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Investigating the Role of p53 in the Germ Cell Apoptotic Pathway

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Chapter 1. Background

DNA and the damage that can occur to it

All living organisms contain DNA which is responsible for the genetic code that dictates the development and functioning of living organisms. DNA is a polymer that is composed of an alternating sugar (deoxyribose) and phosphate backbone linked by ester bonds. Attached to each sugar group is one of four possible nucleoside bases: adenine (A), thymine (T), cytosine (C) or guanine (G). The bases can be subdivided into two groups: purines (adenine and thymine) and pyrimidines (cytosine and guanine). A purine base is always bound to its corresponding pyrimidine base through hydrogen bonds (A binds to T and C binds to G). The base sequences can code for proteins that are responsible for various structures or processes within the cell [\(38\)](#).

When a cell is exposed to radiation, stress or toxic chemicals lesions in the DNA can occur. These lesions include: adducts, thymine dimers, single and double strand breaks (SSB and DSB respectively). DNA adducts occur when DNA is covalently bound to a foreign chemical. Since the DNA forms a new bond to the foreign chemical, old bonds are broken, which lead to improper nucleotide binding. Thymine dimers are caused by ultra violet (UV) light exposure. The UV light is absorbed by thymine and can lead to a double bond

between two adjacent thymine nucleotides. Single and double strand breaks are when one or both DNA strands have a complete break in the strand. Having damaged DNA is extremely dangerous for the cell and often interferes with the cell cycle ([17](#), [22](#), [40](#)).

The cell cycle is divided into four stages: G1, S, G2 and M; as shown in figure 1. G1 (gap1) is the first growth phase where the cell absorbs nutrients and will grow in size. Eventually the cell will grow enough and enter S phase. During S (synthesis) phase the cell replicates its DNA so that it has two copies of each chromosome. Following S phase there is a G2 (gap 2) phase where the cell continues to grow. Eventually the cell will be large enough to enter M phase. During M phase the cell undergoes mitosis and divides into two cells (38).

The cell cycle is regulated by proteins called cyclins and cyclin-dependent kinases (CDK). Cyclins are the regulatory subunit of the hetero-dimeric protein kinase that regulates the cell cycle. CDK's are the catalytic subunit, but they cannot function without their proper cyclin. Once a CDK binds with its corresponding cyclin the complex becomes activated and can de/phosphorylate other proteins, which in turn can cause the cell to enter the next phase of the cell cycle (12, 23).

Figure 1. Cell growth and division cycle. All normal cells follow the same cycle of: growth, replication of DNA, more growth then division.

[http://bhs.smusd.org/bhsnew/academicprog/science/vaughn/Student%20Projects/Paul%20&%20Marcus/Cell_Replication.html]

Adducts in the strand can interfere with DNA replication or transcription, which can result in several consequences. If an error occurs in the replication process it can result in improper replication, leading to mutated DNA or the inhibition of replication. Without the replication of its DNA, the cell would not be able to divide properly. If the mutated DNA was to be used for transcription then it could result in the production of an improper sequence of mRNA. If the

improper sequence of RNA is transcribed then the amino acid sequences will be altered during translation. This can result in absent or malfunctioning proteins, many of which are crucial to cell survival. Most forms of DNA damage would ultimately result in the death of the cell or under severe damage, the whole organism (40).

DNA repair pathways

Damage to DNA can initiate a DNA repair pathway. Before the DNA can be repaired the cell cycle is first arrested. Once the cell cycle has been stopped, several proteins will then begin to repair the damaged DNA. Once the DNA has been repaired, the cell cycle will then continue as normal. These are complex pathways and they involve many different proteins and steps.

For example, in the case of a DNA single or double strand break, various proteins can recognize the break and initiate different DNA damage response pathways. The proteins Ataxia telangiectasia mutated (ATM) or ataxia telangiectasia Rad3 (ATR) recognize the lesion and can phosphorylate the histone variant H2AX. The phosphorylated H2AX is called γ -H2AX. γ -H2AX then phosphorylates ATM, which in turn can activate several other downstream proteins such as Breast Cancer 1 (BRCA1), p53 binding pair 1

(53BP1) and check point kinases 1 and 2 (CHK1/2); as shown in figure 2.

These proteins are involved with initiating the cellular response to DNA DSBs, but each plays a different role (2, 11, 19, 25).

CHK proteins can phosphorylate the cell division cycle 2 (cdc2) proteins. Cdc2 is a CDK, and it becomes inhibited when it's phosphorylated by the CHK protein. When cdc2 is inhibited the cell can not proceed to the next stage, leading to cell cycle arrest.

The 53BP1 is responsible for phosphorylating and activating p53 which can also play a role in cell cycle arrest. Shortly after p53 becomes phosphorylated it leads to an up-regulation of p21, which leads to G1-S phase arrest. After the cell cycle has been arrested, BRCA1 can participate in the repair of the damaged DNA, but its mechanism is not completely understood. Akt1, a pro-survival protein, can also be linked to repair pathways by helping initiate repair which ultimately leads to cell survival (4, 7).

Figure 2. Proposed double strand break response pathway. A double strand break is detected and can lead to the activation of several different proteins. Through a series of protein [phosphorylations](http://www.nature.com/ncb/journal/v4/n12/fig_tab/ncb1202-e277_F1.html) the cell cycle arrests and DNA repair begins.

[http://www.nature.com/ncb/journal/v4/n12/fig_tab/ncb1202-e277_F1.html]

Akt1 (also known as Protein Kinase B) is a kinase that promotes cellular growth and proliferation. When a growth factor binds to its receptor it produces PI3K (phosphoinositide 3-kinase) which recruits Akt1 to the cell membrane where it is phosphorylated by PDK-1 and PDK-2 (phosphoinositide-dependent kinase). Once Akt-1 has been phosphorylated it will phosphorylate several proteins which lead to gene transcription. This will lead to the activation of several genes associated with cell growth (mTOR, survivin, NF-Kappa B) and inhibition of genes associated with apoptosis (BAD, BAX) (27). Many tumors can arise from over expression of Akt1, and even cause cancers [\(10, 36\)](#). One important gene that Akt1 can activate is MDM2. This protein will bind to [p53](#), inhibit it, and eventually lead to its degradation by ubiquitination. This pathway is depicted in figure 3. This is one of the major reasons some tumors can grow out of control, as without [p53](#) there is little that can stop the cells from growing (32). By blocking the Akt1 pathway, it may enable the tumorigenic cells to become sensitized to [p53](#) and ultimately undergo apoptosis (17, 18, 29, 30).

Figure 3. Akt1 pathways. Growth hormone binds to its receptor and leads to the activation of PI3K. The activated PI3K then activates PI3-4-5 P, which then phosphorylates Akt1. This phosphorylation of Akt1 can cause several other proteins to be activated or inhibited. Mdm2 is an important protein because its phosphorylation can lead to the destruction of p53. p53 can activate phosphatase and tensin homolog (PTEN), which inhibits the activation of PI3-4-5 P. As a result cell survival is maintained and apoptosis is inhibited.

[http://p53.free.fr/p53_info/p53_cancer.html]

Apoptotic response pathways

Sometimes a cell will not be able to repair DNA damage. This will result in a different response pathway known as the apoptotic pathway. Similar to the DNA repair response, the apoptotic response can involve several different pathways involving many different proteins. p53 is one of, if not the most important tumor suppressor protein and is often responsible for initiating apoptosis. There are two main apoptotic pathways, the intrinsic and extrinsic pathway and p53 is involved in both (35).

In the intrinsic pathway p53 can play a role by activating pro-apoptotic genes such as BCL-2 associated X protein (BAX), p53 unregulated modulator of apoptosis (PUMA) and FAS receptor. The expression of PUMA is regulated by p53 and its increased levels lead to apoptosis. PUMA affects the mitochondria of the cell by leading to the expression of BAX. BAX belongs to the BCL-2 (B cell lymphoma 2) family of pro- or anti-apoptotic proteins that regulate the outer

mitochondrial membrane. Although these pathways are not completely understood, it is believed that certain signals can activate or inhibit specific members of the BCL-2 family causing a change in the mitochondrial membrane permeability (45).

As the mitochondrial membrane becomes more permeable, it will lead to the release of cytochrome C from the mitochondria. Cytochrome C is usually involved in oxidative phosphorylation, but it can initiate apoptosis when it is released from the mitochondria into the cytoplasm. Outside of the mitochondria, the cytochrome C binds with apoptotic protease activating factor 1 (Apaf-1) and the nucleotide dATP forming a ternary protein called an apoptosome. The apoptosome can then bind to and cleave caspase-9, which can activate other caspases leading to apoptosis (1). This pathway is depicted in figure 4.

Figure 4. Proposed model of some possible intrinsic apoptotic pathways. As a result of DNA damage, oncogene expression or stress an intrinsic apoptotic pathway may be initiated. p53 becomes active which can result in apoptosis through pathways involving other proteins and the mitochondria.
[\[http://www.biochemsoctrans.org/bst/029/0684/bst0290684f01.htm\]](http://www.biochemsoctrans.org/bst/029/0684/bst0290684f01.htm)

The extrinsic pathway of apoptosis can also be initiated by p53 by up-regulating FAS-L transcription/translation. When FAS ligand (FAS-L) binds to the FAS receptor on the plasma

membrane it induces trimerization of Fas and the recruitment of Fas-associated protein with death domain (FADD) via interactions between the death domains of Fas and FADD. The C terminal death domain is the part of the protein that interacts with the FAS receptor, and the N terminal death effector domain (DED) recruits and cleaves procaspase 8. Procaspase 8 binds to the DED of FADD which leads to the activation of caspase 8. Activated caspase 8 can lead to apoptosis in one of two ways.

The first way is by directly cleaving pro-caspase 3, which produces caspase 3. Caspase 3 then cleaves inhibitor of Caspase Activated Deoxyribonuclease (ICAD), which leads to the release of Caspase Activated Deoxyribonuclease (CAD). CAD then enters the nucleus and cleaves the DNA, leading to apoptosis. The second way caspase 8 can initiate apoptosis is by cleaving the protein BH3 interacting domain death agonist [BID], leading to its truncated version tBID. tBID then binds to mitochondrial receptors which lead to the release of cytochrome C. Outside of the mitochondria cytochrome c binds with Apaf-1 and dATP forming an apoptosome. The apoptosome can then bind to and cleave caspase-9, which once activated can activate other caspases leading to apoptosis. These pathways are shown in figure 5. This second pathway incorporates some of the intrinsic

pathway, which shows that there are many different apoptotic pathways and some of them can crosstalk (13).

Figure 5. Fas apoptotic pathways. When Fas Ligand binds to Fas it recruits FADD, which then cleaves pro-Caspase-8 forming caspase-8. Caspase-8 can either directly cleave pro-caspase-3 or it can indirectly by incorporating the mitochondria. Caspase-3 can then initiate apoptosis.

[<http://www.biochemsoctrans.org/bst/029/0684/bst0290684f01.htm>]

One thing in common with most of these responses is the involvement of the p53 protein. p53 can be involved in both the repair and apoptotic pathway and often is responsible for the transition from repair to cell death. Although the mechanism is uncertain, it is believed that there is some sort of p53 threshold that initiates this transition. When a certain amount of p53 accumulates over time the transition from DNA repair to the apoptotic pathway will be initiated. This would explain why apoptosis is initiated after repair fails. If repair is successful then p53 will be degraded, but if repair fails then p53 will continue to accumulate and apoptosis will eventually be initiated (20,26).

Although p53 is the main initiator of the apoptotic pathway, the pathway can still be initiated in its absence. p53 deficient cells can still undergo apoptosis. This indicates that there are p53-independent apoptotic pathways. One such pathway is the Jun N-terminal Kinase (JNK) p38 pathway. After exposure to UV light or cisplatin, DNA adducts begin to accumulate,

which lead to the activation of p38 and JNK. As p38 and JNK levels increase they lead to the up-regulation of activator protein-1 (AP-1). AP-1 causes an up-regulation of FAS-L which in turn initiates apoptosis. This pathway is shown in figure 6. Several experiments have been performed that help support this theory by showing that: 1) cells that have been treated with inhibitors of JNK and/or p38 inhibit cisplatin induced apoptosis and 2) cells that have been treated with FAS inhibitors exhibit decreased cisplatin induced apoptosis (24, 27, 32).

Figure 6. JNK/p38 Apoptotic Pathway. UV light or cisplatin exposure causes the formation of DNA adducts. These adducts lead to a rise in JNK and p38, which in turn lead to an up-regulation of AP-1. AP-1 leads to the transcription of FAS-L, which will lead to apoptosis.

Male reproductive tract and gametogenesis

The male reproductive tract is comprised of four main sections: the testes are responsible for producing sperm, the epididymis is where spermatozoa undergo maturation, the penis

which is where urine and semen are released, and the ductus deferens which is the tube in which semen is transported to the penis. In addition there are several accessory glands located along the male reproductive tract. The prostate gland and the seminal vesicles store and produce a clear, slightly alkaline fluid that combines with sperm to form semen. The alkalinity aids in neutralizing the acidic fluids of the vaginal tract, therefore increasing the odds of the sperm surviving. The prostate also helps regulate the flow of urine from the bladder to the penis. The mouse also has a preputial gland that is responsible for releasing pheromones, which are chemicals that are used to attract a female mouse. These tissues are shown in figure 7.

Figure 7. Over view of the male mouse reproductive tract.

[<http://www.informatics.jax.org/greenbook/figures/figure13-45.shtml>]

The sperm cells are produced in the testes within the coiled seminiferous tubules. The seminiferous tubules are lined with Sertoli cells that facilitate the development of sperm cells. The sperm first begin as mitotic germinal cells called spermatogonia that are located near the basal lamina. Once the germinal cell has divided several times, it will begin to migrate toward the

lumen and will become a primary spermatocyte. In order to continue toward the lumen the spermatogonia have to be able to pass through tight junctions between Sertoli cells. The Sertoli cells are very tightly joined and very few things can pass between them. Primary spermatocytes will replicate DNA, and will begin to undergo meiosis I. After meiosis I the cells will be haploid secondary spermatocytes. These cells will undergo meiosis II and will result in four haploid cells called spermatids. As the spermatids continue to migrate toward the lumen of the seminiferous tubule they begin to grow a tail, shed excess cytoplasm and membrane, and begin to develop an acrosome (a part of the sperm head that contains enzymes used to break down the eggs membrane). Eventually the spermatids will become spermatozoa and will be released into the lumen of the seminiferous tubules. The spermatozoa will travel down the lumen until they reach the epididymis where they will fully mature into spermatids. This development is shown in figure 8. When the male ejaculates, the sperm will travel from the epididymus down the ductus deferens and out the penis.

Sperm production is tightly monitored by Sertoli cells, which provide structural and secretory support. Sperm cells develop between the Sertoli cells but they must first pass through the tightly regulated blood-testis barrier. The barrier consists of the tight junction between two Sertoli cells and is responsible for keeping the developing germ cells away from the blood stream. Since meiosis produces haploid cells, the germ cells are genetically different from the rest of the somatic cells and the body's immune system may recognize them as foreign cells and could attack them.

In addition to structurally supporting the germ cells, the Sertoli cells provide the developing germ cells with the chemicals and nutrients they need in order properly develop. Some of these include inhibins & activins that inhibit or lead to the production of follicle stimulating hormones (FSH) respectively. FSH is produced in the anterior pituitary gland and is controlled by the release of gonadotropin releasing hormone (GnRH) from the hypothalamus. FSH stimulates the Sertoli cells to produce androgen-binding protein (ABP). ABP is a glycoprotein that binds to androgen

hormones (AH) such as testosterone (T) or dihydrotestosterone (DHT). The binding of these proteins causes them to become less lipophilic, resulting in a higher concentration of T and DHT in the testes. T and DHT are required for sperm production, so having a high concentration of them within the tubules is very important.

Although the Sertoli cells are responsible for supplying the germ cells with T and DHT, these hormones are not produced within the Sertoli cells. Androgen hormones (AH) are produced in the Leydig cells that surround the seminiferous tubules in the interstitial tissue. Their production is stimulated by the release of Luteinizing Hormone (LH) from the anterior pituitary gland. LH increases the activity of the cholesterol desmolase, which converts cholesterol to pregnenolone, which will eventually be converted to T after a series of reactions. The release of FSH also affects the Leydig cells by upregulating LH receptors (LHR), therefore leading to increased androgen production.

Sperm production is a tightly regulated process which involves several negative feedback loops between the hypothalamus, pituitary gland, Leydig and Sertoli cells. As androgen levels

decrease it the hypothalamus produces additional GnRH, stimulating the pituitary gland to produce more LH and FSH. The increased levels of LH and FSH lead to increased production of androgen hormones that aid in spermatogenesis. If any of the androgen hormone [\(AH\)](#) levels become too high, the hypothalamus will recognize it, it will stop producing GnRH, which will lead to lower AH levels (9, 14).

Figure 8. Testis and Spermatogenesis. A cross section of a testicle shows many coiled seminiferous tubules. Inside the tubules sperm are being produced. Sperm start as a

diploid, germinal cell that eventually mature into haploid spermatozoa.

[<http://faculty.sunydutchess.edu/Scala/Bio102/PDF/Spermatogenesis.jpg>]

Summary and Hypothesis

p53 is a major participant of cell cycle arrest and apoptotic [signaling](#) pathways. Akt1 plays the opposite role, as it initiates growth and proliferation and can indirectly inhibit p53.

Although p53 and Akt1 play opposite roles, both are important in the response to DNA lesions. If either protein malfunctions it can lead to many problems, one being cancer. If Akt1 is mutated it can lead to improper DNA repair or uncontrolled proliferation, and [p53](#) that is impaired can not assist in initiating DNA repair or apoptosis.

Many treatments of testicular cancer can cause the patient permanent testicular injury and in some cases sterility. By manipulating [p53](#) or Akt1 it may be possible to initiate apoptosis and cause the desired cancerous cells to die. If [p53](#) functions in germ cells the same way it does in somatic cells,

in theory it may be possible to treat testicular cancer by causing the tumor cells to stop growing and undergo apoptosis. [By comparing how relative p53 and apoptotic levels change over time it will help to better understand the importance of p53 in germ cell apoptotic pathways.](#) My hypothesis is that p53 levels and consequently apoptotic levels will increase (especially in the p53 wild type group compared to the knockout one) after induced DNA damage. This research will hopefully aid in the understanding of the p53 protein, and possibly help in future treatments of cancer (6, 17, 29).

Chapter 2. Effect of Radiation Exposure on p53 in Mouse Germ Cells

Ionizing radiation is composed of energized waves of particles, released from an atom or nucleus that can ionize an atom through atomic interactions. Some of these particles can include photons (γ radiation), electrons (β radiation), and alpha particles (α radiation). α and β radiation are not very penetrating and can be easily deflected. Since α and β radiation can barely penetrate, they will be more dangerous if they are trapped inside the body. γ radiation is less ionizing but

requires more extensive shielding since it is more penetrating. These particles are only considered to be ionizing if they have enough energy and if they actually interact with an atom [\(42\)](#).

These interactions can lead to the gain or loss of an electron, therefore altering the atom to have a different charge. If the atom is to gain an electron, the electron must have enough energy to pass the highest shell of the atom. If it does pass the barrier the electron will eventually emit the excess energy and will remain at its lowest energy state. If the electron does not pass the barrier it will be reflected by the electrostatic force. In order for the atom to lose an electron, the electron must have enough energy to pass over or through the highest potential orbital. If an electron becomes excited but does not have enough energy to pass the potential barrier it will temporarily remain at the closest orbital. The electron will eventually radiate out the absorbed energy, and will return to its original state.

When DNA is irradiated its atoms can undergo a change in charge, which will in turn cause a change in the molecule's interactions. These changes can cause the DNA molecules to bind improperly, resulting in a DNA lesion or strand break. The damaged DNA will lead to one of three fates for the cell. The first is the damage is recognized and the cell will be able to repair it. The second fate is that the damage is too extensive and repair will fail; as a result the cell will undergo apoptosis. The third fate is that the lesion goes unrecognized and will remain in

the DNA resulting in a mutation. This is a typical response if p53 is mutated or absent. One mutation may not be harmful but several mutations can accumulate over time. Eventually enough mutations can occur that the cell becomes cancerous and can lead to a tumor. For the purposes of these experiments radiation will be used to induce apoptosis in testicular cells (15, 16, 17, 42).

Materials and Methods

Animals- p53 deficient and wild type mice were obtained by breeding p53 heterozygous mice (strain B6.129S2-Trp53^{tm1Tyj}) purchased initially from the Jackson Laboratory (Bar Harbor, ME). Mice were given water and standard lab chow *ad libitum*. The temperature of the animal room was kept at constant room temperature (23.3 ± 2 °C and 30-70% humidity) and exposed to 12 hour alternating light and dark cycles. All procedures were performed in accordance with Brown University's institutional animal and use committee in compliance with the guidelines established by the NIH.

Radiation Exposure- Adult 8-week-old p53 wild-type (+/+), heterozygous (+/-) and p53-deficient (-/-) mice were used for experiments. Mice were given half-body irradiation at a single dose of 5 Gy with a delivery rate of 6.84 Gy/min using a cesium source (J.L Sheperd, Mark I Irradiator Glendale, CA). Mice were restrained in a polystyrene chamber, and the upper body was shielded using lead. Control and irradiated mice were sacrificed by carbon dioxide asphyxiation at 1, 3, 6, 12 and 24 hours post irradiation, and their testes were extracted.

Western Blotting - Testes from control, 1, 3, 6, 12, and 24 hour post radiation exposed mice were detunicated, weighed, and homogenized in three volumes of ice-cold RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (P2714, Sigma) by 10 strokes in a Dounce homogenizer. Samples were incubated on ice for 30 minutes. The homogenate was then centrifuged at 13,500 X g for 10 minutes at 4°C. For western blotting, 10 µL of testis supernatant were separated by molecular weight by 10% SDS page, and transferred to Immobilon-P membrane. Blocking solution (20mM Tris pH 4.4, 137 mM NaCl, 10% nonfat dry milk) was added to the membrane for 60 minutes. Primary antibodies to serine 15 phosphorylated p53 were diluted in blocking solution and added to the membranes

at 4°C overnight. After washing with PBS 0.1% Tween-20, horseradish peroxidase-coupled secondary anti-body diluted in blocking solution were incubated with the membranes for 1 hour at room temperature. Membranes were washed three times with PBS-Tween (0.1%) and secondary antibody was detected by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech).

DNA Extraction for genotyping- At 21 days old, the mice were held between thumb and index finger and about ¼ cm of the mouse's tail was snipped off. The DNA was then extracted using a "QIAGEN DNeasy kit." The tail snip was placed in a 1.5 mL microfuge tube with 180 µL ATL (tissue lysis) buffer and 20 µL of proteinase K. The tube was then placed in a temperature bath overnight in order to dissolve the tail. The following day 400 µL of AL-ethanol buffer was added and the microfuge tube was vortexed. The mixture was then pipetted into the DNeasy mini spin column that is placed in a 2mL collection tube and centrifuged at 8000 rpm for 1 minute. The flow-through and collection tube were discarded and the mini spin column was placed in a new collection tube. 500 µL of buffer AW1 (wash buffer 1) was then added and the tubes were centrifuged again at 8000 rpm for 1 minute. The flow-through and collection tube were discarded and the mini spin column was again placed in a new collection tube. 500 µL of buffer AW2

(wash buffer 2) was then added and the tubes were centrifuged at 14,000 rpm for 3 minutes. The flow-through and collection tube were then discarded and the mini spin column was placed in a new 1.5 mL microfuge tube. 200 μ L of DEPC treated water was then added to the tube and centrifuged at 8000 rpm for 1 minute. The easy spin column was discarded and the extracted DNA was diluted in 200 μ L of DEPC treated water in a microfuge tube.

Polymerase Chain Reaction (PCR) - DNA extracted from the previous protocol was added to several reagents to produce the PCR cocktail. These reagents include: DEPC treated water, primers for the p53 gene, magnesium chloride, nucleotide bases, PCR buffer solution, and **Taq** polymerase. Once the mix was prepared, 20 μ L of it was added to 5 μ L of DNA, and that solution was pipetted in a PCR tube. The tube was then placed in the PCR machine and went through an initial cycle of 94°C for 5 minutes. The machine then goes through a second cycle thirty five times which consists of three steps: 94°C for 30 seconds, 61°C for 45 seconds and 72°C for 45 seconds. The PCR then went through a third cycle of 72°C for 2 minutes and then the final hold of 4°C.

Apoptosis Staining (Apoptag) - p53 +/+, +/- and -/- mice were sacrificed at 0, 1, 3, 6, 12 and 24 hours. Their testes were then extracted, embedded in freezing

medium and then flash frozen in liquid nitrogen. The testes were then sliced at 10 μ m using a cryostat into thin sections and mounted to slides. 10% neutral buffered formalin followed by an ethanol/acetic acid mixture, and finally a 3% hydrogen peroxide solution (with a PBS rinse between each step) was used to help fix the section to the slide. After the hydrogen peroxide was washed off equilibrium buffer was quickly applied to each section, and then blotted dry. Terminal Deoxynucleotidyl Transferase (TdT) enzyme was then mixed with reaction buffer at a 1:2 ratio and a couple drops were applied to each section. TdT catalyzes the addition of nucleotides that are secondarily labeled with a marker onto the fragmented DNA associated with apoptosis. Cover slips were then applied to the sections, and then they were incubated at 37°C in a humidification chamber for an hour. The slides were then removed from the chamber, rinsed with PBS and then stop buffer was added to halt the TdT enzyme reaction. The stop buffer is rinsed off and anti-digoxigenin-peroxidase was applied to the sections, which were then incubated at 37°C in a humidification chamber for 40 minutes. The slides were then removed, rinsed off and 3,3'-diaminobenzidide (DAB) was applied in order to add the brown stain for apoptosis. After 5 minutes the DAB was washed off and the cells are counterstained with methyl green for 10 minutes. The methyl green was then rinsed off and the slides are then dipped in isopropanol for 2 minutes. The slides were then dipped in Citrosolve clearing agent for 2 minutes and then blotted dry.

Cover slips are applied to the sections which are then examined under a light microscope.

RESULTS

Experiment 2.1: Body/Testes weights of irradiated mice

Irradiated mice were sacrificed and their body weight (BW) and combined testes weight (TW) were measured in grams. The average and standard error of the mean for the weights were calculated in each group. The body and testes weights did not vary much between groups and there was no statistical difference between the groups. Data is shown in table 1.

	WT	HET	KO
BW (g)	22.63±0.36	23.16±0.4	23.47±0.4
TW (g)	0.167±0.004	0.179±0.004	0.183±0.005

Table 1. The body and combined testes weights $\pm$ SEM of mice following radiation exposure. BW: body weight, TW: combined testes weight, WT: wild type, HET: heterozygous, KO: knockout. Sample size varied from 19 mice for the WT and KO and 26 mice in the HET group. Statistical analysis was performed using One way ANOVA and Dunnett's test.

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Experiment 2.2: p53 Levels Over Time in Irradiated Mice

Mouse testes were homogenized and their protein levels were measured by a protein assay. The samples were then run through a SDS PAGE gel and using a western blot, p53 levels were compared to β -Actin as a standard. Based on the western results p53 levels in wild type mice were shown to increase after radiation exposure. p53 increased around 3 hours and especially at the 6 hour time point, but by 12 hours levels had considerably decreased. By 24 hours the p53 levels returned to normal. The concentration units were determined by comparing the

protein levels at the time point to the initial amount (For example the 2.6 concentration at time point 3hrs means that p53 levels were 2.6 times as much as the amount at the initial 0 hr time point). This data is shown in figure 9.

B

Figure 9. Western gel for p53 levels at varying time intervals post radiation.
A. Picture of a western blot for p53 and β -Actin concentrations at several hours after radiation exposure. **B.** p53 levels increase at 3 hours and 6 hours. Levels then decrease between 12-24 hours, until p53 levels return to normal. Same size varied from 1-2 mice as depicted in the above blot.

Experiment 2.3. Apoptotic levels in irradiated mice testes

Mouse testes were frozen in [tissue freezing](#) medium and sections were sliced [at 10 \$\mu\$ m](#) at minus [15 degrees](#) Celsius onto slides using a [Jung 5000 brand](#) cryostat (***). The slides were stained for apoptosis using an apoptag (TUNEL) staining kit. The incidence of apoptosis in radiation treated [mouse testis](#) was determined by

counting the number of seminiferous tubules in cross sections of testis which exhibited more than 4 cells [that have undergone apoptosis](#). For each genotype/time point between 6 and 10 tubules were observed. Apoptag results showed increased apoptosis levels in all genotypes after radiation exposure. Apoptotic levels didn't increase much until about a 2 fold change around the 6 hour time points, and then increased several fold at 12 hours. By 24 hours apoptotic levels began to decrease but were still relatively high. Generally the WT mice had the highest amount of apoptosis, followed by the HET group, and the KO mice had the least amount of apoptosis.

Figure 10. Apoptotic levels at varying time intervals post radiation.
A. Pictures of tubule cross sections at 12 hours after radiation exposure for p53 wild type and knockout mice. **B.** Representative examples (from a sample size of 3 mice for each bar) of TUNEL stained testis sections from p53 +/+ and p53 -/- are shown above. The percentage of seminiferous tubules in each treatment group which exhibited more than 4 TUNEL positive cells is plotted against the Time after treatment. Each bar represents the average of 6-10 testis sections from 2-3 mice. Statistical analysis was performed using One way ANOVA and Dunnett's test. *p<0.05, ^p<0.01 compared to the p53 +/+ group.

Conclusion

The results of the experiments indicate that p53 is affected by ionizing radiation. Using the Apoptag kit, apoptotic levels were observed. The sections showed that several hours post irradiation there was an increase in apoptotic levels in all 3 genotypes, but significantly less apoptosis in mice that were deficient of p53 compared to the wild type. [These results further support](#) that p53 is an important apoptotic factor, and without it apoptosis is greatly inhibited.

The Western blot results showed that p53 levels began to increase around 3 hours up to 6-12 hours post irradiation, and then begin to decrease afterward. These results support the hypothesis that p53 is being up-regulated to perform cell cycle arrest/apoptosis, and then down-regulated after repair or apoptosis has been completed.

Based upon the results, p53 does play a major role in initiating radiation induced apoptosis in mouse germ cells. Apoptosis still did occur in p53 KO mice, which means that apoptosis does still occur in the absence of p53 but its levels are considerably decreased.

Chapter 3. Effect of cisplatin exposure on p53 in mouse germ cells

Cisplatin is a platinum based chemotherapy drug that is used to treat many types of cancer. Cisplatin has a central platinum atom that is bonded to two chlorine atoms and two ammonia molecules. Cisplatin is administered intravenously in a saline solution. While in the bloodstream the high amounts of chloride ions enable the cisplatin to remain inactive. Cisplatin can then be absorbed into the cell by either passive diffusion or active transport [\(34\)](#).

Once the cisplatin is inside the cell where there is a much lower chloride concentration, the lower chloride concentration and high water concentrations allow one of the chlorine atoms to become displaced with a water molecule, resulting in the charged molecule $[\text{PtCl}(\text{H}_2\text{O})(\text{NH}_3)_2]^+$. The water molecule that is attached can become replaced with one of the nitrogen groups in DNA that is not bound to any hydrogen atoms. This displacement can result in the molecule being linked to the

DNA strand. Once the cisplatin is attached to a base, the other chlorine atom will become displaced with another water molecule enabling it to bind to a different base. The double binding of cisplatin can result in either intra or inter strand cross-linkage. This crosslink can interfere with the replication process by blocking the action of DNA polymerase. This arrest of DNA polymerase then initiates the DNA repair pathways, which will attempt to replace the linked nucleotides. The cisplatin molecule is strongly bound to the DNA and it is unlikely that the cell will be able to repair the DNA damage. The failure to repair the damaged DNA will eventually result in the initiation of the apoptotic pathways and the ultimate death of the cell (33,41).

Cisplatin is used to treat cancer by preventing replication and initiating apoptosis in the affected cells. Since the DNA crosslink is noticed during replication, the constantly replicating cancer cells are more likely to be affected by the cisplatin treatment. Also the faster dividing cells have a

smaller window of time for repair, so it takes less time for apoptosis to be initiated [\(21\)](#).

Although cisplatin may kill the cancerous cells, it can also affect normal cells in the body, which may lead to several possible side effects. Some of these effects include: kidney and nerve damage, hair and hearing loss, nausea, sterility, vomiting and electrolyte loss. Many of these side effects can be treated with medicine and supplements but unfortunately some may not and can result in permanent organ failure [\(34\)](#).

Cisplatin will be used in this [study](#) to induce strand cross-links in the DNA. The cross-links will initiate DNA repair and apoptosis so that the role of p53 in the apoptotic pathways can be better understood. It would be expected that p53 deficient mice will be more resistant to repair and apoptosis, since p53 is a major participant in both the DNA repair and the apoptotic pathways (44).

Figure 11. Cisplatin molecule. The cisplatin molecule is composed of a central platinum atom bonded to a pair of chlorine atoms, and a pair of ammonia molecules.

Materials and Methods

Animals- p53 deficient and wild type mice were obtained by breeding p53 heterozygous mice (strain B6.129S2-Trp53^{tm1Tyj}) purchased initially from the Jackson Laboratory (Bar Harbor, ME). Mice were given water and standard lab chow *ad libitum*. The temperature of the animal room was kept at constant room temperature (23.3 ± 2 °C and 30-70% humidity) and exposed to 12 hour alternating light and dark cycles. All procedures were performed in accordance with Rhode Island College's institutional animal and use committee in compliance with the guidelines established by the NIH.

Cisplatin Exposure- 60 day old mice were administered 10mg/kg of cisplatin in 0.9% saline solution [via](#) I.P. injection. Cisplatin exposed mice were then sacrificed

by carbon dioxide asphyxiation at varying time points: 24, 48 and 72 hours post injection, and their testes were extracted.

Western Blotting - Testes from control, 24, 48 and 72 hour post cisplatin exposed mice were detunicated, weighed, and homogenized in three volumes of ice-cold RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (P2714, Sigma) by 10 strokes in a Dounce homogenizer. Samples were incubated on ice for 30 minutes. The homogenate was then centrifuged at 13,500 X g for 10 minutes at 4°C. For western blotting, 10 µL of testis supernatant were separated by molecular weight by 10% SDS page, and transferred to Immobilon-P membrane. Blocking solution (20mM Tris pH 4.4, 137 mM NaCl, 10% nonfat dry milk) was added to the membrane for 60 minutes. Primary antibodies to serine 15 phosphorylated p53 and Vinculin were diluted in blocking solution and added to the membranes at 4°C overnight. After washing with PBS 0.1% Tween-20, horseradish peroxidase-coupled secondary anti-body diluted in blocking solution were incubated with the membranes for 1 hour at room temperature. Membranes were washed three times with PBS-Tween (0.1%) and secondary antibody was detected by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech).

DNA extraction for genotyping- At 21 days old, the mice were held between thumb and index finger and about ¼ cm of the mouse's tail was snipped off. The DNA was then extracted using a "QIAGEN DNeasy kit." The tail snip was placed in a 1.5 mL microfuge tube with 180 µL ATL (tissue lysis) buffer and 20 µL of proteinase K. The tube was then placed in a temperature bath overnight in order to dissolve the tail. The following day 400 µL of AL-ethanol buffer was added and the microfuge tube was vortexed. The mixture was then pipetted into the DNeasy mini spin column that is placed in a 2mL collection tube and centrifuged at 8000 rpm for 1 minute. The flow-through and collection tube were discarded and the mini spin column was placed in a new collection tube. 500 µL of buffer AW1 (wash buffer 1) was then added and the tubes were centrifuged again at 8000 rpm for 1 minute. The flow-through and collection tube were discarded and the mini spin column was again placed in a new collection tube. 500 µL of buffer AW2 (wash buffer 2) was then added and the tubes were centrifuged at 14,000 rpm for 3 minutes. The flow-through and collection tube were then discarded and the mini spin column was placed in a new 1.5 mL microfuge tube. 200 µL of DEPC treated water was then added to the tube and centrifuged at 8000 rpm for 1 minute. The easy spin column was discarded and the extracted DNA was diluted in 200 µL of DEPC treated water in a microfuge tube.

Polymerase Chain Reaction (PCR) - DNA extracted from the previous protocol

was added to several reagents to produce the PCR cocktail. These reagents include: DEPC treated water, primers for the p53 gene, magnesium chloride, nucleotide bases, PCR buffer solution, and **Taq** polymerase. Once the mix was prepared, 20 µL of it was added to 5µL of DNA, and that solution was pipetted in a PCR tube. The tube was then placed in the PCR machine and went through an initial cycle of 94°C for 5 minutes. The machine then goes through a second cycle thirty five times which consists of three steps: 94°C for 30 seconds, 61°C for 45 seconds and 72°C for 45 seconds. The PCR then went through a third cycle of 72°C for 2 minutes and then the final hold of 4°C.

Apoptosis Staining (Apoptag) - p53 wild type, heterozygous, and knockout mice

were sacrificed at 0, 24, 48 and 72 hours after cisplatin exposure. Their testes were then extracted, embedded in freezing medium and then flash frozen in liquid nitrogen. The testes were then sliced **10 µm** thick **using a cryostat** into thin sections and mounted to slides. 10% neutral buffered formalin followed by an ethanol/acetic acid mixture, and finally a 3% hydrogen peroxide solution (with a PBS rinse between each step) was used to help fix the section to the slide. After the hydrogen peroxide was washed off equilibrium buffer was quickly applied to

each section, and then blotted dry. Terminal Deoxynucleotidyl Transferase (TdT) enzyme was then mixed with reaction buffer at a 1:2 ratio and a couple drops were applied to each section. TdT catalyzes the addition of nucleotides that are labeled with a marker onto the fragmented DNA associated with apoptosis. Cover slips were then applied to the sections, and then they were incubated at 37°C in a humidification chamber for an hour. The slides were then removed from the chamber, rinsed with PBS and then stop buffer was added to halt the TdT enzyme reaction. The stop buffer is rinsed off and anti-digoxigenin-peroxidase was applied to the sections, which were then incubated at 37°C in a humidification chamber for 40 minutes. The slides were then removed, rinsed off and 3,3'-diaminobenzidine (DAB) was applied in order to add the brown stain for apoptosis. After 5 minutes the DAB was washed off and the cells are counterstained with methyl green for 10 minutes. The methyl green was then rinsed off and the slides are then dipped in isopropanol for 2 minutes. The slides were then dipped in Citrosolve clearing agent for 2 minutes and then blotted dry. Cover slips are applied to the sections which are then looked at under a light microscope.

Cisplatin Results

Experiment 3.1: Body/Testes weights for cisplatin exposed mice

Cisplatin injected mice were sacrificed and their body weight (BW) and testes weight (TW) were measured. The average and standard error of the mean (SEM) were calculated for each group. The body weights did not vary between groups but the testes weights did. It appears that the testes weights were greatest in the KO groups, and the WT and HET groups were about the same. This data is shown in table 2.

	<u>WT</u>	<u>HET</u>	<u>KO</u>
<u>BW</u>	<u>WT 26.82</u>	<u>HET 26.39</u>	<u>KO 27.52</u>
<u>BW (g)</u>	<u>26.82±0.77</u>	<u>26.39±1.35</u>	<u>27.52±0.593</u>
<u>SEM</u>	<u>0.77</u>	<u>1.35</u>	<u>0.593</u>
<u>TW</u>	<u>0.092±0.004</u>	<u>0.091±0.004</u>	<u>0.11±0.003</u>
<u>TW (g)</u>	<u>0.092</u>	<u>0.091</u>	<u>0.11</u>
<u>SEM</u>	<u>0.0041</u>	<u>0.0035</u>	<u>0.0027</u>

Table 2. Body and testes weights ± SEM of mice following cisplatin exposure. BW: body weight, TW: combined testes weight, WT: wild type, HET: heterozygous, KO: knockout. Same size was 7 mice for each genotype group. Statistical analysis was performed using One way ANOVA and Dunnett's test. *p<0.05 compared to the p53 +/- group

Experiment 3.2: p53 protein levels in cisplatin exposed mice

Mouse testes were homogenized and their protein levels were measured by assay. The samples were then [run on a SDS gel and western blotted to compare](#) p53 levels at varying time intervals using vinculin as a standard.

Based on the western results p53 levels in wild type mice were shown to increase after cisplatin exposure. p53 levels started out low and by 24 hours they started to increase. p53 levels then increased more by 48 hours and then began to decrease at 72 hours. This data is displayed in figure 13.

[A.](#)

B.

Figure 13. Western gel for p53 levels at varying time intervals post cisplatin exposure.

A. Picture of western blots for p53 and vinculin levels at various hours after cisplatin injection. **B.** p53 levels are low at 0 hrs and then increase at 24

hours, increase more by 48 hours, and then began to decrease a bit by 72 hours.

Sample size varied between 3-6 mice per time point, and was run on 2 blots.

Experiment 3.3 Apoptotic levels in cisplatin exposed mice.

[Cisplatin exposed](#) p53 +/+, +/- and -/- [mice](#) testes were frozen in [tissue freezing](#) medium and sections were sliced [10 µm thick at](#) minus [15 degrees](#) Celsius_onto slides using a [TBS brand](#) cryostat machine. The slides were stained for apoptosis using an Apoptag kit. Apoptag results showed increased apoptosis levels in all genotypes after cisplatin exposure. Cisplatin apoptotic levels increased around the 24 hour time points, increased more at 48 hours, and then began to decrease at 72 hours. The WT mice had the highest amount of apoptosis, followed by the heterozygous group, and the KO mice had the least amount of apoptosis.

This data is shown in figure 12.

p53 $+/+$ 48 hour

p53 $-/-$ 48 hour

A

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Figure 12. Apoptotic levels at varying time intervals post cisplatin injection

A. Pictures of tubules 48 hours after cisplatin injection for p53 wild type and knockout groups. B. Representative examples (from a sample size of 3 mice for each bar) of

TUNEL stained testis sections from p53 $+/+$ and p53 $-/-$ are shown above. The percentage of seminiferous tubules in each treatment group which exhibited more than 4 TUNEL positive cells is plotted against the time after treatment. Each bar represents the average of 6-10 testis sections from 2-3 mice. Statistical analysis was performed using One way ANOVA and Dunnett's test. * $p < 0.05$ compared to the p53 $+/+$ group

Conclusion

The results of the experiments indicate that p53 and apoptotic levels are affected by cisplatin. Using the Apoptag kit, apoptotic levels were observed. The sections showed that 24+ hours post injection there was significantly less apoptosis in mice that were deficient of p53 compared to the wild type. [These results further support](#) that p53 is an important apoptotic factor, and without it apoptosis can be inhibited.

The Western results showed that p53 levels increased in response to cisplatin exposure. Levels began to increase 24 hours after cisplatin exposure, increased even more by 48 hours and then began to decrease by 72 hours. These results support that hypothesis that p53 is being up-regulated to perform cell cycle arrest/apoptosis, and then down-regulated after repair or apoptosis has been completed.

Based upon the results, p53 does play a role in initiating cisplatin induced apoptosis in mouse germ cells. Apoptosis still did occur in p53 KO mice, which means that apoptosis does still occur in the absence of p53 but its levels [are](#) considerably decreased.

Chapter 4. Discussion

Testes and Body Weights

Shortly after being sacrificed the mice had their body weights and testes weights measured and recorded in grams. The mean (+/- SEM) body and testes weights were calculated for each group. This was done to see if apoptosis had an effect on testes or body mass. In the radiation exposed group the body and testes weights did not vary greatly between groups. Although there was no significant difference, it appeared that the KO weights were greater than the other groups. The body weights in the cisplatin treated groups were generally greater than the ones in the radiation group, but the testes weights in the cisplatin group were generally less than the ones in the radiation group. Cisplatin exposure leads to significantly decreased testis weight and could explain the difference between the groups (34). The body weights in the cisplatin group were about the same, but the testes weights were not. The testes weights in the KO group were significantly higher than in the WT or Het groups. A possible explanation is that apoptosis would lead to decreased testis weights due to the loss of germ cells. This would explain why the KO groups (which had overall less apoptosis) would also weigh the most.

Table 3. The body and testes weights of sacrificed mice.

Mean $\pm$ SEM body weight and combined testes weights for mice treated with radiation or cisplatin. BW: body weight, TW: testes weight, WT: wild type, HET: heterozygous, KO: knockout. Statistical analysis was performed using one way ANOVA and Dunnett's test. *p<0.05 compared to the p53 +/- group.

	<u>Radiation</u>			<u>Cisplatin</u>		
	<u>WT</u>	<u>HET</u>	<u>KO</u>	<u>WT</u>	<u>HET</u>	<u>KO</u>
<u>BW</u>	22.63 $\pm$ 0.36	23.16 $\pm$ 0.4	23.47 $\pm$ 0.4	26.82 $\pm$ 0.77	26.39 $\pm$ 1.35	27.52 $\pm$ 0.59
<u>TW</u>	0.167 $\pm$ 0.004	0.179 $\pm$ 0.004	0.183 $\pm$ 0.005	0.092 $\pm$ 0.004	0.091 $\pm$ 0.004	0.11 $\pm$ 0.003*

Western Blot analysis

The western was performed to see how p53 levels changed at varying time points after being exposed to stress. By comparing the p53 levels to the apoptotic levels at corresponding time points, it will help to better understand the role of p53 in germ cell apoptosis.

The western results for radiation showed that the p53 levels did vary at different points post irradiation. The p53 levels began to increase at 3 hours and increased even more at 6 hours. At 12 hours p53 levels had decreased and by 24 hours the levels greatly decreased, and were almost the same as the initial amount.

Western results for cisplatin exposure had similar results. p53 levels were lowest in the 0 hour group, and by 24 hours the p53 levels began to increase, and

even increased some more by 48 hours. By 72 hours the p53 levels were still elevated but it appeared as if they were beginning to decrease. These results are displayed in figure 15.

These results show that p53 levels were affected by induced DNA damage. After DNA was damaged p53 levels increased until a certain point where the levels then began to decrease. This coincides with the theory that p53 is involved in DNA repair and/or apoptotic pathways. p53 levels increase in response to DNA damage and can initiate apoptosis if damage is too extensive. Once p53's job is done its levels then appear to return back to normal.

Figure 15. p53 levels over time for both groups. [Relative concentrations from the](#) Western blot data for both radiation and cisplatin groups were combined into one graph.

Apoptotic Levels

The purpose of staining the cells for apoptosis is to better understand the time frame of apoptosis after inducing DNA damage and p53's possible role in it. Since p53 is believed to be involved in both cell cycle arrest and initiating apoptosis, then if apoptosis is initiated p53 levels should also increase. If a p53 wild type mouse section shows that apoptosis occurred after stress but the p53 KO does not, then it would suggest that p53 plays an important role in apoptosis in the testes. The data from the experiments support this hypothesis. Not only did apoptotic levels increase after radiation or cisplatin exposure, but there were generally increased levels of apoptosis in the WT mice compared to the KO ones.

For the first few hours in the radiation group the apoptotic levels remained about the same. At 6 hours the apoptotic levels began to increase and the difference between the WT/HET groups and the KO group begins to increase. At 12 hours the numbers increased and varied even more among the groups. Finally at 24 hours the overall apoptotic levels begin to decrease, and was lower in the KO group compared to the other two.

The cisplatin group had similar results except they were not as extreme. The 0 hour group had the least amount of apoptotic levels, the 24 hour group's apoptotic levels began to increase and the differences between the WT/HET groups and the KO group also increased. By 48 hours apoptotic levels remained

about the same in the WT/HET groups and the KO group began to decrease.

Finally by 72 hours, apoptotic levels decrease in all three groups, and as expected WT group having the highest apoptotic index, HET having the next lower index, and the KO group having the lowest. This data is shown in figure 14.

These results show that apoptotic levels were affected by both the radiation and cisplatin exposure, and the groups with p53 had significantly more apoptosis than the p53 KO groups. The radiation group's values were not the same as the cisplatin's but they both had the same overall trend. The least amount of apoptosis was in the 0 hr control group and the levels increased over time. Eventually after enough time had elapsed the apoptotic levels appeared to decrease.

Figure 14. Apoptotic levels at varying time points after exposure. Even though the values were different between the radiation and cisplatin groups, they both followed the same trend. Apoptotic levels were lowest at 0 hours and started to increase over time. Eventually after enough time had passed the apoptotic levels started to decrease. p53 levels were also lowest in the p53 -/- groups compared to the other two.

The Model

After mouse testicles were exposed to either ionizing radiation or cisplatin injection p53 levels increased. Apoptosis levels also increased in response to induced DNA damage as well. After enough time had passed after inducing DNA damage, apoptotic and p53 levels began to decrease. This decrease indicates that p53 and apoptotic levels were returning to normal.

These results indicate that there is a correlation between p53 and apoptotic levels after DNA damage was induced. This coincides with the theory that p53 is up-regulated after DNA damage, and if the damage is too extensive then it will initiate apoptosis (10, 19, 32, 39). However apoptosis still did occur in the p53 KO mice, just not as much compared to the mice not deficient of p53. This indicates that although p53 plays an important role in a lot of apoptotic pathways not all pathways require it. A model for possible apoptotic responses to DNA damage is shown in figure 16.

Figure 16. The Model. A proposed model of how DNA damage can lead to apoptosis of cells. DNA damage leads to relatively high levels of apoptosis in the testes in p53^{WT} and HET mice, while p53^{KO} mice had relatively low levels of apoptosis. This shows that p53 plays a major role in most apoptotic pathways. However apoptosis can still occur in its absence, which indicates that there are some apoptotic pathways that do not require p53.

To test this model one could further investigate the apoptotic pathways that either incorporate p53 or do not. To further investigate the p53 apoptotic pathway one could initiate DNA damage and sacrifice the p53 WT mice at determined time intervals. TUNEL staining and western blots could be performed, except for the western blots use antibodies targeted against another kinase believed to be downstream from p53. If the concentrations of these kinases increased along with apoptotic levels it would further support this theory.

A way one could test the pathway that does not incorporate p53 would be to perform TUNEL staining and western blots and use only p53 -/- mice. Different antibodies would be used to see the presence of other kinases that may be involved in one of the apoptotic pathways. Some possible targets would be JNK, p 38 or AP-1. Since the mice would be p53 deficient then apoptosis could only be initiated via the pathways that do not incorporate p53. If the levels of these enzymes increase after DNA damage is initiated and apoptosis occurs, it would indicate that they play a role in the non-p53 apoptotic pathway(s).

My results coincide with similar published work. In other papers p53 and or apoptotic levels increased in response to induced DNA damage in both somatic and germ cells. This increase in p53 and apoptotic levels after DNA damage was induced also coincides with the theory that p53 plays a major role in apoptotic pathways. It is believed that p53 initiates DNA repair, and if it is unsuccessful p53

will then initiate apoptotic pathways. Even though most apoptotic pathways are believed to incorporate p53 some do not, and apoptosis can still occur, just at lower rates (3, 4, 6, 7, 8, 11, 13, 15, 16, 17, 21, 26, 30, 31, 32, 43, 44, 45).

If it is true that p53 does play a major role in apoptosis in germ cells, then it may be possible to induce apoptosis by increasing p53 levels. This could be applied in particular to testicular tumors. Tumors arise from constantly growing and dividing “immortal” cells. If somehow p53 levels were increased in these tumor cells, it may be possible to kill the cells by inducing apoptosis and prevent the tumor from growing. Unfortunately some tumors arise from the cells being non-responsive to p53. If we could better understand the apoptotic pathway(s) that do not incorporate p53, then it may be possible to initiate apoptosis in these cells that are resistant to p53.

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