germ cells initiate cellular changes leading to enhanced germ cell apoptosis after exposure to MEHP. The Fas-FasL signaling pathway plays a major role in initiating germ cell death after MEHP mediated Sertoli cell injury. Injured Sertoli cells were found to have an increased expression of FasL in response to MEHP, thereby triggering apoptosis of Fas expressing germ cells, specifically spermatocytes and round spermatids. However, the factors responsible for sensitizing germ cells to Fas mediated cell death, were undetermined at the time of commencement of the work presented in this dissertation. We hypothesized that the tumor suppressor p53 may play a role in mobilizing Fas receptors to the cell membranes of germ cells and hence sensitize them to FasL mediated cell death in response to MEHP.

Our *in vivo* results utilizing p53+/+ and p53 -/- mice demonstrated that p53 likely played a transcription-independent role in increasing germ cell Fas membrane levels after

MEHP exposure, similar to the demonstration by Bennett *et al.* (Bennett *et al.* 1998) of Fas receptor trafficking upon activation of p53. However, the p53 dependent increases in Fas membrane levels preceded a second apoptotic peak in response to MEHP in the p53+/+ mice, and not the first early peak. Interestingly, this biphasic apoptotic response seemed to be initiated by the activation of caspase-8, a proximal mediator of the death receptor activated pathways in cells. Hence, in the absence of the predicted Fas membrane increases corresponding to the early peak of apoptosis, we looked for alternate explanations for the apparent activation of the Fas pathway in the p53+/+ mice.

We established that protein levels of c-FLIP (L), an inhibitor of caspase-8 activation and hence the Fas pathway, were enhanced only in the p53 -/- mice as early as an hour after exposure to MEHP, without any apparent differences in their transcription compared to levels in p53+/+ mice. Additionally, recent reports have implicated that the relocation of existing Fas receptors into special membrane microdomains called lipid rafts, is adequate for the activation of the Fas pathway in the absence of increased cell membrane localization of these receptors. We were unable to establish such a relocation of Fas into lipid raft domains to explain the sensitivity of p53+/+ mice to MEHP corresponding to the early apoptotic peak at 1.5 h after exposure. These results therefore implicate a role for p53 in the activation of Fas mediated germ cell apoptosis mediated perhaps by p53-dependent degradation of c-FLIP (L), and also perhaps the increased membrane expression of Fas leading to the second peak of apoptosis, but not Fas activation *via* lipid raft localization.

We used an *in vitro* GC-2 germ cell line model, comprising transformed cells with a temperature sensitive p53 mutation, to clarify some of the questions raised by our *in vivo* studies. By changing the temperature at which these cells were maintained, we created cell systems with active or inactive p53. These GC-2 cells were of spermatocytic origin and of the cell type most sensitive to MEHP toxicity *in vivo*. As expected, they were resistant to the direct addition of MEHP. Instead to mimic FasL on injured Sertoli cells that activate germ cell Fas receptors, we utilized the anti-Fas antibody JO2 to prompt the cell death of GC-2 cells. We demonstrated that the activation of p53 in these cells caused an increased membrane localization of Fas receptors and hence their increased sensitivity to cell death in response to JO2. The apparent inability of JO2 to kill GC-2 cells at the p53 non-permissive temperature was correlated to the reduced degradation of c-FLIP (L), as a knockdown of the c-FLIP (L) protein could sensitize these cells to JO2. In the cells at the p53 permissive temperature on the other hand robust ubiquitinylation and hence degradation of the c-FLIP (L) protein occurred after Fas activation. These studies with GC-2 cells confirmed that the activation of the Fas pathway in germ cells, triggered the degradation of c-FLIP (L). However, the direct role of p53 in mediating this degradation could not be established. Instead, p53 was shown to affect the membrane expression levels of Fas, which in turn were found to affect the amount of c-FLIP (L) protein being retained in the cells.

When the results from the *in vivo* and *in vitro* studies are taken together, they establish that the activation of the Fas pathway in germ cells is regulated by the

modification of the protein levels of c-FLIP (L). The reduction in overall levels of germ cell apoptosis in response to MEHP in the p53 -/- mice indicates the involvement of p53 as a stress sensor in germ cell apoptosis. The precise role played by p53 in this model is, however not very clear. The results from the *in vivo* studies do not reflect a strong role for p53 in the alteration of membrane expression levels of Fas, which was postulated to be the key to germ cell sensitivity to increased FasL expression on Sertoli cells in response to MEHP mediated injury. On the other hand, a 10-fold increase in membrane expression of Fas in GC-2 cells is based on the activation of p53, which distinguishes the sensitivity of the p53 permissive and non-permissive cells to JO2 mediated apoptosis.

The Fas membrane expression results from the *in vivo* studies are based on the analysis of membrane levels of Fas in total testicular lysates. The contribution from several different cell types to the analysis may have obscured any significant changes experienced by specific germ cell subtypes such as spermatocytes and round spermatids that are specifically sensitive to the MEHP effects. Furthermore, caspase-8 activation that precedes the two apoptotic peaks in our model is p53-dependent; this implies that death receptor activation in response to MEHP is also p53-dependent. Perhaps by analyzing the membrane expression levels of Fas in enriched germ cell populations, we may resolve the apparent discrepancy in caspase-8 activation and lack of Fas localization to the membrane in p53+/+ mice. Additionally, no analyses of membrane FasL expression were carried out in the p53+/+ and p53 -/- mice. Biphasic changes in FasL expression in response to Sertoli cell injury could account for the activation of caspase-8 in the manner

described in our results. Thus the role of p53 in the membrane localization of both Fas and FasL remains to be clarified.

The degradation of c-FLIP (L) protein upon activation of the Fas receptors in germ cells was established from experiments using GC-2 cells. However, the involvement of p53 in mediating this degradation directly could not be proven. The absence of any significant differences in membrane Fas levels between testicular cells of p53+/+ and p53 -/- mice, led us to initially believe that the retention of the c-FLIP (L) protein in response to MEHP in the p53 -/- mice indicated a role for p53 in the degradation of c-FLIP. p53 transcribes E3 ligases including Mdm2 and PirH2 (Leng *et al.* 2003; Michael and Oren 2003). E3 ligases are proteins that attach ubiquitins to specific proteins targeting them for degradation by the proteasome. It was therefore speculated that p53 could potentially aid in the transcription of the E3 ligase specific for c-FLIP (L). The *in vitro* results however implicate the amount of membrane Fas expression to be the factor limiting c-FLIP (L) degradation; GC-2 cells at the non-permissive p53 temperature express much less Fas at the cell membrane and may not recruit enough c-FLIP molecules to the DISC to ensure their degradation. To resolve the role of p53 status on c-FLIP ubiquitinylation and degradation, future studies should involve the utilization of two cell lines with comparable membrane Fas expression. This will enable us to rule out the contribution from Fas related effects.

Another potential line of research would be to identify the E3 ligase specific for the c-FLIP (L) protein. Although several researchers have identified c-FLIP as a potential target for degradation/removal in cancer cells, in order to sensitize them to death from TRAIL or anti-Fas treatment (Baumler *et al.* 2003; Rippo *et al.* 2004; Zhang *et al.* 2004; Watanabe *et al.* 2005), no research has been done to determine how c-FLIP is degraded physiologically to mediate death receptor activation. Our data suggests that c-FLIP is actively degraded when it is recruited to the DISC, i.e. after Fas activation. Determining how this degradation is brought about would be an interesting question to answer. Recently, it was shown that certain adaptor proteins that are involved in mediating TNFR and other death receptor signaling, called TNF-R-associated factors (TRAFs), especially TRAF2 and TRAF6 are E3 ubiquitin ligases (Shi and Kehrl 2003; Sun *et al.* 2004). Other reports have indicated that the activation of Fas in Jurkat T cells and Raji B cells, leading to the formation of DISC and recruitment of c-FLIP (L), promotes NFκB and ERK signaling through the recruitment of TRAF1 and 2, and RIP and Raf-1 to c-FLIP (Kataoka *et al.* 2000). Additionally, when c-FLIP (L) is processed by caspase-8 at the DISC, the N-terminal fragment of c-FLIP (p43) more readily interacts with TRAF2 to mediate NFκB signaling (Kataoka and Tschopp 2004). Thus, the potential exists for TRAF2 to be the specific E3 ligase for c-FLIP, as TRAF6 does not bind to c-FLIP.

Having identified what may be the potential mechanism by which MEHP toxicity ensures cell death of primary spermatocytes and round spermatids, future studies also should be aimed at addressing the reason for the sensitivity of these specific cell types.

The answer may be related to the cell-type specific expression of c-FLIP (L) and its modification in response to Fas activation. The continuous expression of the p53 protein in spermatocytes and perhaps its ability to influence both the membrane levels of Fas and the stability of c-FLIP may be the key to this mechanism, as the half life of p53 in most other cells is typically only 30 min (Schwartz *et al.* 1993). Spermatocytes may be more sensitive to the paracrine activation of Fas by Sertoli cell FasL, due to the ability of the constitutively expressed p53 in the spermatocytes to be activated after the withdrawal of physical or hormonal support by Sertoli cells after toxicant-induced injury. To summarize our results, a schematic is provided in **Fig. 6.1** as a representation of our current model predicting the mechanism by which the Fas pathway may initiate testicular germ cell apoptosis in response to MEHP.

Even after several decades of research the exact mechanism by which MEHP mediates Sertoli cell injury remains elusive. Several recent studies have only added to the confusion with the suggestion of several additional mechanisms by which MEHP may affect Sertoli cell functions. For instance, in addition to the physical changes such as formation of vacuoles, and collapse of microtubule and vimentin filaments in Sertoli cells, recent data suggest that molecules such as flamingo 1 involved in cell adhesion between germ cells and Sertoli cells are also affected by exposure to MEHP (Richburg *et al.* 2002). Additionally, exposure to DEHP was found to cause a decrease in testicular levels of antioxidants such as free thiols, GSH and ascorbic acid, and also led to the generation of reactive oxygen species (ROS) in the testis (Kasahara *et al.* 2002).

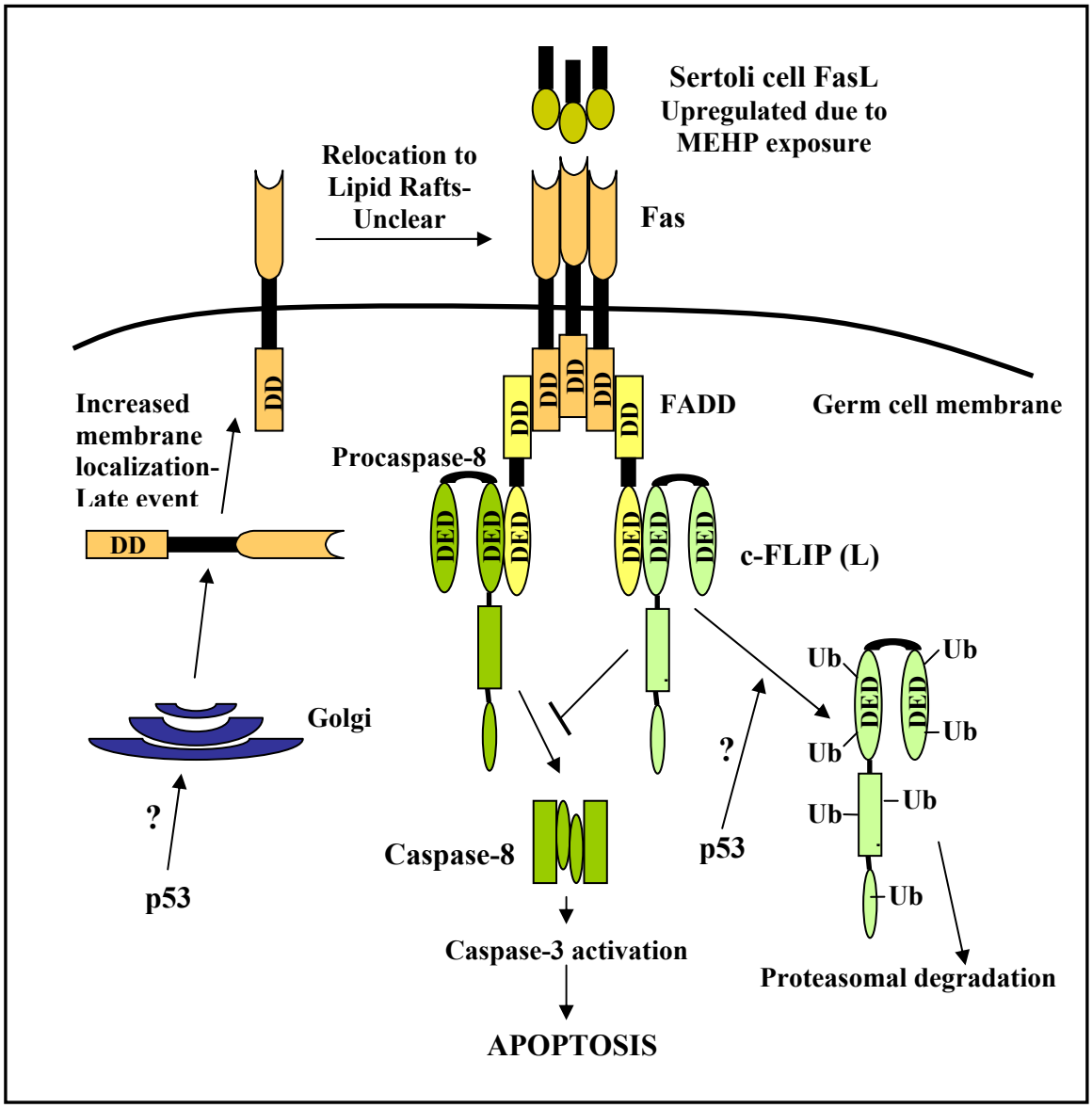

Fig. 6.1. Representation of the proposed model for Fas mediated germ cell apoptosis after testicular exposure to MEHP. Our results from both our *in vivo* and *in vitro* studies when taken together, indicate that p53 in germ cells in response to MEHP-induced Sertoli cell injury, promotes Fas activated germ cell apoptosis by instigating the degradation of c-FLIP (L) protein *via* its ubiquitinylation.

The generation of ROS in Sertoli cells could potentially activate the stress activated kinase JNK or c-Jun N terminal kinase (Chen *et al.* 2001). Active JNK phosphorylates the c-Jun protein which is typically a component of the activator protein-1 or AP-1 transcription factor. This transcription factor is one of the transcriptional regulators of FasL expression (Kavurma and Khachigian 2003). Thus, ROS generation in the testis in response to exposure to MEHP may activate JNK and cause the increased transcription of FasL, which is an observed phenomenon in this toxicity model. Additionally, due to the effects of MEHP in causing peroxisomal proliferation in the liver, it has also been investigated for its ability to stimulate the activation of peroxisome proliferator-activated receptors or PPAR's in the testis. MEHP increased the nuclear localization of PPAR α and γ receptors and prevented the localization of the retinoic acid receptor α (RARα) also to the nuclei of Sertoli cells (Bhattacharya *et al.* 2005). The relevance of these changes has yet to be elucidated. MEHP induced PPAR activation leads to the suppression of aromatase transcription in granulosa cells of the ovary, and hence reduced estradiol production (Lovekamp-Swan and Davis 2003). On the other hand, RARα deficient mice develop testicular degeneration and sterility and the absence of nuclear localization of this protein prevents its activation (Lufkin *et al.* 1993). The activation or non-activation of the three nuclear receptors adds to the growing list of potential ways by which MEHP affects Sertoli cells, which only makes it harder to determine the exact mechanism of action of this toxicant.

Ultimately the ability of MEHP to affect a number of Sertoli cell functions, perhaps by a single mechanism or by several different ones, is responsible for the disruption of the microenvironment in the adluminal compartment of seminiferous tubules. Human exposure to phthalates from the environment may not cause testicular toxicity on the scale demonstrated by the rodent studies. However, the utility of these studies lies in the appreciation of the importance of Sertoli cells as support cells, and hence their contribution toward the proper functioning of spermatogenesis which eventually affects the fertility of the organism. The ability of germ cells to respond to the diminished capacity of Sertoli cells remains a very critical homeostatic mechanism within the testis. One must remember that not all chemical or physical agents that the male human populace is exposed to will cause disruption of Sertoli cell functions. Nevertheless, the identification of the cellular and molecular factors that affect germ cell death in response to Sertoli cell injuries are equally important to the understanding of how various environmental and perhaps even chemotherapeutic agents eventually affect male fertility.

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