

**The Effects of Carcinogens and Irradiation on Cells and Tissues
of the Eastern Red Spotted Newt (*Notophthalmus viridescens*)**

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ABSTRACT

Newts, such as *Notophthalmus viridescens*, can regenerate many structures after amputation or injury and have also shown a refractory response to the formation of cancer in tissues that have regenerative capabilities. The mechanisms behind this latter ability have surprisingly not been studied. In the current study, *N. viridescens* were exposed to a variety of carcinogens in tissue that cannot regenerate with the intention of inducing tumour formation. After testing multiple carcinogens, multiple sites of injection, and two different modes of delivery, no tumours were generated.

Consequently, *in vitro* assays were developed in order to better understand this ability of newt cells to evade transformation. Mouse and newt muscle cells were exposed to DNA damaging agents, such as irradiation and carcinogens, in culture and their response was monitored with respect to the DNA damage response proteins γ -H2AX, p53, and phospho-p53. These proteins are important as they help prevent mutations in the genome from being passed on to daughter cells and potentially generating cells that proliferate uncontrollably, a hallmark of cancer. Preliminary results suggest that after irradiation, γ -H2AX is present in newt cells for a considerably longer period of time in comparison to mouse cells. p53, as well as phospho-p53, appear to be present at a basal level before and after irradiation in newt cells, whereas mouse cells have a distinct increase upon damage and decrease upon repair. The carcinogen treatments also suggest that newt cells have basal levels of expression of these proteins prior to treatment. These studies suggest that newt cells may have a unique profile of these DNA damage response proteins and may be “primed” to repair any future damage. This is a good first step in understanding what is likely a very complicated explanation for newts’ refractory response to cancer formation.

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AUTHORIZATIONS

Figure 1A: Regeneration in Metazoans.

Originally Figure 2A from *Regeneration in the metazoans: why does it happen?* (Sanchez Alvarado, 2000). Authorization to use this figure was obtained from the author Dr. Sanchez Alvarado as well as the journal BioEssays and publisher John Wiley and Sons (through the Copyright Clearance Center).

Figure 1B: Regeneration in Metazoans.

Originally Figure 1 from *Amphibian Limb Regeneration: Rebuilding a Complex Structure* (Brockes, 1997). Authorization to use a modified version of this figure was obtained from the author Dr. Brockes as well as the journal Science and publisher The American Association for the Advancement of Science (through the Copyright Clearance Center).

Figure 3: The stages of newt forelimb regeneration.

Originally Figure 1 from *Newt Opportunities for Understanding the Dedifferentiation Process* (Chaar et al., 2006). Authorization to use a modified version of this figure was obtained from the author Dr. Tsilfidis.

Figure 4: The variety of accessory growths or supernumerary limbs induced by injection of carcinogenic substances into *Triturus viridescens*.

Originally Figures 1-4 from *Induction of Accessory Limbs and of Sarcoma in the Newt (*Triturus viridescens*) with Carcinogenic Substances* (Breedis, 1952). Authorization has been obtained from Cancer Research (American Association for Cancer Research).

Figure 5: Newt forelimbs after being exposed to different carcinogens via intraperitoneal injection during regeneration.

Originally Figures 2 and 4 from *Teratogenic Effects of Carcinogenic Agents on Limb Regeneration in the Japanese Newt *Cynops pyrrhogaster** (Pfeiffer et al., 1985). Authorization to use a modified version of these figures has been obtained from the author Dr. Pfeiffer and the publisher John Wiley & Sons.

Figure 6: Histological sections of normal newt skin and skin treated with methylcholanthrene for 2 weeks.

Originally Figure 1 from *Newt Squamous Carcinoma Proves Phylogenetic Conservation of Tumors as Caricatures of Tissue Renewal* (Zilakos et al., 1002). Authorization to use a modified version of this figure has been obtained from Cancer Research (American Association for Cancer Research).

Figure 8: Genetic instability causes tumour formation.

Originally Figure 1 from *Genetic instability and darwinian selection in tumours* (Cahill et al., 1999). Authorization to use a modified version of this figure has been obtained from author Dr. Lengauer as well as the journal Trends in Cell Biology and publisher Elsevier.

Figure 9: Brief overview of the DNA damage response and repair pathway. Originally Figure 1 from *Phosphatases in the cellular response to DNA damage* (Freeman et al., 2010). Authorization to use a modified version of this figure has been obtained from author Dr. Monteiro.

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LIST OF ABBREVIATIONS

γ -H2AX	Phospho-histone-H2AX
A1	Newt muscle cell line
ATM	Ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related protein
C2C12	Mouse muscle cell line
DAPI	4'-6'-Diamidino-2-phenylindole dihydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
Gy	Gray
H&E	Hematoxylin and eosin staining
H38.5	Mouse fibroblast cell line
MCA	3-Methylcholanthrene
Mdm2	Murine double minute 2
MEM	Minimum Essential Media
MNNG	N-Methyl-N'-nitrosoguanidine
MS-222	Tricaine methanesulfonate
NQO	4-Nitroquinoline-1-oxide
OCT	Optimal cutting temperature
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI3-K	Phosphatidylinositol 3-kinases
PVDF	Polyvinylidene difluoride
RCF	Relative centrifugal force
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 0.1% Tween-20
WST-1	Cell proliferation/viability reagent/assay

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1. INTRODUCTION

Newts, small amphibians from the salamander family, have been an organism of interest to scientists since the late 1700's when Lazzaro Spallanzani began studying their amazing ability to regenerate (Brockes, Kumar et al. 2001). Since Spallanzani's early observations, many scientists have become captivated with these little creatures and the not so little ability they possess, regeneration. While many different organisms can regenerate structures after amputation or injury, newts are particularly notable as they are vertebrate animals that can regenerate a multitude of structures within their body in adult form. Most other vertebrate species that have regenerative capacities are limited to a small number of structures and potentially only when they are young. Not only do newts possess this broad range regenerative ability throughout adulthood, but they also appear to be refractory to developing cancer. This is of interest to the scientific world which is continuously attempting to better understand how cancer cells operate to allow new treatments to be developed to combat this multifaceted disease. While unlocking the newt's secret to preventing cancer will unfortunately not lead to the end of cancer in our world, understanding how newts can avoid it while most other organisms cannot may give scientists a new outlook or approach to cancer prevention and treatment.

1.1 Regeneration in the Kingdom Animalia

The ability to regenerate, re-grow or repair cells, tissues, and organs, has been documented as a widespread phenomenon in metazoans, although it is not represented in all animal phyla (Brockes 1997; Sanchez Alvarado and Tsonis 2006). Though one phylum may contain one or more species with regenerative capabilities, this does not mean that all species within this phylum have the ability to regenerate, no matter how

closely related to one another they may be (Brockes 1997; Brockes and Kumar 2008).

Figure 1A, a phylogenetic tree of Metazoans that can regenerate, illustrates how many different animals have regenerative abilities and how widely distributed this capacity is throughout the kingdom.

The ability to regenerate is different within each species with respect to the tissues that can be regenerated and the mechanisms and pathways used to regenerate.

Morphallaxis allows an animal to regenerate by rearranging pre-existing tissue without cell proliferation (Sanchez Alvarado 2000). Epimorphosis is a type of regeneration that requires cell proliferation and includes 1) transdifferentiation of cells and tissue into the missing structure, 2) proliferation of already present stem cells followed by differentiation, or 3) dedifferentiation of cells followed by proliferation and subsequent differentiation (Sanchez Alvarado 2000). This latter form of regeneration will be discussed in more detail in section 1.3.

The cnidarian *Hydra*, planarian *Dugesia*, and annelid *Nereis* are all capable of bidirectional regeneration (Figure 1B, A, B and C). Bidirectional refers to their ability to respond to transection by the tail section growing a head, and the head section growing a tail, resulting in two smaller organisms (Brockes 1997). Animals such as the amphibian newt and the reptile lizard are able to undergo unidirectional regeneration (Figure 1B, D, and E). After transection of an appendage such as the tail in lizards or the limb or tail in newts, these animals can regenerate the entire missing structure. Newts are especially interesting as they can regenerate more structures than any other vertebrate animal.

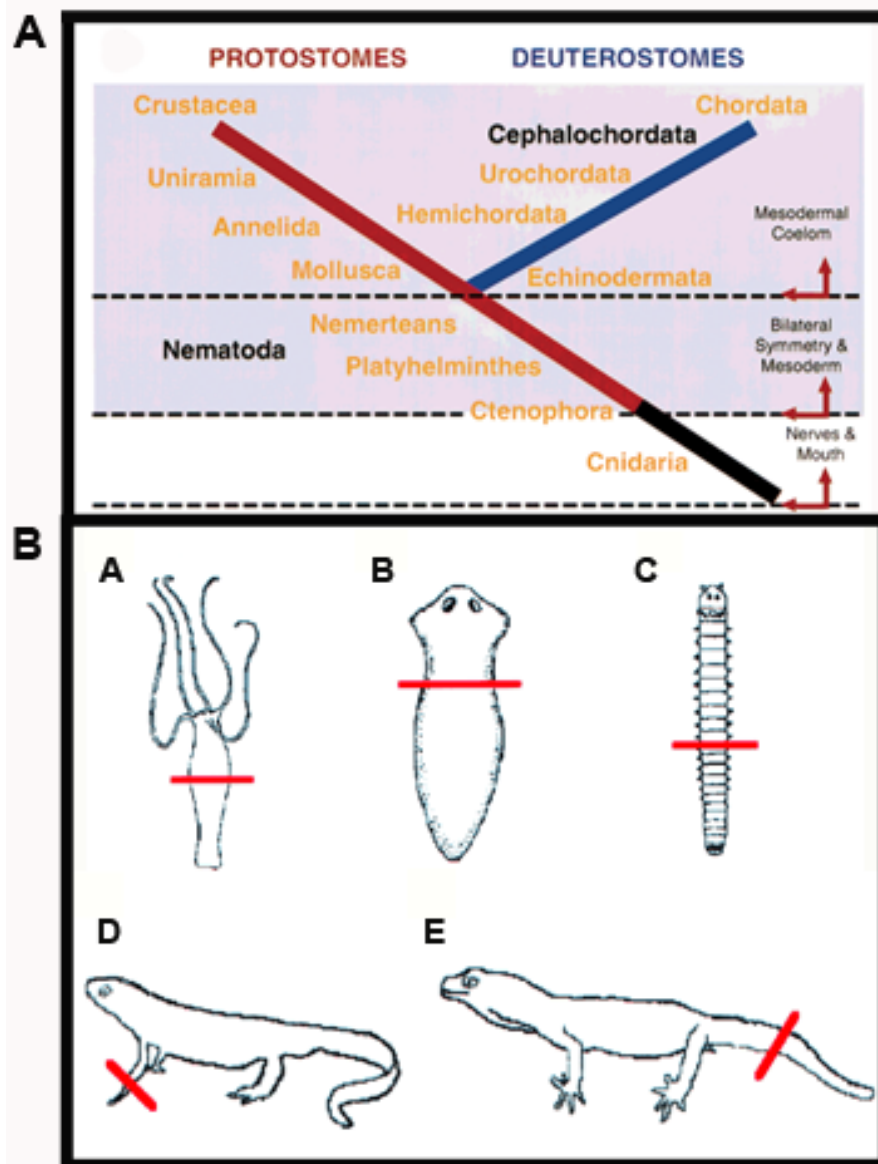


Figure 1: Regeneration in Metazoans. **A)** Phylogenetic tree of Metazoans capable of regeneration (Sanchez Alvarado 2000). The animals within the white box are diploblasts, animals containing the two germ layers (endoderm and ectoderm), and those within the grey area are triploblasts, or those containing three germ layers (endoderm, mesoderm and ectoderm) (Sanchez Alvarado 2000). Phyla that regenerate missing body parts are written in orange, and those written in black have regenerative abilities that have not been well characterized or cannot be agreed upon. The appearance of important morphological features throughout the evolution of Metazoans is illustrated by the dashed lines (Sanchez Alvarado 2000). **B)** Members of the kingdom Animalia capable of regeneration. The cnidarian *Hydra* (A), planarian *Dugesia* (B), annelid *Nereis* (C) are all capable of bidirectional regeneration, or regenerating from each site of transection. The urodele newt (D) and reptile lizard (E) are capable of unidirectional regeneration where an entire appendage is regrown after amputation. Modified from Brockes 1997.

1.2 The Newt

Newts are urodele, or tailed, amphibians that live in an aquatic environment in their adult form. Prior to the adult stage, newts must first pass through an aquatic larval stage as well as a terrestrial juvenile or eft stage. As larvae, the newts undergo metamorphosis and lose their gills allowing them to leave the water and progress to the terrestrial stage (Brockes and Kumar 2005). In the Red-spotted newt (*Notophthalmus viridescens*) used in this study, the eft staged newts are bright orange-red in colour, so are easily distinguishable (Cohen 1995) (Figure 2). This terrestrial stage lasts from one to three years during which the animals become sexually mature. The newts then undergo a second metamorphosis and return to an aquatic lifestyle as sexually mature adults with their colouration changing to an olive green with dorsal red spots (Brockes and Kumar 2005).

In *N. viridescens*, fertilization occurs internally. During mating, the male grasps the female in a grip referred to as amplexus, and manoeuvres her over to the spermatophore (packet of sperm) that he has deposited in the water. The female picks up the spermatophore through her cloaca and can retain the spermatophore for long periods of time. The fertilized eggs are then laid in the water in clusters or stringy masses. These eggs hatch into aquatic larva with external gills and a finlike tail (Cleveland P. Hickman 2003).

1.3 The Regeneration Process in the Newt

Urodele amphibians, such as the newt, possess the remarkable ability to undergo the process of epimorphic regeneration, “the replacement of entire tissues or organs that are morphologically and histologically indistinguishable from the original” (Chaar and Tsilfidis 2006). They are able to regenerate many structures after injury or amputation

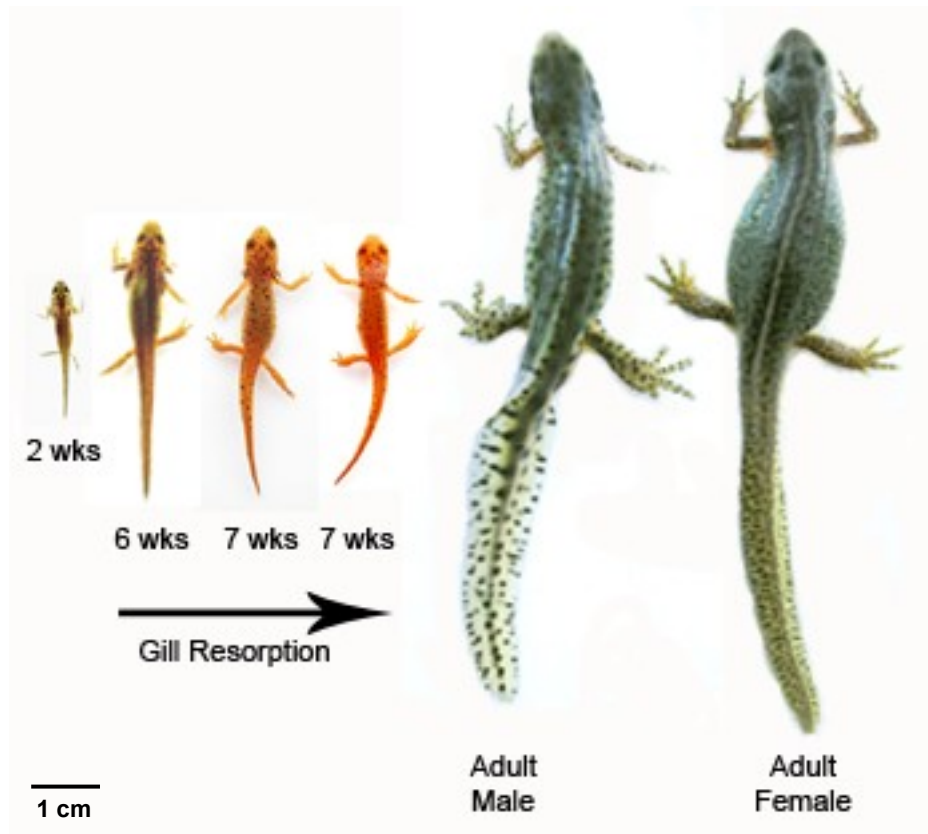


Figure 2: Development of the Eastern Red Spotted Newt, *Notophthalmus viridescens*. This species is used in this study to observe the newts' response to carcinogen exposure.

including their spinal cord, cardiac muscle, lens, retina, limbs, and tail. The molecular controls of this process are not well understood; however, the cellular pathway has been extensively studied and documented (Chaar and Tsilfidis 2006).

The regeneration process begins minutes after amputation with the cessation of bleeding as a result of the formation of a fibrin clot (Figure 3). Epithelial cells then migrate across this fibrin clot, and the wound is covered by a three to four cell layer-thick wound epithelium, approximately nine hours after the initial amputation (Chaar and Tsilfidis 2006). Following this stage is the initiation of dedifferentiation, where fully differentiated cells under the wound epithelium “lose their specialized cytoplasmic characteristics and revert back to a morphologically less specialized or “embryonic-like” state” (Chaar and Tsilfidis 2006). Cells in this less specialized state contain rounded nuclei with distinct nucleoli, increased numbers of free ribosomes, and increased nuclear-cytoplasmic ratios. Dedifferentiated cells collect at the apex of the regenerating tissue and multiply to form a mass called the blastema. After three to four weeks, once a critical mass is achieved, there is progressive redifferentiation from the base of the blastema to its distal tip. Muscle and cartilage are formed first followed by connective tissue, the blood vascular system, and nervous connections. The entire process is complete within approximately two months; the exact time depends on the environmental temperature and the newts’ nutritional intake. After this two month period, the size and coloration of the limb may continue to develop, and it will eventually be indistinguishable from the original structure (Chaar and Tsilfidis 2006).

Some anuran amphibians (for example *Xenopus laevis*, the South-African clawed frog) also have the ability to regenerate many structures, but only while in larval stages

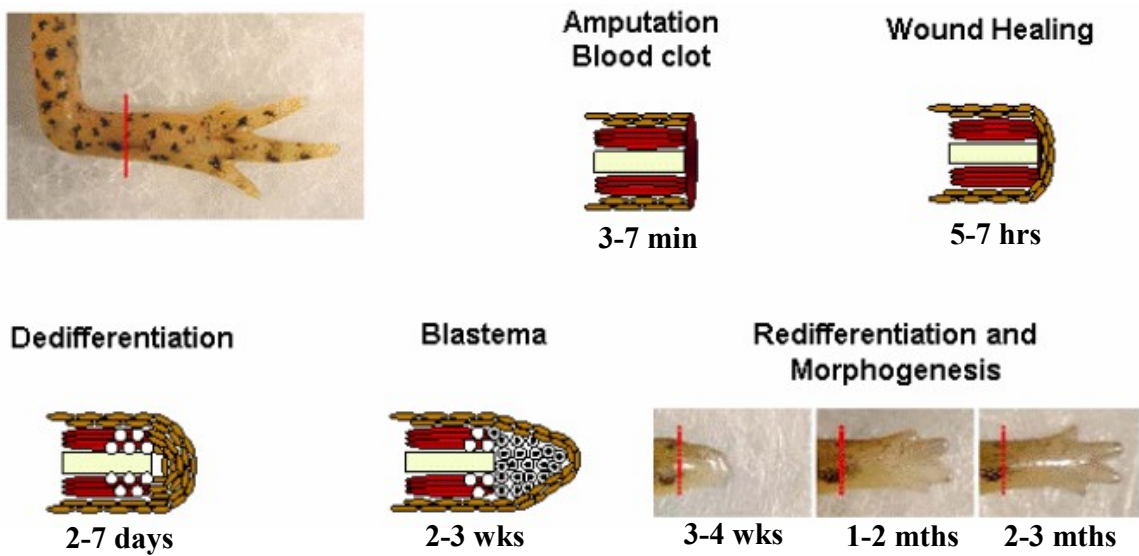


Figure 3: The stages of newt forelimb regeneration. Initially a fibrin clot forms, followed by an epithelial wound cover, thickening of the wound cover as well as dedifferentiation of the cells below it, and proliferation of the dedifferentiated cells to form the blastema. Modified from Chaar et al. 2006.

(Endo, Yoshino et al. 2007). Amputating the hindlimb of *Xenopus* in early stages of development usually results in the regeneration of a complete limb as in newts, but after the five digits of the hindlimb are fully formed during development, only a cartilaginous spike-like structure grows back (Mescher and Neff 2006). Other anuran species also lose the ability to regenerate as development progresses, but to a greater extent than *Xenopus* with no outgrowth at all forming after amputation of a fully developed limb (Mescher and Neff 2006). A possible explanation for this loss is that anurans are unable to produce a functional wound epithelium. Instead, after the epidermis has healed over the amputation site, connective tissue is quickly laid down beneath the epidermis before cell proliferation can occur to establish the blastema (Mescher and Neff 2006). *Xenopus* frogs are an interesting amphibian species as they allow a comparison of the same tissue that can and cannot regenerate within the same species. As adults, *Xenopus* frogs are excellent regeneration models in that they provide an intermediate transition between master regenerators such as the newt and non-regenerating higher vertebrates.

1.4 Previous Studies of Regeneration and Carcinogens

Previous studies have been conducted concerning the effect of carcinogenic substances on newt tissues which possess the ability to regenerate. In one study, a variety of carcinogens were injected into the forelimb of over 500 adult *Triturus viridescens*, red spotted newts (Breedis 1952). Only two of these animals formed malignant tumours, both of which were sarcomas caused by the same substance, methylcholanthrene. All of the other animals had localized proliferation and blastema-like formation in the region of the carcinogen implantation but the blastemas subsequently differentiated into accessory outgrowths. If the injection was in the limb, supernumerary limbs were formed, and if in

the tail, supernumerary tails developed (Breedis 1952) (Figure 4). The blastema-like tissue resembled a cancerous growth early on, but rather than becoming a malignant tumour, some signal within the tissue directed the cells towards differentiation rather than proliferation. These accessory growths did not become larger than their normal counterparts and the only structures that were reduplicated were those found distal to the injection site, although the structures that were duplicated were not always perfect in comparison to the original. When the limbs were amputated in the original limb proximal to the accessory growth, only a normal limb without an accessory growth grew back (Breedis 1952).

Another study looked at the effect of intraperitoneal injections of different carcinogens into *Cynops pyrrhogaster*, the Japanese fire belly newt, during different time points of the regeneration process (Pfeiffer, Nagai et al. 1985). After the forelimb was regenerated, there were deformities such as syndactyly, polydactyly, oligodactyly, and brachydactyly found alone or in combinations on the limb (Figure 5). No incidences of tumour formation were reported elsewhere within these animals even though the intraperitoneal cavity was exposed to the carcinogens injected (Pfeiffer, Nagai et al. 1985).

In a study conducted by Zilakos et al., it was hypothesized that the solution to inducing cancer in the newt might be to implant carcinogens in regeneration-incompetent tissue like the scapular region. Methylcholanthrene, a known carcinogen, was subcutaneously implanted in crystal form in the scapular region of 30-68 newts per trial for 4 trials. Zilakos et al.'s hypothesis was proven to be correct when transplantable epidermal squamous cell carcinomas were visible at 3 weeks post implantation and were

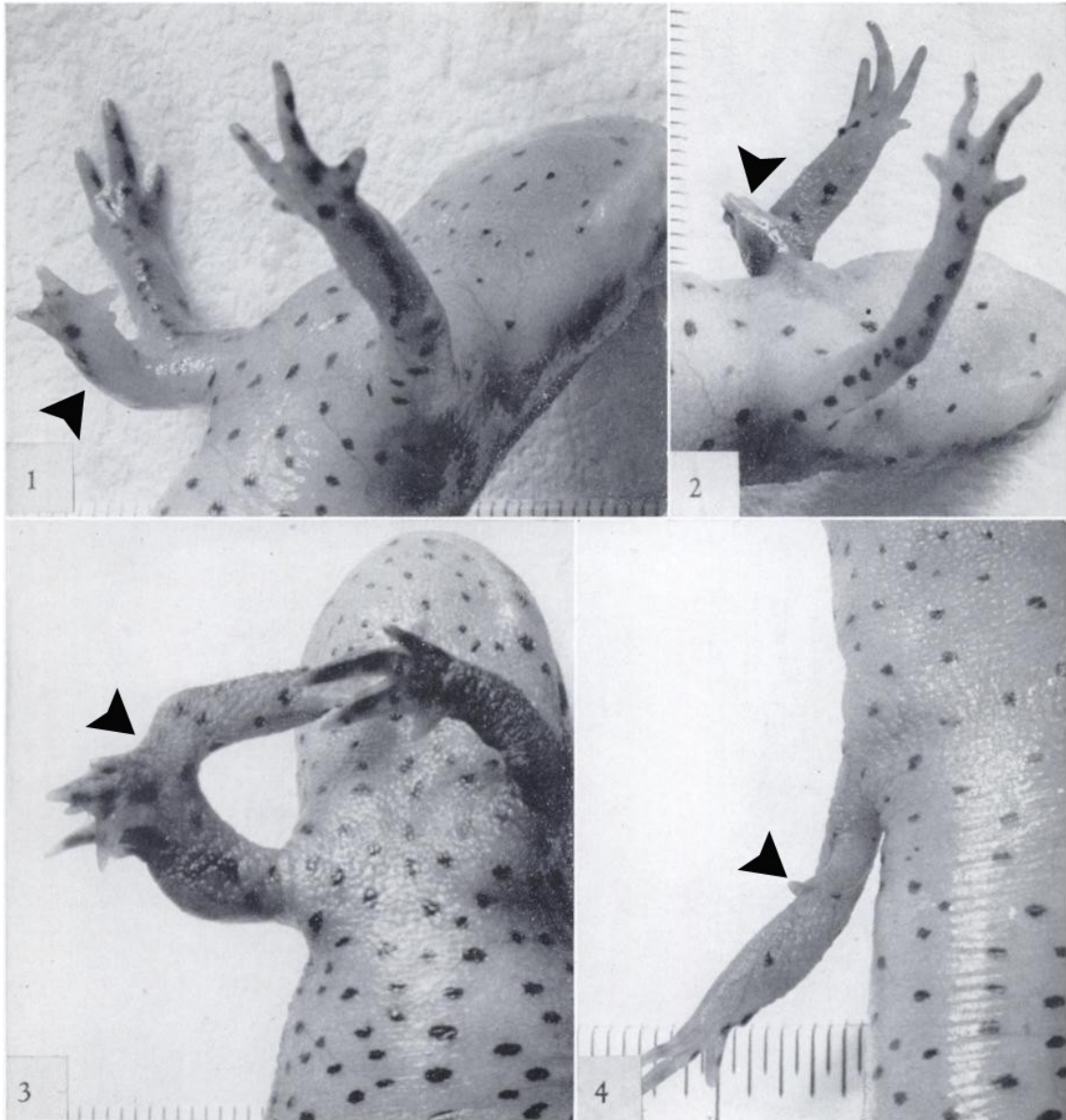


Figure 4: The variety of accessory growths or supernumerary limbs (arrowheads) induced by injection of carcinogenic substances into *Triturus viridescens*. Adapted and reprinted by permission from the American Association for Cancer Research: Breedis, *Induction of Accessory Limbs and of Sarcoma in the Newt (Triturus viridescens)*, Cancer Research, 1952, vol. 12, issue 12, pages 861-6.

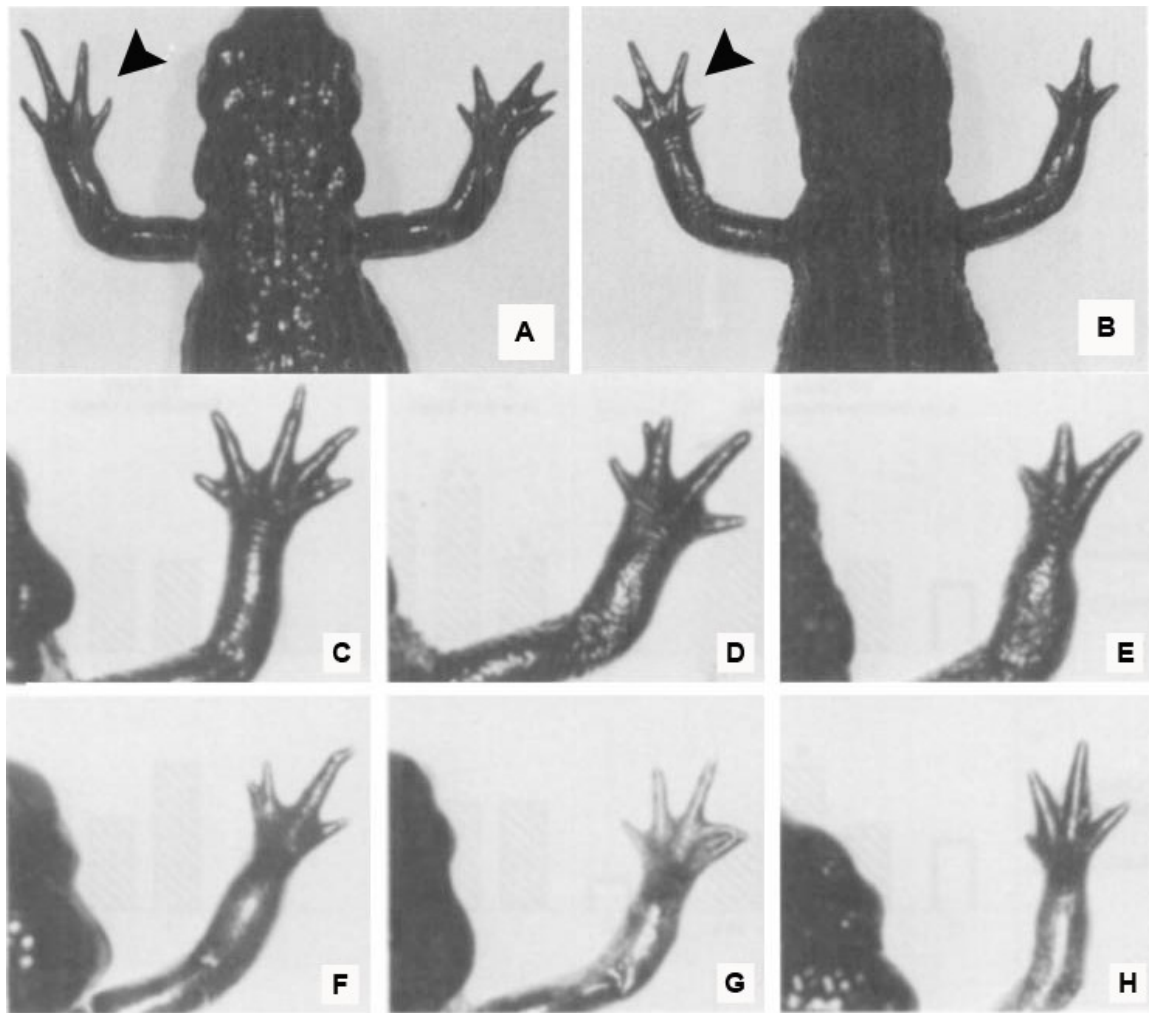


Figure 5: Newt forelimbs after being exposed to different carcinogens via intraperitoneal injection during regeneration. Normal newt limbs are shown by arrowheads in A and B. Mutated limbs are seen on the right in A and B and in C-H. Mutations include polydactyly and syndactyly (C), digital branching (D), and oligodactyly (E). Modified from Pfeiffer et al. 1985. Copyright 1985, John Wiley & Sons. This material is reproduced with permission of John Wiley & Sons, Inc.

induced with a 10-18% incidence (Figure 6). After examination, the tumours were determined to have originated from the epidermis, and when transplanted into other newts, had a 30-40% success rate at inducing tumour formation. Another interesting finding was that skin implanted with methylcholanthrene for only one week also induced tumour formation at a 30% incidence when transplanted into 10 other newts (Zilakos, Tsonis et al. 1992). This study suggests that perhaps newts' ability to evade cancer formation is related to possessing tissue that can regenerate.

Anuran species such as *Xenopus laevis* and *Rana pipiens* have also been used to study the effects of carcinogens in amphibians. Similar experiments have been conducted as in the newts including injection and implantation of carcinogens such as methylcholanthrene and benzopyrene in tadpoles as well as adult frogs (Matos and de Lustig 1973). Lymphosarcomas resulted in many studies, but were not usually seen until 200 or more days post treatment (Balls 1962; Balls 1964).

In many mammalian experiments, phorbol 12-myristate 13-acetate (PMA or more commonly TPA), is used as a cancer promoting reagent to rescue cells from apoptosis (McMahan, Johnson et al. 1999). Interestingly, when this chemical is used on amphibian cells such as *Xenopus laevis* splenocytes, it leads to increased levels of apoptosis (McMahan, Johnson et al. 1999). Apoptosis was found to be higher in PMA treated cells compared to control cells until 48 hours post treatment, where the levels were comparable to the controls (McMahan, Johnson et al. 1999). It has also been found that these cells do not need to enter the cell cycle before undergoing apoptosis (Taylor, Johnson et al. 2003). This direct apoptotic response due to cancer promoting agents may be part of the

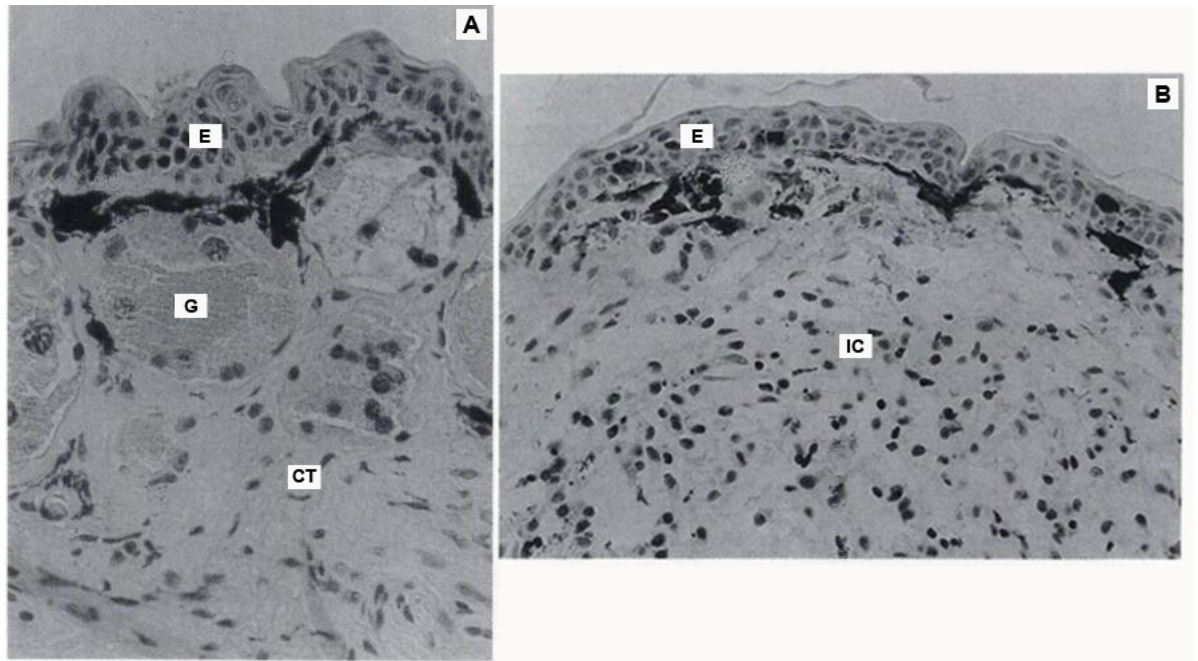


Figure 6: Histological sections of normal newt skin (A) and skin treated with methylcholanthrene for 2 weeks (B). Notice in the treated section B the replacement of glands (G) and connective tissue (CT), as seen in A, with inflammatory cells (IC). E-epidermis; G-gland; CT-connective tissue; IC-inflammatory cells. Modified from Zilakos et al. 1992. Adapted and reprinted by permission from the American Association for Cancer Research: Zilakos, *Newt Squamous Carcinoma Proves Phylogenetic Conservation of Tumors as Caricatures of Tissue Renewal*, Cancer Research, 1992, vol. 52, issue 18, pages 4858-65.

explanation as to why amphibians are more resistant to tumour formation (Taylor, Johnson et al. 2003). If cells possessing genetic instabilities were directly susceptible to apoptosis without entering the cell cycle, then there is no chance of them expressing these instabilities in multiple cells (Taylor, Johnson et al. 2003).

Previous studies conducted in the Tsilfidis lab also suggested a link between regeneration competent tissue and cancer. Regeneration extract was prepared from intact newt forelimbs or tails, as well as from forelimbs or tails that had been allowed to regenerate for four weeks following amputation. These extracts were then used to treat various human cancer cell lines over a period of three days. When human breast cancer cells (MDA 435 MB) were treated for three days with extract derived from the four week regenerated forelimb, the cells were observed to accumulate at the S-phase of the cell cycle (Figure 7A; unpublished results). When human ovarian cancer cells (OV CAR 8) were treated for three days with extract derived from fully differentiated tail tissue, cells were observed to accumulate in the G1 phase of the cell cycle (Figure 7B; unpublished results). If these cells are accumulating at various stages of the cell cycle, they are not passing important cell cycle checkpoints normally and are therefore not proliferating as would normal cancer cells. From these experiments there seems to be another connection between tissue with the ability to regenerate and the control of cell cycle progression.

1.5 Cancer

Cancer is a disease fuelled by genomic instability, with a major hallmark being uncontrolled cell proliferation (Sherr 1996; Ciccia and Elledge 2010). Other traits acquired by cancer cells include resistance to cell death, induction of angiogenesis, activation of invasion and metastasis, and tumour promoting inflammation, just to name

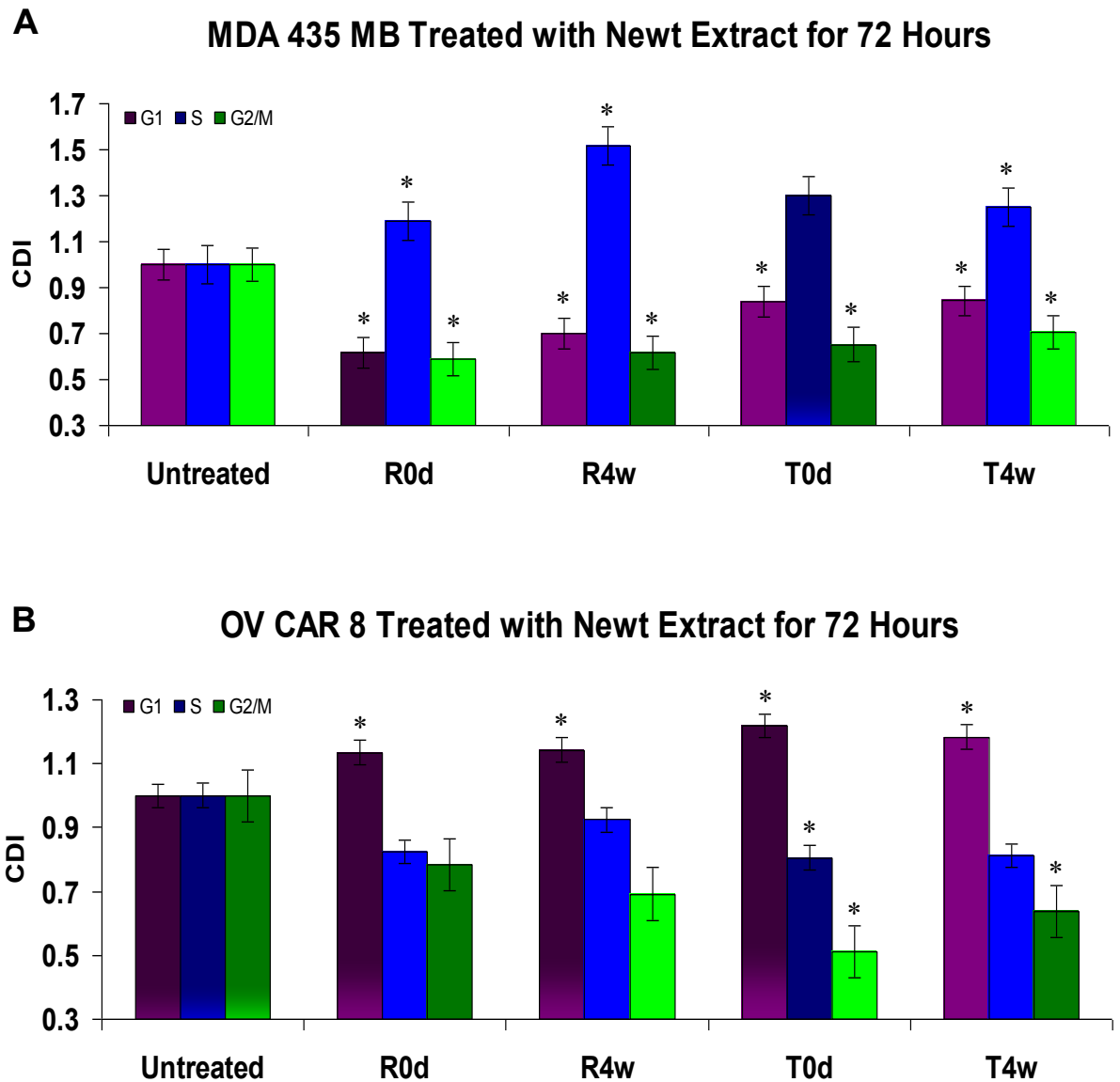


Figure 7: The effect of newt derived regeneration extract on human breast cancer cells (MDA 435 MB) and human ovarian cancer cells (OV CAR 8) after treatment for 72 hours. The regeneration extracts were generated from intact forelimb (R0d), 4 week regenerated forelimb (R4w), intact tail (T0d), and 4 week regenerated tail (T4w). The Cell Division Index (CDI) represents the ratio of treated to untreated cells within different cell cycle stages. **A)** There appears to be an S-phase block in the cells with the R4w extract showing the most significant effect. **B)** The cancer cells appear to exit the cell cycle in the G1/G0 phase with T0d having the most significant effect.

a few (Hanahan and Weinberg 2011). Cancer has become a leading cause of death in the world (Jemal, Bray et al. 2011). Statistics from the International Agency for Research on Cancer found that approximately 12.7 million cancer cases and 7.6 million cancer deaths occurred worldwide in 2008 (Jemal, Bray et al. 2011). According to the World Health Organization (Fact sheet No. 297) tobacco use is a major factor in cancer development, and other main risk factors include poor diet, physical inactivity, and harmful alcohol use, along with genetic predisposition. With these statistics, it is not difficult to see that cancer has become a leading issue in healthcare today with regards to prevention and treatment. Continuous research into the many different forms of cancer itself, as well as other new avenues, such as this study, is necessary in order to develop strategies to combat this disease.

DNA damage leading to cancer can be caused by agents other than lifestyle choices, such as irradiation and chemical agents, as used in this study. Irradiation can be encountered from cosmic radiation as well as medical treatments using X-rays (Ciccia and Elledge 2010). X-rays induce DNA damage through the creation of reactive oxygen species which lead to lesion formation on DNA strands (Bonner, Redon et al. 2008). Chemical agents, or carcinogens, are also used as another method of inducing DNA damage. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) methylates DNA (Mojas, Lopes et al. 2007), 3-Methylcholanthrene (MCA) is a polycyclic aromatic hydrocarbon that binds to DNA to form adducts (Platt, Aderhold et al. 2008) and 4-Nitroquinoline-1-oxide is an alkylating agent that, similar to MCA, also forms DNA adducts (Farber 1982). All of these modifications to the DNA make proper DNA replication difficult

which results in DNA damage (Bonner, Redon et al. 2008) and eventually could lead to uncontrolled cell proliferation.

With lifestyle choices and environmental agents that can lead to mutations in our genetic makeup, it is surprising that cancer is not more prevalent. This is because the transition from normal cell to cancer cell is an extremely complicated process requiring more than a single mutation. Cells have multiple selection barriers to prevent the loss of proliferation control in order to avoid tumour formation (Cahill, Kinzler et al. 1999) (Figure 8). A sequence of random mutations and alterations to DNA that affect cell proliferation and survival genes allow cells to make it through these selection barriers to promote tumour progression (Weinberg 2007).

With all of these different mutations required to induce cancer, it makes treatment very difficult as no two cancer cells or tumours are alike. One of their primary common factors is uncontrolled proliferation. Cancer treatments such as the use of chemotherapeutic drugs target this proliferation, but they are not specific to only cancerous cells and so have many undesired side effects. While cancer treatment and prevention is difficult, knowing how tumours form and progress, the molecular mechanisms associated with cancer formation, along with a large focus on cancer biology in general makes this an exciting time in cancer research. Specialized strategies are being developed to exploit the weaknesses of cancer cells while leaving normal cells intact. Even so, the tremendous heterogeneity of cancer cells, even within a single tumour, makes developing new treatments a very daunting task. Therefore anything scientists can learn about how cancers develop and how some organisms can avoid cancer formation will be very useful to the development of cancer therapies.

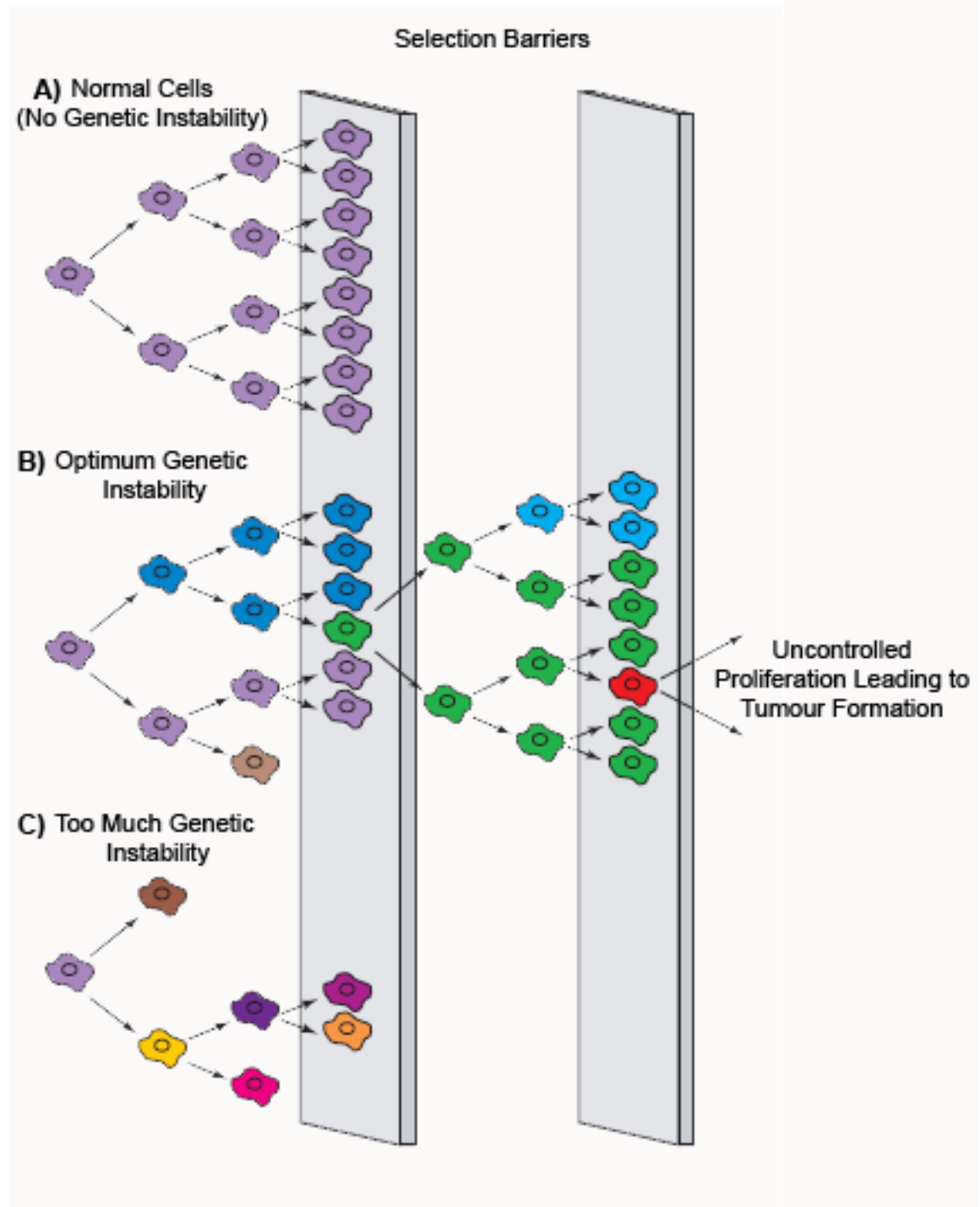


Figure 8: Genetic instability causes tumour formation. **A)** Normal cells with no or low genetic instability have controlled proliferation. **B)** Cells with optimum genetic instability are able to proliferate outside of the selection barriers. When these cells continue to gain genetic mutations it leads to uncontrolled proliferation and eventual tumour formation. **C)** Cells with too much genetic instability are unable to pass the selection barriers and undergo apoptosis. Modified from Cahill et al. 1999.

1.6 DNA Damage Indicator Protein γ -H2AX

Since DNA damage plays such a large role in the development of cancer, it is of interest to consider how newt cells respond to this damage at the protein level. A major player in the DNA damage response pathway is the γ -H2AX protein.

DNA is wrapped around an octamer of histone proteins to form nucleosomes which are in turn arranged in higher order structures to form chromatin. Two different histone proteins from each of four different histone families, H2A, H2B, H3, and H4 make up the eight histone proteins to form this octamer in the nucleosome (Rougakou 1998). The H2A family is broken down into subfamilies, but the one of interest to this study is the H2AX subfamily. When DNA double-strand breaks are formed within a cell, the C-terminal tail of H2AX is rapidly phosphorylated (Figure 9A) at serine-139 by the family of PI3-K like kinases including ATM and ATR (Yuan, Adamski et al. 2010). This phosphorylated version of H2AX is known as γ -H2AX, and is one of the first proteins involved in the DNA damage response pathway (Yuan, Adamski et al. 2010). Phosphorylation occurs minutes after damage, and the γ -H2AX protein helps to sequester other DNA damage response and repair proteins to the site of damage to facilitate repair (Rougakou 1998; Yuan, Adamski et al. 2010). Many H2AX proteins are phosphorylated at the site of a double-strand break which forms a focus. These foci can be observed in a cell with an anti- γ -H2AX protein antibody and used to quantify the amount of double-strand breaks within a cell. The number of visible γ -H2AX foci directly corresponds to the number of double-strand breaks within a cell, ie. in a 1:1 ratio (Fernandez-Capetillo, Lee et al. 2004). When foci are no longer present, this indicates that the double-strand

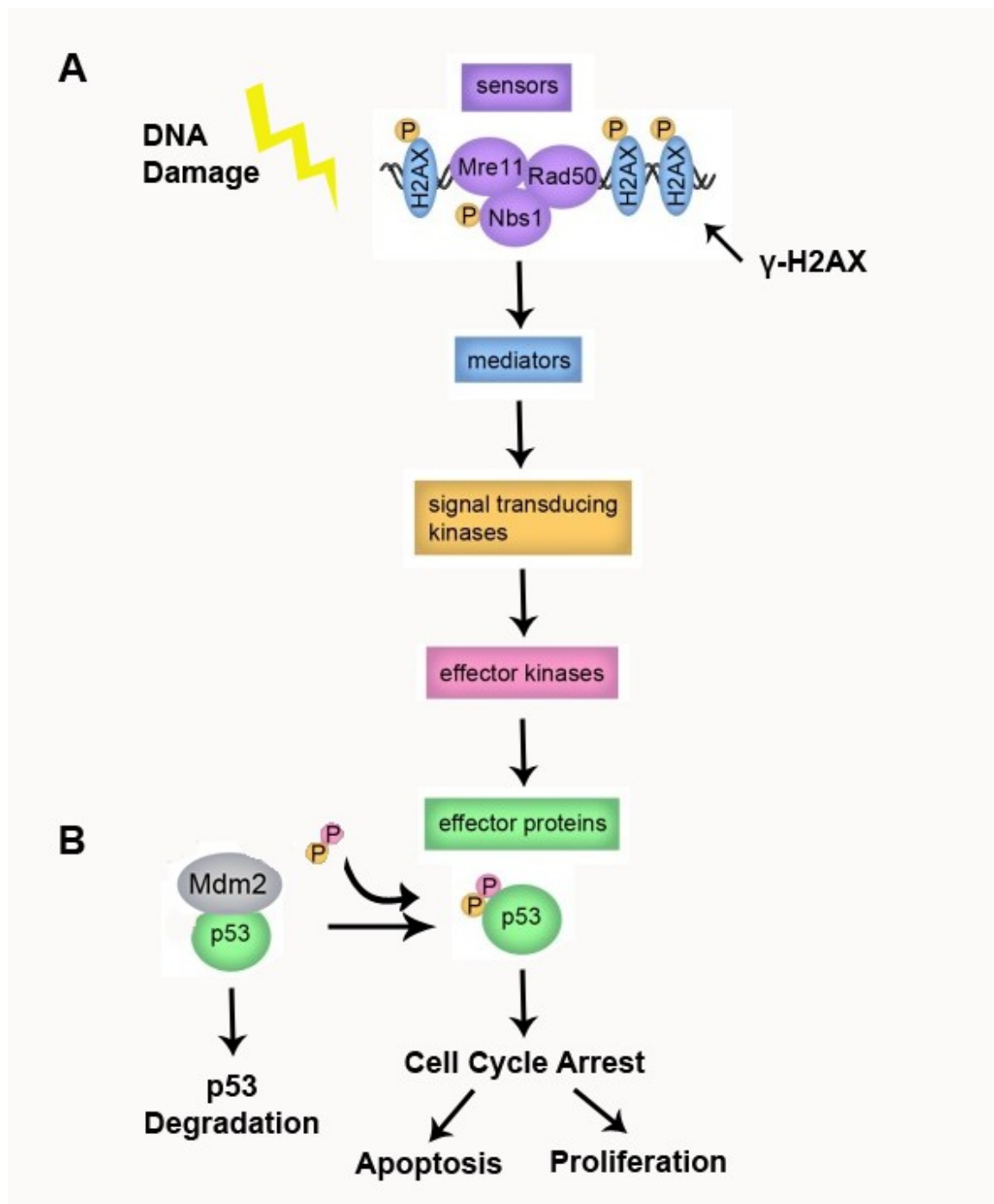


Figure 9: Brief overview of the DNA damage response and repair pathway. **A)** After DNA damage the H2AX histone protein is phosphorylated to form γ -H2AX, which retains repair factors at the damage site. A chain of events leads to the activation of effector proteins. **B)** p53, one of the effector proteins is activated by phosphorylation. p53 is normally targeted for degradation by Mdm2, but when phosphorylated, accumulates in the cell. P-p53 then participates in the cell's decision to continue cycling after sufficient repair, or undergo apoptosis if repair is not possible. Modified from Freeman et al. 2010.

breaks have been repaired, making it a useful assay to observe DNA repair over time *in vitro*.

1.7 Tumour Suppressor Protein p53

Another DNA damage response protein is the p53 protein. The p53 protein is very important in cancer development as it plays a role in cell-cycle control and apoptosis and acts as a tumour suppressor protein. This protein's function is so vital to cells that it had been deemed the "guardian of the genome" and the gene that encodes it is one of the most commonly mutated in cancer cells (Strachan and Read 1999).

The p53 protein is approximately 400 amino acid residues in length with the actual number varying between species. Some of the important domains found within this protein are the transcription activation domain, the core domain, and the C-terminal domain (Okorokov, Sherman et al. 2006). The core domain binds to DNA close to promoters of p53 target genes. p53 has a second DNA-binding domain, unlike some other transcription factors, at its C-terminus which complexes with DNA damage sites such as single and double-strand breaks and mismatched DNA (Okorokov, Sherman et al. 2006).

Under normal conditions very little p53 is found in cells. In a healthy cell, p53 is targeted by Mdm2 for rapid degradation keeping its levels low (Figure 9B). Mdm2 acts as a ubiquitin ligase, adding ubiquitin to p53 protein thereby targeting it for proteasome degradation (Alberts 2002). DNA damage to a cell results in phosphorylation of p53 (P-p53), resulting in an inability to associate with Mdm2, a lack of ubiquitination, and a decrease in p53 degradation allowing the protein to accumulate (Alberts 2002).

One guardian function of p53 is to prevent replication of damaged DNA (Strachan and Read 1999). Upon DNA damage, P-p53 induces the production of other proteins (Weinberg 2007) which all in turn lead to the arrest of cell proliferation until DNA is repaired (Figure 9B). Another guardian function of p53 is programmed cell death, or apoptosis (Strachan and Read 1999). If a cells' DNA is damaged beyond repair, P-p53 promotes the cell to undergo apoptosis to eliminate damaged DNA (Strachan and Read 1999). Without the guardian functions of p53, genetic mutations would be passed on to daughter cells which could threaten the viability and health of the organism that the cells compose (Weinberg 2007).

In cancer cells, mutations to the *p53* gene usually involve the DNA-binding domain. This results in a defective p53 protein that cannot bind target sequences and promote the transcriptional activation of downstream genes (Weinberg 2007). Without this transcriptional activation, important proteins such as p21, which is involved in preventing the G₁/S transition of the cell cycle, are not produced and damaged DNA continues unrepaired through the cell cycle (Alberts 2002).

1.8 Thesis Hypotheses and Objectives

The findings in previous carcinogen and regeneration studies suggest that tissues with regenerative capabilities possess unique anti-cancer properties. From these studies we hypothesized that newt tissue without the ability to regenerate is not refractory to tumour formation by chemical carcinogens. Based on this hypothesis, we developed the following objectives:

1. To induce tumours in non-regeneration capable newt tissues and
2. To characterize the response of newt cells to carcinogens at the molecular level.

The ultimate goal would be to establish a newt tumour cell line and see if the tumours are transplantable to other newts. In addition, we could then study the response of these tumours if a regeneration event is induced in the animal.

To our knowledge, no work has been conducted investigating how newts respond to DNA damage at a molecular level. Due to a lack of information on this subject in the literature, it will be interesting to compare the response of newt cells and mammalian cells to damage at the genetic level. If newt cells are truly refractory to cancer development, one would expect that newt cells would have a unique molecular profile after DNA damage. Any observations gained at the molecular level in newt cells from these experiments may be useful in understanding cancer cell progression and the cancer-refractory nature of newt cells.

2. METHODS

2.1 Animals

All newts used in this study were adult *Notophthalmus viridescens* (Eastern Red Spotted Newts) and were purchased from Charles D. Sullivan Co. Inc. (Nashville, Tennessee) or from Boreal Northwest (St. Catharines, Ontario). They were housed in 22-25 °C dechlorinated water on 12 hour light and dark cycles, and were fed blood worms (Aquatic Foods, California) twice a week.

All frogs used in this study were *Xenopus tropicalis* (Western Clawed Frog) and were a generous gift from Dr. Vance Trudeau from the University of Ottawa Biology Department. The frogs were raised from tadpoles following the protocol found in Appendix 1. Once in froglet form, they were maintained in 25 °C water on 12 hour light and dark cycles and fed crushed frog brittle (Nasco) 2-3 times a week. All animals used in these studies were cared for in accordance with the University of Ottawa Animal Care Committee guidelines.

2.2 Carcinogen Crystal Implantation

Three different carcinogens were used for implantation studies: N-Methyl-N'-Nitrosoguanidine (MNNG; TCI America), 4-Nitroquinoline-1-Oxide (NQO; Sigma), and 3-Methylcholanthrene (MCA; Sigma). Implanting the carcinogens under the skin allows them to exert their mutagenic effects by altering the skin cell's DNA structure. Portions of the chemical attach to the DNA double helix causing difficulties with DNA replication. This could lead to mutations in the cells genetic code allowing for uncontrolled proliferation (Platt, Aderhold et al. 2008).

2.2.1 Forelimb and Scapula Implantation of Three Different Carcinogens

Newts were anaesthetised using 0.5 % Tricaine Methanesulfonate (MS-222; Appendix 1), a common aquatic anaesthetic. The three carcinogens were each implanted into 10 newts by surgical incision, 1-2 mm wide with a razor blade, in the forelimb or scapula region. A scalpel was used to cut under the skin, around the incision site, to create a pocket. Notched forceps (Roboz) were used to lift the skin surrounding the incision and carcinogen crystals were placed in the pocket beneath the skin using #5 forceps (Dumont). Similar amounts of carcinogens were implanted into the animals. The incision was sealed with either super glue (Titan) or New-Skin® Liquid Bandage (Prestige Brands Inc.) and newts were placed in a Petri dish containing half water and half MS-222 for approximately 10 minutes or until the glue dried. The newts were placed in a shallow tank containing dechlorinated water for recovery and then placed in their habitat tanks. Newts were observed 2-3 times per week for 2-4 months for morphological changes due to the carcinogens.

2.2.2 Implantation of MCA into Newt Flank

MCA was implanted into the flank region of 10 newts. The process was carried out as above, with a more superficial incision into the dermis and muscle. The incisions were filled completely with MCA crystals and sealed using New-Skin®. Newts were allowed to recover as in Section 2.2.1 and were observed 2-3 times per week for two months for any morphological changes.

2.2.3 Implantation of MCA into the Flanks of Newts and Frogs

15 newts and 15 frogs were implanted with MCA crystals in their flank. The newts and frogs were anaesthetised using MS-222. As the frogs were not living in

dechlorinated water, their MS-222 was made using half dechlorinated water and half 1:20 Ringers solution at 25 °C. A razor blade was used to make the initial incision in the newts and then a scalpel to generate a pocket for the carcinogen to sit in. Carcinogen crystals were implanted until the flank pocket was full and the incision was sealed with super glue. In frogs, microdissection scissors were used to make the incision which opened up to the internal cavity, so no pocket formation was required. To keep consistency between animals, approximately the same number of crystals were implanted into the frogs as were in the newts. As with newts, frog incisions were sealed with super glue. Animals were observed 2-3 times per week for up to 12 months for any morphological changes.

2.3 Carcinogen Suspension Injection

2.3.1 Injection of MCA and NQO into Newt Scapula

MCA and NQO were suspended in olive oil at concentrations of 0.91 mg/mL and 10 mg/kg respectively by pipetting up and down. A 12 µl drop of the carcinogen suspension was pipetted onto a piece of parafilm and approximately 10 µl was backloaded into a 28 gauge insulin syringe. Olive oil was used as a control injection. Thirty newts were placed in MS-222, anaesthetised, and were injected in the right scapula region; 10 newts were injected with each carcinogen, and an additional 10 animals were used for control injections. The skin above the scapula was pinched with forceps and the syringe was inserted. The skin was released and the carcinogen was injected. Using a 20 gauge needle, a drop of New Skin® was placed on the skin around the still inserted syringe. The syringe was then removed and the newts were placed in a Petri dish containing half MS-222 and half water for a few minutes until the New Skin® had time

to dry. Newts were placed in a plastic tank with a few centimetres of water until they recovered and were then placed in their habitat tanks. The newts were observed 2-3 times per week for up to five months for morphological changes.

2.3.2 Multiple MCA Injections in Newt Flank

MCA was suspended in olive oil at 1.3 mg/mL. A 28 gauge insulin syringe was filled with 250 μ l of the MCA suspension and 10 μ l was injected into each of 10 anaesthetised newts. Injections were made in the right flank between the forelimb and hindlimb. Newts were injected twice; once at time zero and once at 1 week in the same area. For these injections, nothing was used to seal the injection site. The newts were observed 2-3 times per week for two and a half months for morphological changes.

2.3.3 Single High Dose MCA Injection in Newt Flank

MCA was suspended in olive oil at 2.7 mg/mL and was injected once in the right flank between the forelimb and hindlimb. As in the previous injection experiment 10 newts were anaesthetised using MS-222 and injected with 10 μ l of the carcinogen suspension. The syringe was filled as above and injection sites were not sealed. The newts were observed 2-3 times per week for three months for morphological changes.

2.3.4 MCA Injection in Newt Flank and Hindlimb Observed Over Five Weeks

MCA crystals were suspended in olive oil at 2.7 mg/mL by heating at 65 °C for 1 minute, vortexing, and incubating at 37 °C for 1 hour. The suspension was drawn into a 1 mL syringe with a 25 gauge needle, approximately 500 μ l total. Approximately 10 μ l of the suspension was injected into each of 40 anaesthetised newts; 20 in the right hindlimb, and 20 in the right flank between the forelimb and hindlimb. 10 μ l of olive oil was injected as a control on the contralateral side of each animal. Three animals from

each group were anaesthetised in MS-222 and sacrificed every week for a total of five weeks. The tissue from each week was processed as described in section 2.6. The number of animals collected from each group only totalled 15, but 20 were injected to account for the animals that might not survive the five week period.

2.4 Cell Culture

Mouse muscle cells (C2C12) and mouse fibroblast cells (H38.5) were cultured using Dulbecco's Modified Eagle's Medium (DMEM; HyClone) supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone or Biowest), 2mM L-glutamine (Gibco), 100 units/mL penicillin-0.1mg/mL streptomycin (Gibco) and 1mM sodium pyruvate (Sigma). The cells were cultured at 37 °C and 5% CO₂.

Newt primary muscle cells (A1) were cultured using Minimum Essential Media (MEM; HyClone) supplemented with 10% heat inactivated FBS, 24% tissue culture grade water, 2mM L-glutamine, and 1x antibiotic-antimycotic (Gibco). The cells were cultured at 25 °C and 3% CO₂. Plates used to grow A1 cells were coated with 0.75% gelatin (Sigma), made in Milli-Q (Millipore) water and filter sterilized. Plates had gelatin added for five minutes with any excess aspirated off before the addition of media to the plate.

2.5 Cell Treatment

2.5.1 Irradiation

Irradiation treatments were carried out using a Pantak HF-320 irradiator (250 kV, 12.5 mA; Pantak Ltd., East Haven CT, USA). The equation used to determine the amount of irradiation time needed for a certain number of Gray (Gy) was determined by

different calibrations of the machine based on the culture dishes used and were as follows:

$$\text{Time (sec)} = 0.7514 \times \text{Dose (cGy)} + 3.482 \text{ (4-chamber plate)}$$

$$\text{Time (sec)} = 0.744 \times \text{Dose (cGy)} + 3.23 \text{ (10 cm plate)}$$

2.5.2 Carcinogen Treatment

Carcinogen treatments were carried out using MCA partially solubilised in DMSO (Dimethyl Sulfoxide; Sigma) to form a suspension. A 2 mM suspension was made from an 80 mM stock suspension and diluted in the appropriate culture media to 20 μ M per culture plate. DMSO was used as a control in culture media at 1:100.

2.6 Tissue Processing and Histology

2.6.1 OCT Embedment

For tissue sampling in newts or frogs, the animal was anaesthetised in MS-222, sacrificed and the tissue was dissected. Tissue was fixed in 4% paraformaldehyde (PFA) at 4 °C for approximately 2 hours, rinsed for 20 minutes in 1x PBS (Phosphate Buffered Saline) and placed in 30 % sucrose overnight. It was then incubated in 1:1 30% Sucrose: Optimal Cutting Temperature (OCT) compound (Tissue-Tek) at 4 °C overnight. The tissue was transferred to a plastic base mold filled with 1:1 30% sucrose:OCT and then frozen on a Petri dish floating on liquid nitrogen. The molds were kept at -80 °C until sectioned. The tissue was sectioned at a thickness of 10 μ m on a Shandon Cryotome FSE (Thermo Electron Corporation), air dried for 2 hours and stored at -20 °C with dessicant. A complete protocol can be found in Appendix 1.

2.6.2 Paraffin Embedment

Tissue was fixed in 4% PFA at 4 °C for approximately two hours. The tissue was then run through a series of dehydration steps followed by substitution and embedment

with paraffin wax (Paraplast Tissue Embedding Media; McCormick Scientific; Appendix 1). The paraffin blocks containing the tissue samples were kept at room temperature until sectioned. Samples were sectioned at a thickness of 10 μm using a rotary microtome (Spencer 820, American Optical Company) and stored at room temperature.

2.6.3 Decalcification

Hindlimb samples were cut into 2 pieces to ensure maximized surface area exposure to the fixative. The samples were placed in PFA at 4 °C for 2 hours, rinsed in 50% ethanol and placed into Jenkins decalcifying solution for 5 days (Appendix 1). After decalcification, tissue was washed with 100% ethanol and embedded in paraffin wax as above.

2.6.4 Staining

Paraffin embedded sections were de-waxed. Paraffin and OCT sections were rehydrated. Slides were post fixed in 4% PFA, washed, and then stained with haematoxylin and eosin (Appendix 1). The slides were viewed using a Zeiss Axioskop II light microscope (Zeiss; Oberkochen, Germany).

2.6.5 Lump Tissue Processing and Cell Extraction

Two animals showing a morphological response to carcinogen, in the form of a lump beneath the skin, had the tissue photographed, and excised. The excised tissue was rinsed with 4% sodium hypochlorite and immediately rinsed with 80% PBS. The lump was cut into two pieces. The one which included the spine was fixed and embedded in paraffin as described in section 2.6.2. The other piece was processed to attempt to explant the cells into culture. The epidermis was removed and the remaining tissue was cut into 7 pieces; each piece was placed in one well of a 12 well plate (Corning) coated

with 0.75% gelatin. Each piece of tissue had a drop of media placed on it; just enough to cover the tissue. The 12 well plate was placed in a 25 °C, 3% CO₂ incubator overnight. A second drop of media was added to each piece the next morning. At 24 hours, 1 mL of media was added slowly to the side of the well and the tissue was left for 72 hours at 25 °C. At 72 hours, media was gently removed using a pipette and 1 mL of fresh media was again added slowly to the side of the well. This was repeated every 48 hours for the duration of the culture of the explanted tissue.

2.6.6 Immunocytochemistry after Irradiation

C2C12 and A1 cells were plated in 4-chamber plates (BD Falcon) at 1×10^5 and 7×10^4 cells per chamber respectively, with 500 µl of media in each well. 24 hours post plating, cells were irradiated with 1 Gy and fixed with 300 µl of 4% PFA in each chamber at 15 minutes, 1, 2, 4, 6 and 24 hours after irradiation. Cells were blocked in 5% goat serum (Jackson ImmunoResearch) for 30 minutes. Mouse anti-phospho-histone-H2AX primary antibody (1:400; Upstate) was added overnight at 4 °C to cells in a humidified chamber with the lids of the chamber plates removed. Cells were incubated with goat anti-mouse Cy3 secondary antibody (1:800; Jackson ImmunoResearch) for 30 minutes in the absence of light and counterstained with DAPI (1:10 000; Sigma). See Appendix 1 for full immunocytochemistry protocol. The slides were viewed using fluorescence microscopy (Zeiss Axioskop II; Zeiss; Oberkochen, Germany).

2.7 Protein Extraction and Western Blot

2.7.1 Irradiated Cells

C2C12, A1, and H38.5 cells were plated in 35 mm culture plates with 2 mL of their respective media at 9×10^4 , 6×10^4 , and 2×10^5 cells per dish respectively. 24 hours

after plating, cells were treated with 10 Gy of irradiation. Proteins were extracted at 10 minutes, 30 minutes, 1, 2, 4, 6, and 8 hours post irradiation by washing the cells in their respective PBS and adding 150 μ l of nuclear lysis buffer containing Protease Inhibitor Cocktail (Roche; Appendix 1). Plates were shaken on ice for 5 minutes and cells removed with a cell scraper. Samples were collected into a microcentrifuge tube and centrifuged at 13 400 RCF for 15 minutes at room temperature. The supernatant was transferred to a clean tube and stored at -80 °C. Protein concentration was determined using the DC protein assay (BioRad) and 15.5 μ g of sample protein as well as Precision Plus Protein Dual Color Standard marker (BioRad) were electrophoresed onto a 12 or 15% SDS-PAGE gel. Protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore) and the membranes were allowed to dry for at least two hours. Blots were reactivated with methanol for 15 seconds, washed with ddH₂O for two minutes and blocked for one hour in 5% skim milk powder in TBS-0.1% Tween 20 (TBS-T). Phospho-H2AX protein was detected by probing the blots with mouse anti-phospho-histone H2AX primary antibody (1:1000; Millipore), and a horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody (1:5000; GE Healthcare). Phospho-p53 protein was detected by probing the blots with rabbit anti-P-p53 primary antibody (1:1000; Cell Signaling), and a horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:5000; GE Healthcare). p53 was detected by probing the blots with rabbit anti-p53 primary antibody (1:1000, Cell Signaling), and a horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:5000; GE Healthcare).

SuperSignal West Pico Chemiluminescent Substrate detection kit (Pierce) was used to detect protein on the blots. Following detection, blots were washed four times with TBS-T and stripped using 0.2 M NaOH for 5 min. The blots were washed again four times with TBS-T, blocked for one hour and re-probed for rabbit anti- β -actin loading control primary antibody (1:5000; Abcam), and a horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:5000; GE Healthcare). Protein was detected with the SuperSignal kit as described above.

After films were developed they were scanned into digital versions using a Gel Doc™ XR+ Molecular Imager® (BioRad; California, USA). Densitometry of the bands was calculated using Image Lab™ Software (Biorad; California, USA). The intensity of each band was taken within a box of a predetermined size with local background subtraction. This intensity was then divided by its corresponding loading control (β -actin) intensity so that all bands were normalized to the amount of protein loaded.

2.7.2 Carcinogen Treated Cells

C2C12, A1, and H38.5 cells were plated in 60 mm culture dishes at 6×10^4 , 12×10^4 , and 14×10^4 cells per dish respectively, with 5 mL of media in each dish. 24 hours after plating, cells were treated with DMSO or MCA for 24 hours after which they were washed with their respective PBS and replaced with their respective media to recover. Proteins were extracted at 10 minutes, 30 minutes, 1, 2, 4, 6, and 8 hours after recovery for MCA treatments and 10 minutes and 8 hours after recovery for DMSO treatments by washing in PBS and adding 200 μ l of nuclear lysis buffer (Appendix 1). Protein extraction, gel electrophoresis, and gel transfer were completed as in section 2.7.1 with the exception that the lysed protein was spun down at 4 °C rather than room temperature.

Phospho-H2AX protein was detected by probing the blots with mouse anti-phospho-histone-H2AX primary antibody (1:1000), and a horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody (A1-1:5000, C2C12 and H38.5-1:2500). Phospho-p53 was detected by probing the blots with rabbit anti-P-p53 primary antibody (A1-1:1000, C2C12 and H38.5-1:500), and a horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (A1-1:5000, C2C12 and H38.5-1:2500). p53 was detected by probing the blots with rabbit anti-p53 primary antibody (1:500), and a horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:1000). Protein detection, blot stripping, reprobing with β -actin, and densitometry were all completed as in Section 2.7.1.

2.8 Clonogenic Assay

C2C12 cells were plated on 0.75% gelatin coated 10 cm culture dishes and irradiated 24 hours post plating according to Table 1A. Cells were mixed well before plating to ensure a single cell suspension as the cells need to be spread out and plated individually. The cells were allowed to adhere to the plates for approximately three hours in a 37 °C incubator before irradiation and the media was changed 24 hours post plating. All plates were fixed five days post plating when colonies of 50 or more cells were visible in the non-irradiated (0 Gy) cultures.

A1 cells were plated on 0.75% gelatin coated 10 cm culture dishes and irradiated 24 hours later according to Table 1B. All cells, irradiated or not, were left at room temperature for the duration of the irradiation treatments so that all of the cells would be exposed to the same conditions. The media was changed 24 hours post irradiation and then twice a week for the duration of the experiment. Cells were fixed when colonies of

Table 1: A) Cell plating numbers, irradiation dosages, and time in irradiator for clonogenic assay using C2C12 cells

Irradiation Dosage (Gy)	Time in Irradiator	Number of C2C12 Cells Plated	
		Plate 1	Plate 2
0	-----	100	200
2	2 min 32 sec	200	400
5	6 min 15 sec	500	1000
10	12 min 14 sec	10 000	20 000

B) Cell plating numbers, irradiation dosages, and time in irradiator for clonogenic assay using A1 cells

Irradiation Dosage (Gy)	Time in Irradiator	Number of A1 Cells Plated	
		Plate 1	Plate 2
0	-----	1000	2000
2	2 min 32 sec	2000	4000
5	6 min 15 sec	5000	10000
10	12 min 14 sec	50 000	60 000

50 or more cells were observed on the plates.

Cells were fixed according to a protocol used by Guda et al. (Guda, Natale et al. 2007). Media was removed from the plates and replaced with 3 mL of 2% methylene blue (Sigma) in 50 % ethanol. The stain was incubated at room temperature for five minutes and then poured off into a waste beaker. Dishes were dipped into a square glass pyrex dish filled with water slowly and repeatedly until the only blue visible on the plate was the colonies themselves. The plates were then placed in a fumehood to dry after which the colonies could be counted.

The cells response to irradiation was calculated using equations as found in Nature Protocols-Clonogenic Assay of Cells *in vitro* by Franken et al. (Franken, Rodermond et al. 2006).

$$\text{Plating Efficiency (PE)} = \frac{\text{no. colonies formed}}{\text{no. cells seeded}} \times 100\%$$

$$\text{Surviving Fraction (SF)} = \frac{\text{no. colonies formed after treatment}}{\text{no. cells seeded} \times \text{PE}}$$

2.9 Cell Proliferation Assay

2.9.1 Irradiated Cells

C2C12, A1, and H38.5 cells were plated in triplicate for both control and irradiated conditions in 35 mm culture plates with 2 mL of media at 3×10^4 , 7×10^4 , and 7×10^4 cells per plate respectively. Only three 35 mm dishes could fit in the irradiator at one time so one plate of each cell type was treated at the same time. Prior to irradiation the culture media was changed, but replaced with only 1 mL (to decrease the amount of WST-1 reagent used). 24 hours after plating cells adhered to their culture dishes and were irradiated with 10 Gy. Irradiation was repeated two more times for the remaining

two sets of cells. Untreated control cells were brought to the irradiator room to mimic the external conditions of treated cells. 100 μ l of WST-1 reagent (1:10; Roche) was added to A1 cells four hours after irradiation and to C2C12 and H38.5 cells 7.5 hours post irradiation, rocking each plate back and forth a few times after the addition. Eight hours after irradiation, 110 μ l of media was taken from each plate and placed in a 96-well flat bottom plate. Any bubbles in the wells were popped using a needle. 100 μ l of respective media and 10 μ l of WST-1 reagent were used as a blank. Sample absorbances were read on a plate reader (BioTek Synergy HT, BioTek) at 440 nm and 690 nm. The blanks' absorbances were subtracted from the samples' absorbances and the average absorbance for each cell type control and irradiated was calculated. The significance between control and irradiated averages was calculated using the student T-test.

2.9.2 Carcinogen Treated Cells

Cells were plated as in Section 2.9.1. The cells were plated in triplicate for both untreated, DMSO treated and carcinogen treated conditions. Cells were treated at 24 hours with either 20 μ M MCA dissolved in DMSO or DMSO alone at 1:100 diluted in growth media. Untreated controls were given a fresh media change. 24 hours post treatment of MCA or DMSO, cells were washed with PBS and fresh media was added to the plates. Before addition of the WST-1 reagent, 1 mL of media was removed from each plate (again to reduce the amount of WST-1 reagent needed). The WST-1 reagent was added and the absorbances were read and calculations completed in the same manner as found above (Section 2.9.1).

3. RESULTS

3.1 Carcinogen Implantation

3.1.1 Forelimb and Scapula Implantation of Three Different Carcinogens

The first carcinogen implantation experiment involved the subcutaneous implantation of crystals of MNNG, NQO, or MCA in the right forelimb or scapula area of the newt. These carcinogens were chosen as they were frequently used in many previous studies examining the effects of carcinogens on the regeneration process. Animals implanted with MNNG in the forelimb were observed for four months after surgery. After two weeks, one of the animals had noticeable degeneration of the implanted limb. The skin on the forelimb had regressed leaving bone visible (Figure 10A). Seven weeks post implantation, seven animals were remaining; all with a lack of normal skin pigment in the forelimb. This left the skin almost transparent with a redness resembling a pooling of blood beneath the skin surface. (Figure 10B and C). Newts implanted with MNNG in the scapula were observed for two months. Some animals showed redness, and later on white/depigmented skin around the implantation sites but had no other visible external response to the carcinogen (Figure 11).

None of the animals implanted with NQO in the forelimb showed a morphological response to the carcinogen (Table 2). Two of 10 newts implanted with NQO in the scapula region formed small discoloured lumps at the implant site in response to the carcinogen as early as one week post implantation. These scapula implanted animals differed from the MNNG scapula implanted samples as the redness surrounded a small lump at the implant site, and was more spread out over the scapula as well as less defined. Tissue samples were taken at nine days and seven weeks post

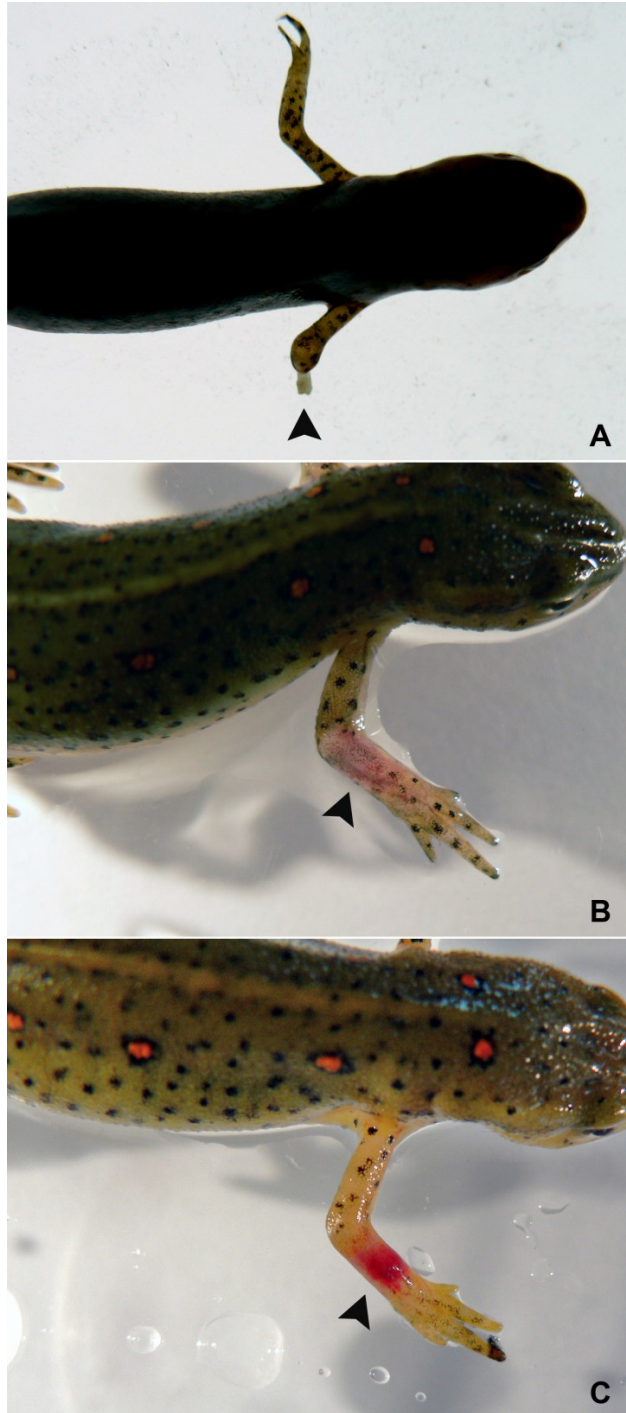


Figure 10: Morphological response of newt forelimbs implanted with MNNG. **A)** Right limb two weeks post implantation has degenerated and bone is exposed. **B)** Right limb three weeks post implantation shows skin thinning with visible blood vessels at the implant site. **C)** Right limb five weeks post implantation shows skin thinning with redness at the implant site.

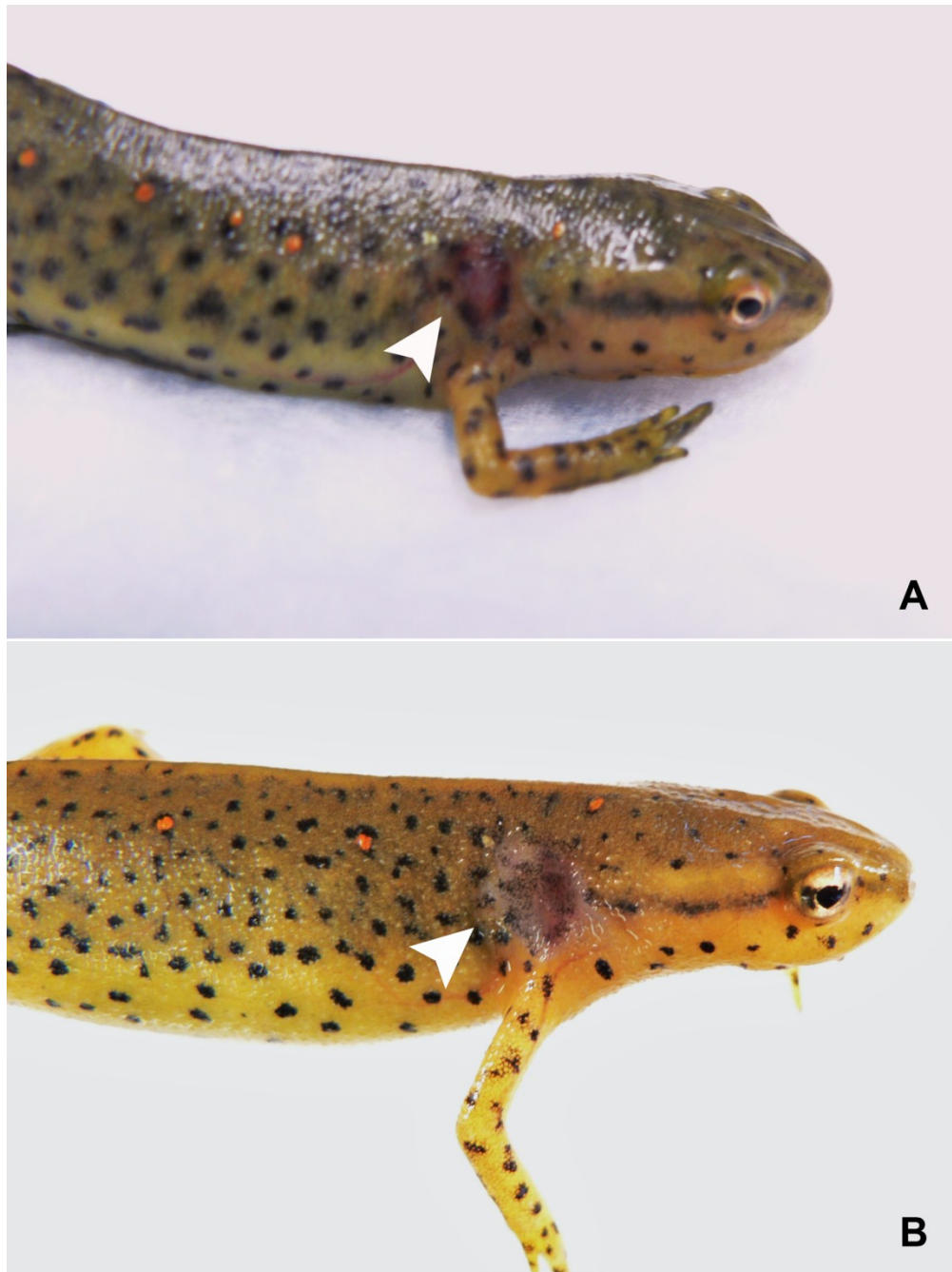


Figure 11: External morphological response of newt scapula implanted with MNNG. **A)** Two weeks post implantation slight redness visible around the administration site. **B)** Six weeks post implantation the skin at the implant site is still red and inflamed but with a white or depigmented ring around the site.

Table 2: Observational summary of newts implanted with three different carcinogens in the forelimb and scapula region.

Carcinogen Implanted	Implant Site	Duration of Observation (months)	# Newts Implanted	# Newts With “Tumour Like Growth”	Observations
MNNG	Forelimb	4	10	0	Redness and loss of pigment at implant site (Figure 10)
MNNG	Scapula	2	10	0	Redness at implant site (Figure 11)
NQO	Forelimb	2	10	0	None
NQO	Scapula	2.5	10	2	Lump Formation (Figure 12)
MCA	Forelimb	2.5	10	0	None
MCA	Scapula	2.5	10	2	Slight lump at implant site

implantation. The samples were fixed, sectioned, stained with haematoxylin and eosin (H&E) and a cancer pathologist, Dr. Manijeh Daneshmand, was consulted. No cancerous tissue or cells were found (Figure 12).

Of the 10 newts implanted with MCA in the forelimb, none showed a response to the presence of carcinogen. Two animals implanted with MCA in the scapula region had a very small lump form at the implantation site, but these did not continue to grow (Table 2).

3.1.2 Implantation of MCA into Newt Flank

Since none of the implantations of carcinogens into the forelimb or scapula of the newts resulted in tumour formation, it was hypothesized that this might be due to these sites being within or close to “regeneration fields”, i.e. regions of the body with regenerative ability. For this reason, the next set of carcinogen implantations were done in the flank of the animal, which does not have the ability to regenerate and is not part of a “regeneration field”.

None of the 10 animals implanted with MCA in the flank and followed for two months responded to the carcinogen with any visible changes. All animals continued to function and survive as if they were untreated newts (Table 3A).

3.1.3 Implantation of MCA into the Flanks of Newts and Frogs

None of the newts treated with MCA in the flank for two months showed any externally visible effects. For this reason, a second experiment was conducted with MCA implantation into the flank of 15 newts, but these animals were followed for a period of 12 months. In addition, 15 *Xenopus tropicalis* froglets were also implanted with MCA in the flank in order to determine if the lack of tumour formation was newt-specific.

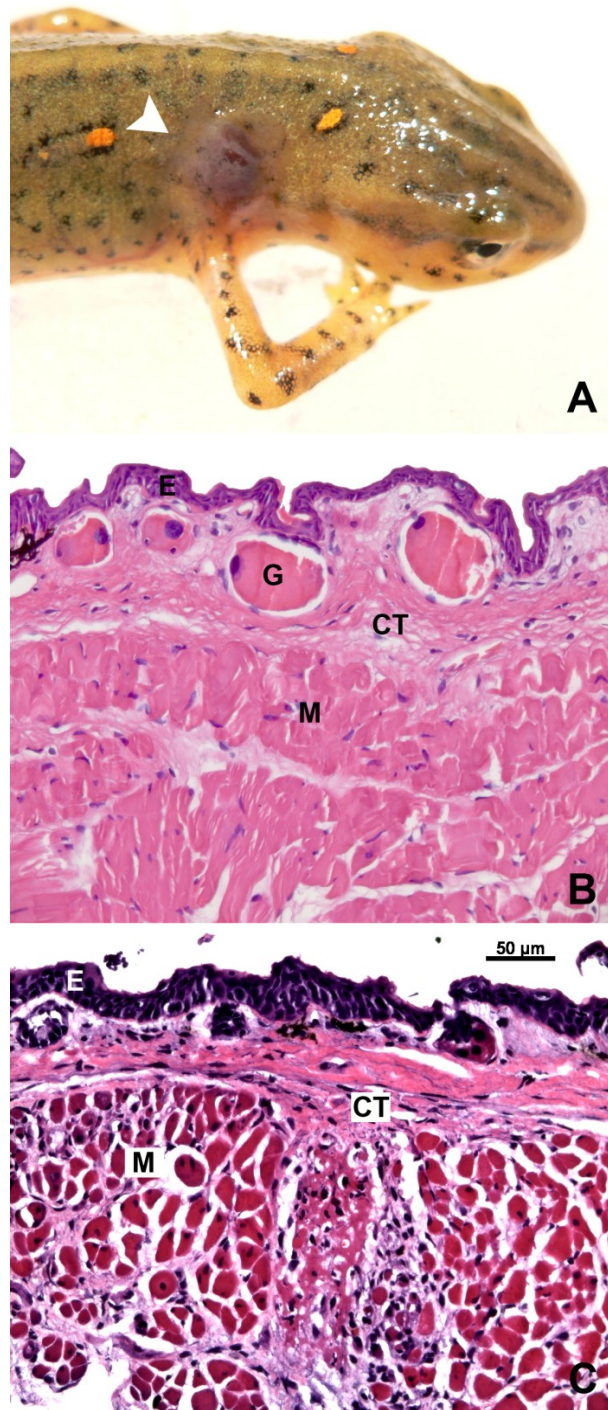


Figure 12: External morphological response and histological sections of newt scapula tissue implanted with NQO, 7 weeks post implant. **A)** Lump visible above newt scapula. **B)** Control tissue section after H&E staining. **C)** Implanted scapula tissue section after H&E staining. Superficial layers appear to be slightly thickened, but no unusual cells seen. E-epidermis; G-gland; CT-connective tissue; M-muscle.

Table 3: A) Observational summary of newts implanted with MCA in the flank.

Carcinogen	Duration of Observation (months)	# Newts Implanted	# Newts With “Tumour Like Growth”	Observations
MCA	2	10	0	None

B) Observational summary of newts and frogs implanted with MCA in the flank.

Carcinogen	Animal Implanted	Duration of Observation (months)	# Animals Implanted	# Animals With “Tumour Like Growth”	Observations
MCA	Newt	12	15	0	None
MCA	Frog	12	15	5	-Pigmented spots (Figure 13) -Lump formation (Figure 14)

None of the 15 newts implanted with MCA in the flank for 12 months responded to the carcinogen with any visible changes (Table 3B). They continued to function and survive as untreated newts would with three of the newts surviving one year post implantation; the remaining animals died of causes unrelated to the carcinogen implantation at various time points after the implantation procedure. Of the 12 newts that died prior to the 12 month end point, none showed any signs of morphological change to the carcinogen implanted, and the three newts remaining at the one year mark also did not show any external signs of a response. Just over two years post implantation, the one remaining newt died due to a fungal infection. This newt developed a dark spot on its right flank near its hindlimb approximately 7 months previously, but this spot did not increase in size and was thus not sampled.

Thirteen frogs remained 12 months post MCA implantation in the flank. One animal formed a dark spot of skin resembling a mole directly opposite to the implantation site on the dorsal side of the animal, four months post implantation. This darkened tissue along with the implantation site were sampled, fixed and stained with haematoxylin-eosin eight months post implantation. When the sections were observed, the darkened tissue did not seem to differ in any way from control frog tissue other than having more pigment beneath the epidermis (Figure 13). The section of the implantation site showed an abundance of dermal glands, but no abnormal cells.

A second frog formed a lump directly beneath the incision site nine months post implantation. This tissue was sampled from the animal, fixed and stained (Figure 14). When the tissue was removed, it appeared to be vascularised, and there was a slight haemorrhage when it was detached from the underlying tissues. The tissue sections

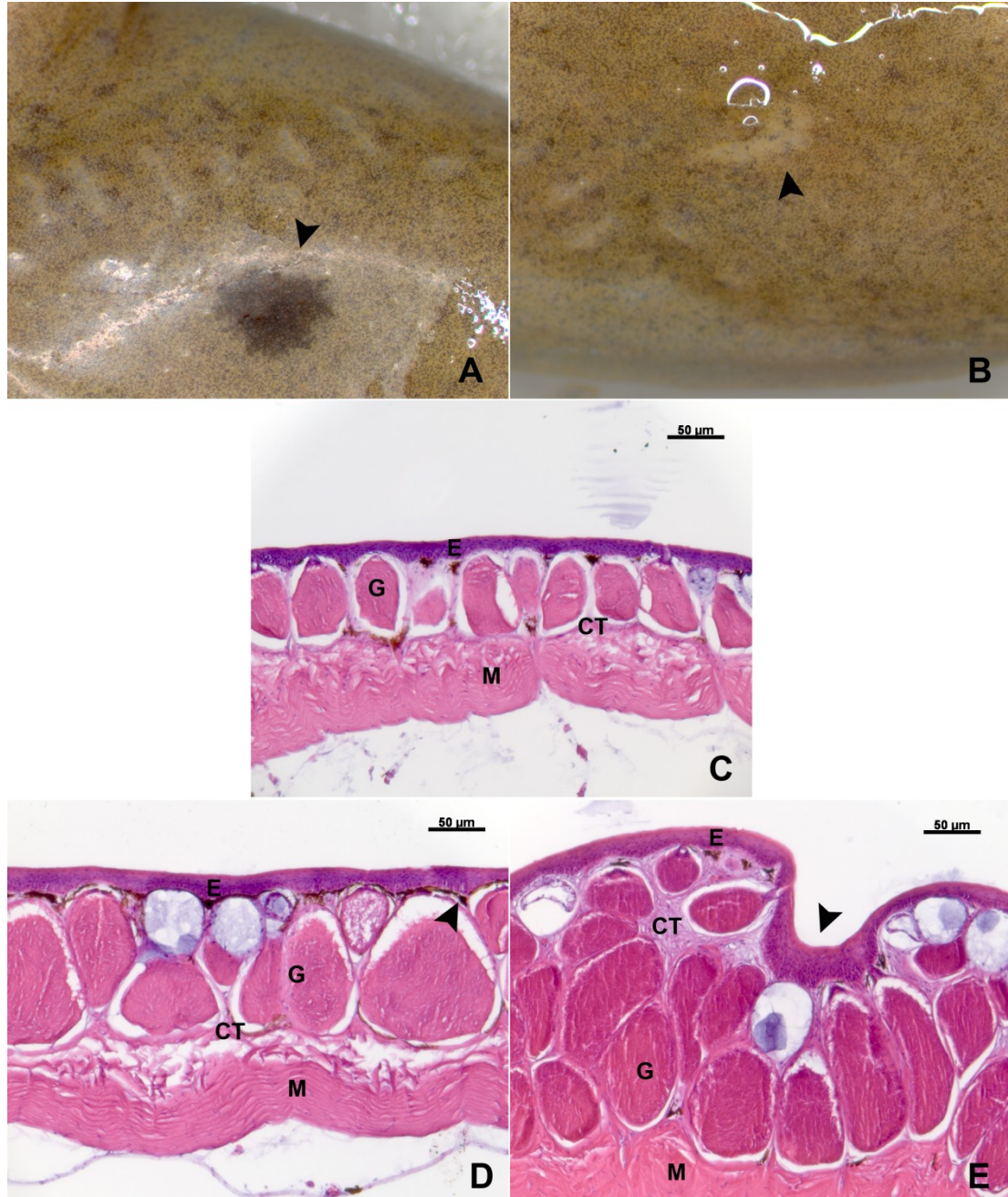


Figure 13: External views and histological sections of *Xenopus tropicalis* flank implanted with MCA four months previously. **A)** Shows the dark pigmentation on the dorsal side of the animal. Interestingly, this pigmentation was not found at the site of the carcinogen implantation. **B)** Shows the dorsal implantation site directly opposite the darkened spot. **C)** Control frog tissue after H&E staining. **D)** An H&E section of the pigmentation. Notice the accumulation of pigment cells beneath the epidermis (arrowhead). **E)** An H&E section of the incision site of implantation (arrowhead). Notice the abundance of dermal glands beneath the epidermis and the thickened epidermis at the incision site. E-epidermis; G-gland; CT-connective tissue; M-muscle.

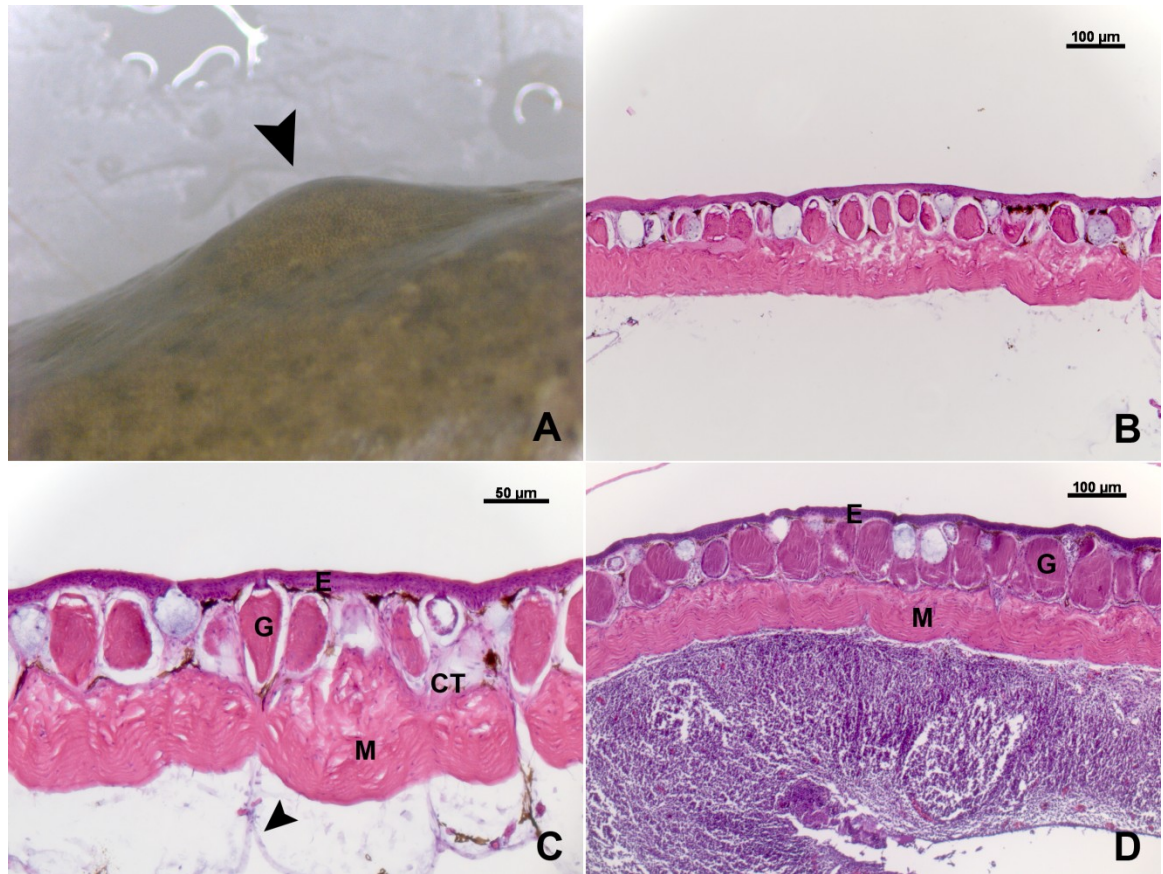


Figure 14: External view and histological sections of a lump of tissue found in *Xenopus tropicalis* flank 9 months post sub cutaneous implantation of MCA. **A)** The lump as seen in the frog. **B)** Control frog skin tissue stained with H&E. **C)** Control frog skin tissue stained with H&E. **D)** Lump tissue stained with H&E. Notice the mass of cells (purple) located beneath the muscle layer. E-epidermis; G-gland; CT-connective tissue; M-muscle.

showed a mass of cells located beneath the muscle layer. The cells in the mass appeared to be hyperproliferated versions of cells normally located beneath the muscle layer in control frog tissue sections (Figure 14C, arrowhead) and were described as a sub-dermal mass, or neoplasia by oncology pathologist Dr. Daneshmand.

Similar lump formation was seen in another frog at 11 months post implantation. The tissue was sampled at 14 months post implantation and the same mass of cells was found beneath the epidermis as seen in Figure 14D. Two other frogs presented with pigmentation spots at various locations on their body, as seen above (Figure 13). All three of these animals seemed to be functioning normally with no indication of any physical or behavioural problems.

Two years post implantation there were 12 frogs remaining. The pigmentation on two of the frogs did not appear to change in size or shape. Some of the animals still had their incision site scar visible, but no response to the implantations was observed. All 12 frogs were sacrificed two years post implantation. A brief autopsy revealed no external or internal visible masses resulting from the carcinogen treatment.

3.2 Carcinogen Injection

3.2.1 Injection of MCA and NQO into Newt Scapula

In the experiments discussed above, implantation of carcinogen crystals into the subcutaneous space did not yield any visible tumours in newts. In order to eliminate the possibility that this was due to a lack of carcinogen diffusion and penetration into the underlying tissues, for the next set of experiments, carcinogens were suspended in vehicle and injected subcutaneously. This was only done for NQO and MCA since MNNG did not show any effects in the previous experiments.

Ten animals were injected with 10 μ l of either NQO (suspended in olive oil at a concentration of 10 mg/kg) or MCA (dissolved in olive oil at a concentration of 0.91 mg/mL) and were observed for two to five months. Two of the NQO injected animals and one of the MCA injected animals formed a very slight external lump three weeks post injection (Table 4). The raised area in the scapula region did not continue to develop and so tissue samples were not taken. The animals were observed for two to five months with no additional growth or changes seen.

3.2.2 Multiple MCA Injections in Newt Flank

Since altering the mode of carcinogen delivery from implantation to injection did not yield any different results, injections were conducted in the flank, as with the latter implantation experiments, to avoid the potential effects of a “regeneration field”. Ten newts were twice injected with 10 μ l of MCA (suspended in olive oil at a concentration of 1.3 mg/mL), with injections one week apart. These animals were observed for two and a half months for any externally visible response to the carcinogen.

Two of the animals showed a slight response to the carcinogen two weeks post injection (Table 5A). Since these raised areas in the flank did not progress into larger lumps, no tissue samples were taken.

3.2.3 Single High Dose MCA Injection in Newt Flank

This experiment was run concurrently with the multiple newt injection experiment described above. Instead of two low dose MCA injections, one high dose MCA injection was administered. 10 newts were injected once in the flank with 10 μ l of MCA (suspended in olive oil at a concentration of 2.7 mg/mL) and observed for three months. One newt showed a morphological response to the MCA injection in the form of a lump

Table 4: Observational summary of newts injected with two different carcinogens in the scapula region.

Carcinogen	Carcinogen Conc.	Injection Site	Duration of Observation (months)	# Newts Injected	# Newts With “Tumour Like Growth”	Observations
NQO	10 mg/kg	Scapula	5	10	2	Very small lump
MCA	0.91 mg/mL	Scapula	2	10	1	Very small lump
Olive Oil	-----	Scapula	5	10	0	None

Table 5: A) Observational summary of newts injected with MCA in the flank two times with injections one week apart

Carcinogen	Carcinogen Conc.	Injection Site	Duration of Observation (months)	# Newts Injected	# Newts With “Tumour Like Growth”	Observations
MCA	1.3 mg/mL 2x	Flank	2.5	10	2	Slight lump at injection site

B) Observational summary of newts injected with MCA at a high dose

Carcinogen	Carcinogen Conc.	Injection Site	Duration of Observation (months)	# Newts Injected	# Newts With “Tumour Like Growth”	Observations
MCA	2.7 mg/mL	Flank	3	10	1	Thickened tissue layers (Figure 15)

three weeks post injection (Table 5B). This tissue was sampled four weeks post injection. Some oedema was present before fixation as indicated by the spaces in between muscle fibres (Figure 15), and there was an increase in epithelial cell layers.

While there were no cancerous cells present in the lump tissue (this was confirmed by oncology pathologist Dr. Daneshmand), it was interesting to note that all of the layers in the tissue were thicker but yet still in organized and defined layers (Figure 15).

Tissue sampled for this experiment was fixed in PFA, bisected, and one piece embedded in OCT and one in paraffin. In comparing the H&E sections of both OCT and paraffin embedded specimens, the paraffin embedded samples appeared to have better kept their tissue structure and organization during sectioning and staining. Therefore PFA fixation and paraffin embedment was used from this point on to preserve samples.

3.2.4 MCA Injection in Newt Flank and Hindlimb Observed Over Five Weeks

With the absence of tumour formation in the previous implantation and injection experiments, it was hypothesized that newts may have an initial response to carcinogen exposure in the form of proliferating cells, but then perhaps are able to control and differentiate these cells, or clear them completely from the body. In order to monitor potential early responses to carcinogen, twenty newts were injected in either the flank or hindlimb with MCA (suspended in olive oil at a concentration of 2.7 mg/mL) and three animals from each group were sampled each week for a total of five weeks. Twenty animals were injected with olive oil alone for each injection area as a control. This would allow the animals' initial response as well as their response over time to the carcinogen to be monitored. Three newts injected with MCA in the hindlimb, as well as the flank, were

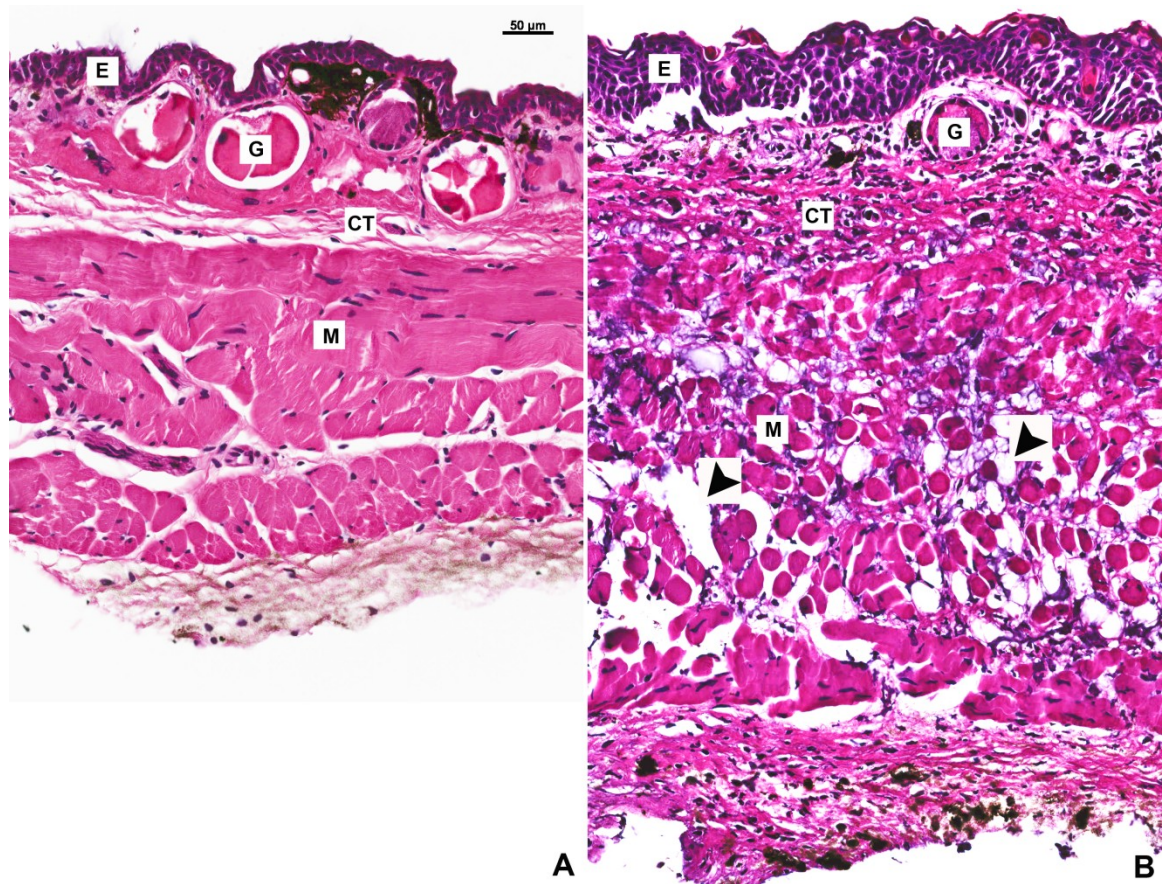


Figure 15: Histological sections of newt flank tissue injected with 2.7 mg/ml of MCA dissolved in olive oil, with H&E staining 4 weeks post injection. **A)** Control flank tissue section. **B)** Injected flank tissue section. Note the thickened but well defined tissue layers in B and spaces between muscle fibres (arrowheads) with no oncogenic cells present. E-epidermis; G-gland; CT-connective tissue; M-muscle.

approximated for tissue sampling. As the hindlimb tissue still contained the tibia and fibula, a decalcification step using Jenkins solution (Appendix 1) was necessary to allow sectioning of bone without tearing.

Extra newts were injected in each of the flank and hindlimb groups to ensure enough animals survived for sampling over the course of five weeks. Two of the flank injected newts were remaining after the initial five weeks, and were observed until 22 weeks post injection at which point they were sampled. Prior to sampling there were no external signs of a response to the carcinogen.

Only one of all of the injected animals showed a visible external response to the carcinogen. One hindlimb injected newt developed a lump dorsally between its forelimbs at 19 days post injection (Figure 16). This lump was dissected out including the spine at the same time as the hindlimbs were taken for sampling, two days after it was initially observed. Half of the lump tissue was explanted into culture (Figure 16B). The tissue was maintained in culture for over one month, but no cells were seen to migrate from the tissue onto the culture plate, so the tissue was discarded. The other half was fixed, sectioned and stained (Figure 16C). While the tissue layers appeared to be slightly thicker than normal newt tissue, there was no evidence of tumour formation, nor were there any cells that looked different than are seen in normal newt flank tissue (Table 6).

The H&E stained sections from over the course of the five week experiment were observed and Dr. Daneshmand was consulted. There were interesting proliferations found in flank treated samples and a few flank olive oil injected samples that Dr. Daneshmand compared to a basal cell carcinoma found in humans. Unfortunately, these proliferations were also found in untreated normal newt flank tissue (Figure 17). The

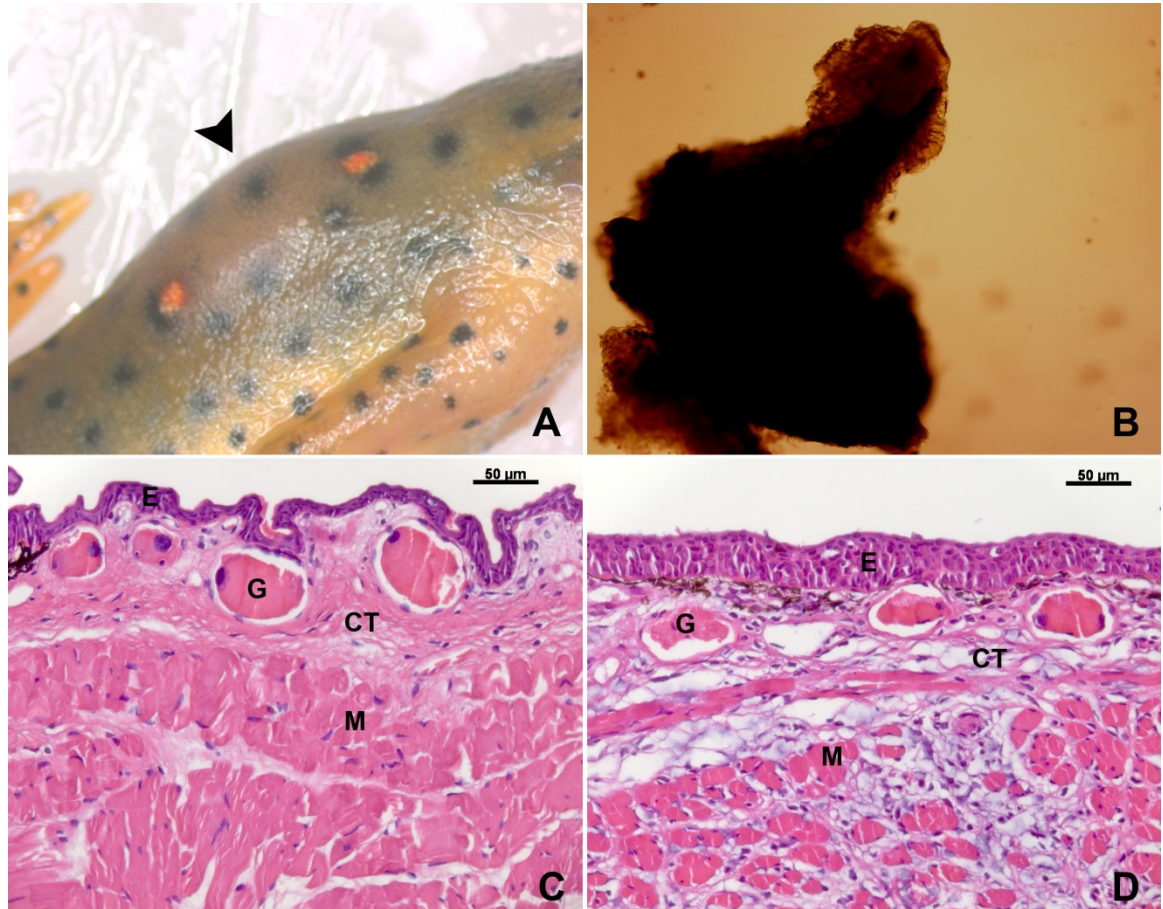


Figure 16: An externally visible lump found dorsally between the forelimbs in a newt 21 days post injection with MCA in the hind limb. **A)** External view of the lump before dissection from the newt. **B)** Lump tissue after being explanted into cell culture. Notice no cells migrating from the tissue. **C)** Control newt tissue H&E stained. **D)** Lump tissue after H&E staining. Notice the thickened epidermis (E) and down growth of connective tissue. E-epidermis; G- gland; CT-connective tissue; M-muscle.

Table 6: Observational summary of newts injected with MCA in the flank and hindlimb and sampled once a week for five weeks

Carcinogen	Carcinogen Conc.	Injection Site	Duration of Observation (weeks)	# Newts Injected	# Newts With “Tumour Like Formation”	Observations
MCA	2.7 mg/mL	Flank (R)	5	20	0	Epithelial Proliferations (Figure 17)
MCA	2.7 mg/mL	Hindlimb (R)	5	20	1	Slight lump found dorsally between forelimbs
Olive Oil	-----	Flank (L) Hindlimb (L)	5	40	0	None

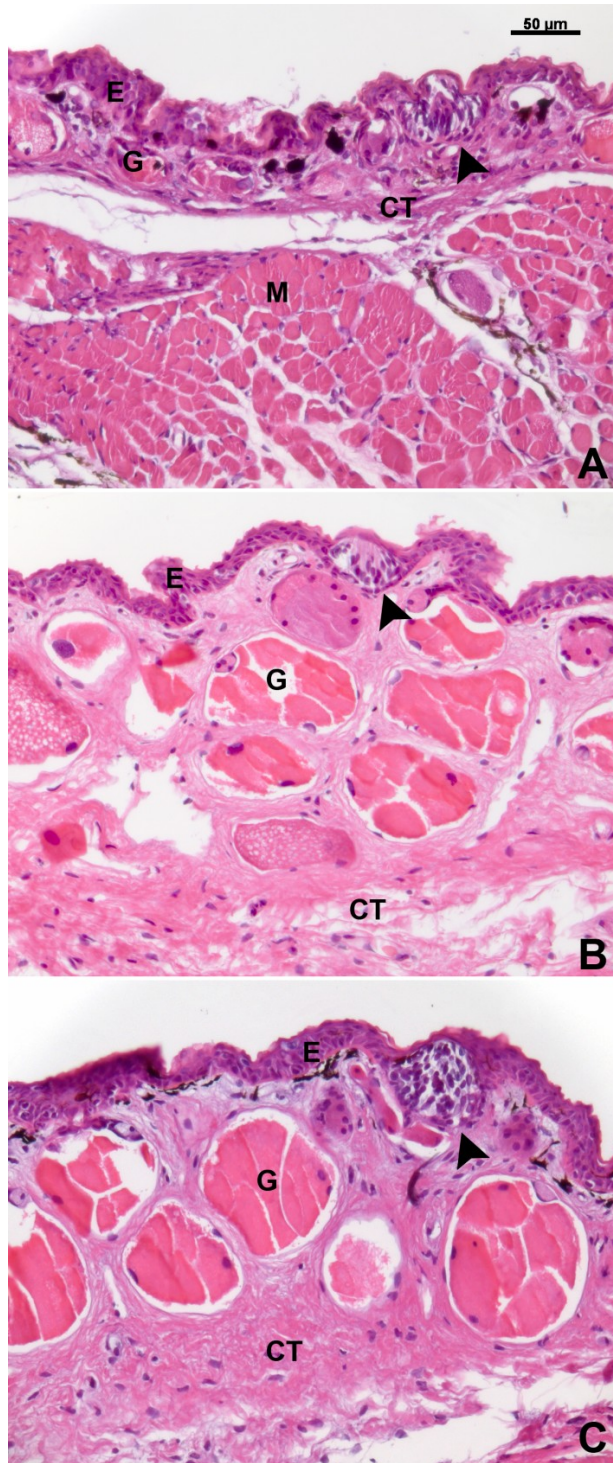


Figure 17: Newt flank tissue 1 week post injection of MCA after H&E staining. **A)** Control newt flank section without any injection. **B)** Section of olive oil injected control newt tissue. **C)** Section of MCA injected newt tissue. The arrowheads indicate an odd proliferation within the epidermis in all samples. E-epidermis; G- gland; CT-connective tissue.

hindlimb samples showed no difference between the olive oil injected and the carcinogen injected samples (data not shown).

3.3 *In vitro* Irradiation Treatments

Given that there was no tumour formation in the *in vivo* carcinogen studies, a new method of studying the newts' interesting response, or lack of one, to cancer causing agents was developed. Irradiation was chosen as a new method of causing DNA damage to newt and mouse cells in culture. With this experimental design, the response of newt and mouse muscle cells with respect to DNA damage could be compared.

3.3.1 Clonogenic Assays

Clonogenic assays were conducted on C2C12 and A1 cells to determine and compare their proliferation response after irradiation. Due to the very slow cycling time of A1 cells, clonogenic assays were too difficult to complete in this cell line. As a result of such drastically different cell cycle times between the two cell lines, any comparisons made between the two would be virtually meaningless. Therefore, this avenue of studying newt and mouse muscle cells response to irradiation was not further pursued.

3.3.2 γ -H2AX Protein Expression after Irradiation Using Immunocytochemistry and Western Blot Analysis

Exposure to irradiation causes double strand breaks within the DNA of a cell which can lead to uncontrolled proliferation. At the site of these DNA double strand breaks, histone protein H2AX is phosphorylated giving the γ -H2AX form of the protein. These phosphorylated proteins are visible as foci within the nucleus when using immunocytochemistry, allowing the protein's expression before and after exposure to irradiation, to be monitored.

C2C12 and A1 cells were irradiated with 1 Gy and their expression of γ -H2AX was monitored over time using immunocytochemistry. When stained for γ -H2AX expression, C2C12 unirradiated control cells were indistinguishable from irradiated cells (Figure 18). The A1 cells showed a similar response (Figure 18). This suggests that 1 Gy of irradiation is not sufficient to induce enough damage to distinguish a difference between control and irradiated cells. For this reason, a dose response was conducted in the next set of experiments below.

When C2C12 and A1 cells were initially irradiated with 1 Gy, there was a lack of γ -H2AX expression with both immunocytochemistry and western blot analysis. As a result of this, an irradiation dose response was conducted. C2C12, A1, and H38.5 cells were irradiated with 1, 5, or 10 Gy and immediately lysed. The western blot showed no expression of γ -H2AX in control cells and cells irradiated with 1 Gy (Figure 19). There was a slight γ -H2AX band at 5 Gy, and a distinct band at 10 Gy in all cell lines. From these results, 10 Gy was chosen as the irradiation dosage for all subsequent experiments.

When treated with 10 Gy of irradiation, C2C12 cells showed a slight peak of γ -H2AX expression at 30 minutes post irradiation which then decreased back to control cell levels by 4 hours post irradiation (Figures 20 A and B). H38.5 cells showed a similar trend as the C2C12 cells but with much higher γ -H2AX expression at 30 minutes and 1 hour post irradiation. A1 cells seemed to have a basal level of γ -H2AX expression in their normal unirradiated cells. After irradiation, γ -H2AX levels increased with a peak at 2 hours post irradiation. The protein levels then decreased back down to control levels, or even slightly below by 8 hours post irradiation.

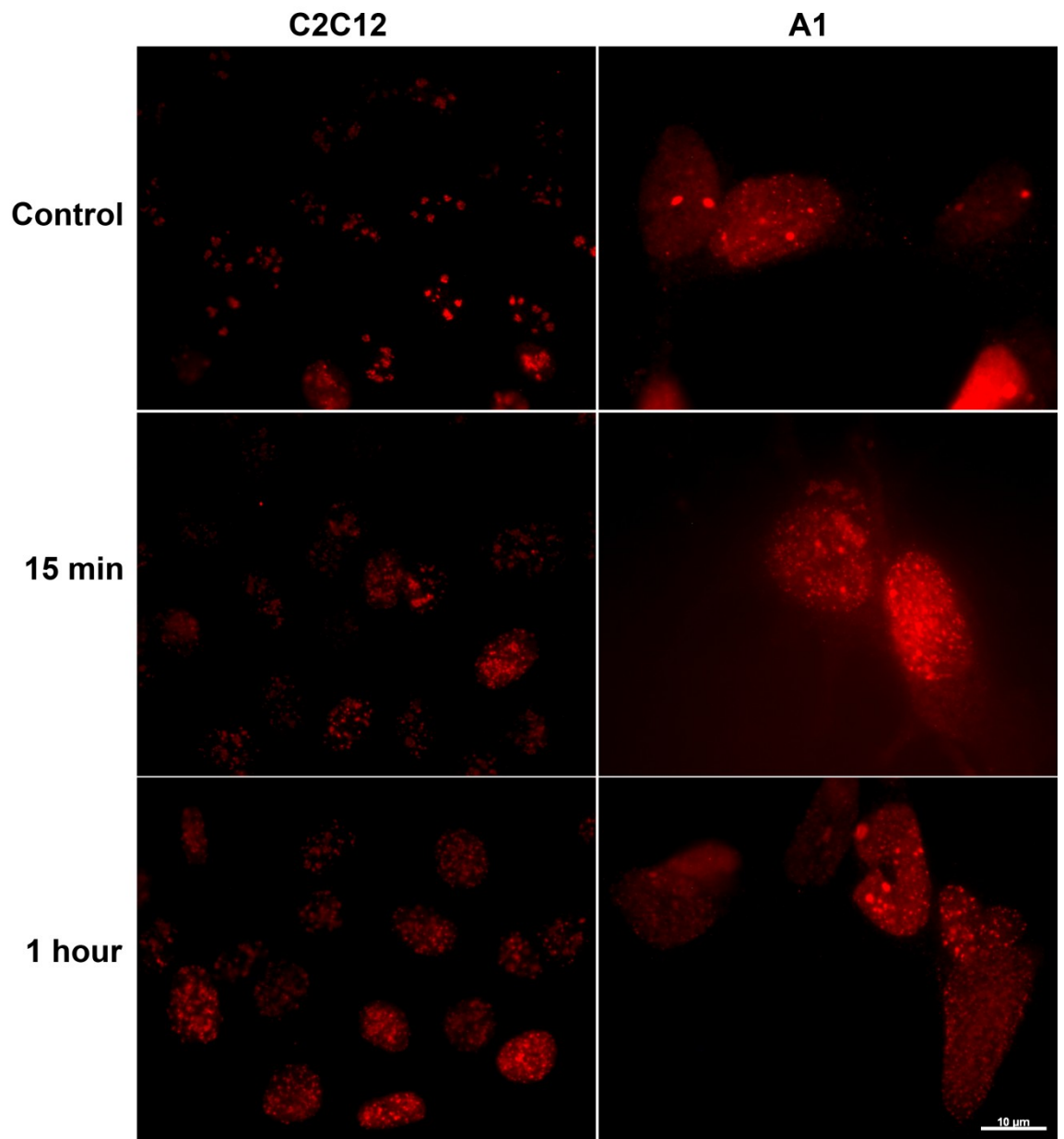


Figure 18: Fluorescence images of C2C12 and A1 control cells and cells irradiated with 1 Gy. Cells were allowed to recover for 15 minutes or 1 hour and were stained for γ -H2AX. Foci are visible in both control and irradiated cells making them indistinguishable from one another.

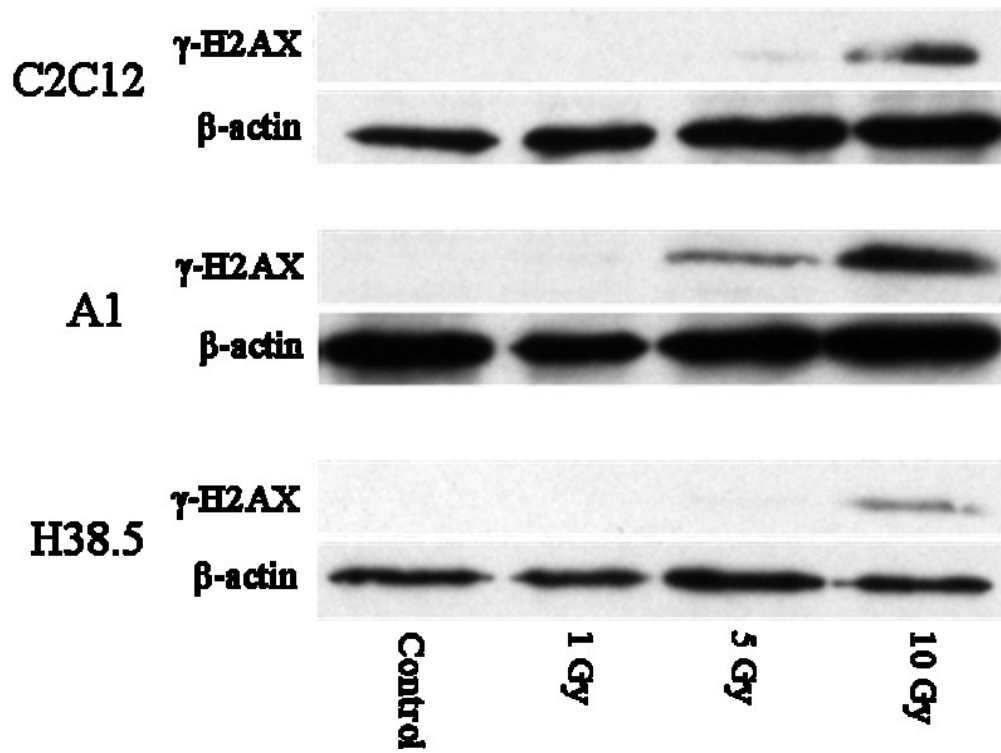


Figure 19: Western blots showing γ -H2AX expression in C2C12, A1, and H38.5 cells immediately after irradiation with varying irradiation dosages. β -actin is used as a loading control.

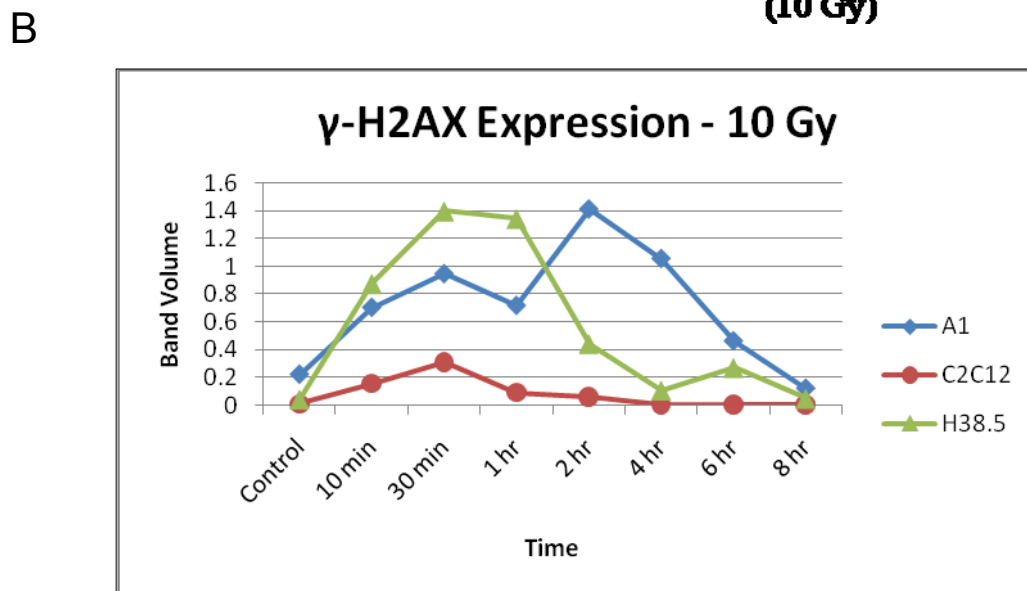
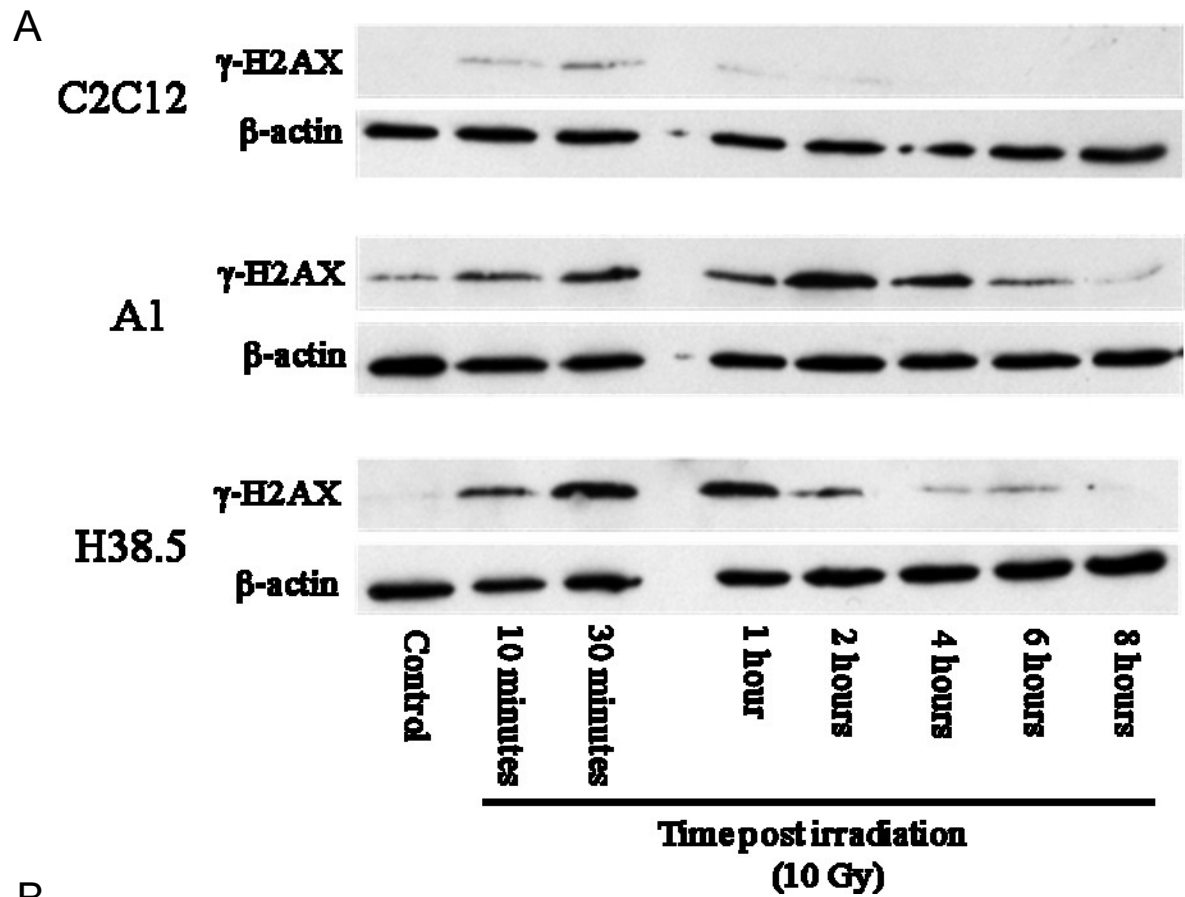


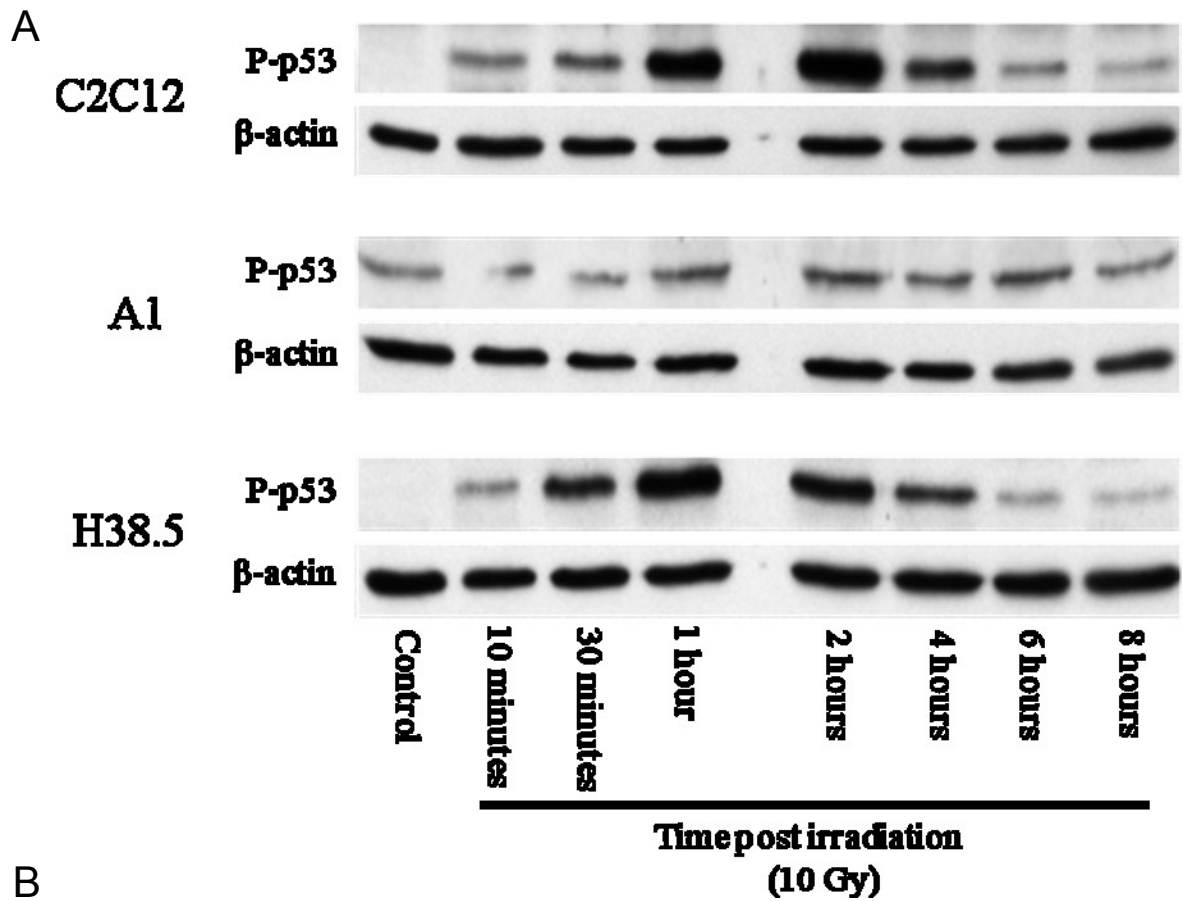
Figure 20: γ -H2AX protein expression in C2C12, A1, and H38.5 cells over time after being exposed to 10 Gy of irradiation. **A)** Western blots showing γ -H2AX protein expression and β -actin as a loading control. **B)** Densitometry showing γ -H2AX protein expression over time.

3.3.3 P-p53 and p53 Protein Expression after Irradiation Using Western Blot Analysis

The p53 protein is very important in cell cycle regulation and so plays a role in preventing cancer within a tissue. While normally degraded, p53 protein levels are increased and the protein is phosphorylated upon DNA damage. p53 and its phosphorylated version, P-p53, are proteins that play a role in cancer prevention and can be compared in newt and mouse cells over time after irradiation.

P-p53 expression increased after irradiation in C2C12 cells, peaking at 1 hour and then steadily decreased back down to control cell levels at 8 hours (Figure 21). H38.5 cells showed the same trend as the C2C12 cells, but with lower expression levels. A1 cells again had a basal level of P-p53 protein expression in control cells which increased at 1 hour, remained elevated up to 6 hours and came back down close to control cell levels at 8 hours. Similar to the γ -H2AX expression, A1 cells appeared to have a delayed response in comparison to H38.5 and C2C12 cells.

C2C12 and H38.5 cells again showed similar trends with respect to p53 expression post irradiation (Figure 22). Both cell lines showed the highest amount of p53 protein at 1 hour after irradiation and similar amounts still at 2 hours. Expression then decreased over time with a large drop at 6 hours post irradiation for the C2C12 cells, but a gradual decline for the H38.5 cells. The H38.5 cells did not make it back down to control cell levels of p53 protein by 8 hours after irradiation. A1 cells had a basal level of p53 protein expression in control cells, and even after irradiation appeared to be relatively unchanged over time.



B

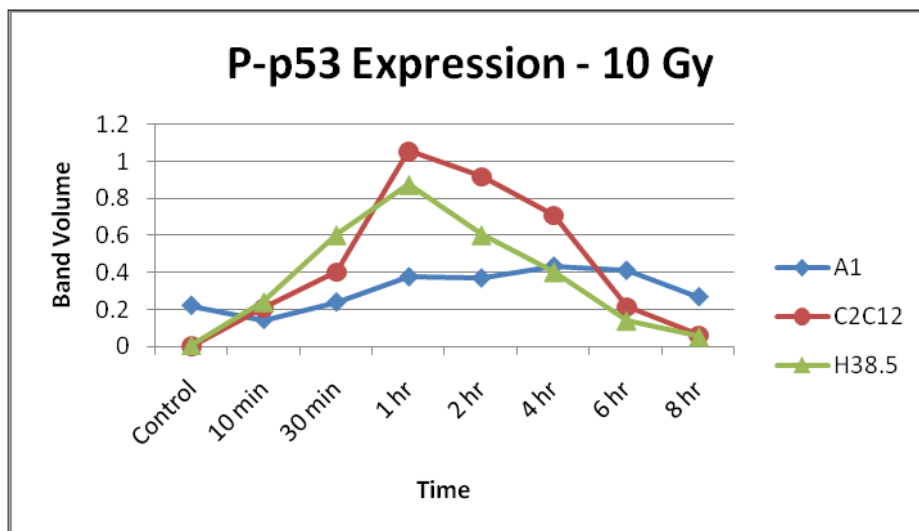


Figure 21: P-p53 protein expression in C2C12, A1, and H38.5 cells over time after being exposed to 10 Gy of irradiation. **A)** Western blots showing P-p53 expression with β -actin as a loading control. **B)** Densitometry showing P-p53 protein expression over time.

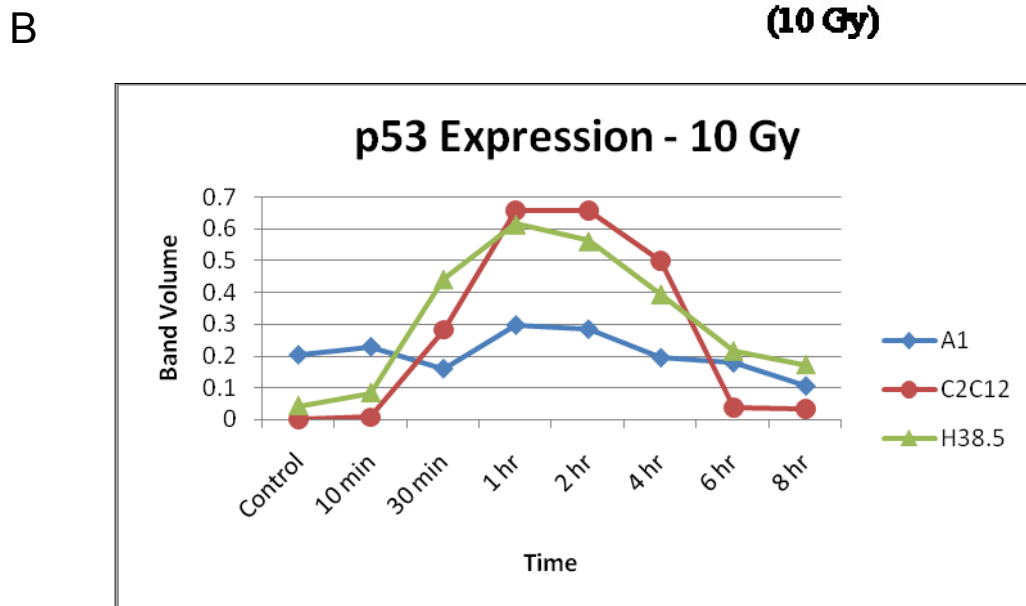
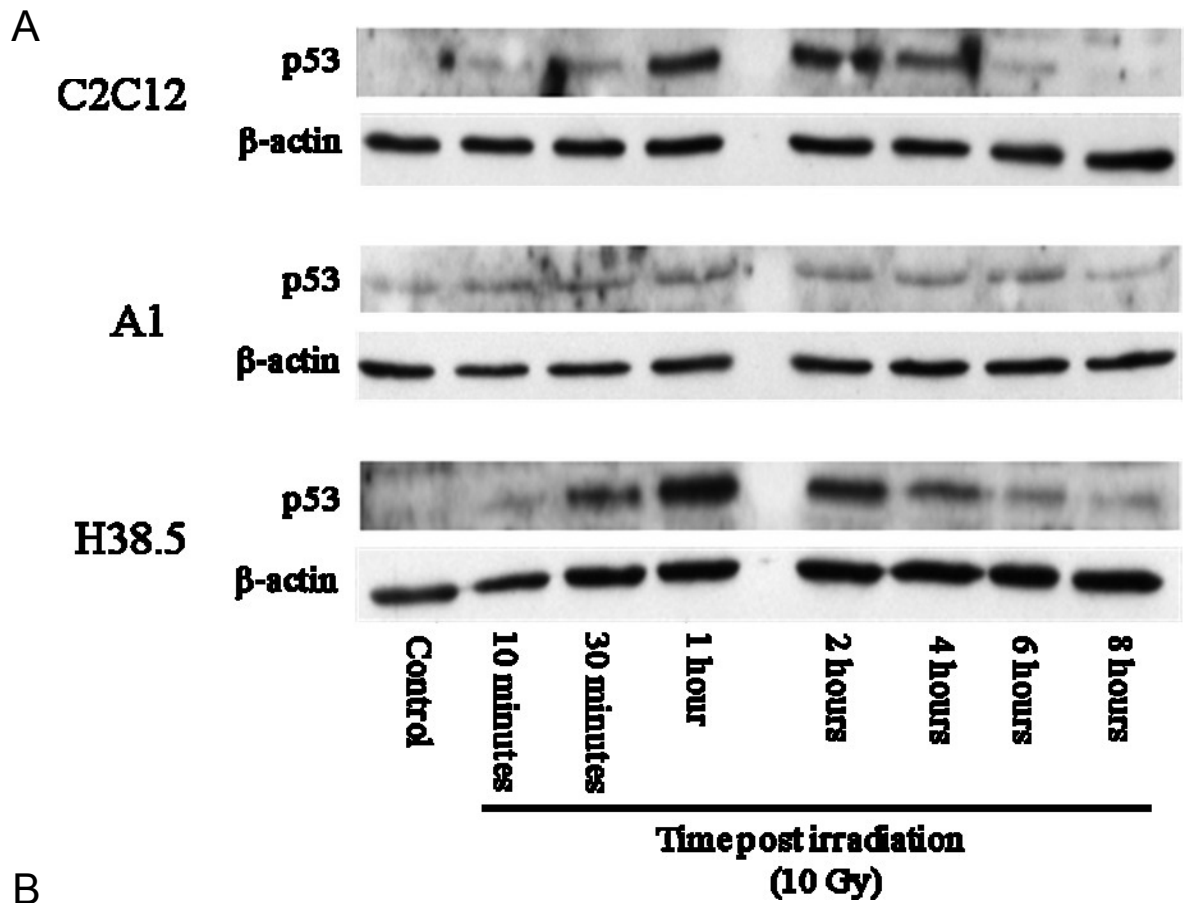


Figure 22: p53 protein expression in C2C12, A1, and H38.5 cells over time after being exposed to 10 Gy of irradiation. **A)** Western blots showing p53 expression with β -actin as a loading control. **B)** Densitometry showing p53 expression over time.

3.3.4 Cell Proliferation Assay

In order to determine if the changes in levels of γ -H2AX, P-p53, or p53 were due to differential expression after damage in the different cell types or due to a loss of protein resulting from cell death, a cell viability assay was conducted.

The cell viability for irradiated and control unirradiated C2C12, A1, and H38.5 cells was compared using WST-1 reagent. WST-1 is a colorimetric assay based on a cells' ability to cleave a salt found within the reagent resulting in a colour change of the cell media. Absorbance readings can be used to compare the viability of cells with or without treatment.

Absorbances read at 440nm for each cell type with or without irradiation showed no significant difference between the two groups in any of the cell lines (Figure 23). This indicated that any change in protein expression found in these cells was accurate and was not as a result of decreasing protein levels due to cell death.

3.4 Carcinogen Treatments

After obtaining differing results between mammalian and newt cells using irradiation as a DNA damaging agent, the carcinogen MCA was also used to attempt to induce DNA repair proteins in culture. 20 μ M of MCA suspended in DMSO was used to treat C2C12, A1, and H38.5 cells in culture for 24 hours and the expression of different DNA damage repair proteins such as γ -H2AX, P-p53, and p53 was monitored over time after removal of the carcinogen from the culture medium.

Cell Viability in Three Cell Types 8 hours after 10 Gy of Irradiation

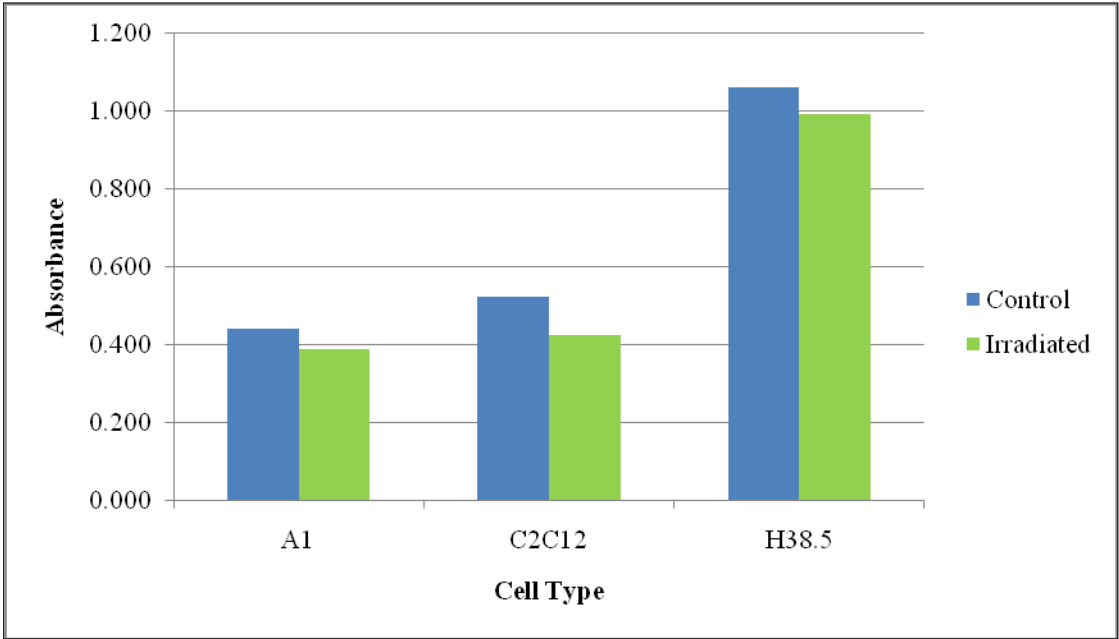


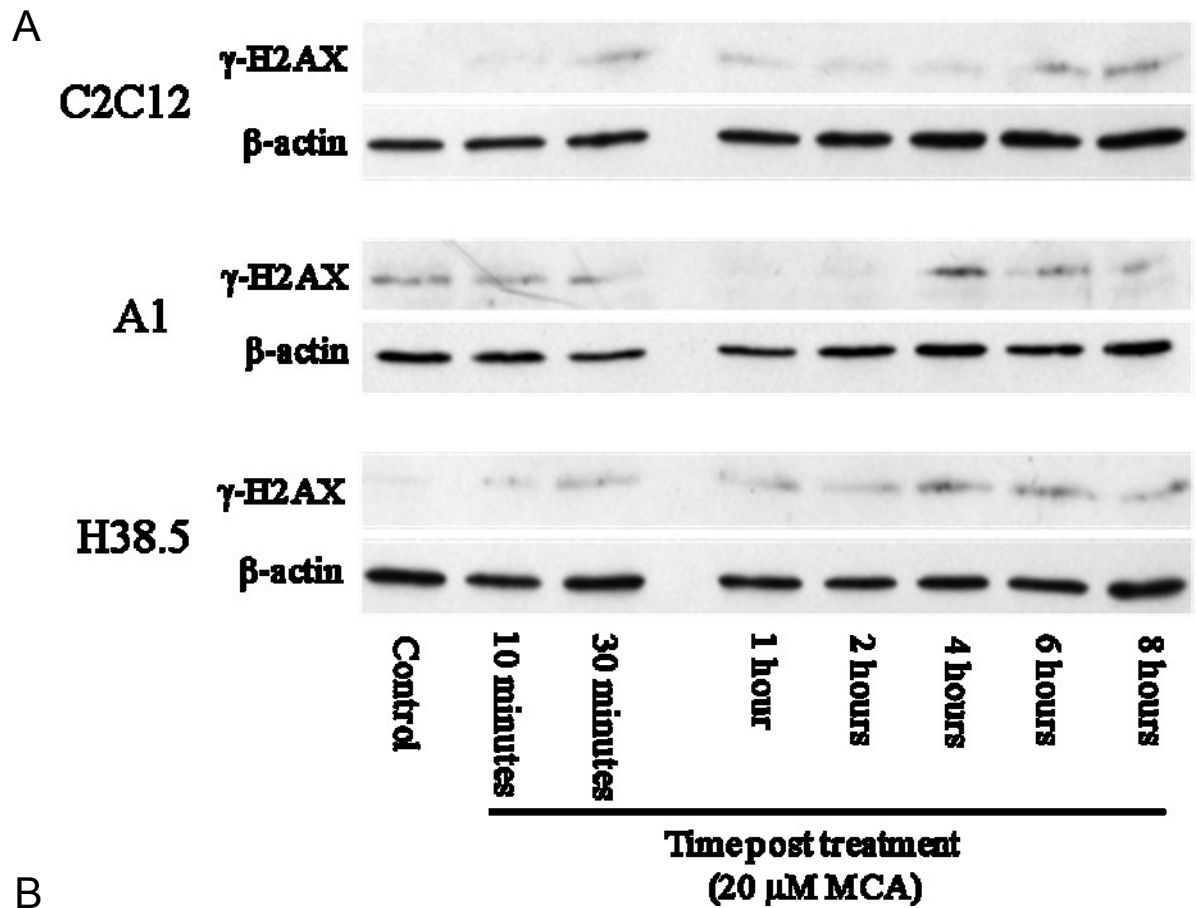
Figure 23: The average WST-1 absorbance at 440 nm for A1, C2C12, and H38.5 cells 8 hours after 10 Gy of irradiation. No significance was found between control and irradiated cells in any cell line using the students T-test. ($p > 0.05$, $n = 3$)

3.4.1 γ -H2AX, P-p53, and p53 Expression after MCA Treatment Using Western Blot Analysis

Untreated A1 cells showed a high level of γ -H2AX protein, but after MCA treatment, cells lost this expression over time until there was almost no protein left at 1 hour after removal of the carcinogen (Figure 24). γ -H2AX protein then had a peak of expression back to untreated cell levels at 4 hours which then decreased over the next 4 hours until the final 8 hour time point. In C2C12 cells, MCA induced expression of γ -H2AX which persisted until at least 8 hours after the carcinogen was removed. H38.5 cells appear to have a peak of γ -H2AX expression at 4 hours after the removal of carcinogen and this then declines at 8 hours.

A1 and C2C12 cells showed similar protein expression profiles for the P-p53 protein (Figure 25). Both cells lines had P-p53 protein levels around untreated cell levels for the duration of the time course after MCA treatment. H38.5 cells on the other hand appeared to have a large peak of P-p53 expression at 6 hours which then declined again at 8 hours.

p53 appeared to have a basal level of expression in all cell types regardless of the time after removal of the carcinogen (Figure 26). It is important to note that these results need to be repeated and that the carcinogen treatment protocol needs to be optimized before any conclusions can be drawn.



B

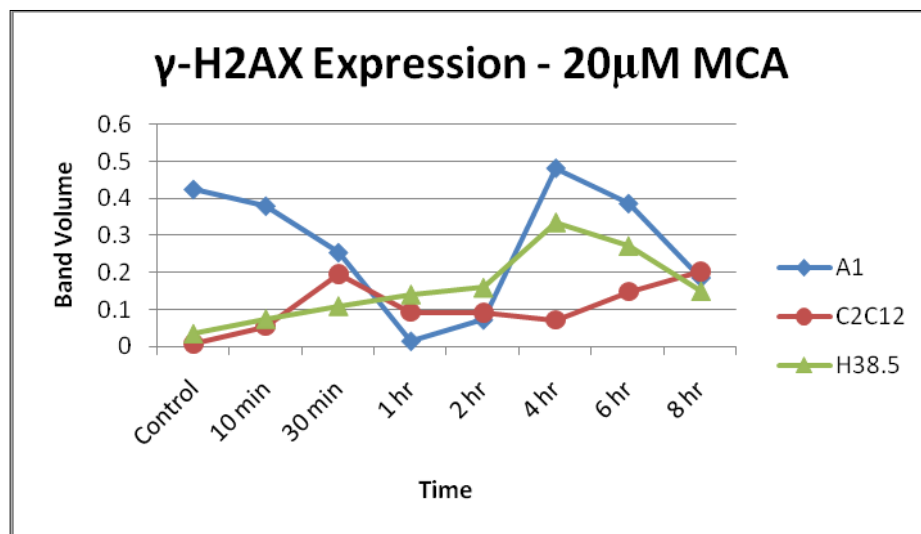


Figure 24: γ -H2AX protein expression in C2C12, A1, and H38.5 cells over time after being exposed to 20 μ M of MCA for 24 hours. **A)** Western blots showing γ -H2AX protein expression and β -actin as a loading control. **B)** Densitometry showing γ -H2AX protein expression over time.

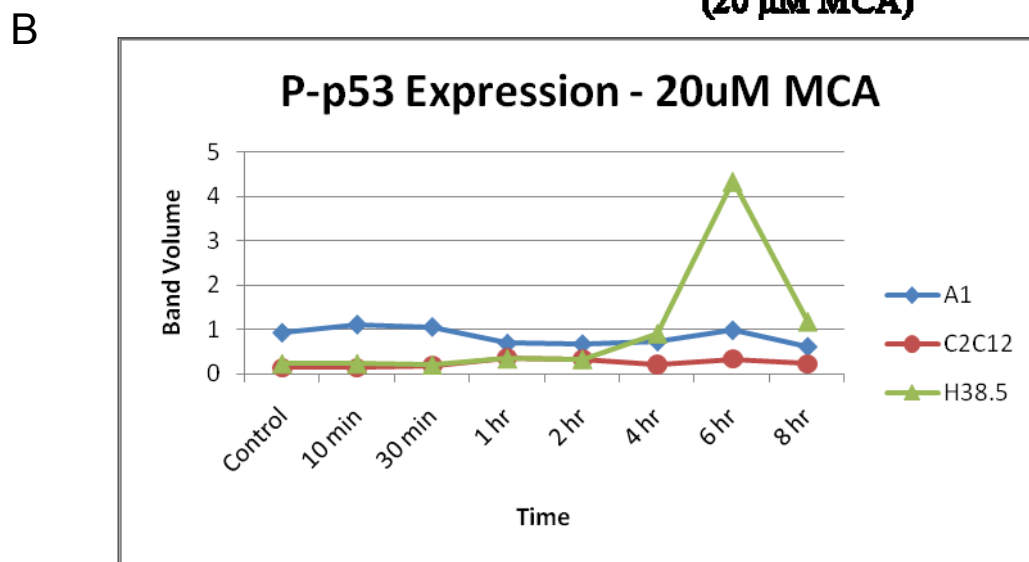
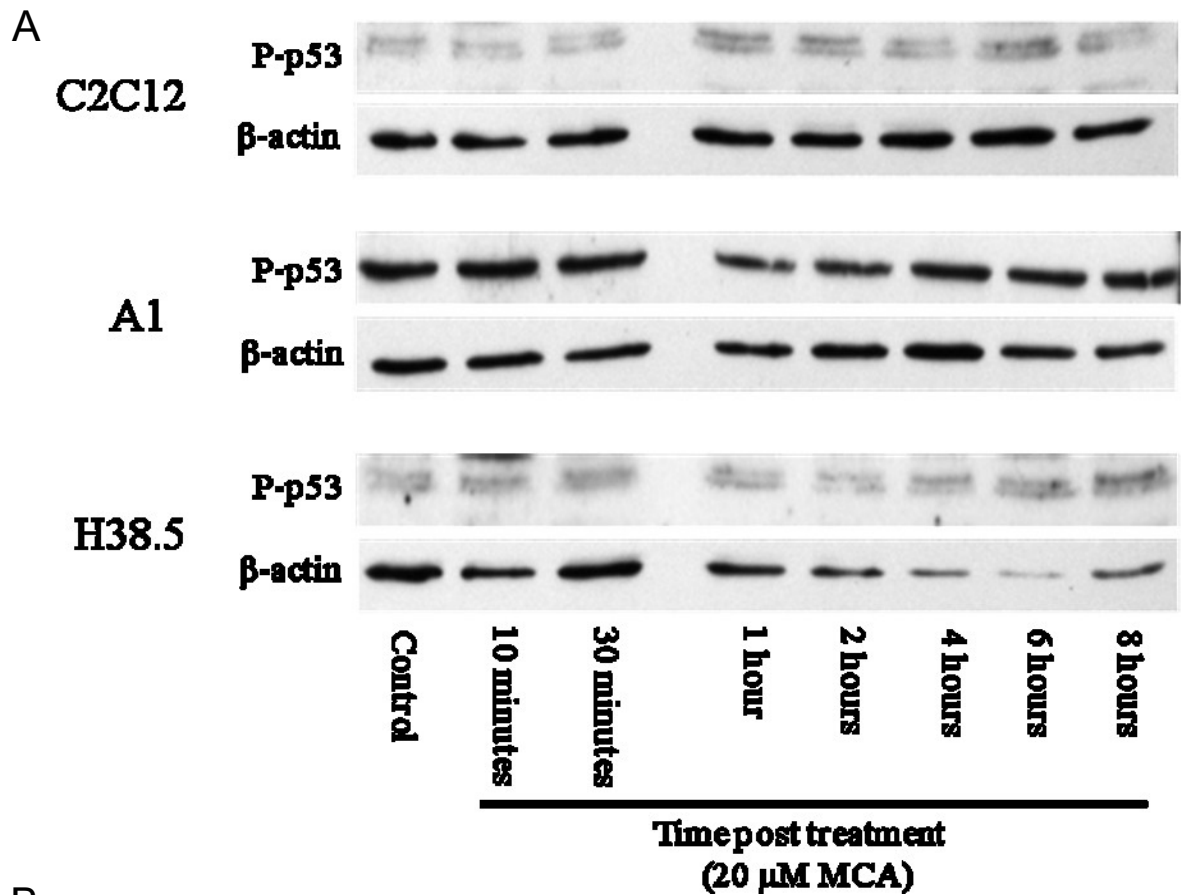


Figure 25: P-p53 protein expression in C2C12, A1, and H38.5 cells over time after being exposed to 20 μM of MCA for 24 hours. **A)** Western blots showing P-p53 expression with β-actin as a loading control. **B)** Densitometry showing P-p53 protein expression over time.

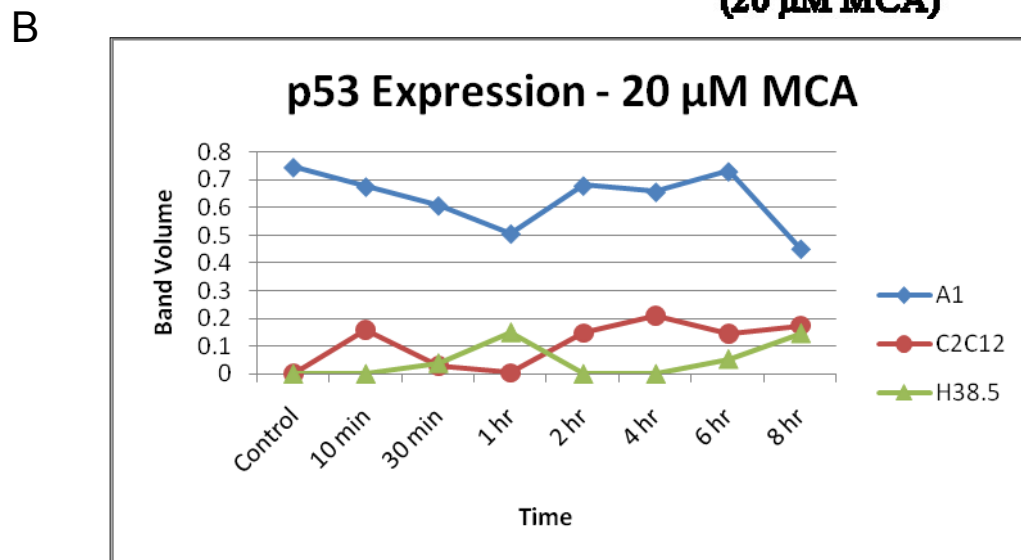
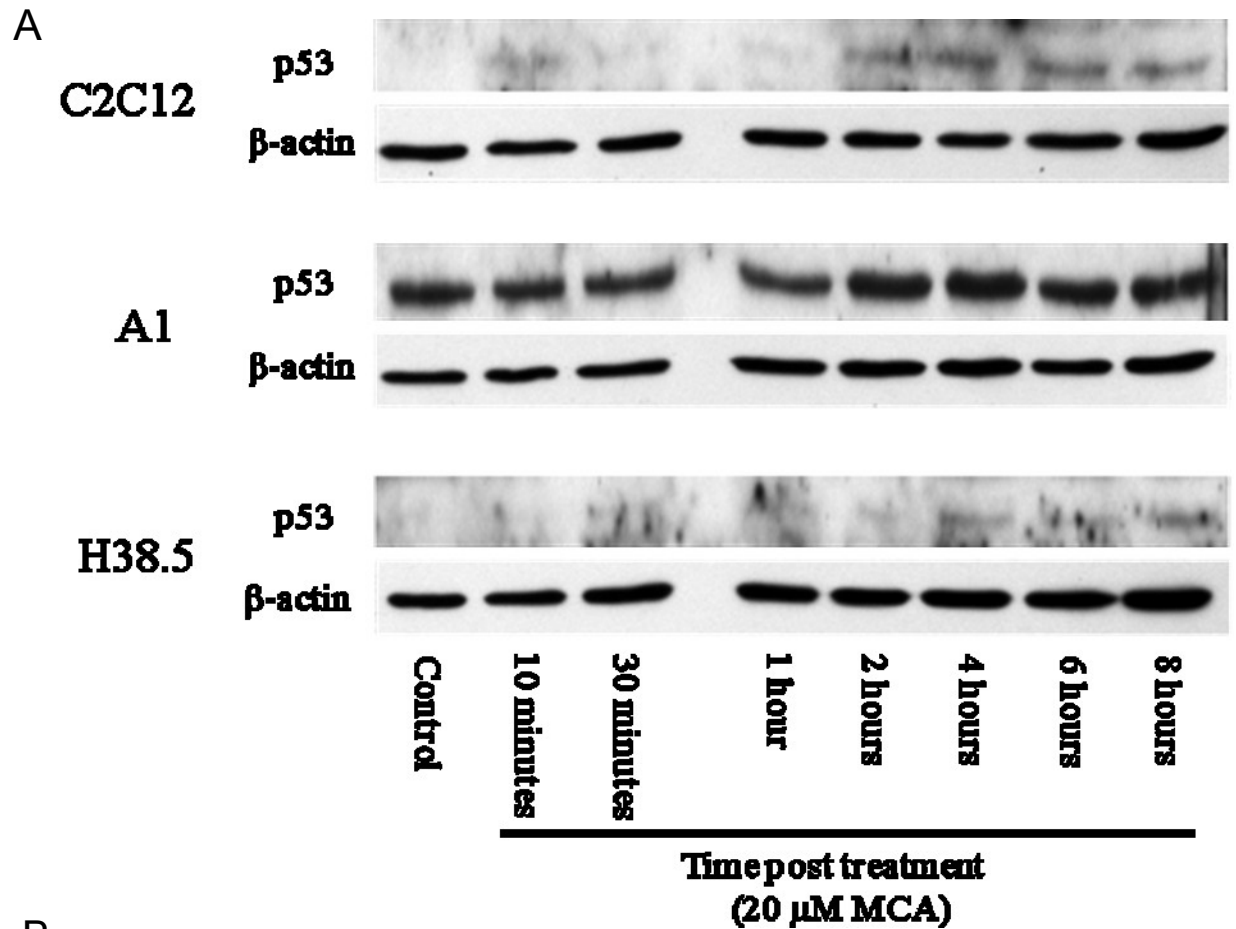


Figure 26: p53 protein expression in C2C12, A1, and H38.5 cells over time after treatment with 20 μM MCA for 24 hours. **A)** Western blots showing p53 expression with β-actin as a loading control. **B)** Densitometry showing p53 expression over time.

3.4.2 Cell Proliferation Assay

The viability of cells treated with MCA in DMSO, or DMSO alone, was compared to their untreated counterparts for each cell line. Average absorbance's at 440 nm showed no significant difference between any of the treatments and their controls (Figure 27) in any cell line, indicating no loss of protein expression due to cell death.

Cell Viability in Three Cell Types 8 Hours after Treatment with 20 μ M MCA or DMSO for 24 Hours

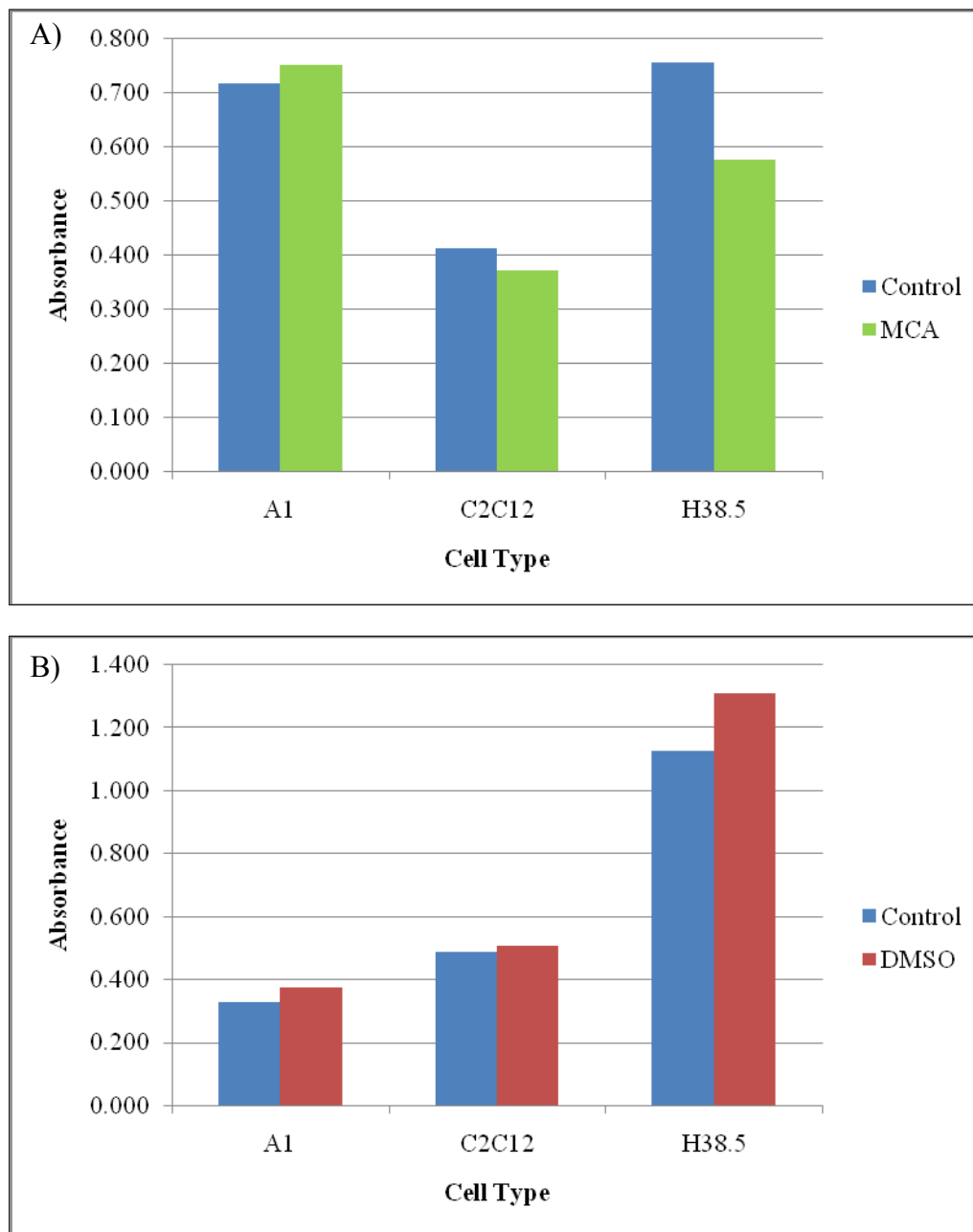


Figure 27: The average WST-1 absorbance at 440 nm for A1, C2C12, and H38.5 cells 8 hours after 24 hours treatment with MCA in DMSO (A), or with DMSO alone (B). No significance was found between control and treated cells in any cell line for either treatment. ($p > 0.05$, $n = 3$, T-test)

4. DISCUSSION

The ultimate goal of this project was to induce tumour formation in the newt in order to study the relationship between the regeneration process and the newts' susceptibility to tumour growth. As a first step towards this goal, we attempted to repeat the work of Zilakos et al. (1992) and to induce tumours in newts in regions of the body that lacked regenerative ability (such as the scapula and flank) (Zilakos, Tsonis et al. 1992). If these experiments had been successful, we would then have determined 1) if the regeneration of a limb or a tail would have any effects on the developing tumour, 2) if tumours were transplantable between animals and, 3) if we could explant the tumours into tissue culture to obtain newt tumour lines. Since no tumours were generated in this study, other experiments investigating the differences between newt and mammalian tissue at a DNA damage response protein level were conducted as an alternate look at the newt's resistance to cancer.

It is interesting to note that this study found that tumour-like formations are possible in animals that can regenerate to a lesser degree, such as *Xenopus tropicalis*, when exposed to similar carcinogen conditions as in the newt. From this finding, the big question is what do newts have that these other organisms do not, when it comes to tumour resistance? While the experiments conducted looking at newt vs. mammalian cells at a protein level may only scratch the surface, they are the beginning steps of answering this question at a DNA damage response level.

4.1 Carcinogen Implantation and Injection *in vivo*

Of the more than 150 newts implanted or injected with a variety of carcinogens at different sites on the newt body, none formed any tumours. This was expected in the

tissues with regenerative capabilities, such as the forelimb, from previous literature (Breedis 1952; Pfeiffer, Nagai et al. 1985), but not in tissue that cannot regenerate, such as the scapula or flank region (Zilakos, Tsonis et al. 1992).

The inability to induce tumour formation in tissue without regenerative capabilities was surprising considering Zilakos et al. (1992) were able to induce tumour formation in this tissue in 10-18% of animals. The Zilakos study did not go into great detail concerning the surgical methods used to implant the carcinogens. It is possible that there were differing implantation methods between this study, and those used by Zilakos, that resulted in our inability to induce tumour formation. Attempts to contact the primary authors to inquire about the surgical procedure were unsuccessful.

Carcinogens were chosen from the most common one's used in regeneration research, as well as the one's that had induced tumour formation or regeneration abnormalities in amphibians in the previous literature (Matos and de Lustig 1973; Tsonis and Eguchi 1981; Tsonis 1983; Pfeiffer, Nagai et al. 1985). The amount of carcinogen implanted into newts and frogs was approximated to be the same within experiments, generally with as much carcinogen as possible being implanted beneath the epidermis. The injection concentrations were determined from previous literature of carcinogen injections conducted in mice (Nettesheim, Snyder et al. 1979; Sobolewski and Bankowski 1984; Yano, Ishikawa et al. 1993; Takagi, Matsuzaki et al. 2001). Tumour inductions in these studies ranged from 4-50% with different variables and methods found in each study, but the carcinogen concentrations used were a starting point for the newt injection experiments. In murine studies, carcinogen administration can be followed by painting the skin with the pro-inflammatory and tumour initiator 12-*O*-

tetradecanoylphorbol-13-acetate (TPA), also known as phorbol 12-myristate 13-acetate (PMA) to promote tumour formation (Bolmer and Wolf 1982; Filler, Roberts et al. 2007). Due to the aquatic habitat of newts, painting the skin with a tumour promoter was not practical. None of the previous studies examining the effects of carcinogens on regeneration used TPA. Moreover, amphibian studies showed that the use of TPA *in vitro* with *Xenopus laevis* splenocytes induced apoptosis rather than cell proliferation (McMahan, Johnson et al. 1999; Ruben, Johnson et al. 2000; Taylor, Johnson et al. 2003). While these studies have not been conducted in the newt, it is interesting to note that typical carcinogen and tumour initiation and promotion protocols, as used in mammalian species, may not be as effective in amphibian species which have even a limited regenerative capacity. This may suggest some explanation as to why newts are not susceptible to cancer formation. Should newt cells react to promotion of cell proliferation in the same way as *Xenopus* splenocytes, then cells that could convey genetic mutations may be eliminated from the population by apoptosis before they have a chance to pass on mutations to their progeny, thereby eliminating an avenue for potential tumour formation. Perhaps the proliferation promoting chemicals' action on cells is controlled much like blastema cell proliferation is controlled.

Although no tumours were formed in newts, growths were produced after exposure to carcinogen. Externally visible growths were found in newts implanted with NQO in the scapula or injected with MCA in the flank, and frogs implanted with MCA in the flank. In all newt cases, these growths showed thickened tissue layers throughout the epidermal and dermal layers (Figures 12 and 15). In the case of newts implanted in the scapula region with NQO, the two resulting growths showed slight thickening of the

epidermis, as well as the muscle layer beneath the dermis. One newt injected with 2.7 mg/mL of MCA in the flank had a growth form that when sectioned showed all layers of epidermis, dermis and muscle thickened, but yet in their own defined layers. Overall, we tried different carcinogens (MCA, NQO, and MNNG), different concentrations, and different sites of administration (scapula, forelimb, flank, and hindlimb). None of the experimental paradigms were successful in inducing tumour formation in newts. It is possible that tissues without regenerative abilities possess the controls found in tissues that can regenerate, such as the forelimb or tail. If this is the case, then all tissues found within the newt would be less likely to have uncontrolled cell proliferation resulting in tumour formation, due to the newt's ability to regulate and control cell proliferation, as seen in the regeneration blastema.

It is interesting to note that after frogs were exposed to MCA implanted in the flank, two animals out of 15 formed growths directly beneath the implant site. When sectioned and stained, these growths showed neoplasia, or a mass of cells beneath the muscle layer that was not present in control tissue. However, these growths were not seen until 9 and 11 months after carcinogen exposure. Unfortunately, only three newts survived until the 9 month time point, when neoplasia was found in frogs. This makes definitive conclusions somewhat difficult. Nevertheless, some comparisons are possible. Breedis (1952) followed experimental newts for over a year after carcinogen exposure and yet was only able to induce two tumours in over 500 newts injected (an incidence of 0.4%) (Breedis 1952). In our studies, we were able to induce neoplasias in 2 out of 15 *Xenopus tropicalis* frogs (approximately 13%) using similar carcinogens and similar

modes of administration. This suggests that tumours are more easily formed in a species that has a reduced ability to regenerate.

From these observations, one might link the refractory response to tumour formation in the newt to their ability to regenerate. Results from this study showing the ability to induce neoplasia in *Xenopus tropicalis*, but only growths consisting of thickened tissue layers in the newt allow for a hypothesis. Since post metamorphosis frogs can regenerate to a very limited degree, but newts possess regenerative capabilities always, perhaps proliferating cells generated by carcinogens in the newt are controlled the same way as proliferating cells are controlled in the blastema. If this is the case, then the growths containing thickened cell layers could be comparable to a blastema. Blastema cells are formed and induced to proliferate in response to injury, and the cells seen in the growths on the newts were induced to proliferate due to chemical carcinogen stimulus; the important similarity is that both types of proliferating cells have their growth controlled by the surrounding newt tissue. Cells within a carcinogen induced skin growth may have responded to the carcinogen with rapid proliferation, but only proliferated within their respective layers and were contained there. Or potentially a mass of proliferating cells resulting from carcinogen exposure was found in one or multiple tissue layers and was induced to differentiate into the layers normally found within the skin.

This latter hypothesis was tested in a study by Rose et al. (1948). In this study, a frog (*Rana pipiens*) renal tumour was transplanted into a newt (*Triturus viridescens*) forelimb (Rose and Wallingford 1948). Once the tumour cells were established and invading newt tissues, the newt limbs were amputated through the cancerous mass. In

early regeneration stages, histological examination revealed the presence of undifferentiated frog cells interspersed among the regenerating newt tissues. In later stages however, differentiated frog muscle, cartilage, and connective tissues were found in association with the corresponding newt tissue (Rose and Wallingford 1948). Any integrated frog tumour cells proximal to the amputation site remained unchanged. In the regenerating tissue the foreign cancer cells were guided by the newt tissue down a differentiation pathway, illustrating the potential control that tissue with the ability to regenerate has over proliferating cells, and that newt tissue in general may possess.

Resistance to cancer formation in the newt may also be related to their different method of wound healing in comparison to mammals. In mammals the first response to injury is inflammation, which lasts for 4-6 days followed by proliferation of cells to cover the wound, which can last up to 14 days. It is in these phases where the mammalian immune system may often finish sealing the wound with a scar (Yokoyama 2008). After limb amputation in newts, the wound is completely covered by a 3-4 cell-layer thick wound epithelium by approximately 9 hours post amputation. This wound epithelium thickens to form the apical epithelial cap beneath which cells begin to dedifferentiate and proliferate (Chaar and Tsilfidis 2006). This rapid healing of the wound proceeds without scar formation and minimizes the inflammatory response to the injury (Han, Yang et al. 2005).

Two noticeable differences between mammalian and newt wound healing are the presence or absence of scarring as well as the duration of inflammation. Mammals form scars after injury whereas newts do not. This could be an important distinction between the two, as scar formation has been related to cancer. Scarring results from the presence

of fibroblasts and their differentiated form of myofibroblasts during the later stages of wound healing. If the myofibroblasts persist at the wound site, there is excessive formation of scar tissue. The presence of fibrotic wounds increases the risk of cancer in many tissues by increasing cell proliferation (Radisky, Kenny et al. 2007), and a distinguishing feature of advanced stage epithelial cancer, or carcinoma, is the presence of a large number of fibroblasts and myofibroblasts (Schafer and Werner 2008).

Chronic inflammatory conditions have also been linked to a predisposition to cancer. When tissues fail to properly activate and inactivate inflammation, it can lead to oxidative stress causing protein and DNA alterations and the increased risk of cancer development (de Visser, Eichten et al. 2006). Since newts do not form scars nor do they have long term inflammation at wound sites, they may decrease their risk of having uncontrolled cell proliferation.

The above studies suggest that following carcinogen exposure, newt cells are able to control their proliferation through differentiation, through their method of wound healing, or through a combination of the two. If this hypothesis is true, it is not possible to tell whether this cell control is due to the newt cells themselves, or the environment the cells are in. It is also not known if the regeneration process reduces the occurrence of cancer in newts, or if newts reduce their susceptibility to cancer through regeneration. Whatever the case may be, these are interesting findings and hypotheses to add to the collection of literature supporting newts as a species refractory to cancer.

4.2 DNA Damage and Repair *in vitro*

4.2.1 Irradiation studies

After many different *in vivo* studies were conducted, an *in vitro* approach was taken to determine if there are differences between newt and mammalian cells with respect to their response to DNA damage. Initially, C2C12 and A1 cell responses to irradiation-induced DNA damage were observed using immunocytochemistry. Both cell types were irradiated with 1 Gy and stained using a primary antibody for γ -H2AX. When the stained cells were examined under the microscope, the irradiated cells were visually indistinguishable from control cells in both cell lines. Foci were visible in both irradiated and control cells. This made monitoring DNA damage repair impossible. As a result of these findings a dose response curve was conducted on C2C12 and A1 cells using 1, 5, and 10 Gy of irradiation and western blots were used to assess γ -H2AX protein levels (Figure 19). Only the 10 Gy treated samples showed γ -H2AX expression in all cell lines. This dosage was used for all subsequent irradiation studies.

After treating all three cell types with 10 Gy and probing for γ -H2AX, p53, and P-p53 the mouse cell lines showed a typical peak of expression at 30 minutes for γ -H2AX, and 1-2 hours for p53 and P-p53. All of these peaks were followed by a quick decline in protein expression or phosphorylation indicating DNA damage repair or cell death. Cell death was ruled out with a WST-1 assay which indicated that there was no difference in cell viability between control and irradiated cells and ensured that any conclusions made were based on protein level changes within cells and not on protein expression changes due to decreased protein presence after substantial cell death.

A1 cells showed a similar peak of expression of γ -H2AX protein after 10 Gy of irradiation but the timing of protein expression was delayed in comparison to the mouse lines, peaking at approximately two hours and taking longer to return to control levels. This slowed decrease was even more interesting in that the γ -H2AX signal was not followed by increased P-p53 expression as seen in the mouse cell lines. The A1 cells showed a very stable amount of p53 and P-p53 protein expression even after DNA damage by irradiation, which is very unusual when compared to the two mouse cell lines.

All western blots were completed successfully once as presented in this study. If repeated in triplicate, it is likely that the densitometry graphs for each protein would have some outliers excluded from them and the trends seen would be more distinct.

When working with newt cells, it is always a challenge to find antibodies that will detect a specific protein since there are no newt antibodies for the proteins of interest in this study. All antibodies in these experiments were mouse specific, and the γ -H2AX and p53 antibodies had not, to our knowledge, been used in newt cells before; the P-p53 antibody, however, had been tested in axolotl and was shown to have specific binding (Villiard, Brinkmann et al. 2007). Since the axolotl is a urodele that is evolutionarily related to the newt, one can assume that this antibody would be specific to newt P-p53. Even though the antibodies to γ -H2AX and p53 were specific to mouse, these are generally well conserved proteins within different species and in the newt sample blots showed very specific bands for γ -H2AX and relatively specific bands for p53.

In the irradiation time course, the A1 cells had protein expression in their control, non-irradiated cells for all three proteins studied. Notably, the γ -H2AX protein was not evident in A1 control cells in the first irradiation experiment (Figure 19) but this was

most likely due to the antibody detection conditions for the western blot. All subsequent blots identified γ -H2AX expression in control A1 cells.

Support for this basal level of DNA damage response proteins comes from a study involving the MRL mouse. The MRL mouse is an interesting tool in regeneration research as it lacks expression of p21, a cell cycle checkpoint protein. MRL mice have been shown to have increased regenerative ability, including the ability to regenerate holes punched into their ear. In one study using MRL mice, it was found that uninjured MRL ear tissue in culture showed basal levels of p53 and γ -H2AX without damaging the cells in any way (Bedelbaeva, Snyder et al. 2010). This suggests that a basal level of DNA damage proteins may be associated with regenerative ability. Cells in tissue that can regenerate may be “primed” to repair damage because of the multiple rounds of proliferation that they normally undergo.

It is also possible that the A1 cells have accumulated damage over time. Not only have the A1 cells been cultured for an extended period of time for these experiments, but the same cell line has been used by all laboratories studying regeneration *in vitro* since the development of A1 newt muscle cells by Ferretti and Brockes in 1988 (Ferretti and Brockes 1988). This is an unfortunate limitation for this series of experiments as any genetic changes to these cells over the years of culturing could be altering the findings in this study. As a result of the continual use of the same cells for so many years, the *in vitro* experiments conducted in this study would have to be repeated using a newly cultured newt muscle cell line to confirm any of the results found.

4.2.2 Carcinogen (MCA) Studies

In order to tie together the *in vivo* carcinogen studies with the *in vitro* irradiation-induced DNA damage studies, cultured cells were treated with carcinogen and DNA damage and repair was assessed. C2C12, A1, and H38.5 cells were treated with MCA in culture for 24 hours and protein expression of γ -H2AX, P-p53, and p53 was monitored at various times after removal of the carcinogen. All cell types showed very weak protein expression for γ -H2AX, p53, and P-p53, at almost all time points after exposure to MCA. The A1 cells again showed protein expression in control cells for all proteins of interest to this study. WST-1 cell proliferation assays conducted with MCA (dissolved in DMSO) or DMSO alone treated cells showed no difference between control and treated cells. This indicates that neither the MCA nor the DMSO in the treatments was causing cell death.

It is likely that the conditions in this carcinogen assay were not optimal to induce DNA damage. The assay was developed from literature that had shown increased p53 and P-p53 expression after exposure to MCA (Kwon, Ueda et al. 2002), but in a cancer cell line rather than a regular mouse cell line. It is possible that 24 hours of treatment with MCA was not the optimal time of treatment, the dosage of MCA chosen was not optimal, or the recovery time after treatment was not long enough to see a change in protein expression.

It was observed that there was a difference in p53 and P-p53 band intensity in the A1 control cells in the 10 Gy and MCA blots. This could be a result of the duration of cell culture, different frozen stocks used to make samples between irradiation and

carcinogen treatments, different antibody conditions used between 10 Gy and MCA blots, or any combination of the three.

If one were to assume that there were no influences from residual damage due to long term culturing of the A1 cells, and that the antibodies used were specific to the proteins of interest in all three cell types used, the results found here are very interesting. While newt cells have the same initial response to DNA double strand breaks induced by irradiation with respect to γ -H2AX formation as the mouse cells do, this phosphorylated histone has a prolonged life span in the newt cells alone. This implies that the newt cells do not repair their DNA as quickly as mouse cells do. Perhaps with this extended time for DNA repair, newt cells are able to do so more efficiently and with greater accuracy. This would decrease the chances for genetic mutations within a cell thereby decreasing its propensity to become cancerous.

The extended presence of γ -H2AX may be related to the amount of H2AX protein present in the first place. The levels of H2AX found within the mammalian histone H2A pool can vary from 2-25% (Fernandez-Capetillo, Lee et al. 2004). This results in H2AX molecules being found in every fifth nucleosome in mammals (Bonner, Redon et al. 2008). The H2AX levels found within the newt cells are unknown, but they may not be the same as mammalian cells. If there is more H2AX present in the newt H2A pool, then perhaps there are more γ -H2AX molecules present around a DNA strand break. Potentially this could provide accumulation of DNA damage response proteins for longer periods of time to ensure proper DNA repair. It could also just result in extended time needed for phosphatases to return γ -H2AX to its normal H2AX form with no implications on the duration or efficiency of repair. It has been found that when H2AX is reduced to

50% of wild-type levels in mice, the decrease in amount of γ -H2AX formed causes the loss of genomic stability maintenance and can lead to increased levels of chromosome aberrations (Fernandez-Capetillo, Lee et al. 2004). If newts do possess more H2AX within their H2A complement, they may be at less risk for genomic instability leading to uncontrolled proliferation and cancer.

While the mouse cells follow a normal response to DNA damage with respect to p53 and P-p53 expression levels, newt cells have a constant level of expression of these proteins before and after DNA damage. This suggests that newts may not use the p53 pathway as mammals do to respond to damage and mediate cell cycle arrest. Should they not use the p53 pathway in response to DNA damage, perhaps they use different family members such as p63 and p73.

From these initial findings there are many more experimental ideas generated. After DNA damage by irradiation, as induced here, do the cells survive long term, or do they undergo apoptosis? Does this have any implications for the duration of γ -H2AX presence in the A1 cells? With the basal level of p53, P-p53, and γ -H2AX present in A1 cells, and if newts use the same DNA damage recognition and repair pathways as mammals, are newts primed and ready to handle DNA damage? What percentage of H2A histones are H2AX in the newt? If newts don't use the p53 pathway to mediate DNA damage response, what do they use? These are just some of the questions that can be explored to try to begin to understand how newts evade carcinogenesis.

4.3 Final Conclusions

The major aim of this study was to begin to understand if and how regeneration might play a role in the newts' refractory response to cancer development. From the experiments conducted, newts appear to be resistant to tumour formation when exposed to a variety of carcinogens through either implantation or injection in tissue with, or without regenerative ability. Interestingly when *Xenopus tropicalis*, a species that loses regenerative capabilities after metamorphosis, were exposed to similar carcinogen conditions, they responded with neoplasia formation. When newt cells were compared to mouse cells at a protein level, they appeared to have extended γ -H2AX expression after DNA damage by irradiation. Moreover, unlike mouse cells, newt cells did not respond to the damage using the p53 pathway as the mouse cells did. These findings lead to many more experimental questions which will have to be addressed before we can understand the basis behind cancer resistance in newt cells and the potential for applying this knowledge to mammalian cells.

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APPENDIX

A1: General Solutions

C2C12 Growth Media (10% Serum)

To make 500 mL:

- 435 mL DMEM (HyClone #SH30081.01)
- 5 mL 100x L-Glutamate (Gibco #25030)
- 5 mL Penicillin/Streptomycin (Gibco # 15140)
- 5 mL Sodium Pyruvate (Sigma # S8636)
- 50 mL Heat Inactivated Fetal Bovine Serum (FBS)

A1 Growth Media (10 % Serum)

To make 500 mL:

- 315 mL MEM (Gibco #11090)
- 120 mL Sterile distilled water
- 50 mL Heat Inactivated FBS
- 5 mL Penicillin/Streptomycin/Fungizone (Gibco #15240-062)
- 5 mL 100x L-Glutamine
- 5 mL ITS (Sigma #1-3146)

Tricaine Methane Sulfonate (MS-222)

To make 1L:

1. Dissolve the following in 1L of de-chlorinated water:
 - 0.5 g 3-aminobenzoic acid ethyl ester
 - 0.6 g sodium hydrogen carbonate (NaHCO_3)

4% Paraformaldehyde (PFA)

To make 500 mL:

1. Heat 400 mL PBS in 65 °C for 20 minutes (PFA cannot get hotter than 65 °C)
2. Add 20g of PFA while stirring in fumehood
3. Add NaOH tablets (approx. 2-3 total) 1 at a time to dissolve the PFA
4. Filter
5. pH to 7.5
6. Top up to 500ml with PBS

Antifade

To make 50 mL:

1. Dissolve the following at 65 °C:
 - 25 mL Tris Buffered Saline (TBS)
 - 25 mL Glycerol (Fisher BP229-1)
 - 0.5 g n-Propyl Gallate (Sigma P-3130)
2. Store at 4 °C

Jenkins Solution

- 4 mL concentrated HCl
- 3 mL glacial acetic acid
- 10 mL chloroform
- 10 mL distilled H₂O
- 72 mL 100% ethanol

Nuclear Lysis Buffer

- 0.5 mL 1 M Tris, pH 7.5
- 1 mL 5 M NaCl
- 1 mL 10% IGEPAL
- 1 mL 10% DOC
- 0.05 mL 20% SDS
- 0.04 mL 0.5 M EDTA
- 1 mini tablet of COMPLETE protease inhibitors
- dH₂O up to 10 mL

A2: Metamorphosis and General Care for *Xenopus tropicalis*

- All animals were raised in glass aquariums in water kept at approximately 25 °C using a tropical aquarium heater

Solutions

Modified Ringers Solution 1X

To make 1L:

Dissolve the following in 1L of de-chlorinated water:

- 5.844 g 0.1M sodium chloride (NaCl)
- 0.134 g 1.8mM potassium chloride (KCl)
- 0.222 g 2.0mM calcium chloride (CaCl₂)
- 0.203 g 1.0mM magnesium chloride (MgCl₂)
- 300 mg sodium hydrogen (NaHCO₃)

Tadpoles

To Change Water:

- Using a siphon with mesh on the end in the tank, remove half of the water in a waste bucket
- Refill the tank to the “Max” line (approximately 10 L) with heated 1:20 Ringers solution diluted in dechlorinated water
- Dispose of waste water down the drain

To Feed:

- Tadpoles need to be fed every day
- Add 1-2 g of Sera Micron Tadpole Food to a 50 mL centrifuge tube filled with approximately 10ml of tadpole tank
- Using a glass pipette and nipple, mix up the tadpole food until dissolved
- Using the same glass pipette, add the food to the tadpole tank

- When forelimbs with digits are visible on the tadpoles, use a net to move them to another tank containing “Metamorphs”

Metamorphs

- Do not feed these animals
- Do not change water
- When metamorphosing animals have turned into froglets i.e. do not have a tail anymore, use a net to transfer them to the froglet tank

Froglets

To Change Water:

- Siphon out as much water as possible into waste water bucket and dispose down the drain
- Add heated 1:20 Ringers solution to the tank
- Slowly add more water diluting out the Ringers solution until only heated dechlorinated water is being added to the tank

To Feed:

- Crush up Frog Brittle (Nasco), approximately 2 pellets/frog and add to tank

A3: Decalcification

1. Place samples in 4% PFA on rocker at 4 °C for approximately 2 hrs
2. Replace PFA with 50% ethanol for 10 minutes
3. Replace ethanol with Jenkins solution
4. Change Jenkins solution 3-4 times in the first 48 hours
5. Replace with fresh Jenkins solution once a day for 5-8 days (not exceeding 10 days in total in Jenkins solution)
6. After 5 days in Jenkins solution rinse samples for 3 hours in 100 % ethanol, replacing with fresh ethanol 3-4 times during that time period
7. Replace ethanol with xylene and leave the samples overnight
8. Change the xylene once over the course of 24 hours and store in xylene until embedded in paraffin

A4: OCT Embedment

1. Place samples in 4% PFA at 4 °C for 2 hours
2. Rinse 3 times in PBS for 20 minutes each wash
3. Add 30% sucrose in 1:1 PBS:OCT embedding medium
4. Leave samples in this mixture until tissue sinks to the bottom of the tube (usually overnight)
5. Freeze limbs in OCT embedding medium floating on liquid nitrogen
6. Store blocks at -80 °C until use
7. Section on cryostat, let slides air dry for 2 hours at room temperature and store slides with dessicant at -80 °C
8. Warm slides at room temperature for at least 30 minutes before opening box

A5: Paraffin Embedment

1. Place samples in 10 mL plastic tubes filled with 4% PFA at 4 °C for approximately 1.5-2 hours
2. Pour off fixative and replace with PBS for 10 minutes at room temperature
3. Repeat PBS washes 2 more times
4. Replace the PBS with 50% ethanol 3 times over 1 hour at room temperature
5. Replace 50% ethanol with 70% ethanol for 20 minutes
6. Repeat 70% ethanol change 2 more times and after the 3rd wash, leave the samples in 70% ethanol overnight at room temperature
7. Incubate samples in 95% ethanol for 20 minutes at room temperature. Repeat 2 more times
8. Incubate samples in 100% ethanol for 20 minutes at room temperature. Repeat 2 more times.
9. Incubate samples in xylene for 10 minutes at room temperature. Repeat 2 more times. (Can leave the samples in xylene until ready to embed in paraffin)
10. Pour off xylene and replace with half fresh xylene, and half liquid paraffin wax
11. Leave samples overnight at 60 °C
12. Pour off xylene/wax mixture and immediately place with molten wax
13. Incubate samples for 1 hour at 60 °C and then replace with molten wax again
14. Samples can be left in molten wax at 60 °C until ready for embedment, or embedded in plastic molds right away

A6: Haematoxylin and Eosin Staining

For paraffin sections:

1. Xylene 10 min
2. Xylene 10 min
3. 100% Ethanol 3 min
4. 100% Ethanol 3 min
5. 95% Ethanol 3 min
6. 95% Ethanol 3 min
7. 70% Ethanol 3 min
8. 50% Ethanol 3 min

For all paraffin and OCT sections:

1. Dip slides in deionized H₂O (dH₂O), lie flat on paper towel 2 min
2. Post fix slides in 4% PFA 3 min
3. Wash out fixative in running tap water 5 min
4. Rinse in dH₂O 1 dip
5. Stain in filtered haematoxylin 15 sec - 5 min
6. Rinse in running tap water until water runs clear 1-3 min
7. Immerse in 70% Ethanol with 5 drops of concentrated HCl 3 dips
8. Rinse in tap water 5 dips
9. Immerse in 1:1 solution of saturated LiCO₃:d H₂O 10 dips
10. Rinse in running tap water 10+ min
11. Counterstain in aqueous Eosin Y 30 sec
12. 50% Ethanol 1 min
13. 70% Ethanol 1 min
14. 95% Ethanol 1 min
15. 100% Ethanol 2 min
16. 100% Ethanol 2 min
17. Xylene 2 min
18. Xylene 2 min
19. Xylene 2 min
20. Mount in permount with a coverslide

A7: Immunocytochemistry

1. Cells fixed in 4% PFA for 20 minutes at room temperature
2. Wash cells for 5 minutes in PBS. Repeat 2 more times
3. Incubate cells in block solution for 30 minutes at room temperature
-Block solution: PBS, 0.2% TritonX-100, 5% goat serum
4. Remove block
5. Add anti-mouse γ -H2AX primary antibody 1:400 in block solution overnight at 4 °C in humidified chamber
6. Wash cells in PBS for 5 minutes. Repeat 2 more times.
7. Add goat anti-mouse Cy3 secondary antibody 1:800 in block solution for 30 minutes at room temperature in the absence of light
8. Wash cells in PBS for 5 minutes. Repeat 2 more times.
9. In the second last PBS wash, add DAPI at 1:10 000
10. Mount in antifade