THE ROLE OF P53 IN NORMAL DEVELOPMENT AND TERATOGEN-INDUCED APOPTOSIS AND BIRTH DEFECTS IN MOUSE EMBRYOS

A Dissertation

by

HIROMI HOSAKO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Toxicology

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Approved by:

Co-Chairs of Committee,	Philip E. Mirkes
	Stephen H. Safe
Committee Members,	Robert C. Burghardt
	Richard H. Finnell
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Intercollegiate Faculty Chair,	Robert C. Burghardt

December 2008

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ABSTRACT

The Role of p53 in Normal Development and Teratogen-Induced Apoptosis and Birth Defects in Mouse Embryos. (December 2008)

Hiromi Hosako, B.S., Texas State University – San Marcos

Co-Chairs of Advisory Committee, Dr. Philip E. Mirkes
Dr. Stephen H. Safe

In the studies described in this dissertation, we investigated the roles of p53 in normal development, teratogen-induced apoptosis, and birth defects. In the first study, the activation of p53 and its target genes, *p21*, *NOXA*, and *PUMA*, were examined during neural tube closure in mouse embryos exposed to hyperthermia (HS) or 4-peroxycyclophosphamide (4CP), teratogens known to induce neural tube defects (NTDs). In the second study, using *p53*-deficient mice, we examined the expression of mRNAs and microRNAs (miRNAs) during neural tube closure. In the third study, the incidence of NTDs was investigated in *p53*- and *p21*-deficient mouse embryos exposed to HS. Finally, we examined the induction of apoptosis in *p53*-deficient mouse embryos exposed to HS.

HS and 4CP induced the activation of p53 by phosphorylation and accumulation of the protein, leading to an increase in *p21* proteins and mRNAs. Although HS and 4CP also induced the expression of *Noxa* and *Puma* mRNAs, no significant increases in NOXA and PUMA proteins were observed, suggesting a possible role of transcription-independent apoptosis. In the second study, we showed that the expression of 388 genes and 5 miRNAs were significantly altered in *p53* -/- compared to *p53* +/+ embryos. Finally, we showed that 10% of *p53* -/- pups exhibit exencephaly, spina bifida, and/or preaxial polydactyly, whereas no malformations were observed among *p21* -/- offspring in the absence of HS. HS resulted in an increased incidence of exencephaly in both *p53* and *p21* null mice indicating that these two proteins act as teratogen suppressors. Our

preliminary data additionally showed that a decreased level of apoptosis was observed in HS-treated embryos lacking a *p53* allele, suggesting that too little apoptosis may be causally linked to NTDs observed in embryos exposed to HS. Taken together, these studies suggest that precise control of apoptosis and cell cycle arrest pathways are critical for neural tube development and the prevention of teratogen-induced NTDs.

DEDICATION

To my Parents and Ivy

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1. INTRODUCTION

1.1. EMBRYONIC DEVELOPMENT

1.1.1. Human and Mouse Embryonic Development

Development of the human embryo and fetus is an amazing but extremely complex process at both the tissue and cellular levels. The gestational period in humans is approximately 38 weeks (266 days), and development of a zygote begins at fertilization about 14 days after the onset of the last menstrual period. After fertilization occurs, the zygote undergoes cleavage as it passes along the fallopian tube toward the uterus, which results in the formation of a blastocyst. Implantation of the blastocyst occurs at the end of the 1st week, leading to a rapid proliferation and differentiation in the 2nd week. Conversion of a bilaminar to a trilaminar embryonic disk, called gastrulation, begins in the 3rd week with the formation of the primitive streak. The period of 3rd to 8th weeks of development is called embryonic (organogenesis) period, during which all of the main organ systems begin to develop. During organogenesis embryos are most vulnerable to various chemical and physical agents (for further discussion, refer to section 1.2), and teratogen exposure during this period may cause major congenital malformations. By the end of the 8th week, the embryo has a distinct human appearance. The interval from the 9th to the 38th week is referred to as the fetal period, and rapid body growth and differentiation of tissues, organs, and systems take place during this period (Moore and Persaud, 2003).

The development of the human embryo and fetus have been observed and described since 1416 BC by Aristotle and Hippocrates (Bard, 1994). In the early 19th century, experimental embryology first started (National Research Council, 2000), and the importance of "inductions," which refers to the ability of one group of cells to influence the fate of another, was first recognized in the 1920s by the transplantation experiments for neural induction by Spemann and Mangold (1924). Although in the

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This dissertation follows the style of *Birth Defects Research A*.

1970s the movements and interactions of tissues and cells were described as developmental mechanisms, the advanced techniques of biochemistry, molecular biology, cell biology, and genetics that were required to understand the molecular mechanisms of embryonic development such as cell-cell signaling and transcriptional regulation were not yet available (National Research Council, 2000). In the past 20 years, the field of embryology has advanced remarkably thanks to these modern technologies, and it is now known that the trillions of cells of an adult animal arise from a single-celled zygote and all of the cells retain the same genetic content. Much of the early molecular work on the development of the embryo was conducted on non-mammalian species because embryos were more accessible and simple (Harkness and Baird, 1997). However, studies of embryonic cells from echinoderms (e.g., sea urchins), nematodes (*C. elegans*), insects (*Drosophila*), and amphibians (*Xenopus*) have led to a discovery that developmental decisions are made based on which chemical signals they receive from other cells (Wolpert, 1969). Subsequently, the signaling pathways involved in developmental decisions were elucidated, and currently seventeen intercellular signaling pathways have been identified (Table 1.1) (National Research Council, 2000). These pathways are repeatedly used at different times and places in the embryo, from various developmental stages and even in the various proliferating and renewing tissues of the adult.

The advancement in molecular techniques made it possible to study mammalian development, especially mouse embryonic development at the cellular level. There are four main reasons why we now pay the greatest attention to one particular species, the mouse, as the developmental system of choice (Bard, 1994). First, the ability to manipulate pre-implantation embryos and to culture early postimplantation embryos is well established. Second, the molecular and cellular biology techniques required for the study of development in mice are now readily available. Third, mouse embryogenesis is the best studied in terms of normal and abnormal human development. Finally, mouse genetics, together with the ability to make custom mutations through transgenic methods, facilitates the investigation of how mutations and specific genes not only play

a role in normal and abnormal mouse development but also by extension the etiology of human congenital defects.

Table 1.1
The Seventeen Intercellular Signaling Pathways*

Period During Development	Signaling Pathway Used
Early development (before organogenesis and cytodifferentiation) and later (during growth and tissue renewal)	1. Wingless-Int pathway 2. Transforming Growth factor β (receptor serine and threonine kinase) pathway 3. Hedgehog pathway 4. Receptor tyrosine kinase (small G proteins) pathway 5. Notch-Delta pathway 6. Cytokine receptor (cytoplasmic tyrosine kinases) pathway (STAT pathway)
Middle and late development (during organogenesis and cytodifferentiation) and later (during growth and tissue renewal)	7. Interleukin-1-Toll nuclear factor-kappa B pathway 8. Nuclear hormone receptor pathway 9. Apoptosis pathway 10. Receptor phosphotyrosine phosphatase pathway
Larval and adult physiology (after cell types have differentiated)	11. Receptor guanylate cyclase pathway 12. Nitric oxide receptor pathway 13. G-protein coupled receptor (large G proteins) pathway 14. Integrin pathway 15. Cadherin pathway 16. Gap junction pathway 17. Ligand-gated cation channel pathway

*From National Research Council, (2000).

1.1.2. Neural Tube Development

Formation of the neural tube, called neurulation, is an important process of embryogenesis in vertebrates that leads to the development of the cell precursors of the brain and spinal cord to form the central nervous system (CNS). Neurulation is an extremely complicated process that requires neural crest cell migration, neuroepithelial proliferation, differentiation, programmed cell death, contraction of apical cytoskeletal microfilaments, and flexing at dorsolateral bending points in the developing neural tube

to form a tube from flat sheet of epithelial cells (Blom et al., 2006). Neurulation occurs in two distinct phases in mammalian embryos: primary neurulation that generates the brain and most of the spinal cord to the caudal neuropore, followed by secondary neurulation that forms the rest of the spinal cord including most of the sacral and all the coccygeal regions (Copp et al., 2003; Detrait et al., 2005; Kibar et al., 2007).

Primary neurulation occurs during the third and fourth weeks of development in humans (Detrait et al., 2005) while in mice the neural tube forms during days 8 to 10 of gestation (Harris and Juriloff, 1999). During primary neurulation the dorsal midline ectoderm of the embryo first thickens and differentiates into the neuroepithelium to form the neural plate (Colas and Schoenwolf, 2001; Detrait et al., 2005). The neural plate first appears at the cranial end of the embryo and differentiates towards the caudal direction. Formation of the neural plate is the result of neural induction, and early studies using *Xenopus* embryos have shown that neural induction is regulated by endogenous bone morphogenetic protein (BMP) antagonists, including Chordin (Sasai et al., 1994), Noggin (Lamb et al., 1993), and Follistatin (Hemmati-Brivanlou et al., 1994). BMP antagonists induce neural induction since ectodermal cells are, by default, programmed to become neuroepithelium, not to become the ectoderm, and this default state is normally inhibited by BMPs expressed throughout the ectoderm (Kibar et al., 2007; Padmanabhan, 2006). In mammals, however, the formation of the neural plate appears to be a more complex process, in which BMP inhibition by fibroblast growth factors (FGFs) and Wnt (Wingless/Int-1) signaling pathways are also required as an early step in neural induction (Bainter et al., 2001; Wilson and Edlund, 2001; Wilson et al., 2001). Recent studies, however, have shown that BMP inhibition is a relatively late step and neural induction involves additional signaling events that need to be identified (Linker and Stern, 2004).

The neural plate then begins to move upward forming the neural fold. The neural plate becomes narrower and longer to form an elongated structure. This movement is called convergent extension, and it occurs by cell movements elongating mediolaterally and by polarized cellular extensions that allow cells to move directionally and to

intercalate in the midline. This change in shape and movement results in convergence toward the midline and extension of the tissue along the anteroposterior axis (Kibar et al., 2007). Convergent extension is known to be regulated by the non-canonical Wnt/frizzled pathways (Lawrence and Morel, 2003).

The neural plate becomes more pronounced and elevated, eventually bending longitudinally along the midline to form neural folds. By day 23 in human embryos and day 8.5 in mouse embryos, the folds rise up dorsally, approach each other and merge together, forming a neural tube open at both ends (Detrait et al., 2005; Kaufman, 1992). Formation of two hinge points is critical for the neural plate bending. The median hinge point (MHP) overlies the notochord along the rostrocaudal axis, and the paired dorsolateral hinge points (DLHPs) are situated at the lateral sides of the neural folds. The MHP, induced by signals from the notochord, is the only bending point at the upper spinal level whereas in the lower spine and cranial region DLHP forms as well as the MHP (Kibar et al., 2007). Studies with cultured mouse embryos have shown that sonic hedgehog (SHH) induced by the notochord inhibits DLHP formation at the upper spinal region (Ybot-Gonzalez et al., 2002). Mechanisms of conversion of columnar cells of the neural tube into wedge-shaped cells, termed apical constriction, are not well understood, but two actin-related genes, *p190RhoGap*, a negative regulator of Rho GTPase involved in actin dynamics (Brouns et al., 2000) and *Shroom*, which is an actin-binding protein (Hildebrand and Soriano, 1999; Martin, 2004), have been shown to mediate this process.

Fusion of the neural folds eventually occurs by the lateral folds coming into contact at the dorsal midline and adhering to each other. The fusion results in the formation of epidermal ectoderm and neuroepithelial and mesenchymal cells of the neural crest (Copp et al., 2003) as well as the breakdown of cells at contact sites by apoptosis (Lawson et al., 1999). The important role of apoptosis in fusion of neural folds has been shown in chick embryos in which caspase inhibitors block neural tube closure (Weil et al., 1997). For further information about apoptosis, refer to section 1.3.

The fusion of neural folds was originally described to be initiated at a single site, extending bidirectionally in a zipper-like fashion to the rostral and caudal neuropores

(Purves and Lichtman, 1985). Currently, several models of neural fold fusion have been proposed based on experimental observations in mice and observation and graphic reconstruction of human embryos from the Kyoto and Carnegie Collections. These studies suggest that three to five and two to five multiple sites of initiation of neural fold fusion exist in mice and humans, respectively (Detrait et al., 2005; Padmanabhan, 2006). The neural fold fusion has been extensively investigated in several species including chick, mouse, rat, hamster, rabbit, and pig. These studies show that although a certain degree of homology is observed between species, there are significant differences between species and even strains, e.g., the number of initiation sites, direction of fusion progression, and sequence and onset of fusion points. The closure site 2, which starts at the prosencephalon-mesencephalon junction, varies in position among different mouse strains, and the position of the site may be associated with the susceptibility of the strains to neural tube defects (Fleming and Copp, 2000). These defects are further discussed in section 1.1.3.2.

Secondary neurulation begins after completion of primary neurulation at the most caudal level, and the neural tube is formed in the tail but without neural folding (Copp et al., 2003; Kibar et al., 2007). The tail bud consists of stem cells from the remnant of the primitive streak, and these cells undergo proliferation and condensation to form the secondary neural tube, eventually fusing with the neural tube formed by primary neurulation (Catala, 2002; Copp et al., 2003). Secondary neurulation forms most of the sacral and all of the coccygeal regions.

As the formation of the neural tube progresses, the differentiation of cells in the CNS also takes place. Neurons, astrocytes, and oligodendrocytes of the CNS arise from proliferating multipotent progenitor cells called neural stem cells (NSC) (Unsicker and Krieglstein, 2006). In the neural tube, NSC are found in the polarized neuroepithelium. This neuroepithelium is referred to as the ventricular zone, the layer facing the lumen of the neural tube. These neural progenitors first produce neurons with gliogenesis following neurogenesis. Neurogenesis proceeds in a rostral-to-caudal direction from the hindbrain to the spinal cord and in a caudal-to-rostral direction from the midbrain to the

forebrain. The production of neurons requires precise control of balance between proliferation, differentiation, and survival by the integration of environmental and intrinsic cues. There are well understood cellular pathways involved in this complex process, e.g., *Notch* genes are expressed in the proliferative regions at specific stages of nervous system development. The Wnt family members stimulate stem cell proliferation not only during brain development, but also during spinal cord neurogenesis. The most prominent member of the family, *Wnt1*, is expressed along the entire antero-posterior axis of the neural tube in the dorsal midline, and in a belt-like fashion at the midbrain-hindbrain boundary (Gavin et al., 1990). *Wnt7a* is also expressed in the germinal layer of the embryonic mouse cortex with several frizzled receptors (Grove et al., 1998; Kim et al., 2001a). *Wnt7b* is expressed in the cortical plate, particularly by deep layer neurons (Kim et al., 2001b; Rubenstein et al., 1999). Other signaling molecules and pathways involved in the development of CNS include SHH pathway for dorso-ventral patterning, FGF pathway for early neurogenesis, axon growth, neuroprotection, and synaptic plasticity, transforming growth factors-α (TGFα), neuregulins, and epidermal growth factors (EGF) for regulating the proliferation of precursor populations in the CNS, BMP for dorso-ventral patterning, specifying regional patterning within the developing neural tube, and neuronal and glial differentiation, and finally, neurotrophins for neuronal maturation (Unsicker and Krieglstein, 2006). Taken together, neural tube development in mammals requires the precise control by numerous cell signaling pathways.

1.1.3. Congenital Malformations

1.1.3.1. Overview of Congenital Malformations

Currently, approximately 2-3% of all live-born babies, which translates into 120,000 every year in the United States, are born with major developmental defects (Holmes, 1997). Those malformations that are life threatening, require major surgery, and present a significant disability account for approximately 70% of neonatal deaths occurring within the first month of age and 22% of the deaths in infants before 15

months of age (Marden et al., 1964). Unfortunately, the causes of more than 50% of the birth defects remain unknown (Table 1.2) (Jelinek, 2005; The Teratology Society, 2005). However, it is known that 23-25% of all birth defects are related to genetic causes and 4-5% are attributable to an exposure to drugs and environmental chemicals. Combined with multifactorial causes resulting from the exposure of genetically predisposed individuals to environmental factors, chemicals and physical agents may contribute to a significant number of developmental malformations (National Research Council, 2000). These agents causing congenital malformations are further discussed in section 1.2.

Table 1.2
The Causes of Birth Defects in Humans*

Known genetic transmission	20 %
Chromosomal aberrations	3 – 5 %
Drugs and environmental chemicals	4 – 5 %
Infections	2 – 3 %
Maternal metabolic imbalances	1 - 2 %
Combinations and interactions	~25 %?
Unknown	65 - 70 %

*Modified from The Teratology Society, (2005) and National Research Council, (2000).

Congenital malformations were already recognized in ancient Assyrian and Babylonian times, and until medieval time superstitious people believed that congenital malformations were supernatural phenomena, i.e., symbols of the devil or miracles. From the late 18th to early 19th century, descriptive studies of congenital malformations predominated. Beginning with the studies of Isidore Geoffroy Saint-Hilaire, who coined the term teratology, the etiology of congenital malformations shifted from theological and metaphysical explanations to purely natural causes (Jelinek, 2005; Schumacher, 2004). During the 20th century, the Teratology Society was formed to promote research

that reveals the causes and improves the diagnosis and treatment of birth defects (The Teratology Society, 2005; Wilson and Warkany, 1985). Since then, the powerful techniques of molecular biology and genetics have been applied to identify teratogens and to understand the biological processes leading to abnormal development and birth defects. Despite enormous efforts and the development of diagnostic tests such as DNA probes, ultrasound devices, and microarrays, the overall incidence of birth defects has not declined. For more than 20 years, major developmental malformations have been the leading single cause of infant mortality in the United States (Petrini et al., 1997). The most common birth defects associated with infant death are heart defects (31.4%), respiratory defects (14.5%), nervous system defects (13.1%), multiple defects associated with chromosomal aberrations (13.4%), and musculoskeletal defects (7.2%). The estimated lifetime cost for children born with spina bifida in California alone exceeds $58 million and for those born with a conotruncal heart defect medical costs exceed $287 million every year, posing enormous emotional and fiscal costs (Waitzman et al., 1994). Therefore, birth defects are a serious health concern that needs to be addressed in our population, and more research is still necessary if we are to understand and prevent congenital malformations.

To understand and prevent congenital malformations, James Wilson set forth six principles of teratogenesis (Wilson, 1973). First published in 1973, these principles of teratogenesis were based upon the available research in developmental biology and experimental teratology, principles that remain valid even today (Jelinek, 2005; Kalter, 2003). These principles are:

1. Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which it interacts with adverse environmental factors.
2. Susceptibility to teratogenesis varies with the developmental stage at the time of exposure to an adverse influence.
3. Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate sequences of abnormal developmental events (pathogenesis).

4. The access of adverse environmental influences to developing tissues depends on the nature of the influences (agent).
5. The four manifestation of deviant development are death, malformation, growth retardation, and functional deficit.
6. Manifestations of deviant development increase in frequency and degree as dosage increases, from the no-effect to the totally lethal level.

Several mechanisms of congenital malformations were proposed; mutation, chromosomal nondisjunction, mitotic interference, altered nucleic acid functions, lack of substrates or precursors, lack of energy sources, enzyme inhibition, altered membrane characteristics, and osmolar imbalance (Wilson, 1973). Recently, molecular mechanisms of teratogenesis, for example, free radicals, apoptosis, and inhibition of histone deacetylase have been proposed, and these mechanisms are discussed in section 1.2.

1.1.3.2. Neural Tube Defects

Neural tube defects (NTDs) are one of the most common congenital malformations in humans with the prevalence of approximately 1-2 in 1000 live births, and their incidence varies among different populations and geographic locations (Copp et al., 2003). NTDs occur very early during pregnancy, and are caused by failure of the neural tube closure during embryogenesis at any level of the rostrocaudal axis. The most common NTDs include anencephaly (a severe form of exencephaly) and spina bifida resulting from the failure of fusion in the cranial and spinal region of the neural tube, respectively. Infants with anencephaly are stillborn or die shortly after birth, whereas infants with spina bifida can survive but often with severe and life-long physical and developmental disabilities (Botto et al., 1999). Various types of spina bifida exist depending on the position of the spinal cord, and condition of vertebra and skin, e.g., spina bifida occulta, spina bifida with meningocele, meningomyelocele, or myeloschisis (Moore and Persaud, 2003; Rossi et al., 2004). Most of these NTDs are due to the failure of primary neurulation, although spina bifida occulta results from the failure of secondary neurulation (Copp et al., 2003). Another rare form of NTD is

craniorachischisis, with a prevalence of approximately 1 in 100,000 births, that results from failure of neural tube closure over the entire body axis. Iniencephaly presents with occipital bone defects, partial or total absence of cervicothoracal vertebrae, and fetal retroflexion (Botto et al., 1999; Moore et al., 1997; Tugrul et al., 2007).

Studies suggest that genetic factors heavily contribute to the etiology of NTDs. The genetic component to NTDs include chromosomal abnormalities (such as trisomy 13, 18, and 21), genetic syndromes (e.g. Meckel syndrome, Fraser syndrome, Gruber syndrome, Waardenburg syndrome, cerebrocostomandibular syndrome, and anal stenosis), ethnic and racial differences in incidence rates, increased risk for a second affected child for couples with one affected infant (3 – 5 fold) and for siblings of affected individuals compared to the general population (10 fold), and the occurrence of familial settings or distributions that do not follow mendelian inheritance (Chen, 2007; Kibar et al., 2007; Padmanabhan, 2006). A recent study indicated that genetic factors for NTDs may be transmitted preferentially from the mother's side of the family (Byrne and Carolan, 2006). To date, however, no single gene has been implicated as a direct cause of NTDs in humans (Harris, 2001).

A large number of experimental and epidemiological studies indicate that the etiology of NTDs also involves environmental factors. Geographic, epidemic, socioeconomic, and seasonal variations in the incidence of birth defects have implicated an important role for environmental factors in the etiology of NTDs (Frey and Hauser, 2003; Mitchell, 2005). In addition, these variations in frequency might point to possible gene-environment interactions at the time of neural tube closure (Padmanabhan, 2006). Animal and epidemiological studies on NTDs provide some evidence that the following agents are possible risk factors of NTDs in humans: physical agents (e.g. x-irradiation and hyperthermia), drugs (e.g. thalidomide, folate antagonists, androgenic hormones, and antiepileptics), substance abuse (e.g. alcohol), chemical agents (e.g. organic mercury, lead), maternal infections (e.g. rubella, cytomegalovirus, *toxoplasmo gondii*, syphilis), and maternal metabolic conditions (e.g. phenylketonuria, diabetes mellitus,

endemic cretinism) (Padmanabhan, 2006). Further discussion about the role of environmental factors in the etiology of NTDs is also found in section 1.2.2.

The pathogenesis of NTDs is believed to involve inhibition of cellular proliferation, disturbance in the shaping of the developing neuroectoderm, or negative changes in vascular development supporting these cells (Bennett and Finnell, 1998). Currently, more than 190 mouse mutant models for NTDs have been utilized to study the mechanisms of NTDs (Harris and Juriloff, 2007). Studies using these mutants show that there are probably different mechanisms specific to each type of NTD, as well as a few common mechanisms among particular types of NTDs (Harris and Juriloff, 1999). In the curly tale (*ct*) model, in which spina bifida occurs in 15-20% of embryos (Embury et al., 1979), the defect appears to be in the adjacent tissue ventral to the posterior neuropore and not in the neural tube folds themselves. In *Cart1, Tcfap2a (Ap2),* and *Apob* mutants, the neural tube fails to elevate because of abnormal and excessive cell death (Harris and Juriloff, 1999). Defective lamina in surface ectoderm and broad floorplate and enlarged notochord also lead to the failure of neural tube closure in *Lama5* and *looptail (Lp)* mutants, respectively (Greene et al., 1998; Hackett et al., 1997). The location and presence of the initiation site for neural tube closure also appear to be the key factors for the certain types of NTDs. Mouse strains with a shift of initiation site 2 towards the rostral direction have a greater predisposition to NTDs, and the exencephaly prone strain, SELH/Bc, even lacks the site (Fleming and Copp, 2000). Strain differences in the susceptibility to NTDs are also found following an exposure to certain agents (Finnell et al., 1986, 1988). At the molecular level, mechanisms proposed include the failure of regulation of the arrangement of actin molecules, the signaling pathway that involves p300 for the regulation of apoptosis, and folic acid metabolism (Harris and Juriloff, 1999).

1.1.3.3. Prevention of Neural Tube Defects

It has been known for more than 20 years that maternal folic acid supplementation prior to and during early pregnancy can reduce the occurrence and recurrence of NTDs by as much as 70% (Czeizel and Dudas, 1992; Finnell et al., 2004; Smithells et al., 1980; Wald and Gilbertson, 1995). Recent data also suggested the protective effects of folic acid for other congenital malformations such as conotruncal heart defects and craniofacial malformations (Berry et al., 1999; Czeizel and Dudas, 1992; Wald et al., 2001). The protective effect in the occurrence of NTDs was observed with 4 mg or 0.8 mg of folic acid supplementation per day in the largest trials, leading to the recommendation that all women who could become pregnant should receive 0.4 mg of folic acid per day (Czeizel and Dudas, 1992; Greene and Copp, 2005). In the United States and subsequently other countries, governments now set health policies that mandate fortification of staple foods. The effectiveness of folic acid fortification has already been shown with a significant reduction (15-50%) in the prevalence of NTDs in the United States, Canada, and Western Australia (Bower et al., 2002; Honein et al., 2001; Persad et al., 2002).

To understand the molecular mechanism of this protective effect of maternal folic acid supplementation, folate uptake, the folate cycles, and the closely-related homocysteine (Hcy) metabolism have been investigated (Fig. 1.1) (van der Linden et al., 2006).

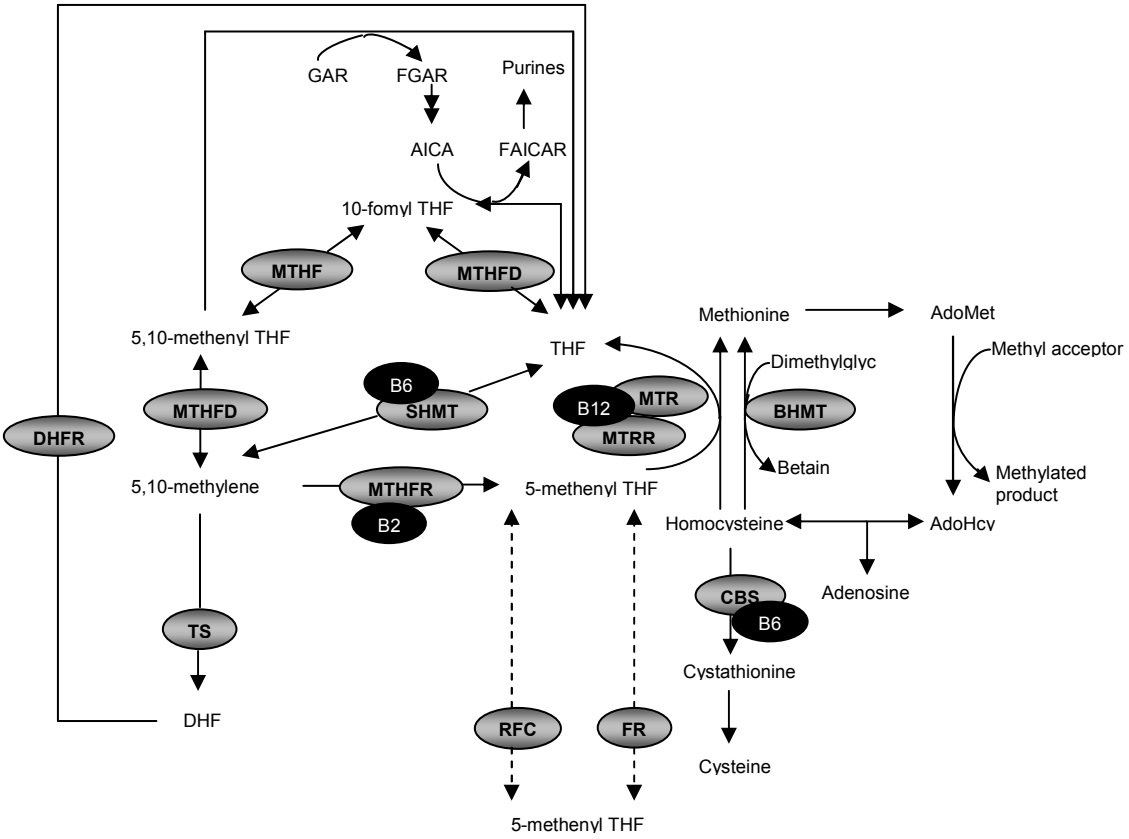

Figure 1.1. Simplified folate pathway and homocysteine metabolism. GAR, glycinamide ribonucleotide; FGAR, formyl glycinamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; FAICAR, formyl 5-aminoimidazole-4-carboxamide ribonucleotide; THF, tetrahydrofolate; DHF, dihydrofolate; DHFR, dihydrofolate reductase; MTHFD, methylenetetrahydrofolate dehydrogenase; TS, thymidylate synthase; SHMT, serine hydroxymethyltransferase; MTHFR, methylenetetrahydrofolate reductase: MTR, methionine synthase; MTRR, methionine synthase reductase; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; RFC, reduced folate carrier; FR, folate receptor, AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; B6, pyridoxal 5'-phosphate; B12, cobalamin; B2, riboflavin. Adapted from van der Linden et al., (2006).

Mutations and polymorphisms in genes encoding proteins directly involved in folic acid uptake and metabolism were screened, and the causal relationship with the risk of NTDs was analyzed. These genes include *folate receptor alpha (FBα)*, *reduced folate carrier (RFC)*, *5, 10-methylene-tetrahydrofolate reductase (MTHFR)*, *cystathionine B-synthase (CBS)*, *methionine synthase (MS)*, *methionine synthase reductase (MTR)*, and *methylenetetrahydrofolate dehydrogenase (MTHFD)* (Barber et al., 1998, 2000; Botto

and Yang, 2000; Brody et al., 1999; De Marco et al., 2000; Finnell et al., 1998; Hol et al., 1998; Lucock et al., 1997; Ou et al., 1996; Shields et al., 1999; van der Put et al., 1995, 1997; Wilson et al., 1999). One of the few polymorphisms that have been identified to be possibly related causally to the NTDs is the *MTHFR* polymorphism. One of the common polymorphisms that occurs is the change at nucleotide C677T, which results in a single amino acid substitution of alanine to valine, and individuals who are homozygous for this gene have 50 – 60% lower enzyme activity at high temperatures as it is a thermolabile form of the wild-type enzyme. These individuals have slightly elevated homocysteine concentrations if their folic acid intake is low. Published studies have shown that infants who are homozygous for the C677T variant have a two to seven fold increased risk for NTDs (Botto and Yang, 2000; van der Put et al., 1995). However, not all of the epidemiological studies found a positive association between this allele and an increased risk for NTDs. Nevertheless, these studies have provided strong evidence that gene-nutrient interactions are significant risk factors for congenital malformations.

Studies using mouse mutant models also suggest that folate metabolism is associated with the risk of NTDs. Mouse models with disrupted folate transport, *folic acid-binding protein 1 (Folbp1)* and *reduced folate carrier 1 (RFC1)* knockouts, have NTDs. However, mutant models for *MTHFR, CBS*, and *MTR* showed no NTD phenotype (Chen et al., 2001; Swanson et al., 2001; Watanabe et al., 1995).

The protective effect of folic acid for NTDs has also been shown in animal studies. Mouse mutants with NTDs including *Cart1*, *splotch*, and *Cited2* showed reduced incidence of NTDs after folic acid supplementation by approximately 60%, 65%, and 84%, respectively (Barbera et al., 2002; Fleming and Copp, 1998; Zhao et al., 1996). In addition to these NTDs that arise from gene mutations, it has been shown that the risk of NTDs induced by teratogenic agents can be also reduced by folic acid supplementation. These agents include valproic acid, hyperthermia and fumonisin (Greene and Copp, 2005; Marasas et al., 2004; Padmanabhan and Shafiullah, 2003; Sadler et al., 2002; Shin and Shiota, 1999; Shiota, 1988). The protective effect for

NTDs has also been shown for inositol, thymidine, arachidonic acid, methionine, and panthothenic acid (Greene and Copp, 2005).

1.1.3.4. Other Congenital Malformations

Cardiovascular defects are the most common defects above all other malformations affecting 1-2% of newborns, and congenital heart defects (CHDs) account for 25% of all congenital malformations and are the leading cause of death in infants under one year of age (Nemer, 2008). Limb defects are one of the most common and visible malformations. They are therefore the most frequently reported phenotypic effects induced by toxicants (Holmes, 2002). Limb malformations are of maltifactorial origin, and known environmental factors that increase the risk of limb defects include thalidomide, warfarin, phenytoin, and valproic acid.

Although not outwardly visible congenital defects, neurological disorders and associated behavioral alterations are now known to be caused by genetic factors and neurodevelopmental toxicants. A variety of environmental chemicals and drugs, known to induce NTDs, are also recognized as neurotoxicants that cause neurodevelopmental problems. These agents include alcohol, retinoic acids, antiepileptic drugs, methyl mercury, cadmium, lead, pesticides, and nicotine (Slikker and Chang, 1998). Animal studies have been performed to understand diagnostic behaviors such as deficits in social interaction, autism, schizophrenia, and obsessive-compulsive disorder (Ridley, 1994). Deficits in social interaction are seen in animals exposed to lead, methylmercury, and maternal deprivation (Burbacher et al., 1990; Laughlin et al., 1991; Seay and Harlow, 1965). Together, the mechanisms of such neurological disorders may be closely related to the mechanisms of NTDs.

1.2. TERATOGENS

1.2.1. Overview of Teratogens

The term teratogen refers to any physical agent, chemical, drug, infectious disease, environmental agent, or maternal condition that produces permanent alterations

in morphology or function of the offspring during prenatal life (Shepard, 1995). There are 65,000 chemicals in the general environment with hundreds or thousands of new chemicals being added every year (Schardein, 1993). Of these, approximately 3,000 have been tested in animals, 1,200 of which cause birth defects in animals, and only 40 of which are known to be teratogenic to humans (Table 1.3) (Shepard, 1995). Known teratogens account for roughly 5-10% of birth defects (Brent and Beckman, 1990; National Research Council, 2000).

1.2.1.1. Thalidomide

From an historical perspective, the concept that chemical or physical agents could cause birth defects in humans is a relatively recent event. The first human teratogen, rubella (German measles), was discovered by an Australian doctor Gregg in 1941 (Schumacher, 2004). Further teratological research was conducted on ionizing irradiation after atomic bombs were dropped on Hiroshima and Nagasaki, Japan, exposing more than 1,500 pregnant women to radiation (Wood et al., 1967a, 1967b, 1967c). These studies provided evidence that the placental barrier between mother and fetus does not fully protect the fetus from teratogens.

Teratological research received significant attention from the general public after the thalidomide disaster (1959 – 1962). This disaster led to the establishment of the Teratology Society and fostered an upsurge in teratological research as well as the establishment of strict safety testing guidelines during pregnancy (Bosch et al., 2008). Thalidomide, 1,3-dioxo-2-[2',6'-dioxopiperidin-3'-yl]-isoindol, is a sedative, hypnotic, and anti-inflammatory medication that was developed by a German pharmaceutical company and was sold from 1957 to 1961 in almost 50 countries under at least 40 different names, e.g., Distaval, Kevadon, Talimol, Nibrol, Sedimide, Quietoplex, Contergan, Neurosedyn, and Softenon (Bosch et al., 2008; Shepard, 1995). Thalidomide was prescribed to pregnant women to avoid morning sickness and to help them sleep.

Table 1.3
List of Teratogenic Agents in Humans*

Radiation	Drugs and Environmental Chemicals
Atomic weapons	Aminopterin and methylaminopterin
Radioiodine	Androgenic hormones
Therapeutic	Busulfan
Infections	Captopril (renal failure)
Cytomegalovirus (CMV)	Chlorobiphenyls
Herpes virus hominisis? I and II	Cocaine
Parvovirus B-19 (Erythema infectiosum)	Coumarin anticoagulants
Rubella virus	Cyclophosphamide
Syphilis	Diethylstilbestrol
Toxoplasmosis	Diphenylhydantoin
Varicella virus	Etretinate
Venezuelan equine encephalitis virus	Iodides and goiter
Maternal Metabolic Imbalance	Lithium
Alcoholism	Mercury, organic
Chronic Villus Sampling (before day 60)	Methimazole and scalp defects
Cretinism, endemic	Methylene blue via intraamniotic injection
Diabetes	Penicillamine
Folic acid deficiency (following gastric by-pass surgery)	13-cis-Retinoic acid (Isotretinoin and Accutane)
Hyperthermia	Tetracyclines
Phenylketonurea	Thalidomide
Rheumatic disease and Sjogren's syndrome and congenital heart block	Toluene abuse
Virilizing tumors	Trimethadione
	Valproic acid

*Modified from Shepard, (1995).

Unfortunately, consumption of thalidomide early in pregnancy caused the births of nearly 10,000 newborns with a severe limb malformation, phocomelia, and other associated defects including defects in external ears and facial hemangioma. After this tragedy, the United States congress enacted laws in 1962, requiring tests for safety during pregnancy before any drug could be approved for use in humans, and, in addition, the FDA published guidelines for teratogenic testing of pharmaceuticals in 1966 (Bosch et al., 2008; Schumacher, 2004). In addition, a pregnancy labeling system was put in place by FDA (FDA, 1979), a system that is currently being updated on the basis of information available since 1979 (Public Affairs Committee of the Teratology Society, 2007). Published studies later have suggested that the S-enantiomer of thalidomide was the main cause of the teratogenic effects (Blaschke et al., 1979), and thalidomide was not

prescribed and sold for decades. Nevertheless, this painful experience with thalidomide has led us to understand the vulnerability of unborn human life and established teratology as an important component of medicine and toxicology (Schumacher, 2004). More recently, the effectiveness of thalidomide against erythema nodusum leprosum as well as other diseases, including cancers and inflammatory diseases, e.g., multiple myeloma, has led the FDA to approve the reintroduction of thalidomide in 2006. To prevent usage by pregnant women, this approval came with strict regulations and a requirement for extensive patient education (Bosch et al., 2008; Richardson et al., 2007).

1.2.1.2. Developmental Toxicity Testing

Currently, several testing methods are used to screen chemicals and other agents for teratogenic effects. In vivo animal techniques have been used for developmental toxicity testing according to international guidelines, and they require pregnant animals such as mice, rats, and rabbits to assess toxic effects to the mother and offspring (Piersma, 2004). In this evaluation method, physiological effects including the morphology and function of the placenta, which is responsible for the exchange of metabolic and gaseous products between the mother and offspring, need to be considered. Four segments of the prenatal development are tested using in vivo methods; preimplantation, organogenesis, fetal development, and lactation period (Schumacher, 2004). Although these tests, along with human epidemiological studies and case reports, have been used by regulatory agencies (U.S. EPA guidelines on prenatal developmental toxicity testing in 1997, and risk assessment in development, reproduction, and neurotoxicology in 1991, 1996, and 1998, respectively) to characterize the potential human health risk in the area of developmental and reproductive toxicity, these approaches to assess human risk are limited because they are time consuming, expensive, and require large number of live animals (Kimmel, 1998; Webster et al., 1997).

Alternative methods to in vivo testing have been developed since the early 1980s, and one of the methods is in vitro whole embryo culture system (WEC). This system, which uses culture bottles to grow embryos in a serum-containing medium under a

steady oxygen level, was developed by New (New, 1978, 1990). This method has several advantages compared to in vivo testing (Webster et al., 1997). Malformations resulting from WEC are directly related to the serum concentration of the agent tested and can be compared to the serum concentration in the human. This comparison is not possible in vivo testing because of the possible pharmacokinetic differences between humans and experimental animals. In vivo testing is also limited by the possibility that metabolites produced in humans and experimental animals may be different. This problem can be bypassed in WEC by adding the metabolite directly. The only major disadvantage in WEC, however, is that the types of malformations that can be induced are limited. This may produce false-negative results for the compounds that exert their teratological effects in very early or late gestation, the period of embryogenesis that can not be tested in the culture system. In addition to WEC, organ culture systems using parts of the embryonic body such as mandible, anlagen of extremities, endocrine organs or tooth buds, have also been used to screen teratogenic compounds (Schumacher, 2004).

Some of the more recently developed in vitro approaches include micromass (MM) cultures of cells derived from dissociated embryos and the embryonic stem cell test (EST) (Faustman, 1988; Spielmann, 1997). The MM technique uses primary cells from mammalian embryos, and the best documented examples include limb bud and brain micromass (Flint and Orton, 1984). The EST approach has the advantage of using established cell lines that resemble early embryos without the necessity to sacrifice pregnant animals and embryos (Rohwedel et al., 2001; Scholz et al., 1999). Alternative methods like WEC, MM, and EST have been used in the drug and chemical industry for screening new chemicals that are structurally related to known embryotoxic chemicals (Scholz et al., 1999), and validation of these methods for developmental toxicity testing have been conducted, including the study carried out by European Centre for the Validation of Alternative Methods (ECVAM) in 2002 (Genschow et al., 2002; Piersma et al., 2004). These techniques, especially EST, therefore, have a great potential to meet a strong demand for alternative in vitro methods to screen a large number of chemicals

for their possible embryotoxicity (Festag et al., 2007). However, it should be noted that these methods do not represent replacements for current animal tests for reproductive toxicity as a whole, although they could provide suitable means for reducing the use of animals in the context of testing strategies.

Although these advanced testing methods, including in vivo and various in vitro techniques, have been routinely used to screen chemicals and other agents for teratogenic effects, human epidemiologic surveillance is and will be our most powerful method to discover human teratogens and to identify the environmental causes of human birth defects (Brent, 2004). The National Birth Defects Prevention Study (NBDPS) initiated by the Centers for Disease Control and Prevention (CDC) in 1997 covers an annual birth population of 482,000 and includes cases identified from birth defect surveillance registries in eight states (Yoon et al., 2001). This study, assisted by clinicians and physicians as well as epidemiologists, abstractors, coordinators, and data managers (Lin et al., 2006), is designed to identify genetic and environmental factors important in the etiology of birth defects (Yoon et al., 2001). However, epidemiologic studies also have some disadvantages, e.g., the occurrence of individual malformations is relatively rare, the fetus is exposed to an array of unknown genetic and environmental factors, the molecular mechanisms that cause most birth defects are unknown, and the defects identified at birth represent only the birth prevalence, not the true incidence of the condition, suggesting that these studies need to be examined carefully with the existing experimental data (Yoon et al., 2001). Together, the establishment of developmental toxicity testing as well as the well-thought experimental design is very important for the identification of teratogens.

1.2.2. Teratogens That Affect Central Nervous System

1.2.2.1. Hyperthermia

Hyperthermia (HS) refers to a body temperature that is above the upper limit of the normal range for the species. It was the first teratogen in animals that was subsequently proven to be teratogenic in humans (Edwards, 2006; Graham et al., 1998).

The normal body temperature range varies by species, e.g., the average temperature is 37 ºC for humans (oral temperature), 38.3 ºC for mice (Shiota, 1988), 39 ºC for sheep and pigs, 39.5 ºC for goats (Blood and Radostits, 1989), and 39.5 ºC for guinea pigs (Edwards, 1969). Therefore, the effects of hyperthermia can be only assessed by the elevation of body temperature above the normal body temperature of the species.

Hyperthermia in humans can be caused by fever related to viral or bacterial illnesses, extreme exercise, saunas, hot tubs, heated beds, and electric blankets (Moretti et al., 2005). Hyperthermia exposure during pregnancy can result in embryonic death, abortion, growth retardation, and various types of malformations, including NTDs (Edwards, 2006). Although the most common outcomes in humans are usually prenatal losses due to an uncontrolled hyperthermia exposure such as a febrile illness, the development of the CNS is known to be particularly sensitive, and published studies have shown that maternal hyperthermia can account for 10-14% of NTD cases (Edwards et al., 2003; Graham et al., 1998; Kline et al., 1985; Shiota, 1982). Epidemiological studies of children with maternal fevers have shown that a 2 ºC elevation for at least 24 hr can result in a range of malformations, and most malformations observed in humans have been confirmed in experimental animals such as guinea pigs, mice, rats, rabbits, sheep, and monkeys (Edwards, 1986; Edwards et al., 2003; Finnell et al., 1992). These defects include NTDs, microphthalmia, cataract, abdominal wall defects, heart defects, microcephaly, mental retardation, talipes, facial limb defects, midface hypoplasia, cleft lip and palate, hypodactyly, renal defects, Möbius syndrome, and arthrogryposis (Edwards, 2006).

It has been questioned whether these defects are caused by metabolic changes in the mother due to the infection and fever and not by the direct action of elevated temperature. This was mostly solved by studies using mouse WEC, showing the association of temperature with the defects observed (Cockroft and New, 1975, 1978; Kimmel et al., 1993; Mirkes, 1985, 1987; Walsh et al., 1987).

Since the outbreak of abortions and congenital malformations found in guinea pigs in 1965 by Marshall J. Edwards (Edwards et al., 1995), studies using experimental

animals and cells have been conducted to understand the effects of hyperthermia in humans and animals, to investigate the pathogenic effects of hyperthermia and mechanisms of congenital malformations such as NTDs, and to identify other agents that might interact with hyperthermia. In earlier studies, thresholds and dose-response relationships for the induction of congenital malformations have been investigated. In rats a 2.5 ºC elevation for 1 hr during early neural tube closure induced an increased incidence of cranio-facial defects, and an elevated temperature of 2-2.5 ºC for 1 hr during early neurogenesis in guinea pigs resulted in an increased incidence of microencephaly (Lary et al., 1983, 1986; Wanner and Edwards, 1983). These studies show that in general, thresholds and dose-response relationship depend on the stage of development at which exposure occurs and vary between species and even between different strains of the same species (Edwards et al., 2003). Finnell, et al. (1986) showed that 44.3% of fetuses of SWV mice exposed to hyperthermia for 10 min at 43 ºC exhibited exencephaly whereas less than 14% of fetuses from LM/Bc, SWR/J, C57BL/6J, and DBA/2J strains of mice were affected. The sensitivity of SWV strain was lost in the offspring from crosses between the SWV and C57BL/6J strain. Different sensitivity among strains has been also reported for teratogens such as valproic acid (Finnell et al., 1988; Naruse et al., 1988), cadmium (Hovland et al., 1999), 2-ethyl hexanoate (Collins et al., 1992), 4-propyl-4-pentenoic acid (Finnell et al., 1988), and carbon dioxide (Collins et al., 1992).

Several possible mechanisms of hyperthermia have been studied, and these include disruption of the cellular processes essential for organogenesis such as cell proliferation, migration, differentiation, apoptosis, and damage to the embryonic vascular system (Edwards et al., 2003). Of these, the best described pathogenic effects and mechanisms is cell death (apoptosis). Further discussion about cell death is found in section 1.3. Edwards was the first investigator to observe cell death in day 21 guinea-pigs exposed to hyperthermia at 42 ºC for 1 hr, with up to 86% of mitotic cells showing damage in the form of nuclear clumping (Edwards et al., 1974). Cell death and inhibition of mitotic activity were also observed in other animals (Shiota, 1988).

Subsequently, it was later shown that this hyperthermia-induced cell death occurs by a process termed apoptosis (Mirkes et al., 1997). In addition, the increased resistance to hyperthermia-induced apoptosis by the induction of thermotolerance was also observed in rats (Mirkes et al., 1997). This thermotolerance was achieved by exposing rat embryos to hyperthermia at 42 ºC for 15 min and then 1 hr later exposing them to 43 ºC for 15 min. Furthermore, the acquisition of thermotolerance showed a correlation with the induction and nuclear translocation of a heat shock protein, Hsp72 (Thayer and Mirkes, 1997). The induction of another heat shock protein, Hsp27, was also observed in embryos exposed to hyperthermia, and the heart of the exposed embryos, where Hsp27 is predominantly expressed, showed resistance to the cytotoxic effects of hyperthermia (Mirkes et al., 1996). The apoptotic pathway observed in embryos exposed to hyperthermia was further examined at the molecular level, and the observation of DNA fragmentation, apoptotic bodies (Mirkes et al., 1997), cytochrome c release (Mirkes and Little, 2000), activation of caspase-3 (Umpierre et al., 2001) and caspase-9 (Little and Mirkes, 2002) have identified the mitochondrial apoptotic pathway (intrinsic pathway) as the main cell death pathway observed (detailed discussion found in section 1.3.4). The upstream events of the mitochondrial apoptotic pathway induced by hyperthermia have been also examined, although it is still not well understood. Global gene analysis using microarrays have identified three p53 target genes that were upregulated by the treatment of hyperthermia, suggesting that p53 might play a role in the induction of apoptosis (Mikheeva et al., 2004).

Animal studies also revealed that some agents interact positively or negatively with hyperthermia to increase or decrease the incidence and severity of malformations. Agents that increase the incidence of malformations experimentally include alcohol (Graham and Ferm, 1985; Shiota, 1988), vitamin A (Ferm and Ferm, 1979), arsenic (Ferm and Kilham, 1977), lead (Edwards and Beatson, 1984), toxemia (Hilbelink et al., 1986), and ultrasound (Angles et al., 1990). Subteratogenic doses of these agents combined with normally harmless temperature can cause birth defects. This might be of particular concern for febrile patients using ultrasound (Edwards, 2006). In contrast,

clinical and experimental studies have shown that folate (Acs et al., 2005; Shin and Shiota, 1999) and multivitamin supplements containing folates (Botto et al., 2002) reduce the risk of damage. Although aspirin and other antipyretic agents also showed protective effects, the experimental studies did not support the clinical studies (Acs et al., 2005; Suarez et al., 2004; Tiboni et al., 1998).

1.2.2.2. Cyclophosphamide

Cyclophosphamide (CP) is an alkylating agent used in the treatment of malignant tumors and autoimmune disorders (Fleming, 1997; Sladek, 1988). CP is also a well studied teratogen that can produce malformations, including exencephaly, cleft palate, syndactyly and kinky tail in mice, and absence of toes and cleft palate in humans. Although the embryotoxicity of CP has been extensively studied in experimental animals, human studies are limited as the number of reported cases is rare and these patients were also exposed to X-ray treatment. CP is a prodrug that needs to be metabolized to exert its therapeutic and embryotoxic effects (Fig. 1.2). CP is mostly metabolized by cytochrome P450 via 4-hydroxylation and *N*-dechloroethylation, with only 10-30% of CP excreted in the urine as original parent compound (Moore, 1991; Sladek, 1988). The P450 enzymes responsible for the 4-hydroxylation are CYP2B6, CYP3A4, and CYP2C, and the major metabolite of CP, 4-hydroxycyclophosphamide yields the active alkylating agent, phosphoramide mustard (PM), and the byproduct, acrolein (AC) (Chang et al., 1993; Huang et al., 2000; Roy et al., 1999). Both PM and AC are active metabolites that interact with cellular macromolecules, and each cause unique teratogenic effects in day 10 rat embryos. PM-treated embryos exhibit abnormalities of the prosencephalon whereas embryos exposed to AC exhibit abnormal axial rotation (Little and Mirkes, 1990; Slott and Hales, 1988). PM binds predominantly to DNA, damaging DNA by forming cross-links, whereas AC binds preferentially to embryonic proteins and does not cause DNA damage at embryotoxic concentrations (Little and Mirkes, 1987, 1990). Subsequently it was shown that the DNA cross-links induced by PM are responsible for the teratogenic effects of CP (Little and Mirkes, 1987).

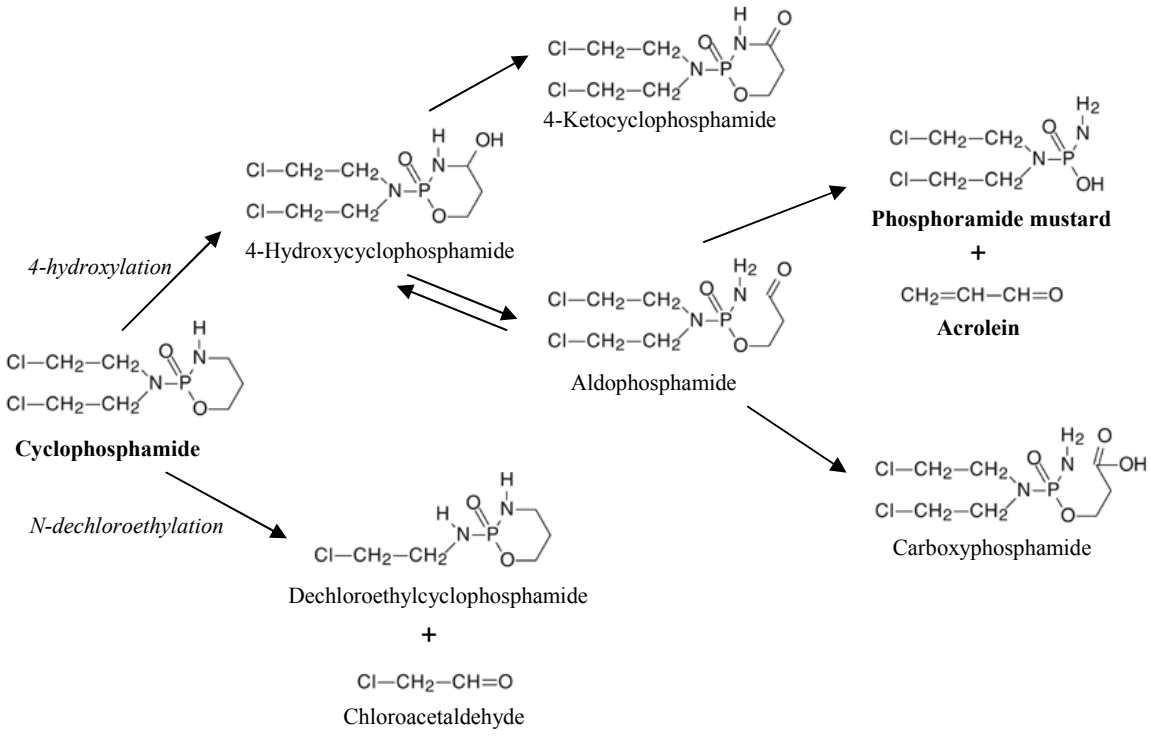

Figure 1.2. Metabolism of cyclophosphamide. Modified de Jonge, et al., (2006) and Rodriguez-Antona and Ingelman-Sundberg, (2006).

One of the known early effects of CP is a perturbation of the cell cycle (Meyn and Murray, 1986). In vitro studies showed that exposure of embryos to PM delayed cell cycle time, particularly the S phase (Mirkes et al., 1989). Other studies have also described effects on the cell cycle after exposure to CP, PM, and AC (D'Incalci et al., 1983; Marano and Puiseux-Dao, 1982; Millar et al., 1986). Recently, the activated analog of CP, 4-hydroperoxycyclophosphamide (4CP), has been shown to activate apoptosis by inducing the mitochondrial apoptotic pathway in mouse embryos (Huang and Hales, 2002; Mirkes and Little, 1998, 2000). In addition, a possible role of p53, a transcription factor that induces apoptosis or cell cycle arrest following a variety of stresses, has been suggested to play a role in apoptosis observed in embryos exposed to CP (Mikheeva et al., 2004; Pekar et al., 2007).

1.2.2.3. Other Teratogens

As discussed in section 1.1.4, other well known teratogens that affect the CNS include, but are not limited to, alcohol, retinoic acid, and folate deficiency (folate metabolism discussed in section 1.1.4.4). Maternal alcohol consumption has been the most common nongenetic cause of mental retardation and can cause a spectrum of physical and mental dysfunction (Abel and Sokol, 1987; Dunty et al., 2001). Fetal alcohol syndrome (FAS), a collection of abnormalities caused by maternal alcohol abuse, occurs in approximately 1-7 in 1000 live births in the United States (Niccols, 2007). Individuals with FAS exhibit pre- and postnatal growth deficiencies, characteristic craniofacial dysmorphology, and varying degrees of CNS dysfunction such as changes in brain structure, cognitive impairments, and behavior problems (Dunty et al., 2001; Niccols, 2007). Although the abnormalities of numerous cellular activities are well studied in animal models and in vitro studies, a large body of evidence indicates that apoptosis is an important pathogenic feature of the FAS (Smith, 1997; Sulik et al., 1988).

Retinoic acid (RA), a metabolite of vitamin A (retinol), is essential not only for the differentiation of progenitor cells to neurons and glia but also for organogenesis, cell proliferation, and apoptosis (Mark et al., 2006; McCaffery et al., 2003). Precise regulation of RA level by controlled synthesis and catabolism is, therefore, essential for normal development. This explains why both excess and deficiency in RA cause developmental malformations and why those areas in which RA signaling takes place are the most sensitive regions for its teratogenicity (Collins and Mao, 1999; McCaffery et al., 2003). The malformations observed in experimental animals include exencephaly, spina bifida, cleft palate, eye defects, hydrocephaly, and shortening of the mandible and maxilla for excess RA and malformations in the eye, urogenital tract, diaphragm, heart, and lung for RA deficiency (Cohlan, 1954; Wilson and Warkany, 1950). The most biologically active retinoids identified are all-trans retinoic acid and its 9-cis isomer. Extensive studies, including studies in vitro, have revealed that retinoid nuclear receptors such as retinoic acid receptor (RAR) and retinoid X receptor (RXR) transduce RA

signals during development, and specific heterodimers are involved in particular developmental processes.

The molecular mechanisms of teratogens that induce NTDs as well as other teratogens have been extensively studied. One of the mechanisms repeatedly identified is apoptosis, and the next section will discuss molecular pathways of apoptosis and apoptosis in normal and abnormal development.

1.3. APOPTOSIS

1.3.1. Molecular Pathways of Apoptosis

Cell death in animals is classified into three types; apoptosis (type I), autophagy (type II), and necrosis (type III) (Krysko et al., 2008). Apoptosis is an active and well controlled process that is important in the maintenance of tissue homeostasis and development. Autophagy is a cytosolic degradation process by which intact organelles or large portion of cytoplasm are engulfed within autophagic vacuoles to ensure the physiological turnover of old and damaged organelles (Galluzzi et al., 2008). Necrosis is distinct from apoptosis and defined as a passive and uncontrolled process that results from high level of physical stress such as injury. During necrosis, rapid and large changes in essential ions, osmolarity, and pH lead to the lysis of the cell and activation of an inflammatory response (Penaloza et al., 2006). Recent findings have changed our classical view of necrosis as a passive and accidental form of cell death to that of actively regulated process, and now it has been observed in parallel with apoptosis rather than as an alternative pathway to apoptosis (Henriquez et al., 2008). In addition, the boundary between apoptosis and autophagy has not been completely clear, largely due to the intrinsic factors among different cell types and the crosstalk among organelles within each type (Lockshin and Zakeri, 2004). In this section, the discussion will focus on molecular pathways of apoptosis and the importance of apoptosis in normal and abnormal development.

Until some of the physiological effects of apoptosis were observed in 1972, cell death in general was believed to be a passive process of cells destined to disintegrate

(Kerr et al., 1972). The observed physiological effects, including cell membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation suggested that cell death is actually an active process that is strictly controlled (Savill and Fadok, 2000). Subsequent studies have shown that chromatin condensation is associated with specific patterns of DNA fragmentation; fragmentation of DNA into large 300- and 50-kb segments and subsequent cleavage between nucleaosomes, resulting in the generation of DNA fragments that are multiples of 185 bp (Ploski and Aplan, 2001; Wyllie, 1980). Concurrently, the cell membrane and the nuclear envelope are thrown into numerous vesicles called apoptotic bodies, which are subsequently engulfed by macrophages or neighboring cells to avoid inflammatory response in the surrounding tissues (Mirkes, 2002; Savill and Fadok, 2000). This process, complementary to mitosis, was termed apoptosis by Currie and colleagues (Kerr et al., 1972).

Fundamental discoveries about the mechanisms of apoptosis at the molecular level were originally made using *C. elegans,* work which was later awarded the Nobel Prize in Medicine in 2002. During the development of *C. elegans*, 1090 cells are generated, of which 131 undergo programmed cell death (programmed cell death is later discussed in section 1.3.2) (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977; Sulston et al., 1983). Genetic analyses of mutated *C. elegans* have identified the basic components of the cell death machinery, which turn out to be widely conserved from nematodes to humans (Metzstein et al., 1998). Two genes that are largely responsible for the cell death were first discovered; *ced-3* (caspase homologue) and *ced-4* (Apaf-1 homologue) (Ellis and Horvitz, 1986). Subsequent screening studies using alleles of *ced-3* and *ced-4* identified another key gene, *ced-9* (antiapoptotic Bcl-2 homologue), that is required to prevent cell death (Hengartner and Horvitz, 1994). Another gene, *egl-1* (BH3 only member homologue), was found to serve as an upstream initiator of cell death (Conradt and Horvitz, 1998). Current evidence suggests that the activation of *egl-1* protein is first induced by upstream death signals, which results in recruitment of *ced-9* to *egl-1*. Following the binding of *egl-1* to *ced-9, ced-9* dissociates from *ced-4* for its

activation, and the free *ced-4* promotes the proteolytic cleavage of the proenzyme form of *ced-3* (Fig. 1.3). The activated *ced-3* finally targets various cellular proteins for

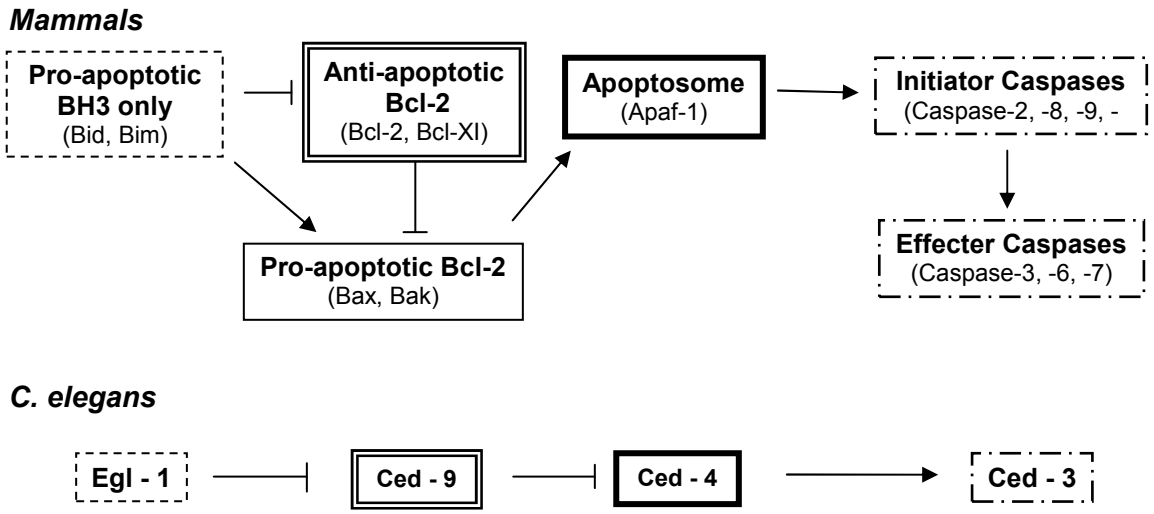

Figure 1.3. Comparison of cell death machinery in mammals to the *C. elegans* system. Modified Jin and El-Deiry, (2005).

cleavage, which results in the structural features of the apoptotic cells. Although the pathway of the apoptotic activation in mammals is analogous to that in *C. elegans*, apoptotic pathways in mammals are much more complex. For example, 14 caspases, the homologues of *ced-3*, have been identified in mammals, of which 7 caspases are known to play critical roles in apoptosis (Mirkes, 2002). Caspases are synthesized as inactive proenzymes (procaspases) that must be proteolyticaly processed to become activated. Caspases that are essential for the regulation and activation of apoptosis are classified into two groups according to their functions and the length of their N-terminal prodomain (Launay et al., 2005; Mirkes, 2002). Caspase-2, -8, -9, and -10, called initiator caspases activate effecter caspases, caspase-3, -6, and -7. Multiple homologues of *ced-9* and *egl-1*, which are antiapoptotic Bcl-2 proteins and proapoptotic BH3 only proteins, respectively, as well as proapoptotic Bcl-2 proteins are also found in mammals.

In mammals, two major apoptotic pathways, the extrinsic pathway (death receptor pathway) and the intrinsic pathway (the mitochondrial pathway), are activated

by a variety of external signals (Fig. 1.4) (Jin and El-Deiry, 2005). The death receptor pathway is activated by extrinsic signals that bind to cell surface receptors whereas the intrinsic pathway is initiated following intrinsic signals such as DNA damage induced by irradiation or chemicals, growth factor deprivation or oxidative stress. These signals activate apoptosis via the interaction with mitochondria (Leist and Jaattela, 2001).

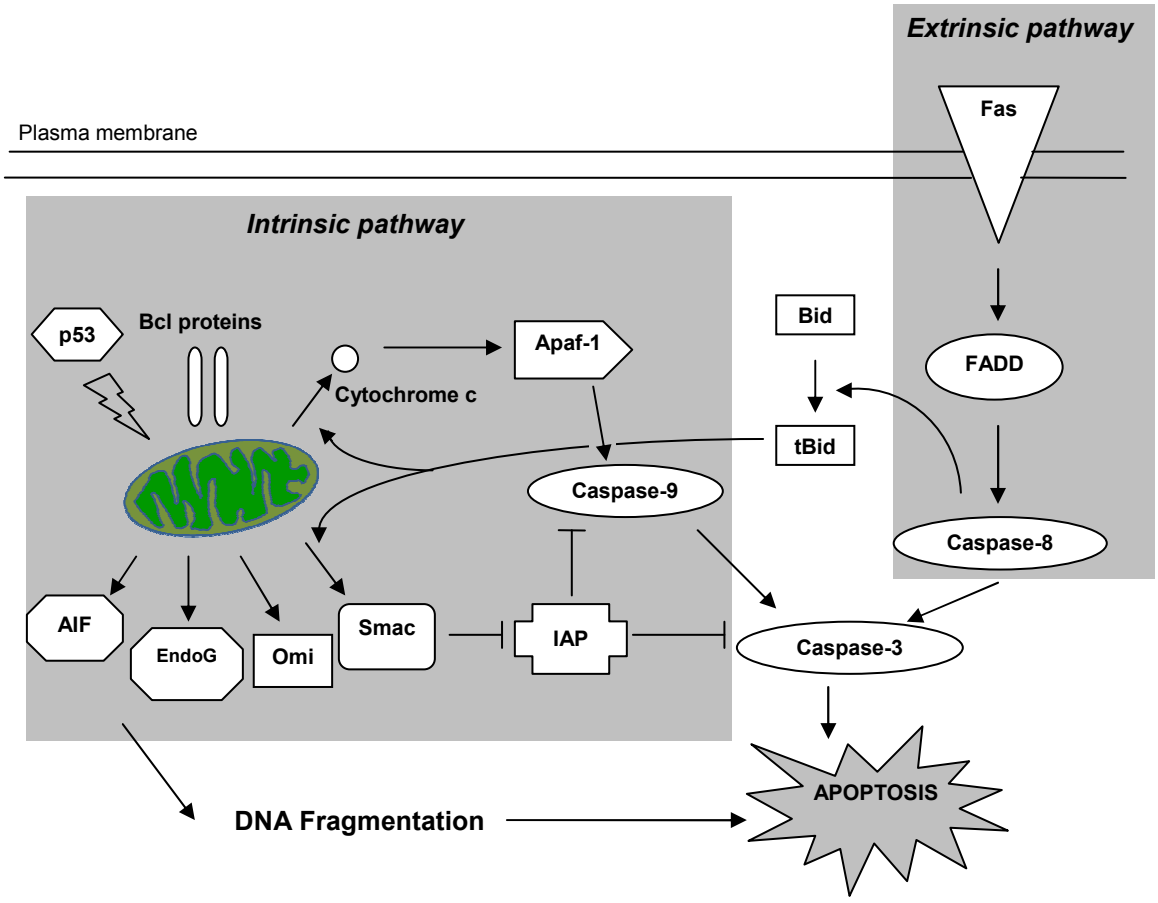

Figure 1.4. Simplified two major apoptotic pathways. Adapted from Shiozaki and Shi, (2004).

The extrinsic pathway is activated by ligand-bound death receptors including Fas, tumor necrosis factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL) receptors (Thorburn, 2004). Apoptosis induced through the extrinsic pathway is critical in several disease processes and has been a major focus in cancer research. The known death receptors all contain an intracellular globular protein interaction domain called a

death domain (DD). Binding of death ligand to the death receptors results in oligomerization of the receptors and recruitment of an adopter protein called Fas-associated death domain (FADD) (Chinnaiyan et al., 1995). FADD contains two domains: DD and a death effector domain (DED). FADD binds to the receptors through interactions between DDs and to procaspase-8 through DED interactions to form a death-inducing signaling complex (DISC). Caspase-8 is auto-cleaved and activated at the DISC, which then leads to the activation of effecter caspases, including caspase-3, -6, and -7 and the subsequent induction of apoptosis (Ashkenazi and Dixit, 1998).

The intrinsic pathways are triggered by various apoptotic signals and initiated inside the cells. The most central regulator of these pathways is mitochondria, and critical event in the intrinsic pathway is mitochondrial outer membrane permeabilization (MOMP) (Jin and El-Deiry, 2005). MOMP is mediated and regulated by Bcl-2 family proteins, and MOMP leads to the release of molecules from mitochondria such as cytochrome c, which is involved in the activation of caspases. Approximately 20 Bcl-2 family proteins have been identified in mammals. They are divided into three groups, but they all contain at least one Bcl-2 Homology (BH) domain. Antiapoptotic members, including Bcl-2, Bcl-Xl, Bcl-w, A1, and Mcl-1 contain four BH domains (BH1, BH2, BH3, and BH4) and prevent cell death by binding to proapoptotic members such as Bax, Bak, and Bok. These proapoptotic proteins also contain three domains (BH1, BH2, and BH3), and present a similar 3D structure with antiapoptotic proteins (Fesik, 2000; Hengartner, 2000). The third group consists of eight proapoptotic proteins that only contain the BH3 domain. These BH3 only proteins include Bid, Bad, Bik, Bim, Bmf, Hrk, Noxa, and Puma. Both proapoptotic groups are necessary for the induction of apoptosis, although BH3 only proteins apparently lie upstream of Bax/Bak because when cells lack Bax and Bak, they fail to induce apoptosis (Cheng et al., 2001; Zong et al., 2001). Death signals first activate BH3 only proteins, which subsequently induce oligomerization of the other proapoptotic group proteins such as Bax and Bak to insert into the outer mitochondrial membrane (OMM). BH3 only proteins can also function to release Bax and Bak from the antiapoptotic Bcl-2 family proteins (Cory and Adams,

2002; Huang and Strasser, 2000). Bax and Bak then induce MOMP, leading to the release of molecules from the mitochondrion through either permeability transition (PT) -dependent or –independent processes. MOMP then leads to the release of soluble mitochondrial proteins, the best studied of which is cytochrome c, a key component of the electron transport chain (Liu et al., 1996). The release of cytochrome c into the cytosol results in the interaction of cytochrome c with Apaf-1, procaspase 9, and ATP/dATP to form a complex called the apoptosome (Li et al., 1997). The apoptosome dimerizes and activates caspase-9. Caspase-9 then activates effector caspases, caspase-3, -6, and -7 leading to the induction of apoptosis. Other proteins released, for example, Smac/DIABLO, HtrA2/Omi, and GSPT1/eRF3, bind inhibitors of apoptosis (IAPs) to neutralize the inhibitory activity of IAPs to promote cytochrome c-dependent caspase activation (Shiozaki and Shi, 2004). IAPs act as a brake for apoptosis by inhibiting activated caspases. A different group of proteins are also released from mitochondria to promote caspase-independent apoptosis. For example, apoptosis inducing factor (AIF) translocates to the nucleus and induces chromatin condensation and large-scale DNA fragmentation (Susin et al., 1999). Another protein, endonuclease G protein (Endo G), is also released in response to apoptotic stimuli and is responsible for the induction of oligonucleosomal DNA fragmentation (Li et al., 2001).

Although these two apoptotic pathways are initiated through different processes, they converge on the activation of effector caspases (Danial and Korsmeyer, 2004). In addition, the death receptor pathway signaling can activate the mitochondrial pathway through the BH3 only protein, Bid. In this pathway, the activated caspase-8 cleaves cytosolic Bid, cleaved Bid (tBid) then translocates to the mitochondria, which leads to cytochrome c release (Li et al., 1998; Luo et al., 1998). Crosstalks between the two pathways also occur at the initiation level. For example, in p53-mediated apoptosis, p53 not only activates the intrinsic apoptotic proteins such as BAX and PUMA leading to the activation of mitochondrial pathway but also upregulates the death receptor pathway proteins like FASL and KILLER/DR5 (Kasibhatla et al., 1998; Wu et al., 1997). Detailed discussion about p53 is found in section 1.4.

1.3.2. Apoptosis in Normal Development (Programmed Cell Death)

Apoptosis occurs in various tissues at different times during development, and the genetically controlled sequence leading to cell death in development is referred to as programmed cell death (PCD) (Mirkes, 2008). Examples of PCD include the neural tube, eye and ear development, cardiovascular development, limb development, deletion of mullerian/wolffian ducts, and kidney development. These widely recognized examples of PCD can be classified into several categories based on their functions; (1) sculpting structures; (2) deleting unwanted structures; (3) controlling cell numbers; and (4) producing differentiated cells without organelles (Jacobson et al., 1997).

The best studied role of PCD is sculpting parts of the body. This includes well-studied limb development, the formation of preamniotic cavity, and fusion of epithelial sheets for the formation of neural tube and lens vesicle. PCD during limb development has been observed in chick and mouse embryos (Ballard and Holt, 1968; Hurle and Colombatti, 1996; Zakeri and Ahuja, 1997). PCD during limb development in chick embryos is observed at the anterior edge of the limb bud and then at the posterior junction of the limb bud and the body wall. At a later stage, PCD is observed in the interdigital mesenchyme. At the molecular level, published studies have identified effecter caspases that are activated and that cytochrome c is released from mitochondria in limb PCD (Umpierre et al., 2001; Zuzarte-Luis et al., 2006). Subsequent studies showed that caspase-2 functions as its initiator caspase and that proapoptotic factor AIF translocates from mitochondria to the nucleus during interdigital PCD, suggesting that PCD during limb development occurs through multiple apoptotic pathways including both caspase-dependent and –independent pathways (Grossmann et al., 2001; Zuzarte-Luis et al., 2006). The role of PCD in the formation of neural tube has been also studied. Inhibition of apoptosis by caspase inhibitors results in the increased incidence of neural tube defects (Weil et al., 1997). The spatial and temporal distribution of PCD in the dorsal midline of the developing chick hindbrain has shown that PCD plays a significant role in morphogenetic thinning during roof plate development (Lawson et al., 1999). It has also been shown that the development of lens vesicle involves PCD of the cells

between the surface ectoderm and the optic vesicle to help trigger invagination and to facilitate separation from the ectoderm (Mohamed and Amemiya, 2003). Furthermore, PCD shapes the inner ear structure in day 5 chick embryo (Avallone et al., 2002). In cardiovascular development, PCD is also essential in creating the four-chambered architecture of the heart (Abdelwahid et al., 2002).

In embryonic development, a number of extra structures are formed that are later eliminated by PCD. Such structures include ones that are needed only at one stage of development, ones that are required only in one sex but not in the other, and ones that were required in an ancestral species but not in extant species (Jacobson et al., 1997). Subplate neurons that are required only during the development of the mammalian cerebral cortex are subsequently removed by PCD. In addition, the Műllerian duct and the Wolffian duct, which develop into female and male reproductive system, respectively, are formed in both sexes. Later, the unnecessary duct for each sex is removed by PCD. Pronephric tubules that function as kidneys in fish and amphibian lavae are formed in mammals but later eliminated by PCD.

In certain organs, cells are produced excessively and then later the number of cells is adjusted by PCD. Neurons and oligodendrocytes, for example, are produced in numbers greater than those in the adult, and are subject to PCD (de la Rosa and de Pablo, 2000). It has been estimated that approximately 50% of the neurons generated are eliminated by PCD (Oppenheim, 1991). The best hypothesis for the biological significance of this pruning process is that neuronal competition for their target serves to adjust the number of neurons and oligodendrocytes to the size of the target (Barres et al., 1992; Oppenheim, 1991).

In the developing lens, the nucleus is removed in the process called nuclear death, leaving the rest of the cell to function as a mature lens cell. A similar phenomenon is observed in mammalian red blood cells and skin keratinocytes, suggesting an involvement of PCD in these processes (Gao et al., 1997; Jacobson et al., 1997). Together, all these examples of PCD clearly indicate that cell death in development is an indispensable part of normal development.

1.3.3. Apoptosis in Abnormal Development

Because of the important role of PCD in normal development, there is a possibility that animals lacking gene(s) critical for PCD develop abnormally. Using knock out mice, it was tested whether any of the apoptotic proteins are essential for the PCD occurring during development. Loss of *caspase-9* and *Apaf-1* resulted in abnormal development, e.g., mouse embryos lacking *caspase-9* showed reduced apoptosis in the developing brain and died around day 11 – 12.5, and embryos lacking *Apaf-1* showed brain defects (e.g. exencephaly) and died on day 16.5 (Cecconi et al., 1998; Hakem et al., 1998; Yoshida et al., 1998). These results suggest that caspase-9 and Apaf-1 are critical for some of the PCD observed in the developing brain (Mirkes, 2008). In contrast, deletion of proapoptotic genes, either *Bax* or *Bak,* showed no effect in development. However, the loss of both *Bax* and *Bak* showed interdigital webbing in the paws, indicating that Bax and Bak together play a role in PCD occurring in the formation of digits (Lindsten et al., 2000). Although all these genes are likely to play critical roles in PCD, caspase-9 and Apaf-1 are essential for at least some PCD in the brain and not in the limbs whereas Bax and Bak play essential role in limb PCD but appear to play no essential role in brain PCD (Mirkes, 2008). These differences could be because these apoptotic proteins are tissue specific. Another possibility is that there are strain-specific effects. Strain differences to abnormalities in knock out mice or abnormalities induced by teratogens have been reported (Finnell et al., 1986; Zheng et al., 1999). Deletion of genes that are not involved in apoptosis also result in abnormal patterns of cell death and development (Zakeri and Ahuja, 1997). *Dominant hemimelia* (*Dh*) mutant mice lacked the features of physiological cell death in their limbs and developed a preaxial polydactyly (Rooze, 1977). *Hemimelic extra toes* (*Hx*) mutant exhibited altered patterns of cell death and polydactyly in all four feet (Knudsen and Kochhar, 1981). Together, these studies suggest that many but not all abnormalities in PCD are causally linked to the development of abnormal development.

1.3.4. Teratogen-Induced Apoptosis

As mentioned in section 1.2, more than 1,200 agents have been known to induce malformations in experimental animals (Shepard, 1995). Although the mechanisms by which these teratogens result in abnormal development are still not well understood, it is known that a number of teratogens induce cell death in tissues that subsequently develop abnormally and give rise to structural and/or functional malformations (Knudsen, 1997; Scott, 1977). Teratogens known to induce cell death include HS, CP (or 4CP), sodium arsenite, staurosporine, retinoic acid (Ali-Khan and Hales, 2003; Okano et al., 2007), folate deficiency (Gu et al., 2002b), cadmium (Fernandez et al., 2003), fumonisin B1 (Jones et al., 2001), ethanol (Dunty et al., 2001), 2-deoxyadenosine (Gao et al., 1994), pentostatin, and N-acetoxy-2-acetyl aminofluorene (Knudsen et al., 1992; Thayer and Mirkes, 1995).

The discussion of teratogen-induced apoptosis, however, will focus on two well known teratogens, HS and CP (please refer to section 1.2.2 for information about these teratogens). These teratogens have been chosen to investigate the molecular mechanisms of teratogen-induced apoptosis because: 1) they represent both chemical and physical teratogens that are well studied, 2) they are both human and animal teratogens, and 3) there is an extensive literature on the biochemistry of HS- and CP-induced cell death.

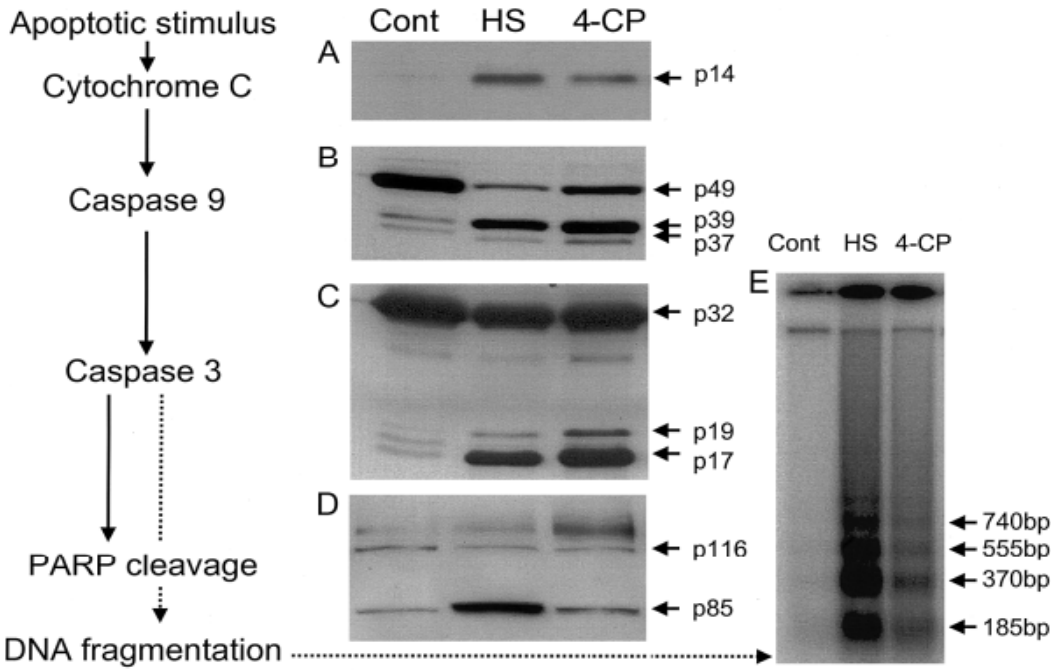

Figure 1.5. Activation of the mitochondrial apoptotic pathway in day 9 mouse embryos exposed to HS and 4CP. Adapted from Mirkes, (2002).

To determine whether HS and CP induce the terminal events of apoptosis, such as the activation of caspase-3, inactivation of the caspase-3 target, poly (ADP-ribose) polymerase (PARP), and DNA fragmentation, day 9 mouse embryos were exposed to these two teratogens *in vitro* (Fig. 1.5) (Mirkes and Little, 1998; Umpierre et al., 2001). In untreated embryos, caspase-3 exists in an inactive form, procaspase-3, which is approximately 32 kDa. Following HS and 4CP exposure (also sodium arsenite), procaspase-3 was cleaved to a larger fragment of approximately 19 kDa (p19) and a smaller fragment of approximately 17 kDa (p17), indicating that caspase-3 is activated by these teratogens. These studies also showed that the enzyme PARP, which is approximately 116 kDa (p116), is cleaved to produce an inactive fragment of approximately 85 kDa (p85). In addition, internucleosomal DNA degradation is observed as a ladder of DNA fragments. Activation of caspase-3 by these teratogens was observed between 2.5 and 5 hr after the exposure whereas the inactivation of PARP and DNA fragmentation occurred 5 hr after the exposure, indicating that apoptotic

signaling pathway occurs within 5 hr after the exposure and that these teratogens activate the terminal portion of the apoptotic pathway. To investigate whether these teratogens activate upstream events in the apoptotic pathway, cytochrome c release from mitochondria was examined (Mirkes and Little, 2000). Results show that all three teratogens induced the release of cytochrome c between 2.5 and 5 hr after the initiation of exposure. To further investigate upstream events in this apoptotic pathway, the activation of caspase-9 was studied (Little and Mirkes, 2002). Results show that in untreated day 9 mouse embryos, caspase-9 exists as its inactive form, procaspase-9, which is approximately 49 kDa (p49). Following the exposure of embryos to HS and 4CP (also staurosporine), the level of procaspase-9 decreased and its cleaved fragments that are approximately 39 kDa (p39) and p37 kDa (p37), increased. Because the generation of p37 fragment occurs through the interaction of cytochrome c and Apaf-1, whereas p39 is generated through a feedback loop involving caspse-3, these results indicate that HS, CP, and staurosporine trigger an apoptotic signal that converges on the mitochondria to induce cytochrome c release. Cytochrome c in cytosol presumably then activates Apaf-1, which, in turn, activates caspase-9. Caspase-9 subsequently activates the key effector caspase, caspase-3. Recent studies have also shown that caspase-3 not only inactivates PARP and promotes DNA fragmentation but also activates caspase-6 and -7 (Little et al., 2003). These studies suggest that intrinsic apoptotic pathway is a critical part of apoptosis induced by some teratogens. Other studies using CP or 4CP have shown that the integral part of the extrinsic pathway, caspase-8 is also activated in the brain and liver of embryos (Torchinsky et al., 2002). In contrast, Huang and Hales (2002), observed the activation of caspase-3, but not caspse-9 and -8 in mouse embryonic limbs cultured with 4CP. These results suggest that the apoptotic signaling induced by teratogens might be tissue specific.

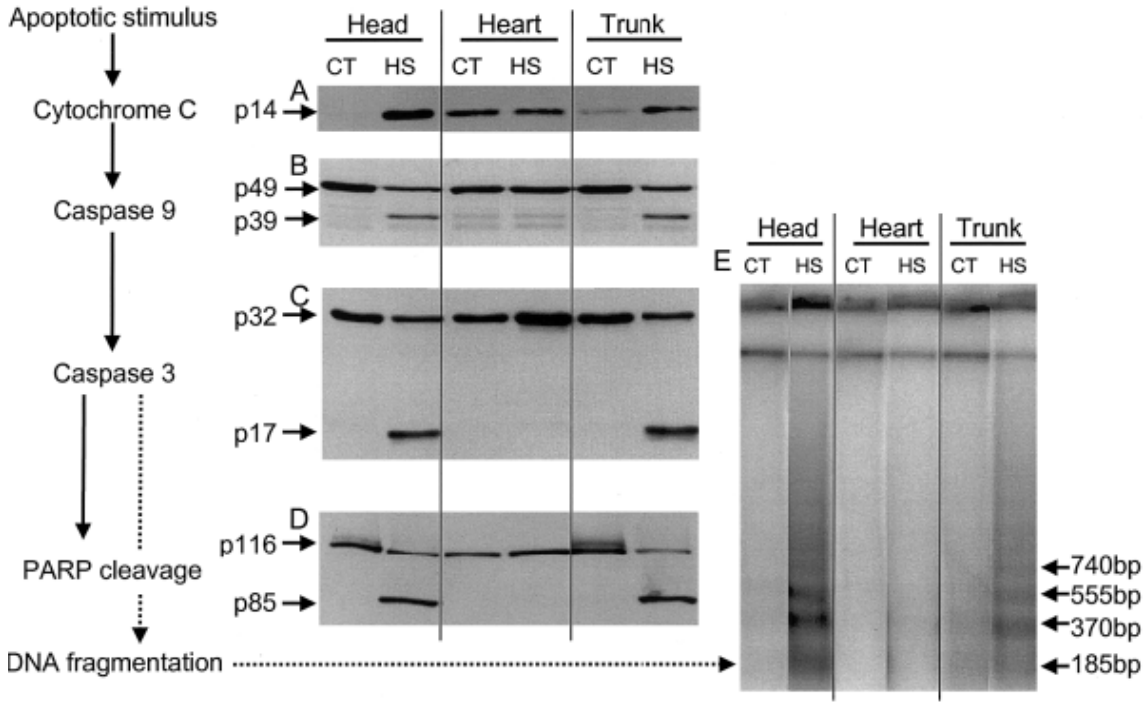

Figure 1.6. Activation of the mitochondrial apoptotic pathway in heads, hearts, and trunks isolated from day 9 mouse embryos exposed to HS. Adapted from Mirkes, (2002).

Published studies also indicate that teratogens induce apoptosis in some cells of the embryos but not others (Fig. 1.6) (Gao et al., 1994; Mirkes, 1985; Mirkes et al., 1985, 1991; Thayer and Mirkes, 1995). Umpierre et al. (2001) has shown using vital dyes and TUNEL staining that teratogen-induced apoptosis is cell specific: some cells in the mouse embryo die, particularly in areas of PCD, whereas other cells survive. Their study revealed that neuroepithelial cells are particularly sensitive to teratogen-induced apoptosis, mesenchymal cells surrounding the neuroepithelium are less sensitive, and cells of the heart are completely resistant (Umpierre et al., 2001). Because none of the terminal events such as cytochrome c release, activation of effector caspases, PARP cleavage, and DNA fragmentation, were observed in the cells of the embryonic heart, these studies indicate that the mitochondrial pathway is disrupted in heart cells upstream of cytochrome c release (Fig. 1.6). The differential sensitivity of embryonic cells to teratogen-induced apoptosis has been also observed following the exposure to N-

acetoxy-2-acetylaminofluorene (Thayer and Mirkes, 1995), ethanol (Dunty et al., 2001), and 2-deoxyadenosine (Gao et al., 1994). Differential sensitivity might be the key to the regulation of teratogen-induced apoptosis.

1.4. TRANSFORMATION RELATED PROTEIN 53

1.4.1. Overview of p53

Transformation related protein 53 (p53), known as the "guardian of the genome," was first identified in 1979 as a cellular protein tightly associated with a DNA tumor virus, simian virus 40 large T protein (SV40LT) (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Vogelstein et al., 2000). The early research on the role of p53 in viral transformation unexpectedly revealed the critical role of p53 in all major types of human cancers. Almost 30 years later, p53 is now recognized as a potent tumor suppressor, whose alteration is found in more than 50% of human cancers (Hollstein et al., 1994). Frequently, even cancers without the mutation of *p53* still have the p53 pathway inactivated by alterations of genes that regulate p53 or by the localization of p53 in the cytoplasm (Levine, 1997). In addition, germline mutations of p53 lead to Li-Fraumeni syndrome, which is a rare cancer predisposition syndrome in humans (Diller and Friend, 1992). Therefore, the role of p53 as a tumor suppressor has been the focus of numerous studies, encompassing over 35,000 publications in Pubmed under the query "p53 and cancer."

p53 functions biochemically as a sequence-specific transcription factor that is essential for maintaining genomic integrity and preventing inappropriate cell proliferation following a variety of stresses that induce cellular damage (Janus et al., 1999). The protein is present at very low levels in unstressed cells because of its fast turnover, which is regulated mainly by the binding of E3 ubiquitin ligase, mouse double minute 2 (MDM2) (Lohrum and Vousden, 1999). In response to cellular stresses such as DNA damage, hypoxia, oncogene overexpression, and nucleotide depletion, p53 is rapidly stabilized and activated by extensive post-translational modifications such as phosphorylation, acetylation, and ubiquitination (Vogelstein et al., 2000; Vousden and

Lu, 2002). The activation of p53 results in the translocation of the protein to the nucleus where it transcriptionally activates its target genes such as *p21*, *Noxa*, *Puma*, *Bax* and *Fas* (transcription-dependent pathway). Active p53 also translocates to the mitochondria or cytosol and interacts with proapoptotic or antiapoptotic proteins (transcription-independent pathway). Both the transcription-dependent and –independent pathways of p53 activate an array of biological responses including apoptosis, cell cycle arrest, senescence, DNA repair, and differentiation (Albrechtsen et al., 1999; Lane, 1992; Levine, 1997; Vogelstein et al., 2000).

The p53 family members p63 and p73 are both structurally and functionally similar to p53 (Stiewe, 2007). Increasing evidence indicates that these homologs may act in concert with p53 in response to DNA damage and the subsequent induction of apoptosis and cell cycle arrest (Jost et al., 1997; Yang et al., 1998). In addition, isoforms of p63 and p73 that lack the N-terminal transactivation domain, ΔNp63 and ΔNp73, were found to be naturally occurring inhibitors of p53 (Fillippovich et al., 2001; Pozniak et al., 2000; Yang et al., 2000). These studies indicate that the interaction and communication of p53 with other p53 family proteins are also important for understanding the function of p53.

The p53 protein also plays roles in normal development. *p53* mutant mice exhibit birth defects such as exencephaly and have a high mortality rate *in utero* in addition to the high cancer predisposition (Armstrong et al., 1995; Donehower et al., 1992; Sah et al., 1995). Published studies have shown that p53 is altered by teratogens such as cadmium and 4CP (Pekar et al., 2007; Sarkar and Sharma, 2003; Yu et al., 2008). Detailed discussion of teratogen-induced activation of p53 is the focus of the study presented in this dissertation.

1.4.2. Molecular Structure of p53

Knowledge of molecular structure of the p53 protein is important to understand how p53 is regulated and activated as well as how it activates various biological pathways. The protein consists of 393 amino acids and contains three functional

domains (Fig. 1.7) (Bode and Dong, 2004). The N-terminus transactivation domain of p53 interacts with a number of proteins, which are required for transactivation activity. For example, a negative regulator of p53, MDM2, interacts with the transactivation domain of p53 to inhibit the transcription activity of p53 (Chen et al., 1995). In humans, the N-terminal portion contains Src homology 3-like (SH3) domain. The SH3 domain is a proline-rich domain that interacts with SIN3, which protects p53 from degradation.

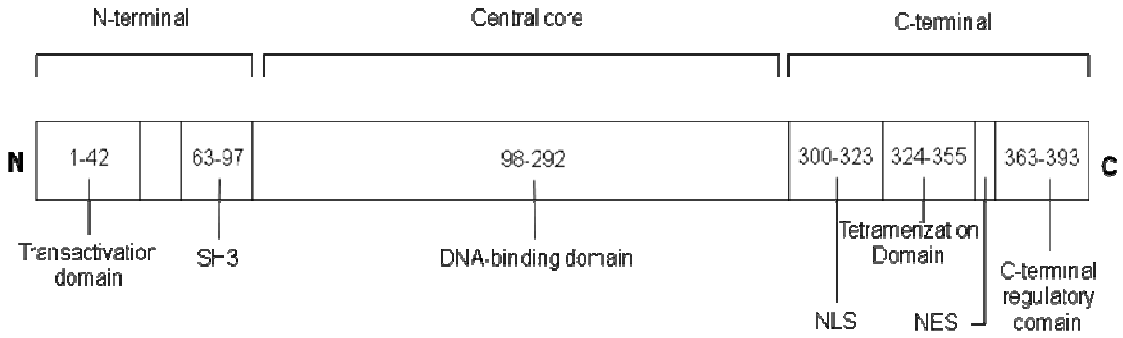

Figure 1.7. Functional domains of p53. Adapted from Bode and Dong, (2004).

The central core DNA-binding domain of p53 is required for the sequence-specific interaction of p53 with its target proteins. DNA-protein binding is facilitated by the four evolutionally conserved regions within the central core domain (Cho et al., 1994) as well as by the binding of proteins such as p53 binding protein 1 and 2 (p53BP1 and p53BP2) to the domain (Derbyshire et al., 2002; Gorina and Pavletich, 1996). The C-terminal portion contains the nuclear localization signal motifs (NLS), which regulate nuclear translocation of p53 from the cytoplasm to the nucleus following DNA damage. In addition, the C-terminus contains an oligomerization domain that promotes the formation of p53 tetramers, nuclear export signals (NES), and the regulatory domain that regulates the core DNA-binding domain.

Because p53 is maintained at low levels in unstressed condition, the stabilization and activation of p53 is essential for the activation of p53-regulated biological pathways. The binding of p53 to other proteins, especially the binding of MDM2 to p53 at the N-terminal transactivation domain, play important roles in the stabilization and activation

of p53. In addition, the alteration of the DNA-binding domain as well as other domains by post-translational modifications is also critical (Cuddihy and Bristow, 2004) (for detailed discussion of post-translational modifications, please refer to section 1.4.3.3).

Mutation of the *p53* often alters the function and structure of p53 or inactivates the gene (Braithwaite et al., 2005). The most frequent mutations identified in p53 (75%) are missense, and these mutations are most often found in the tetramerization domain (3.4%) and the SH3 domain (2.3%) (Hofseth et al., 2004; Olivier et al., 2004). However, 90% of the mutations that inactivate p53 function in cancer cells can be observed in the DNA-binding domain, and these mutations generate a protein that lacks the ability to transcribe p53 target genes. Common polymorphisms of p53 also have been studied, although a great number of these natural variants are found in non-coding regions of the gene (Pietsch et al., 2006). Polymorphisms in *codon 47* and *72*, however, may play a role in p53-dependent apoptosis and cancer progression (Bulavin et al., 1999; Okamura et al., 2001; Sanchez-Prieto et al., 2000; Storey et al., 1998; Takekawa et al., 2000).

1.4.3. Stabilization and Activation of p53

1.4.3.1. Types of Stress That Stabilize and Activate p53

p53 is stabilized and activated by a variety of stresses. A well studied group of agents that increase the level of p53 includes a variety of DNA damaging agents. DNA is often the main target of environmental genotoxins such as ultraviolet light (UV), ionizing radiation, polycyclic aromatic hydrocarbons, and alkylating compounds (Table 1.4) (Roos and Kaina, 2006).

Following DNA damage, ataxia telangiectasia mutated (ATM) or the homologous A-T and Rad3-related (ATR) protein initiate the activation of downstream pathways through phosphorylation of a number of proteins, including p53, MDM2, H2AX, checkpoint kinase 2 (CHK2), and p53BP1 (Ackermann and El-Deiry, 2008).

Table 1.4
Agents That Stabilize and Activate p53 Protein*

Type	Agent
DNA damaging agents	
Irradiation	UV
	Gamma rays
	X-rays
	Alpha particles
Carcinogens	Polycyclic aromatic hydrocarbons
	Mycotoxins
	Heavy metals (Cadmium)
Oxidative/nitrosative stress	Hydrogen peroxyde
	Nitric oxide donors
Cytotoxic drugs	Platinum compounds
	Alkylating agent
	Antimetabolites
	5-FU
	PALA
	Methotrexate
	Tumor antibiotics
	Mitomycin C
	Antinomycin D
	Bleomycin
	Anthracyclins (doxorubicin)
Topoisomerase inhibitors	Camptothecin
Non-genotoxic agents	
Anti-microtubule agents	Taxanes
	Nocodazole
Ribonucleotide depletion	
Hypoxia/anoxia	
Cell adhesion	
Oncogene activation	Overexpression of E1A
Cytokines	TNF-alpha
Hyperthermia	
Proteasome inhibitors	
Senescence/telomere erosion	
Polyamine analogues	

*Adapted from Pluquet and Hainaut, (2001).

Upon phosphorylation, p53 is stabilized and initiates cell cycle arrest or apoptosis. It is believed that low levels of DNA damage triggers the transcription of cell-cycle related genes such as *p21* whereas high levels of DNA damage activate pro-apoptotic genes including *Bax, Puma,* and *Fas* receptor (Roos and Kaina, 2006).

In contrast, exposure to agents that do not induce any detectable level of DNA damage such as hypoxia also results in the accumulation of p53 (Hammond et al., 2002). Hypoxic stress induces p53 protein accumulation and p53-dependent apoptosis. The stabilization and accumulation of p53 occur through the decreased level of MDM2 and possible interaction of p53 with hypoxia inducing factor 1 alpha (HIF1α) (Hammond and Giaccia, 2005). p53-dependent apoptosis is associated with cytochrome c release and the activation of Apaf-1 and caspase-9, suggesting the involvement of the mitochondrial apoptotic pathway (Alarcon et al., 2001). Unlike DNA damaging agents, hypoxia does not induce p53-dependent cell cycle arrest. In addition, hypoxia-induced p53 does not seem to activate well known proapoptotic genes such as *Bax, Noxa,* and *Puma* and instead acts almost entirely as a transrespressor to induce apoptosis, suggesting that hypoxia-induced p53 activates apoptosis differently from DNA damaging agents (Hammond and Giaccia, 2005).

Oncogenic stimuli such as c-myc and RAS induce p19ARF, which associates with MDM2, to allow p53 to be stabilized and activated (Pomerantz et al., 1998; Zhang et al., 1998). Depletion of ribonucleotides (Linke et al., 1996), cytokine stimulation, anchorage, cell-to-cell contact, viruses and various metabolic changes are also known to stabilize and activate p53 (Braithwaite et al., 2005).

1.4.3.2. p53-MDM 2

In the absence of cellular stress, an E3 ubiquitin ligase MDM2 binds to p53 and transports p53 to the cytoplasm for its degradation by the 26S proteasome. MDM2, thus, maintains p53 at very low steady-state levels under unstressed condition and regulates the stabilization and activation of p53. In response to stress, MDM2 itself is ubiquitinated and degraded, releasing p53 and allowing it to be stabilized and activated (Alarcon-Vargas and Ronai, 2002; Fang et al., 2000). MDM2 is also phosphorylated in an ATM-dependent manner, which decreases its ability to interact with p53, thus, stabilizing p53 (Khosravi et al., 1999). Furthermore, MDM2 has the ability to inhibit acetylation of p53 mediated by CREB-binding protein (CBP) or by the p300 protein (Ito et al., 2001; Kobet et al., 2000). Activation of p53 leads to the transactivation of

MDM2, ensuring the autoregulatory feedback loops between Mdm2 and p53 (Harris and Levine, 2005). Loss of *Mdm2* leads to embryo lethal phenotypes that are completely rescued by concomitant deletion of *p53*. These studies indicate that Mdm2 is an important negative regulator of p53 (Jones et al., 1995; Montes de Oca Luna et al., 1995; Parant et al., 2001). Other ubiquitin ligases, PRH2 and COP1 have been also shown to interact with p53 and promote its ubiquitination independent of MDM2 (Harris and Levine, 2005; Leng et al., 2003).

Another Mdm2 family protein, Mdmx is also a key regulator of p53. Recent studies have shown that MDMX also interacts with p53, but controls p53 not by ubiquitinating it but by inhibiting its transactivation activity (Finch et al., 2002). Interestingly, MDM2 and MDMX interact with each other to protect p53 from undergoing MDM2-mediated degradation (Jackson and Berberich, 2000) although MDMX can also stabilize MDM2 by preventing its self-ubiquitination. MDM2 and MDMX are functionally dependent in controlling p53. In the absence of MDMX, MDM2 still retains the ability to control p53 but the capacity of MDM2 to control p53 is reduced due to its shorter half-life (Gu et al., 2002a). MDMX, on the other hand, is dependent on MDM2 to inhibit p53 function (Li et al., 2002). These studies show that both MDM2 and MDMX are critical regulators of p53.

1.4.3.3. Post-Translational Modifications

Other than the modification of MDM2 and other proteins, post-translational modifications of p53 also play critical roles in the regulation of p53 stabilization and activation (Appella and Anderson, 2001). These modifications are known to enhance the DNA-binding activity of p53 as well as influence the interaction between p53 and MDM2 (Harris and Levine, 2005; Lavin and Gueven, 2006). The types of post-translational modifications include phosphorylation, acetylation, methylation, ubiquitination, neddylation, and sumoylation (Appella and Anderson, 2001). The most common modification is phosphorylations, and it accounts for all modifications observed in the N-terminal portion as well as the majority of modifications in the central core and C-terminal portion (Table 1.5). The p53 protein is phorphorylated by a variety of

protein kinases that are activated by different stresses. Published studies have shown that protein kinases that phosphorylate p53 include ATM, ATR, CHK1, CHK2, Jun NH_2-terminal kinase (JNK), p38, and others (Lavin and Gueven, 2006). The most well described phosphorylation site is ser-15 (ser-18 in mouse) carried out by ATM, which occurs in response to double stranded breaks (DSBs) induced by ionizing radiation (Saito et al., 2002). ATM phosphorylates p53 at multiple sites, including ser-6, 9, 15, 20, 46, and thr-18. The most frequently phosphorylated sites are ser-15, 20, 33, and 46. In contrast, phosphorylation at thr-81 is only observed after exposure to UV or H_2O_2. JNK is the only protein kinase identified that phosphorylates p53 at thr-81, a modification that stabilizes and activates p53 (Buschmann et al., 2001).

Phosphorylation of p53 in the N-terminal portion generally influences the affinity of p53 with MDM2. Ser-376 and ser-378 are phosphorylated in resting cells, and are dephosphorylated in response to radiation (Waterman et al., 1998). Dephosphorylation of p53 at ser-376 generates a binding site for 14-3-3 proteins, which then enhances the DNA-binding activity of p53. Modifications at C-terminal region allow the region to unfold, increasing the affinity of p53 for sequence-specific DNA-binding (Selivanova et al., 1998).

Acetylation of lysine at the C-terminal region also enhances DNA-binding activity. The well described acetylation of p53 occurs by binding of the histone acetylase family members p300/CBP (Gu and Roeder, 1997), and the acetylation of p53 by p300/CBP has been shown to enhance its transcriptional activity (Avantaggiati et al., 1997). Generally acetylation stabilizes the p53 protein by preventing ubiquitylation. Ubiquitylation of p53 by MDM2 leads to the degradation of p53, and the ubiquitylation sites are also targets of acetylation. Acetylation of p53, therefore, prevents ubiquitylation and degradation of p53 (Juan et al., 2000; Luo et al., 2000, 2001; Vaziri et al., 2001). Whether acetylation stabilizes and activates p53 remains controversial (Espinosa and Emerson, 2001; Nakamura et al., 2000), and it is believed that this could be due to the differences in the experimental protocols or animals or cell type used.

Table 1.5
Post-Translational Modifications of p53*

Domain	Amino acid/location	Modification	Modifier proteins
Transactivation domain	S 6	P	CK1, ATM, ATR, DNA-PK, SMG1, mTOR
	S 9	P	CK1, ATM, ATR, DNA-PK, SMG1, mTOR, CHK2
	S 15 (S 18)	P	DNA-PK, ATM, ATR, mTOR, SMG1, p38, ERK, CHK2, RSK2, AMPK
	T 18	P	CHK2, VRK1, CK1, ATM
	S 20 (S 23)	P	CHK2, JNK, MAPKAPK2, CHK1, Plk3, ATM
	S 33 (-)	P	p38, CAK, GSK3β, CDK9, CDK5
	(S 34)	P	JNK
	S 37	P	ATR, p38, GSK3β, CAK, DNA-PK, CHK2
	S 46 (-)	P	HIPK2, p38, CHK2, CDK5, PKC, ATM
	T 55	P	TAF1, ERK2
SH3	(T76/86)	---	---
	T 81 (-)	P	JNK
DNA-binding domain	K 120	A	Tip60, hMOF
	S 149	P	CSN
	T 150	P	CSN
	T 155	P	CSN
	S 215	P	STK15, Aurora-A
NLS	K 305	A	p300
	S 313, 314	P	CHK1, CHK2
	S 315	P	CDK2, AURKA, GSK3β, CDK9, STK15
	K 320 (K 317)	A, Nd	PCAF
C-terminal regulatory domain	S 366	P	CHK2
	K 370 (K 367)	A, Ub, Nd, M	MDM2, CBP/p300, SMYD2
	K 372, 373 (K 369, 373)	A, M, Ub, Nd	CBP/p300, MDM2, PCAF, SET9, HCP core protein
	S 376 (S 373)	P	PKC, CHK1, CHK2
	T 377	P	CHK1, CHK2, PKC
	S 378 (S 375)	P	PKC, CHK1, CHK2
	K 381, 382 (K 378, 379)	A, Ub, M	CBP/p300
	K 386 (K 383)	S, Ub	Sumo1, CBP/p300, MDM2
	T 387	P	Chk1, Chk2
	S 392 (S 389)	P	PKR, CK2, FACT, p38, Cdk9

*Combined Apella and Anderson, (2001); Bode and Dong, (2004); Lavin and Gueven, (2006); Xu, (2003).

Recent studies have further revealed that neddylation of p53 by MDM2 may also repress the activation of p53 (Xirodimas et al., 2004). Frequently the target sites of

neddylation are also targeted by ubiquitylation. Ubiquitylation refers to the modification of lysine residues in the C-terminal by ubiquitin-like protein called NEDD8 conjugated to MDM2. It is not known whether neddylation competes with acetylation or ubiquitylation (Bode and Dong, 2004).

Sumoylation of p53 has been also shown to alter p53 transcriptional activity (Melchior and Hengst, 2002). Sumoylation regulates the formation of promyelocytic leukaemia (PML) tumor suppressor protein localized in the nucleoplasm or in subnuclear structures referred to as nuclear bodies (NBs). This protein is implicated in the pathogenesis of acute promyelocytic leukaemia (APL), but the role of sumoylation is not clear. Sumoylation, similar to ubiquitylation, forms an isopeptide bond between the small ubiquitin-like protein SUMO1 and lys-386 of the p53 protein (Gostissa et al., 1999; Rodriguez et al., 1999). Recent studies have shown that MDM2 and ARF-mediated nuclear targeting regulate this modification (Chen and Chen, 2003). Whether sumoylation activates or repress p53, however, remains controversial.

The p53 protein is also altered by ubiquitylation, leading to its degradation. The addition of ubiquitin molecules to p53 marks the protein to be degradated by the 26S proteasome. Studies have shown that MDM2 is involved in the localization and degradation of p53 and that different levels of MDM2 can induce both mono- and poly-ubiquitylation of p53 (Li et al., 2003).

The same modification sites are targeted by different enzymes in response to variety of cellular stresses. Extensive studies have shown that these redundancies of p53 modifications could be that specific stress leads to a distinct modification pattern, or a unique combination of modifications is required for further modifications, which leads to maximal level of activation (Bode and Dong, 2004). The example includes phosphorylation of ser-15 followed by the phosphorylation of thr-18. Phosphorylations at ser-15, thr-18, and ser-20 recruit other factors such as p300, CBP, and P/CAF for its acetylation. It is generally accepted that the functional responses of p53 by its stabilization and activation of p53 are highly orchestrated by phosphorylations at

multiple sites combined with other forms of modifications such as acetylation, deacetylation, ubiquitylation, and neddylation.

1.4.4. Activation of p53 Pathways

1.4.4.1. Transcription-Dependent Pathway

p53 is a transcription factor that activates or represses a significant number of genes related to cell cycle regulation and apoptosis in response to cellular stresses. p53 directly activates the expression of genes through binding to specific DNA sequences containing two adjacent copies of the consensus sequence 5'-RRRCWWGYYY (*N*=0-13)RRRCWWGYYY-3' (R=G or A, W=T or A, Y=C or T, *N*=any base), located in the promoter or introns of the downstream target genes (el-Deiry et al., 1992; Kern et al., 1991). Identification of the p53 target genes have been attempted by using three different methods (Yu and Zhang, 2005). The first approach is the identification of genomic DNA fragments that are bound to p53 based on the reasoning that p53 promotes the activation of the target genes by interacting at their promoter region or introns (Nakamura, 2004; Tokino et al., 1994). The second approach is to systematically identify genes that show different expression in the presence or absence of p53 (el-Deiry, 1998b; Polyak et al., 1997; Sax et al., 2003; Yu et al., 1999; Zhao et al., 2000). The third approach is to identify basic components of p53-regulated pathways through genes that are already known to play roles in these pathways. For example, investigation of the apoptotic pathway showed that p53 transactivates *caspase-6* in response to DNA damage (MacLachlan and El-Deiry, 2002). These approaches have identified several hundred possible genes, although further verification of each gene is necessary to confirm the association (Wei et al., 2006).

Cells undergo growth arrest by the transactivation of cell cycle-related genes induced by p53. For example, well described genes that are activated include the CDK inhibitor *p21*, *GADD45*, *14-3-3σ*, *cyclin G*, and *placenta transforming growth factor beta* (*PTGF-β*) (Sax et al., 2003). *p21* is required for the induction of a G1 arrest whereas *GADD45* and *14-3-3σ* regulate G2/M phase in response to ionizing radiation

(Brugarolas et al., 1995, 1999; Deng et al., 1995). These studies show that p53 can transcriptionally activate cell cycle arrest by preventing both G1/S and G2/M progression.

Apoptosis is another process induced by the activation of p53 after cellular damage. Both the mitochondrial apoptotic pathway and death receptor pathway can be mediated by transactivation of p53 target genes. The p53 target genes involved in the mitochondrial apoptotic pathway includes *Bax*, *Noxa*, *Puma*, *p53AIP1*, *p53-induced genes (PIGs)*, and *Apaf-1*. *Bax* is the first proapoptotic Bcl-2 family member identified to be regulated by p53, and loss of *Bax* accounted for approximately half of the tumor growth observed in a brain tumor model that lacks the p53 gene (Miyashita and Reed, 1995; Symonds et al., 1994; Yin et al., 1997). p53 also transactivates BH3 only proteins, *Noxa* and *Puma*, which function upstream of *Bax* for the induction of apoptosis. *Puma* has been shown to be a critical mediator of p53-dependent apoptosis following DNA damage (Han et al., 2001; Nakano and Vousden, 2001; Yu et al., 2001). *Noxa* also activates p53-dependent apoptosis but to a lesser extent than *Puma* (Oda et al., 2000a; Shibue et al., 2003; Villunger et al., 2003). Studies have also shown that another BH3 only protein *Bid* and a mitochondrial protein encoded by *p53AIP1* are activated by p53 (Oda et al., 2000b; Sax et al., 2002). *PIGs* were originally identified following p53 overexpression (Polyak et al., 1997), and the activation of *PIGs* suppresses tumor formation and induces apoptosis (Gu et al., 2000; Lehar et al., 1996). Studies have shown that PIGs induce apoptosis by generating or recognizing reactive oxygen species (ROS) (Polyak et al., 1997).

The death receptor pathways are activated through the transactivation of *Killer/DR5*, *DR4*, *Fas/Apo-1*, and *Pidd* by p53 (Sun, 2006). In response to chemotherapeutic agents and TRAIL, the extrinsic pathway is activated by the transactivation of *DR 4* and *DR 5* (Liu et al., 2004; Sheikh et al., 1998). *Pidd* also leads to apoptosis through the extrinsic pathway, and it is transactivated by p53 following ionizing radiation in MEFs (Lin et al., 2000). Although *Fas* is also activated by p53, the

induction of *Fas* is tissue specific and often does not require p53 (Bouvard et al., 2000; Muller et al., 1998).

p53 can also transcriptionally represses genes for the induction of apoptosis, such as *Igfr*, *Bcl-2*, and *survivin*, which are cell survival genes (Levine, 1997; Vogelstein et al., 2000). There are three known mechanisms for the activation of apoptosis by repression of these genes (Ho and Benchimol, 2003). First, p53 interferes with the function of DNA-binding transcriptional activators. Second, p53 interferes with the basic components of the transcriptional machinery. Third, p53 alters chromatin structure by recruiting chromatin-modifying factors such as histone deacetylases (HDAC) to reduce promoter accessibility to the transcriptional machinery and activator proteins.

1.4.4.2. Transcription-Independent Pathway

Although p53 is a transcription factor that activates an ever-increasing number of target genes, transcription-independent activity of p53 has been receiving increasing attention (Moll et al., 2005). The initial evidence that p53 might promote transcription-independent apoptosis was discovered in 1994 by using cells expressing a temperature-sensitive mutant p53 protein (Val135) (Caelles et al., 1994). In this study, cells that undergo p53-dependent apoptosis upon the shift of temperature, exhibited apoptosis even after the cells were treated with drugs that inhibit transcription of p53 target genes. Additional studies using cells with transcriptionally inactive mutant p53 proteins have confirmed the transcription-independent apoptosis activated by p53 (Chen et al., 1996; Haupt et al., 1995; Wagner et al., 1994). However, because of an initial lack of any idea about how p53 activates apoptosis through a transcription-independent mechanism, these studies were neglected until recently (Moll et al., 2005). Subsequently, Moll and colleagues (Marchenko et al., 2000; Sansome et al., 2001) showed that translocation of wildtype p53 to mitochondria induces p53-dependent apoptosis *in vivo*. They also showed that mitochondrial p53 binds to BCL-2 and BCL-XL to inhibit their antiapoptotic activity (Chipuk et al., 2004; Mihara et al., 2003). Another group also observed the localization of p53 to the mitochondria (Dumont et al., 2003) and discovered the ability of p53 to bind to proapoptotic protein BAK (Leu et al., 2004).

Other studies have shown that wildtype p53 can also directly activate apoptosis in the cytoplasm by binding to BAX (Chipuk et al., 2004). Furthermore, a proapoptotic protein PUMA, which is transactivated by p53, also binds to p53 to release BCL-XL from p53, suggesting that nuclear and cytoplasmic proapoptotic function of p53 are coupled by PUMA (Chipuk et al., 2005). Interestingly the kinetics of both transcriptional-dependent and –independent apoptosis induced by γ irradiation *in vivo* have revealed that the apoptotic response occurs in two waves (Erster et al., 2004); the first apoptotic response induced by translocation of p53 to mitochondria occurred within 30-60 min after the initial stress response followed by the second apoptotic response induced by transcription of p53 target genes. Another study has suggested that transcription-dependent response induced by low dose irradiation is primarily responsible for the induction of cell cycle arrest whereas the transcription-independent response activated by high dose irradiation leads to the induction of apoptosis (Speidel et al., 2006). Taken together, these studies show that transcription-independent function of p53 is also critical in the induction of apoptosis. Whether p53 is activated in response to teratogen exposures and whether the induction of apoptosis is through transcription-dependent or –independent response is the focus of the research presented in this dissertation (section 2).

1.4.5. p53 Pathways

1.4.5.1. Apoptosis

This topic is discussed in detail in sections 1.3, 1.4.1, 1.4.4, and 5. Please refer to these sections.

1.4.5.2. Cell Cycle Arrest

The cell cycle consists of a series of events by which a cell duplicates its genetic material and divides into two identical daughter cells. The orderly sequence of events consists of four distinct phases. The genetic material is duplicated only once in the S (synthesis) phase, and the cell with duplicated chromosomes is divided equally to the two daughter cells in the M (mitosis) phase. The phase preceding the S phase is called

G1 (gap 1) phase, and phase between the S and M phase is called G2 (gap 2) phase. Most of the terminally differentiated cells in the body of adults, however, are maintained in a phase called G0, which is a non-dividing, resting stage that branches out from the early G1 stage (Tessema et al., 2004). G1 and G2 are critical for the regulation of cell cycle. The shift from one phase to another is strictly controlled by certain classes of cellular proteins. Cyclin dependent kinases (CDKs) are serine/threonine protein kinases bound and activated by cyclins that appear and disappear strictly during the cell cycle phases (Sherr, 1996). Currently 16 cyclins and 9 CDKs have been identified in mammals although not all of them have a defined function in cell cycle regulation (Johnson and Walker, 1999). In addition, the progression of the cell cycle is controlled at several points of cell cycle termed cell-cycle checkpoints (Tessema et al., 2004). Checkpoints ensure the proper DNA replication and chromosome segregation as well as halt the cell cycle in case of DNA damage or errors. The cell cycle is arrested to prevent genomic instability that results from defects in the cell cycle. Cell cycle arrest at the G1/S checkpoint prevents cells from duplicating defective or damaged chromosomes while G2/M checkpoint ensures properly replicated DNA/chromosomes before cells enter into mitosis. The final checkpoint exists at the metaphase of mitosis, which is before the separation of sister chromatid occurs. The checkpoints in the gap phases are mediated by the two families of cyclin kinase inhibitors (CKIs), INK4 family ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$) and/or the CIP/KIP family ($p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$). The INK4 family members only function at G1 checkpoint whereas the CIP/KIP family serves at both checkpoints as well as in all the four cell cycle phases (Sherr, 2000).

In response to genotoxic stress such as ionizing radiation ATM and ATR can induce cell cycle arrest. ATM and ATR activate checkpoint kinases, CHK1 and/or CHK2 (Chaturvedi et al., 1999; Sanchez et al., 1997), which in turn phosphorylates and inhibits CDK1 (Vermeulen et al., 2003). ATM, ATR, CHK1, and CHK2 also phosphorylate and activate p53. Three proteins, p21, 14-3-3σ, and GADD45 are the main cell cycle-related target genes of p53. p21 has the ability to induce cell cycle arrest

at both G1 and G2 checkpoint. It binds and inactivates CDK4, 6/cyclin D and CDK2/cyclin E complexes, resulting in pRB hypophosphorylation and cell cycle arrest at G1 phase (Stewart and Pietenpol, 2001). p21 also arrests cells at G2 checkpoint by inhibiting CDK1/cyclin B1 (Flatt et al., 2000; Innocente et al., 1999). 14-3-3σ binds to CDC25C, which is a phosphatase responsible for the activation of CDKs, or to CDK1/cyclin B complex, which induces export of this complex to the cytoplasm and inhibits the function of these proteins (Abraham, 2001; Furnari et al., 1997; Stewart et al., 2003). The transactivation of 14-3-3σ activates cell cycle arrest only at G2 phase (Nurse, 2002). GADD45 also induces a G2 arrest by altering the expression of cyclin B1 or CDC25C (Wang et al., 1999).

1.4.5.3. Factors That Decide Which Pathway is Activated

How does p53 decide whether cells undergo cell cycle arrest or apoptosis? How does p53 know which genes to turn on or off to achieve the desirable cell fate, for example, apoptosis for cell death or cell cycle arrest for cell survival? This question has been a focus of intensive research (Aylon and Oren, 2007; Harris and Levine, 2005; Laptenko and Prives, 2006), and three important mechanisms for deciding cell fate have been elucidated (Fig. 1.8). Recent studies indicated that different affinities of p53 toward different p53 response elements may affect promoter choice and cell fate (Laptenko and Prives, 2006). Generally growth arrest related genes tend to contain high affinity sites whereas proapoptotic genes seem to contain low affinity sites (Inga et al., 2002), which indicates that cells make a maximum attempt to retain cells for survival. In addition, several co-factors and transcription factors have been discovered that guide p53 to promoters of specific genes in order to induce a particular pathway (Aylon and Oren, 2007). For example, recently discovered apoptosis-stimulating protein of p53 (ASPP) family proteins, ASPP1 and ASPP2, bind to the DNA binding domain of p53 upon cellular damage and specifically enhance p53-induced apoptosis by directing p53 to the promoters of proapoptotic genes such as *Bax* and *PIG3*, but not to the promoters of cell cycle genes and regulatory genes such as *p21* and *Mdm2* (Sullivan and Lu, 2007). Transcription factor BRN3b also binds to p53 to promote the activation of proapoptotic

genes such as *Bax* selectively (Budhram-Mahadeo et al., 2006). In contrast, several different proteins including BRN3a, YB1, and Hzf selectively stimulate cell survival by transcriptionally activating cell cycle related genes such as *p21* and *14-3-3σ* (Aylon and Oren, 2007). Finally, the pattern of post-translational modifications of p53 may well control the promoter choice and cell fate. For example, phosphorylation of ser-46 has been observed to specifically induce p53 proapoptotic genes such as *Noxa*, *p53AIP1*, and *p53RFP* (Nakamura et al., 2006). The life-death decision is also made at lys-320 as well as other lysines located in the C-terminal section of p53 (Le Cam et al., 2006). Acetylation of lys-320 leads to apoptosis whereas ubiquitylation at this site directs cell fate toward growth arrest by activating *p21* and *cyclin G1*. These studies beautifully illustrate how cells decide upon a certain cell fate in response to a variety of stimuli and how strictly p53 is regulated and controlled so as to achieve the desired outcome.

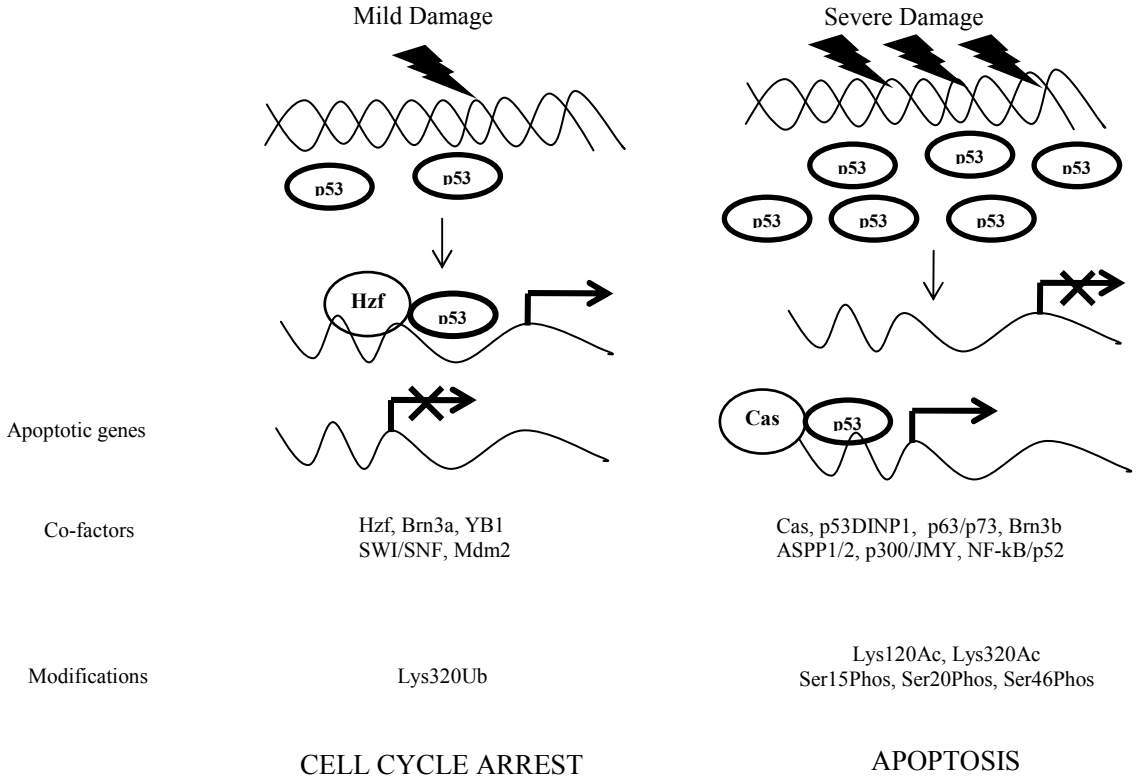

Figure 1.8. Differential regulation of p53 target genes. Adapted from Aylon and Oren, (2007).

1.4.6. p53 and MicroRNAs

1.4.6.1. Overview of MicroRNAs

The recent discovery of small RNA molecules, known as microRNAs (miRNAs), has quickly expanded our view of gene regulation and revealed further complicated mechanisms of gene regulation in eukaryotes (Ambros, 2004; Bartel, 2004; He and Hannon, 2004). miRNAs are small 18- to 24-nucleotide long RNAs that bind to mRNAs and regulate gene expression largely by inhibiting their expression either by interfering with translation or by destabilizing the target mRNAs (Meister and Tuschl, 2004; Pillai et al., 2007). Currently, nearly 800 miRNA have been predicted to exist in humans (Bartel, 2004; Bentwich et al., 2005). A series of steps are involved in the biosynthesis of miRNAs. A long primary miRNA (pri-miRNA) is first translated from non-coding polycistronic regions of the genome by polymerase II (Pol II) (Zhou et al., 2007). These pri-miRNA transcripts, which can exceed 1 kb and give rise to a number of different miRNAs (Du and Zamore, 2005), are then cleaved by the nuclear ribonuclease III (RNase III) Drosha and transported from the nucleus to the cytosol by exportin5. The pre-miRNA, that is now a 70 – 100 nt stem loop, is processed by a second RNase III Dicer to produce a mature miRNA duplex (Du and Zamore, 2005; Kosik, 2006). The duplex unwinds and the single strand is incorporated into the RNA-induced silencing complex (RISC). RISC complex then binds to its target mRNA and silences its expression.

It is now clear that miRNAs play important regulatory roles in developmental timing and patterning, cellular differentiation, proliferation, organogenesis, and apoptosis (Alvarez-Garcia and Miska, 2005; Ambros, 2004). In mouse embryos, miRNAs are expressed in stage- and tissue-specific manner (Mineno et al., 2006; Schulman et al., 2005). In addition, exposures that induce developmental anomalies, such as folate deficiency and sodium arsenite, are known to increase global miRNA expression in human lymphoblastoid cells (Marsit et al., 2006). Furthermore, recently another group of small RNAs called germline-specific PIWI-interacting RNAs (piRNAs), which play roles in spermatogenesis, have been discovered and have been

implicated in the inheritance of epigenetic information. The importance of miRNA in cellular responses to teratogens/xenobiotics as well as in disease pathology, particularly cancer, will be a focus of intensive research. Prediction of target genes using computational algorithms as well as experimental identification of target genes have been also extensively investigated, and a number of on-line sites are publicly available.

1.4.6.2. Regulation of MicroRNAs by p53

Several recent studies have shown that the *miR-34* family miRNAs are induced by p53 and mediate apoptosis in response to DNA damage and oncogenic stress. Using adriamycin as a DNA damaging agent, Chang et al. (2007) showed in colon cancer cells (HCT116) that *miR-34a* exhibited p53-dependent upregulation, and the characterization of the *miR-34a* primary transcript and promoter confirmed that *miR-34a* is directly transactivated by p53. Tarasov et al., (Tarasov et al., 2007) utilized massively parallel sequencing and observed that *miR-34a* is differentially expressed in a p53-dependent manner. In addition, two other studies have further confirmed independently that the transcriptional activation of *miR-34a* promotes p53-mediated apoptosis *in vivo* (Bommer et al., 2007; Raver-Shapira et al., 2007). More studies followed and supported the previous studies as well as the identification of other *miR-34* family, *miR-34b* and *miR-34c* as the transcriptional target of p53 (Corney et al., 2007; He et al., 2007; Tazawa et al., 2007).

1.4.7. Expression of p53 in Mouse Embryos

The importance of p53 in embryonic development was first suggested in 1980 when protein expression of p53 was observed in primary embryo cells of day 12 – 14 mouse but not those in cells of day 16 embryos (Mora et al., 1980). Subsequently, a significant decrease in the level of p53 protein was observed in cells of day 11 embryos, indicating a functional role of p53 in embryogenesis (Chandrasekaran et al., 1981). Decrease in the level of p53 protein was also observed in the similar stage of rat and hamster embryos, suggesting that the expression pattern of p53 in embryos is evolutionary conserved (Chandrasekaran et al., 1981). Decreased level of *p53* mRNA in

mouse and chick was also observed at similar stages (Louis et al., 1988), and further examination of *p53* mRNA using in situ hybridization revealed that in day 8.5 post-coitum (p.c.) and 10.5 p.c. mouse embryos, expression level of p53 mRNA remains high and is detected ubiquitously (Schmid et al., 1991). As differentiation of cells progresses, the expression of p53 mRNA becomes more pronounced in specific tissues including the brain, liver, lung, thymus, intestine, salivary gland and kidney. The level of *p53* mRNA, then, decreases significantly and rapidly when cells are in the terminal differentiation stage beginning on day 11 in the mouse (Rogel et al., 1985). Overall, these studies have shown that in developing embryos the level of p53 protein and mRNA expression both decrease significantly at a certain stage, suggesting an important functional role for the protein during these proliferative stages.

Although the expression levels of p53 differ among embryonic stages and may suggest a functional role of p53 during embryogenesis, the expression of p53 is not always correlated with the activation of p53. In unstressed cells, p53 is believed to be biochemicaly inactive, and post-translational modification and translocation of p53 are often necessary for the activation of p53 (Eizenberg et al., 1996, refer to section 1.4.4). Using *lacZ* transgenic mice in which a bacterial *lacZ* marker gene is regulated by a promoter with a *p53* DNA response element, three studies have shown that in early stages (day 8.5 – 12.5) most of the expressed p53 is inactive, suggesting a post-translational control on the activity of p53. Weak but pronounced expression of active p53, however, was observed in the branchial arches and the periphery of the somites in day 8.5 embryos, branchial arches, the tips of the limb buds, and the midbrain-hindbrain boundary in day 10.5 embryos, and in the forebrain and in the hair follicles of the sensory vibrissae in day 12.5 embryos (Gottlieb et al., 1997). Other studies have also shown that active p53 is expressed in the brain of day 8, 10, 11 embryos and at all later stages (day 15, 17, 19 embryos and newborn mice) (Komarova et al., 1997; MacCallum et al., 1996). These studies using *lacZ* transgenic mice have all shown that although p53 protein and mRNA are highly expressed during early embryogenesis, most of this p53 is inactive, suggesting that p53 protein level is not always correlated well with its

functional activity (Lutzker and Levine, 1996). However, in the early stage of embryogenesis the highest level of p53 expression is in the developing nervous system. Thus, this particular organ may be most susceptible to maldevelopment in the absence of p53 because of an inability to induce apoptosis or arrest cell cycle in neuronal precursor cells at the time of neural tube closure (Choi and Donehower, 1999). Therefore, this temporal and spatial regulation of p53 in embryogenesis supports the idea that p53 may have an important role in the process of embryonic development (Almog and Rotter, 1997). The role of p53 in normal development and gene and miRNA expression differences in *p53*-deficient mouse embryos are the focus of the research presented in this dissertation (section 3).

1.4.8. p53 Transgenic Mice

Further insight into the role of p53 in embryonic development came through the observation of p53 knockout mice. The first abnormality in *p53*-deficient mice was reported by two independent research groups in 1995 (Armstrong et al., 1995; Sah et al., 1995). Although mice lacking *p53* alleles are viable, 2-6% of the offspring exhibit exencephaly resulting from the abnormal neural tube closure. In addition, some *p53*-deficient pups exhibited ocular, limb and tooth defects. Significantly increased mortality *in utero* or soon after birth was also observed. These studies also revealed that the *p53*-deficient mice exhibiting NTDs are predominantly females, a situation also found in human exencephaly cases as well as in other mouse mutants, e.g., *1/Hipk2, Efna5, Marcks, Nif1,* and *xn* mutants (Harris and Juriloff, 2007; Seller, 1987, 1995).

1.4.9. p53 as a Teratogen Enhancer or Suppressor

Although mice lacking *p53* alleles exhibit NTDs, embryos exposed to certain teratogens show the activation of p53. Whether p53 plays a role as a teratogen suppressor or inducer has been controversial, although the role of p53 as a suppressor has been more favored based on the protective role of p53 following a variety of environmental stresses (Choi and Donehower, 1999). Recent studies have shown that

p53 functions as a teratological inducer (Boreham et al., 2002; Narai et al., 2006; Wang, 2001), as well as a teratogen suppressor (Baatout et al., 2002; Bekaert et al., 2005; Moallem and Hales, 1998). For example, it has been shown that the loss of *p53* alleles increase the incidence of fetal resorption and developmental anomalies in mouse embryos exposed to benzo[a]pyrene (Nicol et al., 1995). It was also shown that *p53* -/- embryos showed higher incidence of structural anomalies and fetal death and a fewer number of apoptotic cells than that of *p53* +/+ embryos exposed to radiation (Baatout et al., 2002; Norimura et al., 1996). Others have also shown that the absence of p53 resulted in the increased limb defects but no signs of apoptosis (Moallem and Hales, 1998). These studies suggest that p53 acts as a teratological suppressor. In contrast, eye defects in mouse embryos exposed to 2-chloro-2'-deoxyadenosine (2CdA) was mediated by p53-dependent apoptosis, suggesting that p53 acts as a teratogen inducer (Wubah et al., 1996). Other studies have also shown that the lack of p53-dependent apoptosis resulted in reduced the incident of limb defects and cleft palate (Boreham et al., 2002; Narai et al., 2006; Wang, 2001). Examination of these studies suggests that the role of p53 differs by the type of teratogen as well as the type of malformations evaluated. Most studies that have identified p53 as a teratogen suppressor have examined multiple types of malformations as well as resorptions (Baatout et al., 2002; Bekaert et al., 2005; Nicol et al., 1995; Norimura et al., 1996) whereas studies that have identified p53 as a teratological inducer have investigated specific malformations such as eye defects, limb malformations, or cleft palate (Boreham et al., 2002; Narai et al., 2006; Wang, 2001; Wubah et al., 1996). The role of p53 in teratogen-induced maldevelopment is the focus of the research presented in this dissertation (section 4).

2. TERATOGEN-INDUCED ACTIVATION OF P53 IN EARLY POST-IMPLANTATION MOUSE EMBRYOS*

2.1. OVERVIEW

HS and 4CP activate the mitochondrial apoptotic pathway in day 9 mouse embryos. Previous microarray analyses revealed that several p53 target genes are upregulated after exposure to HS or 4CP, suggesting a role for p53 in teratogen-induced apoptosis. To explore the role of p53, we assessed the activation of p53 in day 9 mouse embryos exposed to HS or 4CP in vitro.

Both teratogens induced the accumulation of p53 and phosphorylation of p53 at ser-15, two hallmarks of p53 activation. HS and 4CP also induced an increase in *Noxa* and *Puma* mRNAs, transcripts of two known proapoptotic p53 target genes; however, these two teratogens did not induce significant increases in NOXA and PUMA proteins, suggesting that p53 does not activate the mitochondrial apoptotic pathway by transcriptionally upregulating the expression of NOXA and PUMA proteins. HS and 4CP also induced the expression of p21 mRNA and protein, suggesting a role for p53 in teratogen-induced cell cycle arrest. Previously, we also showed that HS and 4CP activate the apoptotic pathway in the embryo proper (head and trunk) but not in the heart. We now show that HS and 4CP induce a robust activation of p53 in the embryo proper but an attenuated induction in the heart. HS and 4CP induce the expression of p21 protein in majority of the cells in the embryo; however, expression of NOXA and PUMA proteins were not significantly induced in heads, hearts, or trunks of day 9 embryos. Overall, our results suggest that p53 may play a transcription-dependent role in teratogen-induced cell cycle arrest but a transcription-independent role in teratogen-induced apoptosis in day 9 mouse embryos exposed to HS or 4CP.

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2.2. INTRODUCTION

Over 1,200 chemical and physical agents are known to cause structural and/or functional congenital anomalies in experimental animals (Shepard, 2001). Although the mechanisms by which these agents disrupt normal development are often not well understood, it is known that many teratogens induce cell death in tissues that subsequently develop abnormally and give rise to structural malformations (Knudsen, 1997; Scott, 1977). In addition, teratogens often induce cell death in areas of normal PCD suggesting a mechanistic link between PCD and teratogen-induced cell death (Menkes et al., 1970; Milaire, 1983; Sulik et al., 1988). Finally, mouse mutants, in which genes known to play a role in apoptosis have been deleted, exhibit abnormal levels of apoptosis associated with abnormal development often culminating in structural birth defects (Boya and de la Rosa, 2005). Taken together, available data indicate that the dysregulation of apoptosis is consistently correlated with embryo/fetal lethality and/or birth defects.

Previously, we showed that HS, 4CP, and staurosporine, known animal teratogens, induce apoptosis in early postimplantation rodent embryos by activating the mitochondrial apoptotic pathway. Activation of this pathway is characterized by the release of cytochrome c and the subsequent activation of caspases, cleavage of poly ADP-ribose polymerase, and DNA fragmentation (Little et al., 2003; Little and Mirkes, 2002; Mirkes and Little, 1998, 2000). Thus, at least for this small sampling of teratogens, teratogen-induced apoptosis in early postimplantaion mouse embryos involves activation of the mitochondrial apoptotic pathway.

Using vital dyes and TUNEL staining, we have also shown that teratogen-induced cell death is cell specific, that is, some cells in the mouse embryo die, particularly in areas of normal PCD, while other cells, often neighboring cells, survive (Mirkes and Little, 1998; Umpierre et al., 2001). For example, cells of the embryonic nervous system (neuroepithelial cells) are particularly sensitive to teratogen-induced cell death, whereas mesenchymal cells surrounding the neuroepithelium are less sensitive (Umpierre et al., 2001). In contrast, cells of the embryonic heart are resistant to cell

death induced by a variety of teratogens (Gao et al., 1994; Umpierre et al., 2001). We have also shown that although teratogens activate the apoptotic pathway in sensitive cells, these hallmarks of apoptosis are not activated in cells of the heart (Mirkes and Little, 1998; Umpierre et al., 2001). These results indicate that the mitochondrial apoptotic pathway is blocked in heart cells at the level of the cytochrome c release from mitochondria or at some point upstream of cytochrome c release.

The rapid induction of the mitochondrial apoptotic pathway in teratogen-sensitive neuroepithelial cells and the failure to activate this pathway in teratogen-resistant heart cells suggest that the embryo must possess factors that regulate the efflux of cytochrome c and thereby the activation of the mitochondrial apoptotic pathway. To begin to identify proteins and signaling pathways that regulate cytochrome c release, we compared gene expression patterns in HS- or 4CP-treated and –untreated mouse embryos before and during the activation of the mitochondrial apoptotic pathway, using DNA microarray gene expression profiling. Our studies identified five candidate ‘‘apoptosis-related’’ genes (Mikheeva et al., 2004). Three of these genes, *Mdm2, Gtse1*, and *Cyclin G*, are coordinately upregulated by both HS and 4CP during the first 5 hr after embryos are exposed to these teratogens. Because these three genes are all p53-regulated genes, our microarray data suggested that HS and 4CP both activate p53.

p53 is essential for preventing inappropriate cell proliferation and maintaining genomic integrity following a variety of stresses (Harris and Levine, 2005). The level of p53 is maintained at a low level because of its rapid turnover through proteolysis. Following DNA damage, hypoxia, oncogene expression, and nucleotide depletion, p53 undergoes extensive posttranslational modifications. These modifications result in the accumulation of p53, its translocation into the nucleus, enhanced binding to DNA, and transcriptional activation of its target genes. The protein products of these target genes subsequently regulate a number of cellular processes, the most well studied being cell cycle arrest and apoptosis.

The objectives of the current studies were (1) to assess the kinetics of p53 activation in day 9 embryos exposed to HS or 4CP, (2) to determine whether key p53

proapoptotic or cell cycle arrest genes/proteins are induced by HS and/or 4CP, (3) to assess whether p53 is activated in cells sensitive and resistant to teratogen-induced apoptosis, and (4) to determine whether key p53 proapoptotic or cell cycle arrest genes/proteins are induced by HS and/or 4CP in cells sensitive and resistant to teratogen-induced apoptosis. Our data show that p53 is rapidly activated in day 9 mouse embryos exposed to HS or 4CP and suggest that once activated, p53 plays an important role in teratogen-induced apoptosis and cell cycle arrest.

2.3. MATERIALS AND METHODS

In Vitro Whole-embryo Culture: Primigravida Swiss-Webster mice were obtained from a local supplier. The morning following copulation was designated day 0 of gestation. On the morning of day 9, conceptuses from multiple litters were explanted using the whole rodent embryo culture system established by New (1978) with the following modifications. Embryos from all litters were equally distributed among the different treatment groups. For each treatment group, 10–12 embryos were cultured in 12 ml of media containing 80% heat-inactivated rat serum, 20% Hanks' balanced salt solution (HBSS), 50 U/ml penicillin, 50 μg/ml streptomycin; gassed with a mixture of 20% O_2, 5% CO_2, 75% N_2; and cultured for 1 hr prior to treatment.

Embryo Exposure Conditions: Treatment of the embryos with 4CP, a preactivated analogue of cyclophosphamide (a gift of Michael Colvin, Johns Hopkins University), was initiated by direct addition of freshly prepared 250X solution of 4CP (in HBSS) to the culture medium resulting in a final concentration of 40 μM 4CP. HS-treated embryos were exposed to 43 ºC for 15 min and then returned to 37 ºC (Mirkes, 1985). Embryos were continued in culture with drug or following heat shock for up to 5 hr.

Embryo Collections: At indicated times, treated and control embryos were removed from culture, dissected free of associated membranes and rinsed in cold HBSS. Embryos from each treatment group were pooled prior to the preparation of embryo lysates. For those experiments requiring embryo parts, groups of embryo were further

dissected into heads, hearts, and trunks using a fine lancet and forceps. The dissected head consisted of the prosencephalon and mesencephalon and the trunk consisted of the remainder of the embryo minus the heart. Dissected heads, hearts, and trunks were separately pooled prior to the preparation of head, heart, and trunk lysates.

Western Blot: Following treatment, washed embryos or embryo parts were sonicated in RIPA lysis buffer (10mM HEPES, pH 7.4, 42 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 0.1 mM EGTA, 1% Triton-X100 plus 1 mM PMSF, 1 mM DTT, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 5 μg/ml aprotinin). Aliquots were taken for protein quantification using Bicinchoninic Acid (BCA) assay and equal amounts of protein of each sample in 2X laemmli buffer were applied to 12.5% PAGE (Laemmli, 1970) and transferred to PVDF membranes. Immunoblot analysis was carried out as previously described (Mirkes and Little, 1998) using 3% nonfat dry milk in tris-buffered saline (TBS)/0.5% Tween-20 (TW) for blocking and antibody dilutions. The primary antibodies used were mouse monoclonal anti-Pan p53 at 1:500, rabbit polyclonal anti-human phosphoserine-6, -9, -15, -20, -37, -46, -392 specific p53 (Cell Signaling, Beverly, MA) at 1:1000– 1:2000 (note: human ser-15 is equivalent to mouse serine-18), mouse monoclonal anti-p21 at 1:625 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-NOXA at 1:1000 (Ingenex, San Diego, CA), rabbit polyclonal anti-a PUMA at 1:5000 (Oncogene Science, San Diego, CA), mouse monoclonal anti-GAPDH at 1:1,200,000 (Chemicon, Temecula, CA), and mouse monoclonal anti-actin at 1:300,000 (Sigma, St Louis, MO). Membranes were incubated overnight with primary antibodies and then washed four times with TBS/TW. HRP linked anti-mouse or anti-rabbit secondary antibodies (Amersham Life Sciences, Arlington Heights, IL) were used at 1:3000 for 2 hr, and membranes were washed twice with TBS/TW and three times with TBS. Antigen-antibody complexes were visualized by development with ECL Plus (Amershan Life Sciences) and autoradiography. For image analysis, antigen antibody complexes were detected using the KODAK Image Station 440, and quantification of protein band densities was determined using ImageQuant (Molecular Dynamics,

Sunnyvale, CA). Differences in levels of particular polypeptides were assessed using t-test (Statview 512).

Real-Time PCR: Embryos for real-time PCR were quick-frozen and stored at -80 ºC. Total RNA was isolated from embryos using the RNeasy mini kit (Qiagen,Valencia, CA) according to the manufacturer's instructions. After treatment with 1 U DNase (Promega, Madison, WI) at 37 ºC for 30 min, followed by incubating with stop solution at 65 ºC for 10 min, 2 µg total RNA was reverse transcribed using 0.025 µlg/µl oligo-d(T)15 primer and 0.5 mM dNTP mix (Roche Applied Science, Indianapolis, IN) by heating at 70 ºC for 5 min, followed by cooling on ice. The mRNAwas then copied into cDNA by diluting 10-fold with first-strand buffer containing 100 U SuperScript II (Gibco, Rockville, MD), 10 mM DTT and 1 U/µl RNasin (Promega) and incubated at 45 ºC for 1 hr. The reaction was terminated by placing at 70 ºC for 15 min and then brought to 4 ºC and stored at - 80 ºC until analyzed by real-time PCR. Fluorogenic 5' nuclease assays (TaqMan) were carried out in Functional Genomics Laboratory in the Center for Ecogenetics and Environmental Health at the University of Washington, Seattle, WA, using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems Inc., Foster City, CA). Statistical differences of control to HS or 4CP samples were determined by ANOVA analysis followed by Dunnett's post hoc test.

Immunohistochemistry: Following treatment and embryo collections, embryos were immediately fixed in 4% paraformaldehyde for 2 hr at 4 ºC. The embryos were then dehydrated in several changes in ethanol, embedded in paraffin, sectioned at 5 µm, and mounted on glass slides. Tissue sections were heated at 58 ºC for 20 min, deparaffinized in histoclear, and rehydrated. Sections were then boiled for 20 min with sodium citrate buffer (pH 6.0) for antigen unmasking. Endogenous peroxidase activity was blocked by placing slides in 3% H_2O_2 in dH_2O for 10 min. Slides were briefly rinsed in distilled water and in TBS/TW. Sections were blocked with 1% BSA in TBS for 1 hr. Primary antibody solutions, diluted in blocking solution, were placed on the sections overnight. The ser-15 p53 antibody (Cell Signaling) was prepared against a synthetic

phosphopeptide corresponding to residues surrounding ser-15 of human p53, and the dilution used was 1:50. To confirm the specificity of ser-15 p53, the primary antibody was incubated with the blocking peptide (Cell Signaling) for 30 min at room temperature and then the adsorbed primary antibody was placed on tissue sections and incubated overnight at room temperature. The p21 antibody (Santa Cruz Biotechnology) was prepared against amino acids 1–159 representing full-length p21 of mouse, and the dilution used was 1:50. Slides were washed three times in TBST for 5 min each. Biotinylated donkey anti-rabbit or sheep anti-mouse IgG secondary antibodies (Amersham) were placed at 1:200 for 30 min. The slides were washed as before and incubated in Vectastain Universal Elite ABC Reagent (Vector Laboratories, Burlingame, CA) for 30 min. Antigen-antibody complexes are visualized as brown staining. Slides were then rinsed in water, dehydrated, counterstained with hematoxylin, and coverslipped.

2.4. RESULTS

To investigate the activation of p53, we measured two different but related aspects of teratogen-induced activation of p53, that is, p53 accumulation and site-specific phosphorylation, in day 9 embryos exposed to HS or 4CP. To study p53 accumulation, we used commercially available antibodies that recognize both unphosphorylated (Pan p53) and phosphorylated p53. To determine the kinetics of p53 activation, we assessed total p53 accumulation at 1, 2.5, and 5 hr after initiation of exposure to HS or 4CP (Fig. 2.1). Figure 2.1A is a representative western blot showing that HS induces an apparent increase in total p53 as early as 2.5 hr after exposure. Densitometric analysis of western blots from three independent experiments shows that HS induces a statistically significant increase in total p53 at the 2.5- and 5-hr time points (Fig. 2.1B). We next determined whether another known teratogen, 4CP, also induced the activation of p53 in day 9 mouse embryos. Figure 2.1C shows that 4CP, like HS, induces an apparent increase in total p53 as early as 2.5 hr after exposure to 4CP; however, densitometric analysis of western blots from three independent experiments

indicates that a statistically significant increase is only observed at the 5-hr time point as shown in Figure 2.1D.

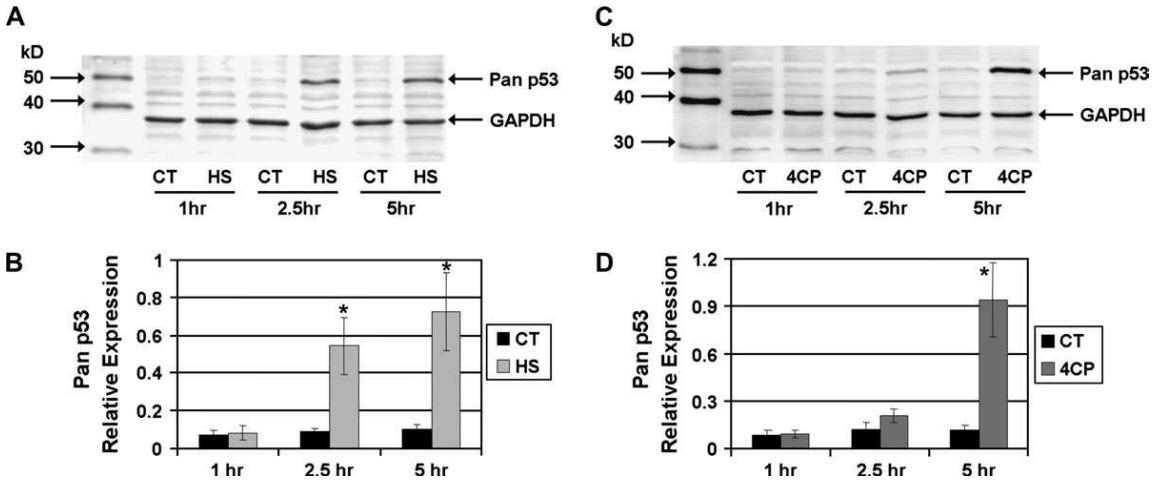

Figure 2.1 Western blot analysis of the time course of total p53 (Pan p53) accumulation in cultured day 9 mouse embryos receiving no treatment (CT), a HS of 43 ºC for 15 min (A), or continuous exposure to 40 µM of 4CP (C). Densitometric analysis of three independent experiments is presented for HS- (B) and 4CP-treated embryos (D). * Indicates a significant difference from CT ($p < 0.05$).

To study site specific phosphorylation of p53, we initially used commercially available antibodies that recognize p53 phosphorylated at specific serine residues (ser-6, -9, -15, -20, -37, -46, -392) and western blot analysis to determine which, if any, sites are phosphorylated in response to HS. Although phospho-specific antibodies to ser-6 (Fig. 2.2, lanes 3 and 4), ser-9 (Fig. 2.2, lanes 5 and 6), ser-20 (Fig. 2.2, lanes 9 and 10), ser-37 (Fig. 2.2, lanes 11 and 12), and ser-46 (Fig. 2.2, lanes 13 and 14) p53 failed to detect an increase in phosphorylated p53, the phospho-specific ser-15 p53 antibody (Fig. 2.2, lanes 7 and 8) detected a HS-induced increase in ser-15 p53. In addition, there may be a slight increase in ser-392 p53 (Fig. 2.2, lanes 15 and 16) induced by HS.

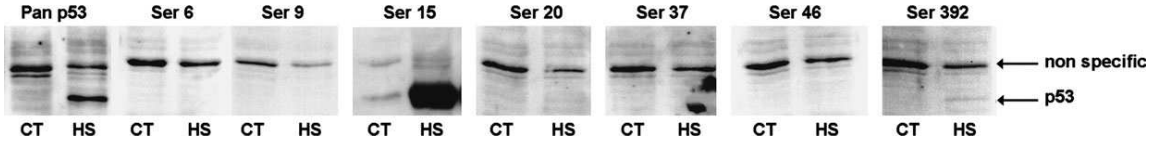

Figure 2.2. Western blot analysis of phosphorylation of p53. Lysates from control and heat-shock–treated (HS, 43 ºC for 15 min) cultured day 9 mouse embryos were applied to 12.5% PAGE, transferred to PVDF membranes, and probed with Pan p53, ser-6 p53, ser-9 p53, ser-15 p53, ser-20 p53, ser-37 p53, ser-46 p53, or ser-392 p53 antibodies.

On the basis of these preliminary results, we next assessed the kinetics of HS- and 4CP-induced increases in ser-15 p53. Figure 2.3A is a representative western blot showing that HS induces an apparent increase in ser-15 p53 as early as 2.5 hr after exposure. Densitometric analysis of western blots from three independent experiments shows that HS induces a statistically significant increase in ser-15 p53 (Fig. 2.3B) at the 2.5- and 5-hr time points. Figure 2.3C shows that 4CP, like HS, also induces an apparent increase in ser-15 p53; however, densitometric analysis of western blots from three independent experiments indicates that a statistically significant increase is only observed at the 5-hr time point (Fig. 2.3D).

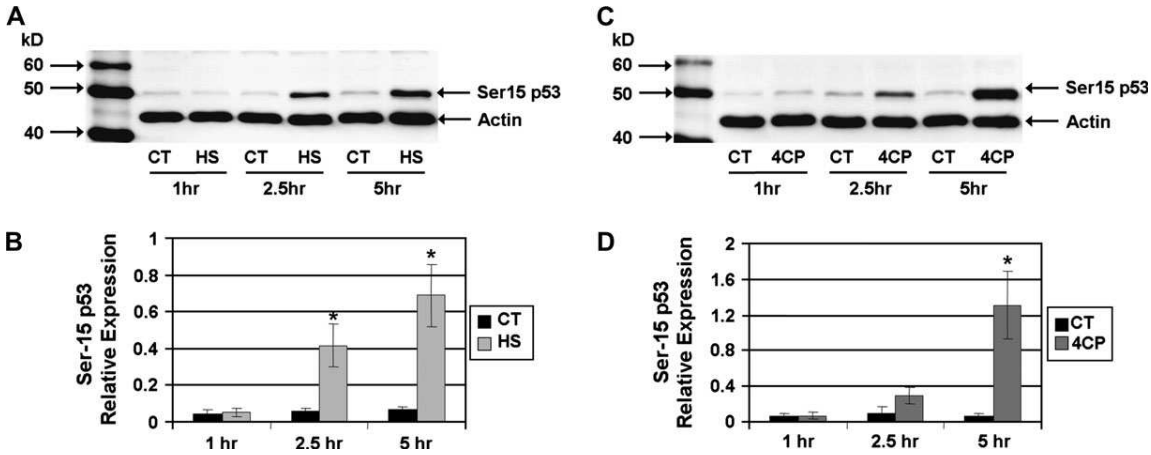

Figure 2.3. Western blot analysis of the time course of ser-15 p53 phosphorylation in cultured day 9 mouse embryos receiving no treatment (CT), a HS of 43 ºC for 15 min (A), or continuous exposure to 40 μM of 4CP (C). Densitometric analysis from three independent experiments is presented for HS (B) - and 4CP-treated embryos (D). * Indicates a significant difference from CT ($p < 0.05$).

Having shown that HS and 4CP both activate p53, we next sought to determine whether these two teratogens activated both arms of the p53 pathway, that is, one leading to cell cycle arrest and the other to apoptosis. To do this, we used real-time PCR and western blot analysis to quantitate the levels of *p21*, a major p53 target gene involved in cell cycle arrest, and *Noxa* and *Puma*, two ‘‘proapoptotic’’ p53 target genes.

With respect to the cell cycle arrest arm of the p53 pathway, both HS and 4CP induce a significant increase in *p21* mRNA but only at the 5-hr time point (Figs. 2.4A and B). Using western blot analysis, we show that p21 protein levels mirror *p21* mRNA levels, with a significant increase in HS- and 4CP-induced p21 levels at the 5-hr time point (Fig. 2.4C and D, respectively).

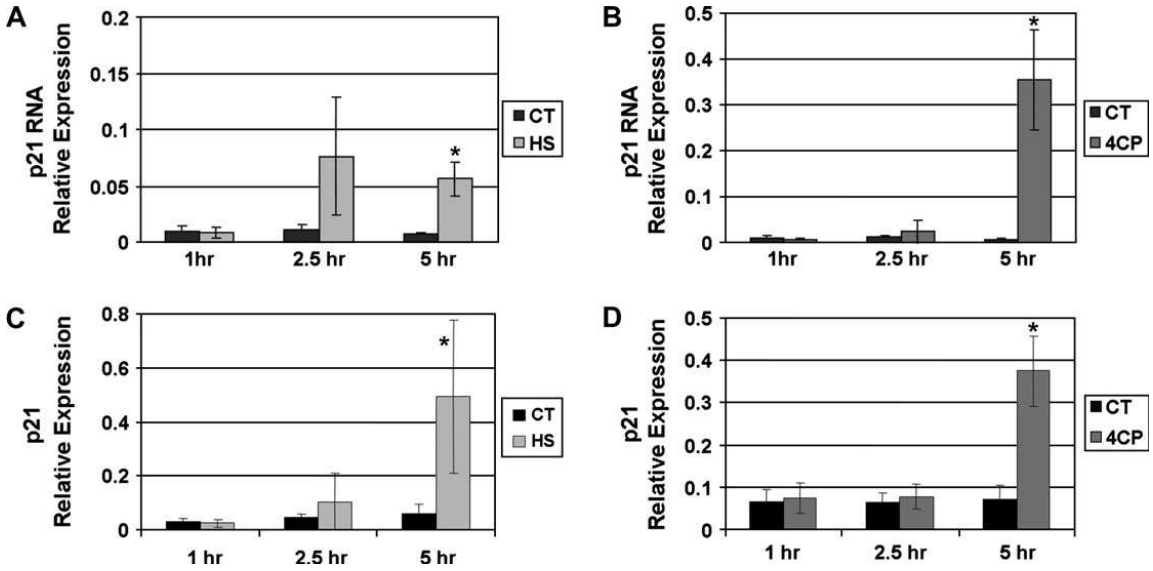

Figure 2.4. p21 mRNA (A and B) and protein expression (C and D) in cultured day 9 mouse embryos receiving no treatment (CT), a HS (43 ºC for 15 min), or continuous exposure to 40 μM of 4CP. Densitometric analysis from three independent western blot experiments is presented for HS(B)- and 4CP-treated embryos (C). * Indicates a significant difference from CT ($p < 0.05$).

With respect to the apoptosis arm of the p53 pathway, HS and 4CP induce apparent increases in Noxa mRNA at the 2.5- and 5-hr time points; however, only the HS- and 4CP-induced increases at the 5-hr time point are statistically significant (Fig. 2.5A). Similarly, HS and 4CP also induce apparent increases in Puma mRNA at the 2.5- and 5-hr time points; however, only the HS-induced increase at 5 hr and 4CP-induced increase at 2.5 and 5 hr are statistically significant (Fig. 2.5B).

Although HS and 4CP induced increases in Noxa and Puma mRNAs at the 5-hr time point, western blot analysis did not show significant increases in NOXA or PUMA protein at any of the time points studied (Fig. 2.6A–H). It should be noted that although other studies have identified several isoforms of PUMA (α, β, γ, and δ), only PUMA α is expressed in the day 9 mouse embryo.

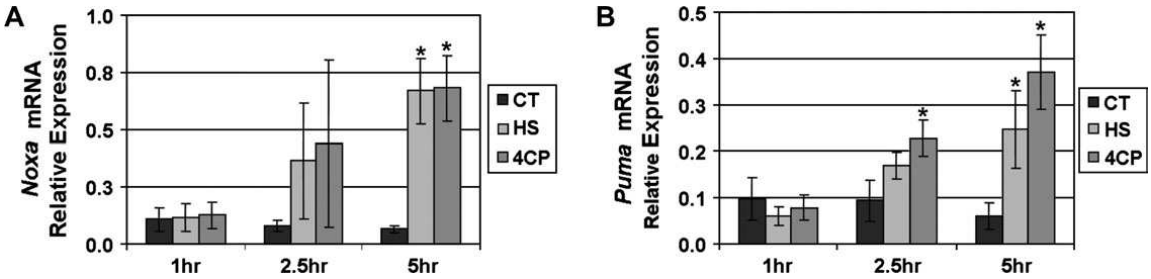

Figure 2.5. Noxa (A) and Puma (B) mRNA expression in cultured day 9 mouse embryos receiving no treatment (CT), a HS (43 ºC for 15 min), or continuous exposure to 40 μM of 4CP. * Indicates a significant difference from CT ($p < 0.05$).

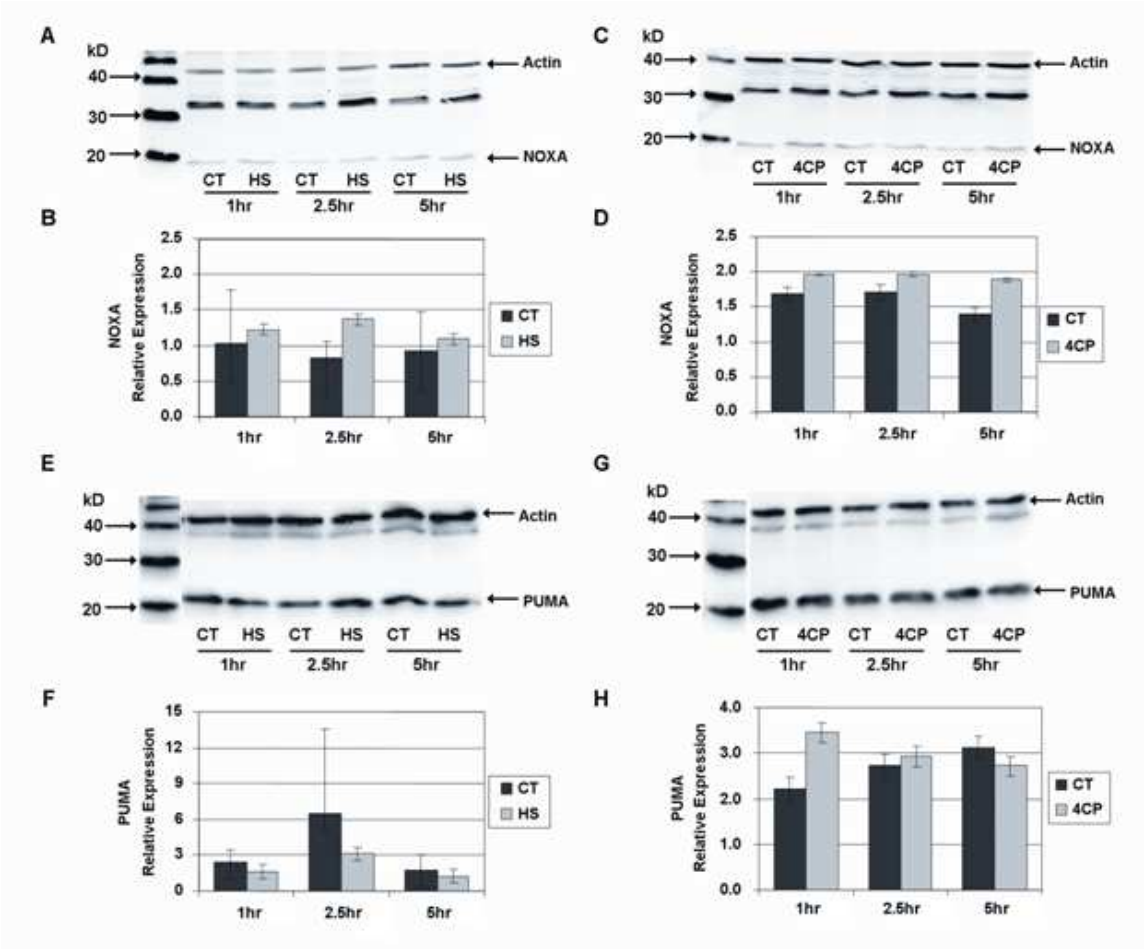

Figure 2.6. Western blot analysis of the time course of NOXA (A–D) and PUMA (E–H) protein expression in cultured day 9 mouse embryos receiving no treatment (CT), a HS of 43 ºC for 15 min (A and E), or continuous exposure to 40 μM of 4CP (C and G). Densitometric analysis from three independent experiments is presented for HS (B and F)- and 4CP (D and H)-treated embryos.

Published research from our laboratory has shown that although HS and 4CP activate the mitochondrial apoptotic pathway leading to apoptosis in day 9 mouse embryos, activation of this pathway does not occur in cells of the embryonic heart, which are resistant to teratogen-induced apoptosis. On the basis of this information, we next compared the activation of p53 in day 9 mouse embryo hearts (resistant tissue) and the day 9 mouse embryo head and trunk (sensitive tissues). Figure 2.7A presents a representative western blot showing HS-induced increases in total p53 in heads and trunks but not hearts. Densitometric analysis of western blots from three independent

experiments shows that HS induces a statistically significant increase in total p53 in heads and trunks (Fig. 2.7B). In the heart, HS appears to induce a modest increase in total p53; however, this increase is not statistically significant. The results for total p53 are mirrored by those for ser-15, that is, significant increases in ser-15 p53 in heads and trunks and a modest but not a significant increase in ser-15 p53 in hearts (Fig. 2.7C and D).

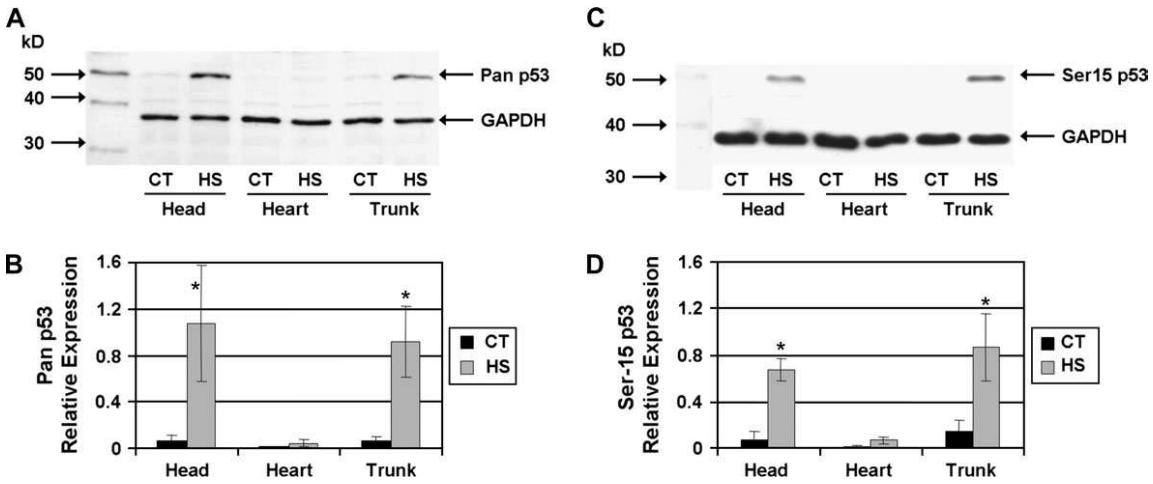

Figure 2.7. Western blot analysis of Pan p53 accumulation (A) and ser-15 p53 phosphorylation (C) in cultured day 9 embryo heads, hearts, and trunks receiving HS exposure. Lysates of heads, hearts, and trunks were prepared from cultured day 9 mouse embryos at 5 hr after receiving no treatment (CT) or HS (43 ºC for 15 min). Densitometric analysis from three independent experiments is presented for Pan p53 (B) and ser-15 p53 (D). * Indicates a significant difference from CT ($p < 0.05$).

Although the western blot data clearly show that HS induces a robust activation of p53 in the day 9 mouse embryo head and trunk but only a modest increase in the heart, this approach does not provide any information concerning HS-induced activation of p53 in specific cells/tissues of the head and trunk. To obtain such data, we used an anti–ser-15 p53 antibody and immunohistochemistry (Fig. 2.8). Figure 2.8A is a parasagittal section from a day 9 control embryo. Occasional p53-positive cells (brown staining) are observed in the neuroepithelium (arrows in Fig. 2.8B) but not in the heart (Fig. 2.8C) or trunk (Fig. 2.8D). In contrast, many more p53-positive cells are observed in a comparable parasagittal section from a day 9 embryo exposed to HS and harvested 5 hr after exposure (Fig. 2.8E). Although p53-positive cells are present in all tissues of the embryonic head and trunk, for example, outer epithelium, neuroepithelium, loose mesenchyme, and somites, the majority of cells in any one tissue are unstained. Higher magnification photomicrographs of the prosencephalon (Fig. 2.8F) and the trunk region (Fig. 2.8H) show robust staining in cells and different tissues, for example, epithelial, mesenchymal, and neural cells in the head. Even higher magnification photomicrographs show that this staining is nuclear (data not shown). In contrast, heart cells exhibit either no apparent staining or much reduced staining intensity (Fig. 2.8G). The lack of staining in Figure 2.8I–L, in which an adjacent section was stained with the anti–ser-15 p53 antibody that had been adsorbed with the peptide used to make the primary antibody, confirms the specificity of the primary antibody. Overall, these immunohistochemical data confirm the western blot data showing that HS-induced activation is robust in some cells of the embryo proper but attenuated in cells of the heart.

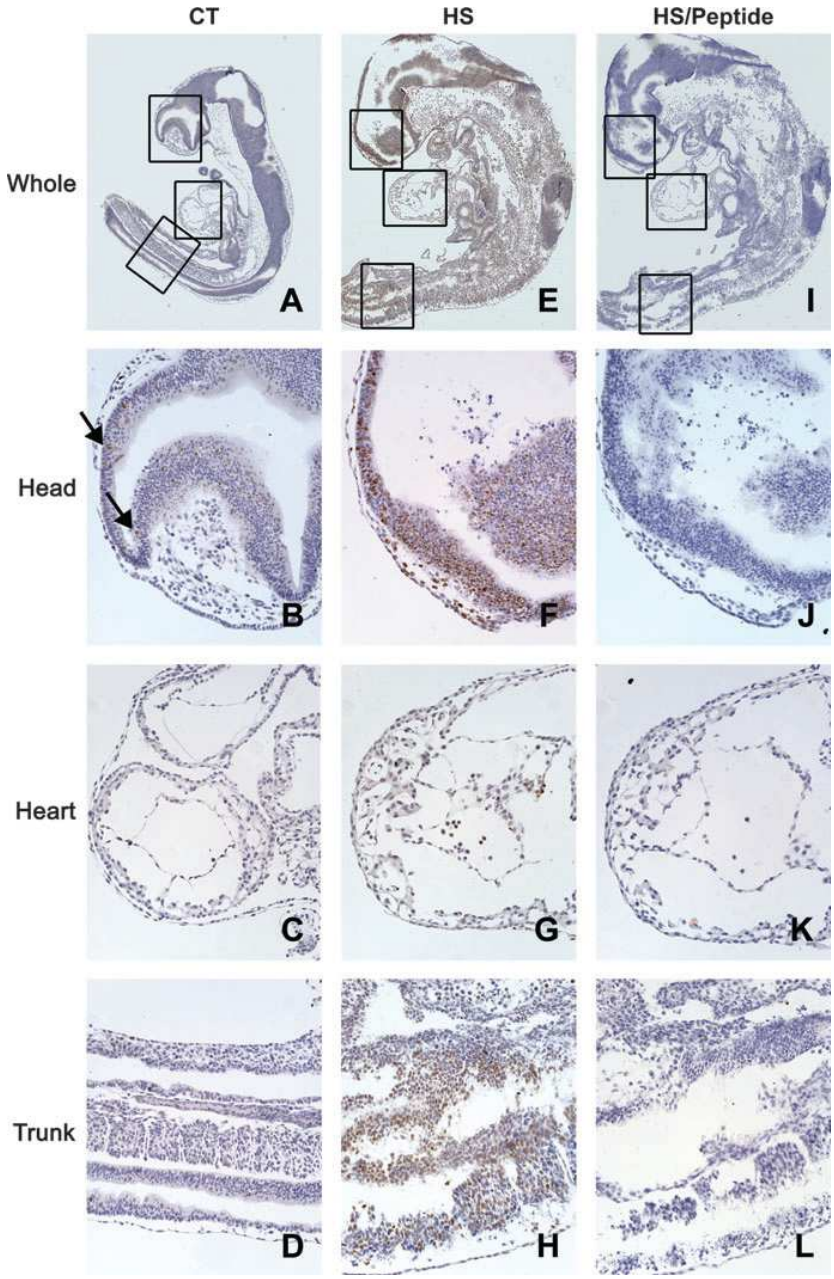

Figure 2.8. Immunohistochemical detection of ser-15 p53 in cultured day 9 mouse embryos receiving no treatment (CT) or a HS of 43 ºC for 15 min. Magnified images of the head (B), heart (C), and trunk (D) of the control embryo (A) are presented. The arrows indicate immunoreactive cells. Magnified images of the head (F), heart (G), and trunk (H) of HS-treated embryo (E) are presented. To show the specificity of the antibody, section of the HS-treated embryo was stained with an anti–ser-15 p53 antibody that had been incubated with the peptide (I). Magnified images of the head (J), heart (K), and trunk (L) are presented.

A western blot analysis of the effects of 4CP exposure on p53 activation in day 9 mouse embryo head, heart, and trunk shows that 4CP induces a statistically significant increase in total p53 (Fig. 2.9A and B) and ser-15 p53 (Fig. 2.9C and D) in heads and trunks. Unlike the situation in HS-treated embryos, 4CP induced an attenuated but statistically significant increase in total and ser-15 p53 levels in the heart (Fig. 2.9B and D).

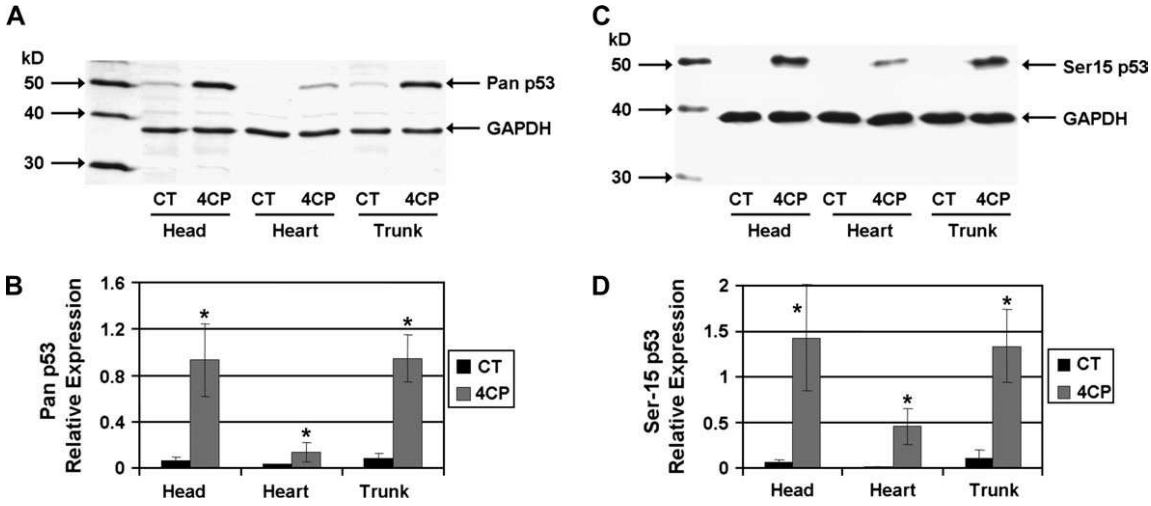

Figure 2.9. Western blot analysis of Pan p53 accumulation (A) and ser-15 p53 phosphorylation (C) in cultured day 9 embryo heads, hearts, and trunks receiving 4CP exposure. Lysates of heads, hearts, and trunks were prepared from day 9 mouse embryos at 5 hr after receiving no treatment (CT) or continuous exposure to 40 μMof 4CP. Densitometric analysis from three independent experiments is presented for Pan p53 (B) and ser-15 p53 (D). * Indicates a significant difference from CT ($p < 0.05$).

Having shown that HS and 4CP induce a robust activation of p53 in the head and trunk and a more attenuated activation in the heart, we next sought to determine the extent to which these two teratogens activated both arms of the p53 pathway, that is, one leading to cell cycle arrest and the other to apoptosis. The induction of p21 in heads, hearts, and trunks is presented in Figure 2.10. HS-induced activation of p53 in the heads and trunks (Fig. 2.7) is correlated with a statistically significant induction of p21 (Figs. 2.10A and B). Similarly, 4CP-induced activation of p53 in the heads and trunks (Fig. 2.9) is correlated with a statistically significant induction of p21 (Fig. 2.10C and D).

Although there is no apparent activation of p53 in the heart (Fig. 2.7) and an attenuated but significant activation of p53 in heart cells from 4CP-treated embryos (Fig. 2.9), HS and 4CP both induced a statistically significant induction of p21 in the heart (Fig. 2.10B and D).

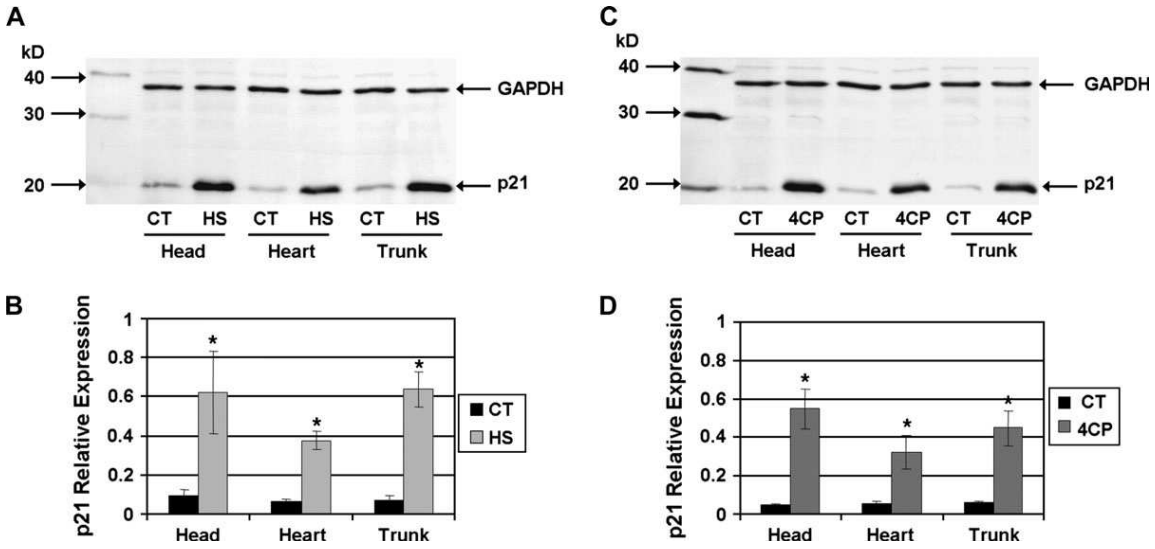

Figure 2.10. Western blot analysis of p21 (A and C) in cultured day 9 embryo heads, hearts, and trunks. Lysates of heads, hearts, and trunks were prepared from cultured day 9 mouse embryos at 5 hr after receiving no treatment (CT), a HS of 43 ºC for 15 min (A), or continuous exposure to 40 μM 4CP (C). Densitometric analysis from three independent experiments is presented for p21 (B and D). * Indicates a significant difference from CT ($p < 0.05$).

To confirm the increased mRNA and protein expression levels of p21 in day 9 mouse embryos after the exposure to HS, and to provide information concerning HS-induced activation of p21 in specific cells/tissues of the head and trunk, immunohistochemistry was used to stain p21 protein in embryos (Fig. 2.11). Figure 2.11E is a parasagittal section from a day 9 embryo taken 5 hr after exposure to HS and probed with an anti-p21 antibody. Many cells throughout all regions including the heart cells are uniformly stained. Higher magnification photomicropraphs of the prosencepahalon (Fig. 2.11F), heart (Fig. 2.11G), and the trunk region (Fig. 2.11H) show distinct staining in many cells and different tissues. Control embryos (Fig. 2.11A) stained with p21 showed an absence of staining in the head (Fig. 2.11B), heart, (Fig.

2.11C), and trunk (Fig. 2.11D), except for the specific staining of a subset of cells in somites. Overall, these immunohistochemical data show that HS-induced activation is robust in cells of the embryo, including heart cells, and confirm the western blot data.

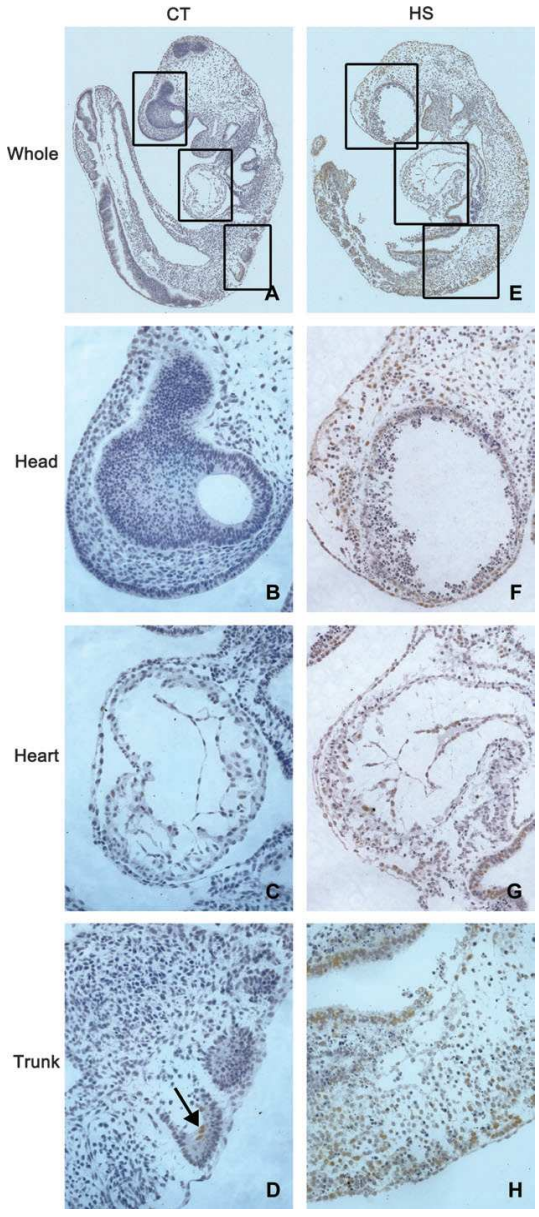

Figure 2.11. Immunohistochemical detection of p21 in cultured day 9 mouse embryos receiving no treatment (CT) or a HS of 43 ºC for 15 min. Magnified images of the head (B), heart (C), and trunk (D) of the control embryo (A) are presented. The arrow indicates immunoreactive cells in somites. Magnified images of the head (F), heart (G), and trunk (H) of the HS-treated embryo (E) are presented.

We next determined whether HS- and 4CP-induced activation of p53 was accompanied by an increased expression of NOXA and PUMA proteins, two proapoptotic proteins known to be transcriptional target genes of activated p53, in heads, hearts, and trunks of day 9 mouse embryos (Fig. 2.12). Western blot analysis (Figs. 2.12A and E) showed that HS does not induce a significant increase in NOXA (Fig. 2.12B) or PUMA (Fig. 2.12F) protein expression in either heads, hearts, or trunks; however, our results do show that NOXA and PUMA proteins are constitutively expressed in heads, hearts, and trunks of unexposed embryos. Similar results were obtained when embryos were exposed to 4CP (Figs. 2.12C, D, G, and H).

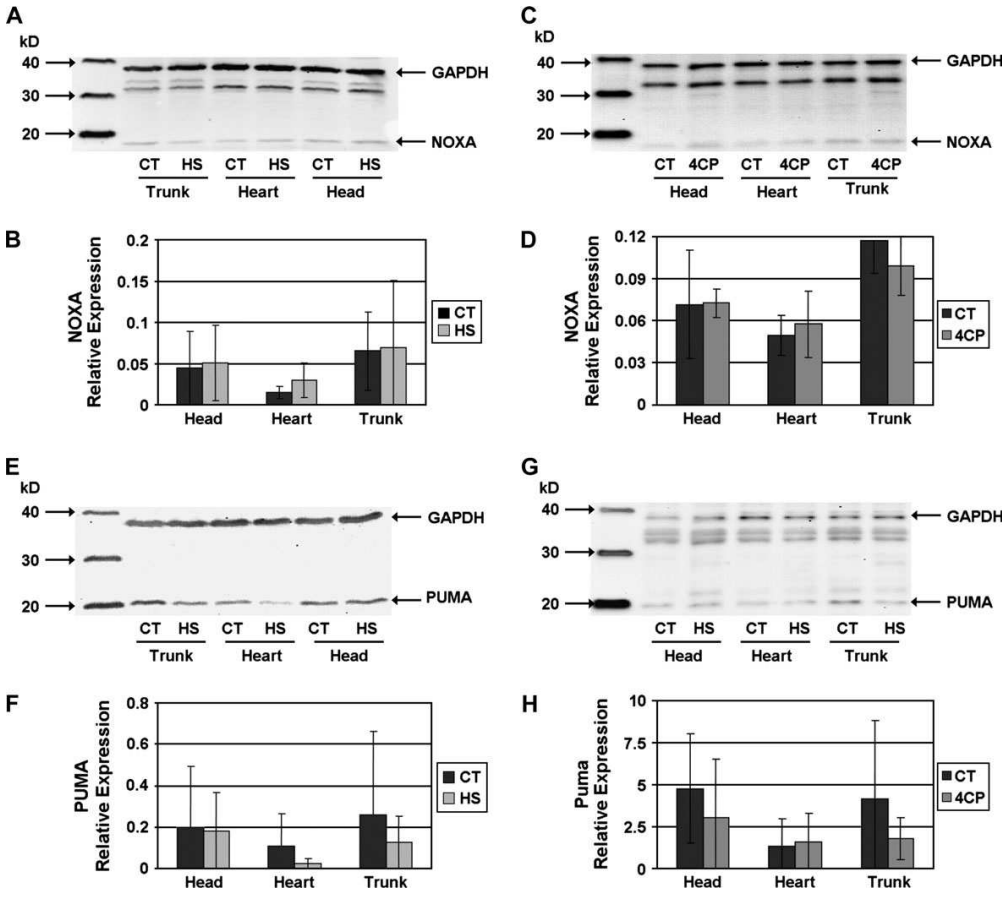

Figure 2.12. Western blot analysis of NOXA (A and C) and PUMA (E and G) in cultured day 9 embryo heads, hearts, and trunks. Lysates of heads, hearts, and trunks were prepared from day 9 mouse embryos at 5 hr after receiving no treatment (CT), a HS (43 ºC for 15 min), or continuous exposure to 40 μM 4CP. Densitometric analysis from three independent experiments is presented for NOXA (B and D) and PUMA (F and H).

2.5. DISCUSSION

Previously published gene expression profiling studies revealed that three known p53 target genes, Mdm2, Gtse1, and Cyclin G, were significantly upregulated in day 9 mouse embryos after exposure to HS or 4CP, thus implicating p53 as an important regulator of teratogen-induced apoptosis (Mikheeva et al., 2004). Called the ‘‘guardian of the genome,’’ p53 is known to play a key role in regulating whether a cell will arrest, undergo apoptosis, senesce, or differentiate in response to various stresses. To achieve this regulatory role, p53 must be activated by a variety of posttranslational modifications, for example, phosphorylation, acetylation, and ubiquitination (Brooks and Gu, 2003). Using a panel of phospho-specific p53 antibodies directed against human ser-6, -9, -15, -20, -37, -46, and -392 p53, we have now shown that p53 is phosphorylated at ser-15 after exposure to HS or 4CP. Phosphorylation at this serine is known to regulate apoptosis because p53- mediated apoptosis is significantly impaired when ser-15 is mutated to alanine (Chao et al., 2003; Sluss et al., 2004). Using an antibody that recognizes phosphorylated and nonphosphorylated p53 (Pan p53), we also showed that the increase in ser-15 p53 is correlated with an increase in total p53. Finally, using ser-15 p53 antibodies and immunohistochemistry, we have shown that activated p53 localizes to the nucleus of stained cells. Together, these results show that HS and 4CP, two teratogens that induce apoptosis in day 9 mouse embryos, also activate p53, a known regulator of apoptosis.

Our data also show that p53 is rapidly activated by both HS and 4CP, with activation occurring between 1 and 2.5 hr after exposure. Thus, p53 is activated before HS- and 4CP-induced release of mitochondrial cytochrome c and activation of the caspase cascade, which occur between 2.5 and 5 hr after exposure to these two teratogens (Little and Mirkes, 2002; Mirkes and Little, 1998, 2000). Although not definitive, the kinetics of p53 activation are consistent with a regulatory role for p53 in teratogen-induced apoptosis. We are currently using p53 mutant mice to determine whether p53 is required for teratogen-induced apoptosis.

Although our data are consistent with a regulatory role for p53 in teratogen-induced apoptosis, the mechanisms by which p53 activates the mitochondrial apoptotic pathway in the day 9 mouse embryo are unclear. One known mechanism, elucidated from cell culture studies, is the transcription-dependent expression of proapoptotic genes. Two of the major downstream targets of p53-mediated apoptosis are Noxa and Puma, transcripts of proapoptotic genes belonging to the Bcl-2 family. At least in some settings, NOXA protein is an essential mediator of p53-dependent apoptosis (Yakovlev et al., 2004) and activates the mitochondrial apoptotic pathway by interacting with Bcl-2 family members resulting in the release of cytochrome c and the activation of caspase-9 (Oda et al., 2000a; Seo et al., 2003). Other studies suggest that PUMA protein interacts with BCL-2 and BCL-XL and thereby induces mitochondrial membrane potential change, cytochrome c release, and caspase activation (Chipuk et al., 2005; Yu and Zhang, 2003; Yu et al., 2001).

Our results indicate that although HS and 4CP both induce increased expression of Noxa and Puma mRNAs, the increased expression of these mRNAs is not coupled with an increased expression of NOXA and PUMA proteins. Although we cannot explain the apparent disconnect between mRNA and protein levels, the explanation may involve a timing issue. Our data show that the increases in Noxa and Puma mRNA levels are significant at the 2.5- and 5-hr time points, latter being the latest time point studied in terms of NOXA and PUMA protein levels. Thus, there may not have been sufficient time for the increases in Noxa and Puma mRNAs to be translated into increases in their respective proteins that could be detected by western blot analysis. Had we measured NOXA and PUMA protein levels at some later time point, we may have observed the expected increases. Nonetheless, even if this were observed, increases in NOXA and PUMA proteins at times later than 5 hr would not be relevant to the initial activation of the mitochondrial apoptotic pathway, which is activated at some time between 2.5 and 5 hr after exposure to these teratogens. Thus, our data suggest that p53-mediated upregulation of NOXA and PUMA proteins is not involved in HS- and 4CPinduced activation of the mitochondrial apoptotic pathway.

Although upregulation of NOXA and PUMA proteins may not be required, these proteins may still play a role in the activation of the mitochondrial apoptotic pathway. Of interest, our data show that NOXA and PUMA proteins are constitutively expressed in the day 9 embryos in the absence of any teratogenic exposure. The function of these proteins in mouse development is unknown but do not appear to be required for normal development because Noxa and Puma null mice are born at the expected frequency and exhibit a normal phenotype (Villunger et al., 2003). How the proapoptotic activity of NOXA and PUMA is blocked is also unknown; however, it may be that these proteins are sequestered in an inactive form that is then activated in response to appropriate apoptotic stimuli. The constitutive expression of proapoptotic Bcl-2 family members that are sequestered and then activated in response to various apoptotic stimuli is well documented. Examples include binding to other proteins (BIM and BMF binding to dynein motor complex), cleavage (inactive BID cleaved to active tBID by caspase-8), and phosphorylation-induced binding of BAD to 14-3-3 (Gross et al., 1999; Li et al., 1998; Puthalakath et al., 1999, 2001; Zha et al., 1996). However, we are not aware of any published data showing that NOXA or PUMA proteins are sequestered in the absence of an apoptotic stimulus and then activated after an appropriate cell death signal.

Even if p53-mediated upregulation of NOXA and PUMA proteins does not play a role in activating the mitochondrial apoptotic pathway in teratogen-exposed mouse embryos, p53 is known to upregulate other proapoptotic proteins, for example, BAX, p53AIP, and PIGs. Whether any of these or other p53 target genes play a role in HS- and 4CP-induced activation of the mitochondrial pathway is unknown; however, our studies have shown that there is no significant increase in BAX protein levels in mouse embryos exposed to HS or 4CP (unpublished data). Although p53 may regulate teratogen-induced apoptosis in the mouse embryo by transcriptionally upregulating proapoptotic target genes, we presently do not have any data to support this possibility.

Alternatively, recent evidence has uncovered a transcription independent role for p53 in the regulation of apoptosis. Early studies, using cancer cell lines, reported that apoptosis occurred in the presence of inhibitors of transcription and translation or in cells

expressing p53 mutants with abrogated transactivation activity (Caelles et al., 1994; Jimenez et al., 2000). Subsequent studies have shown that after apoptotic stimuli, activated p53 rapidly translocates to mitochondria where it physically interacts with BCL-2 and BCL-XL to antagonize their antiapoptotic activities or BAX and BAK to promote their proapoptotic activities (Chipuk et al., 2004; Leu et al., 2004; Marchenko et al., 2000; Mihara et al., 2003; Schuler et al., 2000). Current studies are underway to determine whether p53 may have a transcription- independent role in inducing apoptosis in mouse embryos exposed to HS or 4CP.

Our data also show that HS and 4CP induce the upregulation of cyclin-dependent kinase p21 mRNA and protein. Moreover, results from our immunohistochemical analysis indicate that p21 protein is upregulated in most, if not all, cells of the day 9 mouse embryo after exposure to HS. Because p21 is a known p53 target that plays a central role in arresting the cell cycle after various genotoxic stresses (Harris and Levine, 2005; Taylor and Stark, 2001), our results suggest that cells of the day 9 mouse embryo have activated the cell cycle arrest arm of the p53 pathway in response to teratogenic exposures. Although we have not shown that HS induces cell cycle arrest in early postimplantation rodent embryos, we have shown that phosphoramide mustard, the major teratogenic metabolite of 4CP, induces alterations in the cell cycle in postimplantation rat embryos (Little and Mirkes, 1992; Mirkes et al., 1989). In addition, Chernoff et al. (1989) and Francis et al. (1990) have shown that cyclophosphamide induced a dose-dependent increase in the percentage of limb bud cells in the S phase of the cell cycle. Together, these results demonstrate that cyclophosphamide 4CP induce alterations in the cell cycle in early postimplantation mouse embryos exposed in vitro or in vivo.

Published data consistently show that teratogens induce apoptosis in some cells of the embryos and not others (Gao et al., 1994; Mirkes, 1985; Mirkes et al., 1985, 1991; Thayer and Mirkes, 1995). With respect to HS- and 4CP-induced apoptosis, cells within the neuroepithelium and neural crest cells that have migrated from the neuroepithelium are the most sensitive to teratogen-induced cell death, whereas surrounding

mesenchymal cells as well as epithelial cells are less sensitive. In contrast, cells of the heart are completely resistant to teratogen-induced apoptosis (Umpierre et al., 2001). In addition, activation of the mitochondrial apoptotic pathway, characterized by cytochrome c release, activation of caspases, and the induction of DNA fragmentation, is completely blocked in heart cells from day 9 mouse embryos (Little and Mirkes, 2002; Mirkes and Little, 1998, 2000). In the present studies, we now show that heart cell resistance is associated with significant attenuation of the activation of p53 in heart cells. Despite the attenuated activation of p53 in heart cells in response to teratogenic exposures, our data indicate that both HS and 4CP induce increased levels of p21 in heart cells. These results suggest that p53 is activated in the heart and when activated subsequently upregulates the expression of p21, thereby arresting heart cells. One caveat, however, is that p21 expression can also be induced via p53- independent mechanisms (O'Reilly, 2005). Using p53 null mice, we are currently determining whether HS- and 4CPinduced upregulation of p21 is p53-dependent.

Our results showing robust activation of p53 in cells sensitive to teratogen-induced apoptosis and attenuated activation of p53 in cells resistant to teratogen-induced apoptosis, leads to the hypothesis that high levels of activated p53 induce apoptosis, whereas low levels of activation lead to cell cycle arrest. This hypothesis is supported by studies showing that high amounts of ectopic p53 induce apoptosis, whereas lower amounts result in cell cycle arrest (Chen et al., 1998; Lokshin et al., 2005; Ronen et al., 1996). More recently, Speidel et al. (2006) showed that low levels of UV-irradiation, which led to a relatively low-level activation of p53, induced temporary cell cycle arrest, whereas high levels of UV-irradiation, which induced a more robust activation of p53, led to apoptosis. Although our results are consistent with the cell culture data suggesting that low levels of p53 activation culminate in cell cycle arrest whereas more robust activation of p53 results in apoptosis, additional research will be required to determine whether the sensitivity/resistance of specific cells to teratogen induced apoptosis in the day 9 mouse embryo is determined by the extent to which p53 is activated.

3. GENE AND MIRNA EXPRESSION IN P53-DEFICIENT DAY 8.5 MOUSE EMBRYOS*

3.1. OVERVIEW

NTDs are one of the most common human birth defects in the United States. In animal studies, deletion of *p53* leads to a significant increase in embryos that exhibit exencephaly. To study the molecular-level alternations in *p53*-deficient embryos, we identified genes and miRNAs whose expressions were modified by deletion of *p53* in day 8.5 mouse embryos. Mouse embryos from *p53* heterozygous crosses were collected, genotyped, and embryos of similar genotype (+/+; +/-; -/-) were pooled. RNA from the pooled samples was isolated to determine mRNA and miRNA expression levels using Whole Genome Bioarrays and Low Density Arrays, respectively. In *p53* -/- embryos, 388 genes showed statistically significant alteration in gene expression of more than 2 fold compared to *p53* +/+ embryos. Expression of *p53* and p53 target genes, such as *p21* and *cyclin G1,* were significantly down-regulated in *p53* -/- embryos. In contrast, expression of other p53 target genes, *Mdm2, Noxa*, and *Puma,* were unchanged. We have also shown that 6 genes, known to cause NTDs when deleted, were down-regulated in *p53* -/- embryos. Finally, 5 miRNAs also showed statistically significant alterations in expression levels in *p53* -/- embryos compared to *p53* +/+ embryos. Combined analysis of the experimental data and two publicly available algorithms identified putative target genes of these miRNAs. Our data have identified genes and miRNAs that may be involved in the mechanisms underlining NTDs and begin to define the developmental role of p53 in the etiology of NTDs.

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*Reprinted from "Gene and miRNA Expression in p53-Deficient Day 8.5 Mouse Embryos" by Hiromi Hosako, Gail S. Martin, Marianne Barrier, Yian A. Chen, Ivan V. Ivanov, and Philip E. Mirkes. *Birth Defect Research A*. Accepted.

3.2. INTRODUCTION

NTDs represent a group of serious congenital malformations resulting from failure of neural tube closure during early development and are one of the most common human birth defects, with a prevalence of approximately 1 in 1000 live births in the United States (Copp et al., 2003). Of these, the most common NTDs are anencephaly/exencephaly and spina bifida resulting from the failure of neural tube closure in the cranial and spinal region, respectively. The etiology of NTDs is complex and is known to involve both genetic and environmental factors. In humans, genetic factors such as chromosomal abnormalities and single-gene disorders, including Fraser syndrome and Waardenburg syndrome, are associated with NTDs (Padmanabhan, 2006). Although no single gene mutation has been shown to be solely responsible for human NTDs, animal studies have identified more than 190 genes that cause NTDs when deleted (Harris and Juriloff, 2007). Examples of environmental factors that contribute to NTDs include geographic and temporal variations as well as physical and chemical agents, such as x-irradiation, hyperthermia, antiepileptic drugs, thalidomide, and folate antagonists (Mitchell, 2005; Padmanabhan, 2006). In addition, gene-environment and gene-gene interactions are also known to play a role in the etiology of NTDs. For example, maternal folic acid supplementation interacts with the presence of gene variants directly involved in folic acid metabolism and uptake in determining the risk of NTDs (Cabrera et al., 2004). Mutation in *methylene tetrahydrofolate reductase* (*MTHFR*) 677C → T, which plays a critical role in homocysteine metabolism, is associated with decreased MTHFR activity, low plasma folate, and high plasma homocysteine; dietary and supplemental folate have been reported to modify the penetrance of this polymorphism (Koch et al., 1998; Shaw et al., 1998; Volcik et al., 2003).

Animal studies have also shown that the transcription factor p53 plays a role in the etiology of NTDs (Harris and Juriloff, 2007). Early studies (Armstrong et al., 1995; Sah et al., 1995) reported that although mice lacking *p53* are viable, a significant portion of *p53* -/- mice (2~6%) exhibit abnormal neural tube closure resulting in exencephaly as

well as a significant increase in mortality *in utero* or soon after birth. In addition, these studies revealed that the *p53* -/- mice exhibiting NTDs are predominantly females, which is also found in human exencephaly cases as well as in other mouse mutants, e.g., *1/Hipk2, Efna5, Marcks, Nif1,* and *xn* mutants (Harris and Juriloff, 2007; Seller, 1987, 1995). It should be noted, however, that the penetrance of exencephaly in *p53* -/- embryos is not complete.

Called "the guardian of the genome", p53 is essential for preventing inappropriate cell proliferation and maintaining genomic integrity. Activation of p53 can lead to apoptosis, cell cycle arrest, DNA repair, senescence, and differentiation following a variety of stresses (Hofseth et al., 2004; Lane, 1992; Levine et al., 2006). To be stabilized and activated, p53 undergoes either a single or series of post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, and neddylation (Harris and Levine, 2005; Xirodimas et al., 2004). Interaction of p53 with other proteins, for example, MDM2 (Lakin and Jackson, 1999) and PIN1 in the N-terminal transactivation domain of p53 (Ryan and Vousden, 2002; Zacchi et al., 2002) also influence the stabilization and activation of p53. Once activated, p53 translocates to the nucleus and activates numerous target genes that involve multiple pathways. Some of the genes activated by p53 are transcription factors, which further activate multiple genes (Levine et al., 2006). Although the list of p53 target genes now contains several hundred genes (Wei et al., 2006), only selected sets of genes are activated to achieve the desirable outcome in a specific situation. Recent studies indicated that promoter choice and cell fate may well depend on the level of activated p53 protein, indicating that different affinities of p53 exist toward different p53 response elements (Laptenko and Prives, 2006). Moreover, several binding proteins, including ASPP, BRN3, and YB1, which guide p53 to the promoters of specific genes in order to induce a particular pathway, have been discovered (Budhram-Mahadeo et al., 2006; Homer et al., 2005; Sullivan and Lu, 2007). In addition, recent studies revealed that p53 can also translocate to mitochondria, where it directly binds to anti-apoptotic proteins BCL2 and BCL-XL, and the pro-apoptotic proteins, BAX, BAK, and BAD to

activate the mitochondrial apoptotic pathway, suggesting further complexity of molecular functions of p53 (Chipuk et al., 2004; Jiang et al., 2006; Leu et al., 2004; Mihara et al., 2003).

In mouse embryos, both p53 mRNA and protein are ubiquitously expressed until mid gestation (Louis et al., 1988; Rogel et al., 1985; Schmid et al., 1991). At later organogenesis stages, however, the expression levels decrease rapidly and only specific differentiating tissues show the expression of p53. Although p53 is expressed in developing embryos, most p53 protein in embryos is relatively inactive, suggesting post-translational control of p53 activity (Gottlieb et al., 1997; Komarova et al., 1997; MacCallum et al., 1996). Weak but pronounced expression of active p53, however, is observed in the brain of day 8 - 11 embryos, suggesting that the developing brain may be most susceptible in the absence of p53 given the importance of apotosis, cell proliferation, and differentiation in the process of neural tube closure (Choi and Donehower, 1999). In addition, it is not well understood which p53 target genes are regulated and the extent to which they are activated by p53 in the developing brain.

Although it is well known that mRNA transcription and post-transcriptional processing are key determinants in the regulation of gene expression, a recently discovered class of small RNA molecules, known as microRNAs (miRNAs), have been shown to regulate gene expression in mammals, with about 800 miRNAs currently predicted to exist in humans (Bartel, 2004; Bentwich et al., 2005). These 18-24 nt RNAs bind to mRNAs and inhibit their expression either by interfering with translation or by destabilizing the target mRNAs (Meister and Tuschl, 2004; Pillai et al., 2007). Although little is known about the biological function of miRNAs in mammals, recent studies have suggested that miRNAs play important regulatory roles in developmental timing and patterning, cellular differentiation, proliferation, organogenesis, and apoptosis (Alvarez-Garcia and Miska, 2005; Ambros, 2004). In mouse embryos, miRNAs are expressed in stage- and tissue-specific manner (Mineno et al., 2006; Schulman et al., 2005). In addition, exposures that induce developmental anomalies, such as folate deficiency and sodium arsenite, are known to increase global miRNA expression in human

lymphoblastoid cells (Marsit et al., 2006). Moreover, the *miR-34* family has been recently shown to be induced by p53 and to mediate apoptosis in response to DNA damage and oncogenic stress (Bommer et al., 2007; Chang et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007).

To begin to dissect the mechanistic links between loss of *p53* and the etiology of NTDs, we set forth three objectives. First, we used DNA whole genome microarrays to compare mRNA expression profiles in day 8.5 *p53* +/+, +/-, and -/- mouse embryos. Second, we used miRNA low density arrays to compare miRNA expression profiles in day 8.5 *p53* +/+, +/-, and -/- mouse embryos. Finally, we ran a stepwise regression using our experimental data and used two computational algorithms to examine potential involvement of specific miRNAs in the alteration of mRNAs that are disregulated in *p53* -/- embryos.

3.3. MATERIALS AND METHODS

Experimental Animals: All animals were maintained in accordance with animal use protocol approved by the Institutional Animal Care and Use Committee at Texas A&M University. A pair of *p53* +/- mice (a gift of Lawrence A. Donehower, Baylor College of Medicine) with C57BL6-Tyrc-Brd background (Zheng et al., 2002) was used for breeding. Mice were kept with freely available pellet food and water at 21-23 ˚C and 12-h light/dark cycle (6:00-18:00). Virgin *p53* +/- females were cohabited with *p53* +/- males and checked the following morning for the vaginal plug. Upon detecting a plug, 12 a.m. was designated GD 0.

Embryo Collections and Genotyping PCR: Embryos were explanted and their yolk sac was separated in ice-cold Hanks' balanced salt solution on GD 8.5. Each embryo was placed in a separate PCR tube with RNAlater (Ambion, Austin, TX) and stored at -20 °C. The yolk sac dissected from each embryo was put in a PCR tube with 7.5 µl of tail lysis buffer (25 mM NaOH and 0.2 mM EDTA), and heated at 95 °C for 20 min. The same volume of Tris-HCl (40 mM) was added to the tube, and 3 µl of the yolk sac sample was used for *p53* genotyping of its matching embryo. Primers used for PCR

genotyping were: *p53* forward primer (in the intron 1): 5'-GTGTGTGAAATGGTGGATGG-3', *p53* reverse primer (in the intron 3/4): 5'-AGGTGATGGCTGTGGATG-3', *puro* forward primer: 5'-ATGACCGAGTACAAGCCCAC-3', and the *puro* reverse primer: 5'-GCGTGAGGAAGAGTTCTTGC-3'. The expected length of each band was 1147 bp for *p53* and 166 bp for *puro* gene. The PCR program consisted of one cycle of 2 min at 95 ˚C, 40 cycles of 40 s at 95 ˚C, 40 s at 65 ˚C, and 60 s at 72 ˚C, followed by one cycle of 7 min at 72 ˚C. Samples were visualized on a 1.5% agarose electrophoresis gel after staining with ethidium bromide.

Total RNA Preparation: Based on the PCR results, 3-7 embryos of similar genotype from multiple litters were pooled in RNAlater to obtain quadruplicate samples of each of three genotypes. Total RNA was extracted from each sample using mirVana™ miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. Concentration and quality of the total RNA were assessed using a Nanodrop (Nanodrop, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), respectively. The RNA samples were stored at -80 ˚C until analyzed.

Codelink Mouse Whole Genome Bioarray: Microarray hybridization and scanning were performed by the Genomics Core Facility of the Center for Environmental and Rural Health at Texas A&M University. Quadruplicate total RNA samples of each genotype were used to generate biotin-labeled cRNA via a modified Eberwine RNA amplification protocol. Labeled cRNA was applied to the CodeLink Mouse Whole Genome Bioarray, which contains 34,967 unique probe sequences, for 18 hr (GE Healthcare, Waukesha, WI). After incubation, the slide was washed, stained, and scanned. Array images were processed using CodeLink system software. Raw CodeLink data output were imported into GeneSpring GX 7.3. (Agilent Technologies) and normalized by setting all measurements <0.01 to 0.01, normalized to the 50th percentile of all measurements on each array, and to the median of all measurements on each gene. The twelve data sets were then assigned to three genotypes. To remove unreliable measurements, quality control on gene level was performed for 1) data

identified as present or marginal in at least 6 of the 12 samples, 2) no unknown flag designation in more than 4 of the 12 samples, 3) signal intensity greater than background in at least 4 of the 12 samples, and 4) control signal in at least 3 in 4 samples per genotype group greater than the ratio of the fixed error to proportional error for that genotype group. Lists of genes that showed alterations in expression in *p53* +/- and -/- embryos compared to *p53* +/+ embryos were created using fold change filters (x>2 fold and 2>x>1.5 fold). To identify statistically altered genes in *p53* +/- and -/- embryos compared to *p53* +/+ embryos, a one-way Welch t-test ($p<0.05$) was used. Gene annotations were acquired using the accession numbers provided with the arrays and the GeneSpider function in GeneSpring. Our microarray data have been deposited in Gene Expression Omnibus (GEO), with the accession number GE11321. Genes with *p53* consensus DNA binding sites in the list of genes with significant alteration were identified according to the directory catalogue of putative p53 DNA-binding elements in the laboratory of Statistical Genetics at Rockefeller University (Hoh et al., 2002).

Quantitative Real-Time PCR: To confirm changes in the gene expression detected by microarray analysis, real-time PCR was performed. Of the quadruplicate total RNA samples used for microarray, three replicates of each genotype were used. Total RNA (1-10 μg) was reverse-transcribed using 25 ng/μl oligo$(dT)_{15}$ primer and 9.6 μM random hexamers by heating at 65 °C for 5 min, followed by cooling on ice. The mRNA was then copied into cDNA by 300 U SuperScript II RNase H^- reverse transcriptase, 1st strand buffer, 10 mM DTT (Invitrogen, Carlsbad, CA), and 30 U RNase block (Qiagen, Valencia, CA) and incubated at 37 °C for 1 hr. The reaction was terminated by placing at 90 °C for 5 min and then brought to 4 °C and stored at -80 °C until ready to use. Subsequently, 1 μl cDNA was added to 96-well fast plate with master mix (Applied Biosystems, Foster City, CA) and TaqMan probe to carry out fluorogenic 5' nuclease assays (TaqMan) using 7500 Fast Real-Time PCR System (Applied Biosystems). Pre-designed probes of the selected genes were purchased from Applied Biosystems, and relative quantification was performed using ΔΔCt method.

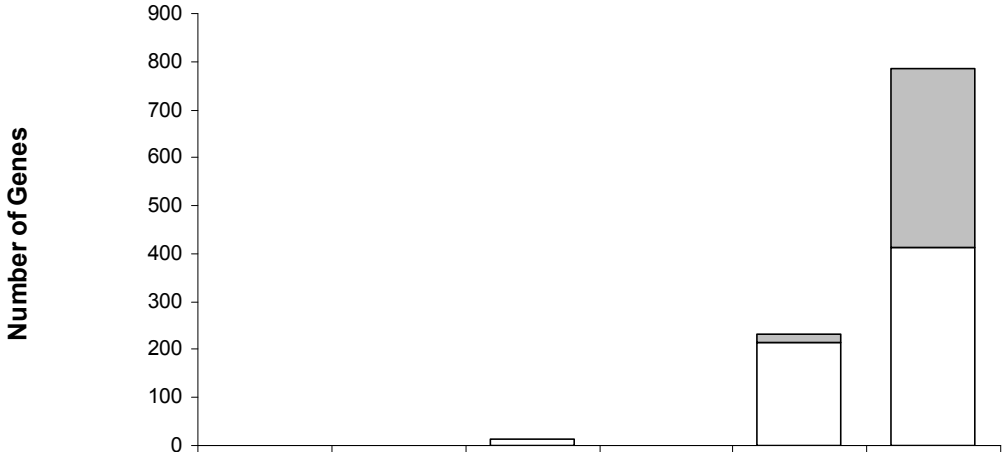
miRNA TaqMan® Low Density Array (LDA): Of the quadruplicate total RNA samples used for microarray, three replicates of *p53* +/+ and -/- samples were used. Total RNA (50 ng) was reverse-transcribed using TaqMan MicroRNA RT kit and Multiplex RT human primer pool (Applied Biosystems) according to the manufacturer's instructions. Diluted cDNAs (62.5 fold) were added to master mix (Applied Biosystems) and applied to miRNA TaqMan LDA human panels (Applied Biosystems) for the simultaneous quantification of 368 miRNAs. miRNA TaqMan assays were carried out using 7900 HT Fast Real-Time PCR System (Applied Biosystems). Relative quantification was performed using ΔΔCt method, and the data were normalized using a miRNA with the least changes between samples. Statistical analysis of the alterations of miRNA expression between *p53* +/+ and -/- samples was performed using t-test ($p<0.05$).

Putative miRNA Target Gene Analysis: To examine potential involvement of specific miRNAs in the alteration of mRNAs that are disregulated in *p53* -/- embryos, significantly down-regulated or up-regulated miRNAs in *p53* -/- embryos compared to *p53* +/+ embryos were first identified based on the experimental data. Potential targets of the selected miRNAs were predicted by two publicly available computational techniques, PicTar (http://pictar.bio.nyu.edu/) and miRBase (http://microrna.sanger.ac.uk/sequences/). These two algorithms use independent computational approaches to identify common targets of miRNAs. PicTar uses experimental results to define probabilities for a mRNA sequence to be a binding site for a given miRNA and requires that the free energy of the entire miRNA:mRNA duplex be below a cutoff value (Krek et al., 2005), whereas miRBase scans sequence database against 3'-untranslated regions predicted from all available species in Ensembl (Griffiths-Jones et al., 2006). We adopted a conservative approach and selected the common predicted targets detected by both algorithms. To compare these predicted targets for the possible interactions that could be specific to our study, we performed a stepwise regression using our experimental mRNA and miRNA data to determine which miRNA or genotype might be responsible for the regulation of the significantly

expressed mRNAs ($p<0.05$). Finally, the predicted target genes identified by both computational methods were compared to the sets of genes identified by our regression model.

3.4. RESULTS

Differentially Expressed Genes Identified by Codelink Bioarray: To examine the expression patterns and identify genes that are expressed differentially among *p53* +/+, +/-, and -/- day 8.5 mouse embryos, microarray gene profiling was used. Data from four independent experiments show that deletion of both *p53* alleles results in a significant alteration of embryonic gene expression, whereas deletion of only one *p53* allele leads to altered expression of only a few genes. Figure 3.1 shows the number of genes with statistically significant changes in expression in *p53* +/- and -/- compared to *p53* +/+ day 8.5 mouse embryos. At the 2-fold cut off, only 2 genes showed a significant increase in gene expression and no gene showed a significant decrease in gene expression in *p53* +/- compared to *p53* +/+ embryos. In *p53* -/- embryos, however, 17 genes showed increased gene expression and 371 showed decreased gene expression compared to *p53* +/+ embryos. When the cut off fold change is decreased to 1.5-fold, a similar trend is observed: only 14 genes showed significant alteration in *p53* +/- embryos, whereas in *p53* -/- embryos 232 genes showed a significant increase in gene expression and 785 genes showed a significant decrease in gene expression. These data show that a significant number of genes are altered when both *p53* alleles are deleted, and among those altered genes at the 2-fold cut off, the majority (77%) are down-regulated in the absence of *p53*.



	p53 +/+ Increased	p53 +/+ Decreased	p53 +/- Increased	p53 +/- Decreased	p53 -/- Increased	p53 -/- Decreased
2<x fold	0	0	2	0	17	371
1.5<x<2 fold	0	0	12	0	215	414

Figure 3.1. p53-regulated gene expression in day 8.5 mouse embryos. Number of genes showing statistically significant increase or decrease in gene expression more than 1.5 fold in deletion of *p53* allele was identified by microarray. Genes with 1.5<x<2 fold change are shown in grey and 2<x fold change are shown in white.

Table 3.1 lists 17 genes that showed increased gene expression of more than 2-fold in *p53* -/- embryos. Supplemental Table 3.2 shows 371 genes with more than 2-fold decrease in gene expression in *p53* -/- embryos. To examine how many of these genes whose expression levels were altered in *p53* -/- embryos are possibly up- or down-regulated directly by p53, we attempted to identify genes that contain putative p53 DNA-binding sites. These genes are shown in Tables 3.1 and 3.2. Out of 388 genes, 43 genes were identified as possible p53 target genes. Thus, about 11% of the disregulated genes could be directly regulated by p53, whereas most of the genes altered in *p53* -/- embryos are indirectly affected by p53.

Table 3.1
Genes Whose Expression Was Increased in *p53* -/- day 8.5 Embryos by More Than Two Fold*

Symbol	Description	Fold change	Putative site(s)
Pp11r	Placental protein 11 related	5.5	
Ezh1	Enhancer of zeste homolog 1 (Drosophila)	3.93	
AI852444	Expressed sequence AI852444	3.64	
2210019E14Rik	RIKEN cDNA 2210019E14 gene	3.59	
Plxna1	Plexin A1	3.44	
Ccr9	Chemokine (C-C motif) receptor 9	2.53	
Baiap1	BAI1-associated protein 1	2.4	
Rock1	Rho-associated coiled-coil forming kinase 1	2.24	X
	0 day neonate head cDNA, RIKEN full-length enriched library, clone:4833411N21 product:unclassifiable, full insert sequence	2.23	
Rbm16	RNA binding motif protein 16	2.14	
Grip1	Glutamate receptor interacting protein 1	2.07	
3110001N23Rik	RIKEN cDNA 3110001N23 gene	2.07	
Ssbp3	Single-stranded DNA binding protein 3	2.06	
	Adult male corpora quadrigemina cDNA, RIKEN full-length enriched library, clone:B230209D03 product:unknown EST, full insert sequence	2.06	
Gnas	RIKEN cDNA A930027G11 gene	2.06	
Laf4	Hypothetical protein A730046J16	2.05	
Adcyap1r1	Adenylate cyclase activating polypeptide 1 receptor 1	2.01	

* Significant difference between day 8.5 *p53* +/+ embryos and *p53* -/- embryos. $p<0.05$.

Table 3.2
Genes Whose Expression Was Decreased in *p53* -/- day 8.5 Embryos by More Than Two Fold*

Symbol	Description	Fold change	Putative site(s)
Trp53bp1	Transformation related protein 53 binding protein 1	23.50	
Pabpc4	Poly(A)-binding protein, cytoplasmic pseudogene	11.39	
Dnmt1	DNA methyltransferase (cytosine-5) 1	10.43	X
Ddx41	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41	8.68	
4933407C03Rik	RIKEN cDNA 4933407C03 gene	8.02	
Bcar1	Breast cancer anti-estrogen resistance 1	7.46	
Trim35	Tripartite motif-containing 35	7.18	
Pkm2	Pyruvate kinase, muscle	7.14	
Fyn	Fyn proto-oncogene	6.89	X
Csk	C-src tyrosine kinase	6.64	X
Pex5	Peroxisome biogenesis factor 5	6.26	
Mcm7	Minichromosome maintenance deficient 7 (S. cerevisiae)	6.15	X
Sars2	Seryl-aminoacyl-tRNA synthetase 2	6.11	
Afg3l1	AFG3(ATPase family gene 3)-like 1 (yeast)	5.34	
Krt2-8	Keratin complex 2, basic, gene 8	5.27	
Mrc2	Mannose receptor, C type 2	5.14	
Tgfbi	Transforming growth factor, beta induced	4.98	X
Zfp162	Zinc finger protein 162	4.94	
Eef2	Eukaryotic translation elongation factor 2	4.92	
B930069K15Rik	RIKEN cDNA B930069K15 gene	4.89	
Sfrs2	Splicing factor, arginine/serine-rich 2 (SC-35)	4.84	
Tcfap4	Transcription factor AP4	4.82	
Sgpl1	Sphingosine phosphate lyase 1	4.81	
H2afx	H2A histone family, member X	4.77	
Prkaca	Protein kinase, cAMP dependent, catalytic, alpha	4.53	X
Hdac7a	Histone deacetylase 7A	4.47	X
Sec61a1	Sec61 alpha 1 subunit (S. cerevisiae)	4.40	
Ddx6	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	4.35	
Pard3	Par-3 (partitioning defective 3) homolog (C. elegans)	4.29	
Rarg	Retinoic acid receptor, gamma	4.18	X
Jarid1a	RIKEN cDNA D230014I24 gene	4.12	
Cdkn1a	*Cyclin-dependent kinase inhibitor 1A (P21)*	4.06	X
Punc	Putative neuronal cell adhesion molecule	4.06	
Pard6g	Par-6 partitioning defective 6 homolog gamma (C. elegans)	4.03	
1700081L11Rik	RIKEN cDNA D030002E05 gene	4.01	
Sall4	Sal-like 4 (Drosophila)	4.01	
Zfp336	Zinc finger protein 336	3.99	
Phgdh	3-phosphoglycerate dehydrogenase	3.98	
F11r	F11 receptor	3.96	
Mapkapk2	RIKEN cDNA 8030443L12 gene	3.96	X
1500041I23Rik	RIKEN cDNA 1500041I23 gene	3.95	
Sfrs2ip	Splicing factor, arginine/serine-rich 2, interacting protein	3.92	
Kif3a	Kinesin family member 3A	3.89	
Tnrc11	Trinucleotide repeat containing 11 (THR-associated protein)	3.87	
Bop1	Block of proliferation 1	3.87	
Itpkb	Inositol 1,4,5-trisphosphate 3-kinase B	3.84	
Wasl	Wiskott-Aldrich syndrome-like (human)	3.80	
C030025P15Rik	RIKEN cDNA C030025P15 gene	3.78	
Herc1	Hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 1	3.78	
5730555F13Rik	RIKEN cDNA 5730555F13 gene	3.77	
Matr3	Matrin 3	3.76	

Table 3.2 Continued

Symbol	Description	Fold change	Putative site(s)
Copeb	Core promoter element binding protein	3.75	X
C130096N06Rik	RIKEN cDNA D130005J21 gene	3.71	
Jarid1a	RIKEN cDNA D230014I24 gene	3.70	
Rbm28	RNA binding motif protein 28	3.68	
Polr3d	Polymerase (RNA) III (DNA directed) polypeptide D	3.65	
Prp19	PRP19/PSO4 homolog (S. cerevisiae)	3.63	
BC065123	CDNA sequence BC065123	3.62	
Nsf	N-ethylmaleimide sensitive fusion protein	3.61	
Brd1	Similar to bromodomain containing protein 1; BR140-like gene	3.60	
Cyp20a1	Cytochrome P450, family 20, subfamily A, polypeptide 1	3.59	
Dyrk3	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3	3.59	
Actn4	Actinin alpha 4	3.57	
Mogat2	Monoacylglycerol O-acyltransferase 2	3.55	
Nol5	Nucleolar protein 5	3.54	
Hyou1	Hypoxia up-regulated 1	3.53	
Lims1	LIM and senescent cell antigen-like domains 1	3.50	
Mrps5	Mitochondrial ribosomal protein S5	3.49	
Fgd1	FYVE, RhoGEF and PH domain containing 1	3.48	
Swap70	SWAP complex protein	3.48	X
Trp53	Transformation related protein 53	3.47	X
Akt2	Thymoma viral proto-oncogene 2	3.46	
Tyro3	TYRO3 protein tyrosine kinase 3	3.45	X
	BY671610 RIKEN full-length enriched, 14.5 days embryo df/df Rathke's pouches Mus musculus cDNA clone K820020G11 3', mRNA sequence.	3.44	
Prss8	Protease, serine, 8 (prostasin)	3.43	
Csnk1a1	Casein kinase 1, alpha 1	3.41	
Lgtn	Ligatin	3.41	
Bbx	Bobby sox homolog (Drosophila)	3.40	
Ttn	Titin	3.39	X
A730098D12Rik	RIKEN cDNA A730098D12 gene	3.38	
Galnt2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2	3.37	
P4ha2	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha II polypeptide	3.36	
Ube2v1	Ubiquitin-conjugating enzyme E2 variant 1	3.35	
A430106J12Rik	RIKEN cDNA A430106J12 gene	3.32	
Ptpn11	Protein tyrosine phosphatase, non-receptor type 11	3.30	X
Scpep1	Serine caroboxypeptidase 1	3.29	
Sfrs2ip	Splicing factor, arginine/serine-rich 2, interacting protein	3.29	
Acin1	Apoptotic chromatin condensation inducer 1	3.26	
Mtdh	Metadherin	3.25	
	Gene model 293, (NCBI)	3.25	
Nfe2l1	Nuclear factor, erythroid derived 2,-like 1	3.24	
Fstl1	Follistatin-like 1	3.22	
Popdc2	Popeye domain containing 2	3.22	
Elmo2	Engulfment and cell motility 2, ced-12 homolog (C. elegans)	3.21	
2610019N13Rik	RIKEN cDNA 2610019N13 gene	3.21	
	AU016710 Mouse two-cell stage embryo cDNA Mus musculus cDNA clone J0728F07 3', mRNA sequence.	3.15	
Smc6l1	SMC6 structural maintenance of chromosomes 6-like 1 (yeast)	3.10	
AW610627	Expressed sequence AW610627	3.09	
Ywhag	3-monooxgenase/tryptophan 5-monooxgenase activation protein, gamma polypeptide	3.09	
Arfgap1	ADP-ribosylation factor GTPase activating protein 1	3.04	

Table 3.2 Continued

Symbol	Description	Fold change	Putative site(s)
C730036B14Rik	RIKEN cDNA C730036B14 gene	3.03	
Nolc1	Nucleolar and coiled-body phosphoprotein 1	3.03	
Vgll4	RIKEN cDNA A530060M17 gene	3.02	
Txndc7	Thioredoxin domain containing 7	3.02	
Falz	Fetal Alzheimer antigen	3.01	
Mbtps1	Membrane-bound transcription factor protease, site 1	3.01	
Eif5a	Eukaryotic translation initiation factor 5A	3.01	
Ephb4	Eph receptor B4	3.00	X
Ppp1r8	Protein phosphatase 1, regulatory (inhibitor) subunit 8	2.96	
1500031N24Rik	RIKEN cDNA 1500031N24 gene	2.95	
Dpagt1	Dolichyl-phosphate (UDP-N-acetylglucosamine) acetylglucosaminephosphotransferase 1 (GlcNAc-1-P transferase)	2.95	
Ptprf	Protein tyrosine phosphatase, receptor type, F	2.94	
Skb1	SKB1 homolog (S. pombe)	2.93	
Tceb3bp1	Transcription elongation factor B polypeptide 3 binding protein 1	2.93	
Cct4	Chaperonin subunit 4 (delta)	2.92	
Itga3	Integrin alpha 3	2.91	X
Hes3	Hairy and enhancer of split 3 (Drosophila)	2.88	
6330545A04Rik	RIKEN cDNA 6330545A04 gene	2.87	
Csnk1g3	Casein kinase 1, gamma 3	2.87	
Tnk2	Tyrosine kinase, non-receptor, 2	2.85	
Nrip1	Nuclear receptor interacting protein 1	2.84	X
Rps6ka1	Ribosomal protein S6 kinase polypeptide 1	2.82	X
Zfp292	Zinc finger protein 292	2.81	
2410003B16Rik	RIKEN cDNA 2410003B16 gene	2.81	
Ccng1	Cyclin G1	2.78	X
Ahcyl1	S-adenosylhomocysteine hydrolase-like 1	2.78	
4632412I24Rik	RIKEN cDNA 4632412I24 gene	2.78	
Skiip	SKI interacting protein	2.78	
2810047L02Rik	RIKEN cDNA 2810047L02 gene	2.77	
	Transcribed locus	2.77	
Sbno1	Sno, strawberry notch homolog 1 (Drosophila)	2.77	
Rxrb	Retinoid X receptor beta	2.77	X
	Transcribed locus	2.76	
C78915	Expressed sequence C78915	2.75	
Dhx29	DEAH (Asp-Glu-Ala-His) box polypeptide 29	2.75	
2700069A02Rik	Echinoderm microtubule associated protein like 5	2.74	
Pdpk1	3-phosphoinositide dependent protein kinase-1	2.74	X
Rab11b	RAB11B, member RAS oncogene family	2.74	X
Sumo2	SMT3 suppressor of mif two 3 homolog 2 (yeast)	2.73	
Col2a1	Procollagen, type II, alpha 1	2.72	X
Pim1	Proviral integration site 1	2.70	X
Klf3	Kruppel-like factor 3 (basic)	2.70	
Mtdh	Metadherin	2.70	
Atp7a	ATPase, Cu++ transporting, alpha polypeptide	2.69	
Eif4ebp2	Eukaryotic translation initiation factor 4E binding protein 2	2.68	
Btbd7	BTB (POZ) domain containing 7	2.68	
E430034L04Rik	RIKEN cDNA E430034L04 gene	2.67	
Tle3	Transducin-like enhancer of split 3, homolog of Drosophila E(spl)	2.67	X
Canx	Calnexin	2.67	
2310061F22Rik	RIKEN cDNA 2310061F22 gene	2.66	
Itm2c	Integral membrane protein 2C	2.66	
Zfp219	Zinc finger protein 219	2.66	
Dhcr24	24-dehydrocholesterol reductase	2.65	

Table 3.2 Continued

Symbol	Description	Fold change	Putative site(s)
Akt1s1	AKT1 substrate 1 (proline-rich)	2.64	
E430012M05Rik	RIKEN cDNA E430012M05 gene	2.64	
1500010G04Rik	RIKEN cDNA 1500010G04 gene	2.63	
8430413D17Rik	RIKEN cDNA 8430413D17 gene	2.62	
Dpysl3	Dihydropyrimidinase-like 3	2.61	
Fpgs	Folylpolyglutamyl synthetase	2.61	
Mns1	Meiosis-specific nuclear structural protein 1	2.61	
Pb1	RIKEN cDNA 2310032M22 gene	2.59	
Sertad2	SERTA domain containing 2	2.59	
Exoc7	Exocyst complex component 7	2.57	
Twistnb	TWIST neighbor	2.55	
6330406L22Rik	RIKEN cDNA 6330406L22 gene	2.55	
Ddah1	Dimethylarginine dimethylaminohydrolase 1	2.54	
Pcyt2	Phosphate cytidylyltransferase 2, ethanolamine	2.54	
Gcs1	Glucosidase 1	2.54	
Jarid2	Jumonji, AT rich interactive domain 2	2.53	
Eif4g1	Eukaryotic translation initiation factor 4, gamma 1	2.53	
Purb	Purine rich element binding protein B	2.53	
5830445C04Rik	RIKEN cDNA 5830445C04 gene	2.53	
	L0029F11-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0029F11 3', mRNA sequence.	2.53	
Gpd2	Glycerol phosphate dehydrogenase 2, mitochondrial	2.53	
Cldn5	Claudin 5	2.52	
Styx	Phosphoserine/threonine/tyrosine interaction protein	2.52	
Hgs	HGF-regulated tyrosine kinase substrate	2.51	
Spg7	Spastic paraplegia 7 homolog (human)	2.51	
Hmg20b	High mobility group 20 B	2.50	
2610034N03Rik	RIKEN cDNA 2610034N03 gene	2.49	
AA960436	Expressed sequence AA960436	2.49	
5830417C01Rik	RIKEN cDNA 5830417C01 gene	2.49	
Sympk	RIKEN cDNA 1500016F02 gene	2.47	
Smad4	MAD homolog 4 (Drosophila)	2.47	
E330018D03Rik	RIKEN cDNA E330018D03 gene	2.47	
Add1	Adducin 1 (alpha)	2.47	X
0610030E20Rik	RIKEN cDNA 0610030E20 gene	2.46	
Fads2	Fatty acid desaturase 2	2.45	
Efnb1	Ephrin B1	2.44	X
4930470D19Rik	RIKEN cDNA 4930470D19 gene	2.44	
B230219D22Rik	RIKEN cDNA B230219D22 gene	2.44	
Gna13	Guanine nucleotide binding protein, alpha 13	2.44	X
2810474O19Rik	RIKEN cDNA 2810474O19 gene	2.43	
Il1r2	Interleukin 1 receptor, type II	2.43	X
BC013667	CDNA sequence BC013667	2.43	
Mcart1	Mitochondrial carrier triple repeat 1	2.42	
Ptbp2	Polypyrimidine tract binding protein 2	2.42	
Pias4	Protein inhibitor of activated STAT, 4	2.42	
6430510M02Rik	RIKEN cDNA 6430510M02 gene	2.42	
	Transcribed locus	2.42	
Tmem2	Transmembrane protein 2	2.42	
4921504I05Rik	RIKEN cDNA 4921504I05 gene	2.41	
Srprb	Signal recognition particle receptor, B subunit	2.41	
A230098A12Rik	RIKEN cDNA A230098A12 gene	2.41	
Lztr1	Leucine-zipper-like transcriptional regulator, 1	2.41	
Mbtd1	Mbt domain containing 1	2.40	

Table 3.2 Continued

Symbol	Description	Fold change	Putative site(s)
Nxn	RIKEN cDNA C230071I02 gene	2.40	
Camkk2	Calcium/calmodulin-dependent protein kinase kinase 2, beta	2.40	
Ppp5c	Protein phosphatase 5, catalytic subunit	2.39	
	LL2in12223T7 Hematopoietic Stem Cell Subtracted Library Mus musculus cDNA 5', mRNA sequence.	2.39	
Tcfe3	Transcription factor E3	2.38	
Dek	DEK oncogene (DNA binding)	2.38	
Htatip	HIV-1 tat interactive protein, homolog (human)	2.38	
Prkcbp1	Protein kinase C binding protein 1	2.38	
1500005K14Rik	RIKEN cDNA 1500005K14 gene	2.37	
C330016O16Rik	RIKEN cDNA C330016O16 gene	2.37	
Stat3	Signal transducer and activator of transcription 3	2.37	X
Trio	RIKEN cDNA 6720464I07 gene	2.37	X
Rai1	Retinoic acid induced 1	2.37	
Elp3	Elongation protein 3 homolog (S. cerevisiae)	2.36	
BC035954	CDNA sequence BC035954	2.36	
Dnpep	Aspartyl aminopeptidase	2.35	
4833418A01Rik	RIKEN cDNA 4833418A01 gene	2.35	
Bat9	HLA-B-associated transcript 9	2.35	
Golgb1	Golgi autoantigen, golgin subfamily b, macrogolgin 1	2.34	
Ablim1	Actin-binding LIM protein 1	2.33	
Ube2d3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	2.33	
Rnpc2	RIKEN cDNA B330012G18 gene	2.33	
Ttc3	Tetratricopeptide repeat domain 3	2.33	
Fbxw7	F-box and WD-40 domain protein 7, archipelago homolog (Drosophila)	2.32	
Suhw4	Suppressor of hairy wing homolog 4 (Drosophila)	2.32	
Kif5b	Kinesin family member 5B	2.32	
Wdhd1	WD repeat and HMG-box DNA binding protein 1	2.32	
Tbx1	T-box 1	2.31	
Snx27	Sorting nexin family member 27	2.31	
2810403A07Rik	RIKEN cDNA 2810403A07 gene	2.31	
Zfp369	Zinc finger protein 369	2.31	
Brd2	Bromodomain containing 2	2.30	
C130052G03Rik	RIKEN cDNA C130052G03 gene	2.30	
Etv5	Ets variant gene 5	2.30	
Neo1	Neogenin	2.29	X
Rad23a	RAD23a homolog (S. cerevisiae)	2.29	
5730478M09Rik	RIKEN cDNA 5730478M09 gene	2.28	
BC024322	CDNA sequence BC024322	2.28	
Lzf	Leucine zipper domain protein	2.28	
Ppap2b	Phosphatidic acid phosphatase type 2B	2.28	
Pik3r2	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2 (p85 beta)	2.28	
Crry	Complement receptor related protein	2.28	
Ppil2	Peptidylprolyl isomerase (cyclophilin)-like 2	2.27	
3300001P08Rik	RIKEN cDNA 3300001P08 gene	2.27	
Ank3	Ankyrin 3, epithelial	2.27	X
Baz2a	Bromodomain adjacent to zinc finger domain, 2A	2.26	
Ube2e3	Ubiquitin-conjugating enzyme E2E 3, UBC4/5 homolog (yeast)	2.26	
Cd34	CD34 antigen	2.26	X
Hoxb9	Homeo box B9	2.25	
Igf2bp1	Insulin-like growth factor 2, binding protein 1	2.25	
Peli1	Pellino 1	2.25	
Rgl2	Ral guanine nucleotide dissociation stimulator-like 2	2.25	

Table 3.2 Continued

Symbol	Description	Fold change	Putative site(s)
BC025600	CDNA sequence BC025600	2.25	
Dvl1	Dishevelled, dsh homolog 1 (Drosophila)	2.25	X
AU019823	Expressed sequence AU019823	2.24	
Epb4.1l5	Erythrocyte protein band 4.1-like 5	2.24	
Hcfc1	Host cell factor C1	2.24	
2900016D05Rik	RIKEN cDNA 2900016D05 gene	2.23	
Cit	Citron	2.22	
Hnrpul1	Heterogeneous nuclear ribonucleoprotein U-like 1	2.22	
Kif2c	Kinesin family member 2C	2.22	
Acads	Acyl-Coenzyme A dehydrogenase, short chain	2.22	
Cpne3	Copine III	2.21	
4833412N02Rik	RIKEN cDNA 4833412N02 gene	2.21	
D330023I04Rik	RIKEN cDNA D330023I04 gene	2.21	
Odd1	Oxidative-stress responsive 1	2.21	
Gm1131	Gene model 1131, (NCBI)	2.20	
Pip5k1b	Phosphatidylinositol-4-phosphate 5-kinase, type 1 beta	2.20	
Ptprk	Protein tyrosine phosphatase, receptor type, K	2.20	
	H4033G10-3 NIA Mouse 7.4K cDNA Clone Set Mus musculus cDNA clone H4033G10 3', mRNA sequence.	2.19	
Abcd4	ATP-binding cassette, sub-family D (ALD), member 4	2.19	
Wnt5a	Wingless-related MMTV integration site 5A	2.19	
Ccnd3	Cyclin D3	2.19	X
Siat5	Sialyltransferase 5	2.19	
BC008163	CDNA sequence BC008163	2.18	
Srebf1	Sterol regulatory element binding factor 1	2.18	
Stat3	Signal transducer and activator of transcription 3	2.18	X
8430422M09Rik	RIKEN cDNA C130002M15 gene	2.18	
BC065120	CDNA sequence BC065120	2.18	
Fcmd	Fukuyama type congenital muscular dystrophy homolog (human)	2.18	
Mpp5	Membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5)	2.18	
	L0910B09-3 NIA Mouse Newborn Kidney cDNA Library (Long) Mus musculus cDNA clone NIA:L0910B09 IMAGE:30000980 3', mRNA sequence.	2.18	
Glcci1	Glucocorticoid induced transcript 1	2.17	
Armc1	Armadillo repeat containing 1	2.17	
Alkbh	AlkB, alkylation repair homolog (E. coli)	2.17	
	Gene model 890, (NCBI)	2.17	
Pja2	Praja 2, RING-H2 motif containing	2.16	
	Transcribed locus, weakly similar to NP_081764.1 Mus musculus 5730493B19Rik gene	2.16	
BC061253	CDNA sequence BC061253	2.16	
Picalm	Phosphatidylinositol binding clathrin assembly protein	2.16	
Aup1	Ancient ubiquitous protein	2.16	
U2af2	U2 small nuclear ribonucleoprotein auxiliary factor (U2AF) 2	2.15	
1700023B02Rik	RIKEN cDNA 1700023B02 gene	2.15	
Arf4	ADP-ribosylation factor 4	2.14	
Vamp3	Vesicle-associated membrane protein 3	2.14	
6-Sep	Septin 6	2.14	
2900073C17Rik	Mus musculus adult male hippocampus cDNA, RIKEN full-length enriched library, clone:2900073C17 product:unclassifiable, full insert sequence.	2.14	
Ahcyl1	S-adenosylhomocysteine hydrolase-like 1	2.13	
Dgat2	Diacylglycerol O-acyltransferase 2	2.13	
Ubp1	Upstream binding protein 1	2.13	

Table 3.2 Continued

Symbol	Description	Fold change	Putative site(s)
Cnot6l	CCR4-NOT transcription complex, subunit 6-like	2.12	
Wrb	Tryptophan rich basic protein	2.12	
	Transcribed locus	2.12	
Zfp313	Zinc finger protein 313	2.11	
Cdca5	Cell division cycle associated 5	2.11	
Ppig	Peptidyl-prolyl isomerase G (cyclophilin G)	2.11	
Cdh3	Cadherin 3	2.10	
Dlg5	RIKEN cDNA 1700067A10 gene	2.10	
2610318C08Rik	RIKEN cDNA 2610318C08 gene	2.10	
Hmga2	High mobility group AT-hook 2	2.10	
2700029M09Rik	RIKEN cDNA 2700029M09 gene	2.10	
Gtpbp1	GTP binding protein 1	2.10	
4921506J03Rik	RIKEN cDNA 4921506J03 gene	2.10	
Cox15	COX15 homolog, cytochrome c oxidase assembly protein (yeast)	2.09	
Pknox1	Pbx/knotted 1 homeobox	2.09	X
Mak10	MAK10 homolog, amino-acid N-acetyltransferase subunit, (S. cerevisiae)	2.09	
AW011752	Expressed sequence AW011752	2.08	
Hsd17b7	Hydroxysteroid (17-beta) dehydrogenase 7	2.08	
Mll5	Myeloid/lymphoid or mixed-lineage leukemia 5	2.08	
Efna2	Ephrin A2	2.07	X
Ddx3x	Fibroblast growth factor inducible 14	2.07	
Ahcy	S-adenosylhomocysteine hydrolase	2.07	
Sas	Sarcoma amplified sequence	2.07	
Fkbp10	FK506 binding protein 10	2.07	
9030408N13Rik	RIKEN cDNA 9030408N13 gene	2.07	
2810410D24Rik	RIKEN cDNA 2810410D24 gene	2.06	
Mef2a	RIKEN cDNA A430081F14 gene	2.06	
Carf	RIKEN cDNA A530050O19 gene	2.06	
Dnajc1	RIKEN cDNA D230036H06 gene	2.06	
	Similar to Polyadenylate-binding protein 4 (Poly(A)-binding protein 4) (PABP 4) (Inducible poly(A)-binding protein) (iPABP) (Activated-platelet protein-1) (APP-1)	2.06	
Sf3a2	Splicing factor 3a, subunit 2	2.05	
Polr2a	Polymerase (RNA) II (DNA directed) polypeptide A	2.05	
1810045K07Rik	RIKEN cDNA 1810045K07 gene	2.05	
Csnk1e	Casein kinase 1, epsilon	2.05	X
Siat6	RIKEN cDNA 9530034E10 gene	2.05	
Nvl	Nuclear VCP-like	2.04	
Glipr2	GLI pathogenesis-related 2	2.04	
Hist1h3d	Histone1, H3d	2.04	
Xrn1	RIKEN cDNA A130001C09 gene	2.04	
Zswim6	Zinc finger, SWIM domain containing 6	2.04	
Pex11a	Peroxisomal biogenesis factor 11a	2.03	
1110001A05Rik	RIKEN cDNA 1110001A05 gene	2.03	
Mia	Melanoma inhibitory activity	2.03	
4631426J05Rik	RIKEN cDNA 4631426J05 gene	2.02	
AI839920	Expressed sequence AI839920	2.02	
Rabl3	RAB, member of RAS oncogene family-like 3	2.02	
Basp1	Brain abundant, membrane attached signal protein 1	2.02	
1110014K08Rik	RIKEN cDNA 1110014K08 gene	2.02	
Slc31a2	Solute carrier family 31, member 2	2.02	
Wars	RIKEN cDNA 5033424D13 gene	2.02	
C130089L09Rik	RIKEN cDNA C130089L09 gene	2.02	
Ccm1	Cerebral cavernous malformations 1	2.02	

Table 3.2 Continued

Symbol	Description	Fold change	Putative site(s)
Fmr1	Fragile X mental retardation syndrome 1 homolog	2.01	
Nfatc3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	2.01	
Ncoa6ip	Nuclear receptor coactivator 6 interacting protein	2.01	
1700024N20Rik	RIKEN cDNA 1700024N20 gene	2.01	
Cdc42ep1	CDC42 effector protein (Rho GTPase binding) 1	2.00	
Cpox	Similar to Cpo protein	2.00	

*Significant difference between day 8.5 *p53* +/+ embryos and *p53* -/- embryos. $p<0.05$.

Examination of Known p53 Target Genes and NTD-related Genes: To verify the gene expression data obtained by microarray gene profiling, selected known target genes of p53, i.e. *cyclin G1* (*ccng1*), *p21* (*cdkn1a*), *Mdm2*, *Puma (Bbc3), Noxa (Pmaip1),* and *p53 (Trp53)* itself, were independently validated using the TaqMan quantitative real-time PCR method (Figs. 3.2 and 3.3). As expected, RT-PCR validated that expression of *p53* was significantly decreased in expression in *p53* +/- and *p53* -/- embryos (Fig. 3.2). Well known p53 target genes, *cyclin G1* and *p21* were also shown to be significantly down-regulated in *p53* -/- embryos. In contrast, RT-PCR verified that other p53 target genes, *Mdm2*, *Noxa*, and *Puma,* did not show significant alteration in *p53* -/- embryos.

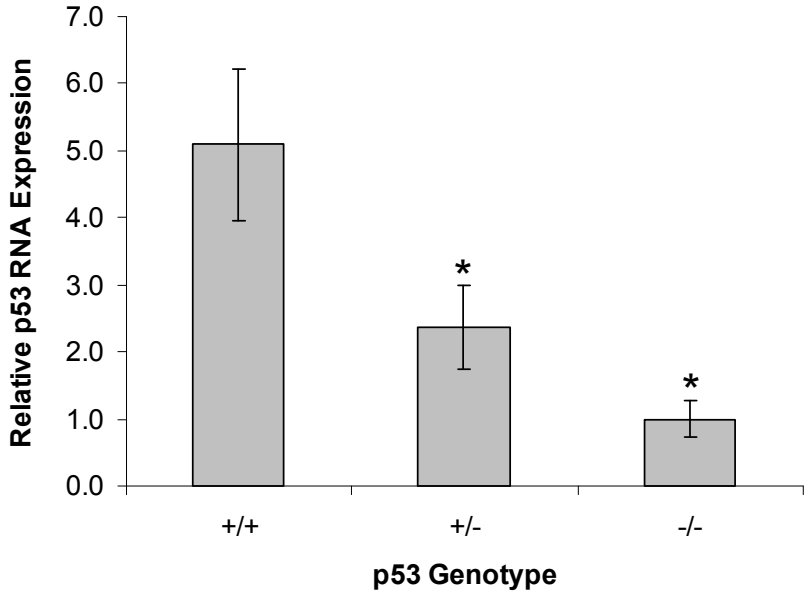


Figure 3.2. RT-PCR verification of *p53* gene expression in day 8.5 mouse embryos. Three replicates of pooled embryos were used for each *p53* genotype. * indicates a significant difference in *p53* gene expression compared to that of *p53* +/+. $p<0.05$.

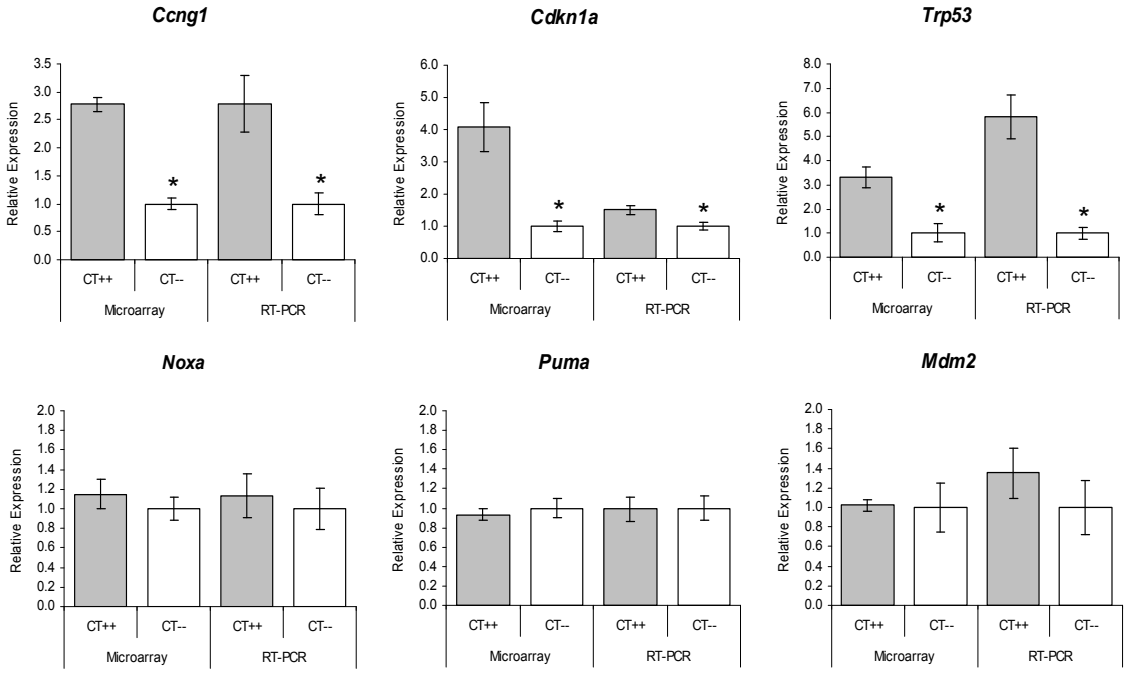


Figure 3.3. p53 and known p53 target gene expression in day 8.5 mouse embryos using microarray and RT-PCR. * indicates a significant difference in gene expression compared to that of *p53* +/+. $p<0.05$.

There are more than 190 genes, including p53, that play a role in the etiology in NTDs when deleted. To determine whether any of these genes are among the 388 genes that showed altered expression in *p53* -/- embryos compared to *p53* +/+ embryos, we searched the gene list presented in supplemental Tables 3.1 and 3.2. Our microarray data showed that the expression of six NTD-related genes, C*sk, Itga3, Jarid2, Prkaca, Rarg,* and *Sall4,* were significantly decreased in *p53* -/- embryos. Similarly, RT-PCR data also showed decreased expression of these genes; however, the results did not achieve statistical significance (Fig. 3.4).

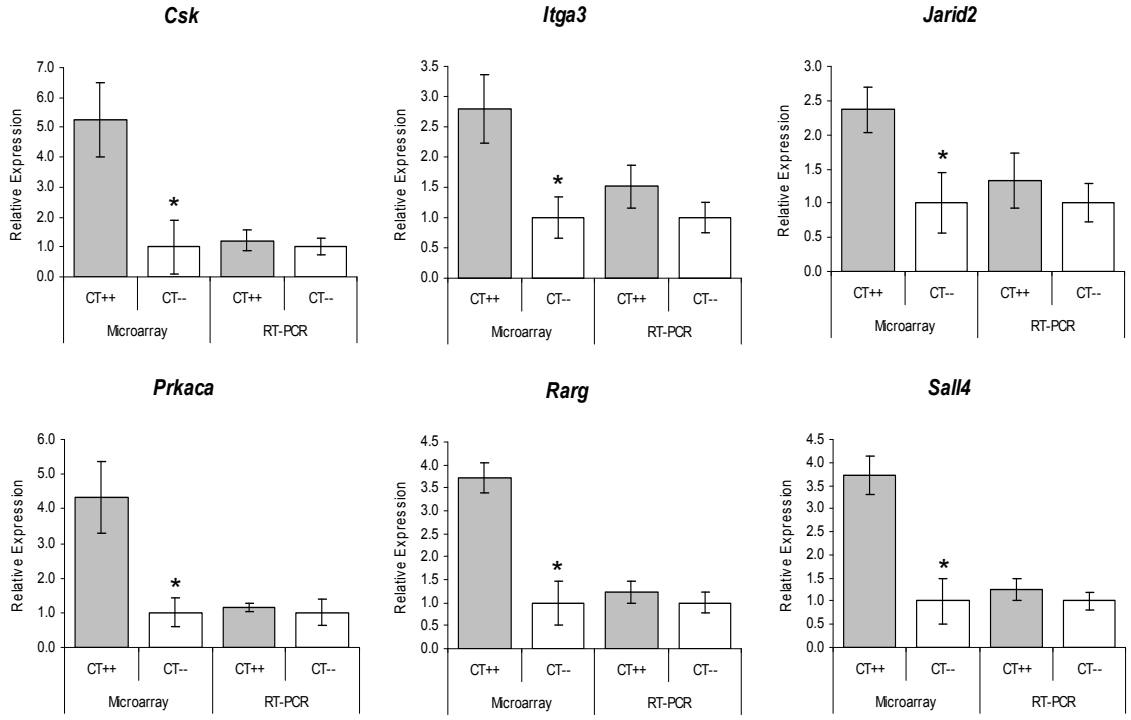


Figure 3.4. Gene expressions of six genes that are known to cause NTDs when deleted in day 8.5 mouse embryos using microarray and RT-PCR. * indicates a significant difference in gene expression compared to that of *p53* +/+. $p<0.05$.

Differentially Expressed miRNAs and Comparison of Putative and Experimental miRNA Target Genes: To compare the miRNA expression pattern in *p53* +/+ and -/- embryos, miRNA LDAs were utilized. Of the 368 miRNAs examined, 165 miRNAs were detected in day 8.5 mouse embryos, and out of these miRNAs, 111

miRNAs have mouse homologs (Table 3.3). This RT-PCR technique further identified three miRNAs, *miR-1, miR-142-3p, miR-301*, that show significantly decreased expression and two miRNAs, *miR-30e-3p* and *miR-331,* with significantly increased expression in *p53* -/- embryos compared to *p53* +/+ embryos (Table 3.4).

With the exception of miR-301, our computational target gene analysis using the predicted targets identified by the two algorithms and the experimental data obtained in this study have revealed several putative target genes of the selected miRNAs (Table 3.4). Table 3.4 lists miRNAs that showed significant alteration in *p53* -/- embryos compared to *p53* +/+ embryos, the fold change and *p* value for their alteration, as well as the predicted target genes that showed a strong correlation with these miRNAs ($p<0.05$).

Table 3.3
Detection of miRNA Expressions in Day 8.5 Embryos

	# of miRNAs Detected	# of miRNAs Not Detected	Total # of miRNAs Investigated
Mouse miRNAs	111	29	140
Total miRNAs (Human)	165	203	368

Table 3.4
miRNA Expression Changes in *p53* -/- Day 8.5 Embryos and Putative Targets of the miRNAs

Name	Fold change	*P*	Putative targets* Up-regulated	Down-regulated
miR-1	-1.53	0.040	*Fyn*	*Rev1*
miR-30e-3p	1.41	0.004	*Ssx2ip, 4930591A17Rik*	*Gnai2, Mafg, Rhebl1*
miR-142-3	-2.14	0.024	*Arntl*	
miR-301	-1.28	0.035	None	
miR-331	1.87	0.006	*Vcp, Gnb2, Tbcld10b, Pcbp4, Ppapdc3*	*Rarg*

*Genes identified by a comparison of the predicted targets identified by two algorithms and the genes identified by our regression model. $p<0.05$.

3.5. DISCUSSION

Our data show that deletion of only one *p53* allele leads to the altered expression of only a few genes. This correlates with our finding that no *p53* +/- fetuses exhibit exencephaly (section 4), suggesting that at least in the developing mouse embryo loss of one *p53* allele and the associated changes in gene expression are not sufficient to induce NTDs. Other studies, however, have shown that loss of one *p53* allele leads to increased tumorigenesis in mice and humans (Lynch and Milner, 2006; Varley et al., 1997; Venkatachalam et al., 1998, 2001). These studies and our data indicate that the reduction of *p53* dosage is sufficient for cancer promotion but not the induction of NTDs. The mechanistic links between *p53* and tumorigenesis and NTDs may also be different. These differences may relate, in part, to different requirements for p53 in the activation of different p53 target gene sets during tumorigenesis and neural tube closure.

Our data also show that deletion of both *p53* alleles results in a significant alteration of embryonic gene expression. Moreover, this altered gene expression correlates with our finding that 11% of *p53* -/- develop NTDs, i.e., exencephaly or spina bifida (data found in section 4). Among the significantly altered genes during the neural tube closure, the majority (77%) are down-regulated in the absence of *p53* and only 11% of the disregulated genes contain putative p53 DNA-binding sites. A similar study in a temperature-sensitive human lung cancer cell line has also shown that the majority of the altered genes are down-regulated in *p53*-deficient cells and less than 20% of the genes altered in p53-deficient cells are primary targets of p53 (Kannan et al., 2001), indicating that p53 activates pathways and exerts its effect through a diverse network of transcriptional changes. Thus, our data suggest that not only the loss of *p53* gene itself and the alteration of expression of p53 target genes but also alteration of "downstream" genes could well contribute to the mechanisms of NTDs.

Interestingly, in *p53* -/- embryos the expression of several p53 target genes, for example, *Mdm2, Noxa*, and *Puma*, was not altered, whereas a few p53 target genes such as *p21* and *cyclin G1* showed decreased expression, indicating that even in normal day 8.5 developing embryos *p21* and *cyclin G1* are activated and regulated by p53. This also

suggests that even in day 8.5 embryos without any stress, p53 is activated to regulate specific target genes. Published research has shown that although a high level of inactive p53 protein can be observed during embryogenesis, active p53 protein is observed in the developing nervous system (Gottlieb et al., 1997; Komarova et al., 1997; MacCallum et al., 1996). Taken together, it is possible that these cell cycle arrest related genes, *p21* and *cyclin G1*, are activated by p53 mainly in the nervous system, where cell cycle arrest is important for the control of cell numbers at the time of neural tube closure (Copp et al., 2003). In contrast, expressions of pro-apoptotic genes such as *Noxa* and *Puma* are unchanged, yet these genes are expressed in *p53* -/- embryos, suggesting that at this embryonic stage, these p53 target genes are not regulated by p53 but could be regulated by transcription factors other than p53. Our data collectively suggest that at the time of neural tube closure, p53 tightly controls its specific transcriptional target genes to exert particular pathways.

To assess the possible contribution of the altered genes to NTDs observed in *p53* -/- embryos, we identified six genes (*Csk, Itga3, Jarid2, Prkaca, Rarg, and Sall4*), known to cause NTDs when they are deleted, that were significantly down-regulated with analysis by microarray expression profiling. RT-PCR analysis to confirm the expression levels of these six genes showed similar decreases in expression; however, these differences were not statistically significant. Nonetheless, our microarray and RT-PCR analysis suggest that these six genes may contribute to the NTDs observed in *p53* -/- embryos. Additional research is necessary to determine whether any or all of these genes play a causal role in the etiology of NTDs in *p53* -/- embryos.

Other genes that could possibly contribute to NTDs in *p53* -/- embryos were selected from Tables 3.1 and 3.2, and grouped into the following categories; cell cycle-related genes, retinoid-related genes, axon guidance-related genes, and ATM-related genes. For example, our microarray analyses have identified two key cell cycle-related genes, *p21* and *cyclin G1,* that are significantly down-regulated in *p53* -/- embryos. Induction of p21 inhibits cyclin D/cyclin-dependent kinase 4 (CDK4) complex, leading to cell cycle arrest at G1 phase (el-Deiry, 1998a). The function of cyclin G1 is not well

known; however, it has been hypothesized that cyclin G1 is a key regulator of the p53-MDM2 network (Okamoto et al., 2002). In addition, it has been suggested that cyclin G1 plays roles in G2/M arrest after cellular stress (Kimura et al., 2001) given that over-expression of cyclin G sensitizes cells to apoptosis in mouse cell lines (Okamoto and Prives, 1999). We have also shown that *cyclin D3*, which promotes cell progression by forming a complex with CDKs (Sherr, 1995), showed a significant decrease in *p53* -/- embryos. Taken together, our results indicate that the lack of *p53* alleles clearly disregulates genes involved in cell cycle progression, suggesting that disregulation of cell cycle may be one of the mechanisms of NTDs observed in *p53* -/- embryos.

Similarly, we have shown that the expression of *retinoic acid receptor gamma* (*Rarg)*, *retinoic X receptor beta (Rxrb)*, and *retinoic acid induced 1* (*Rai1*) genes are also significantly decreased in *p53* -/- embryos. In addition, *retinoic acid receptor alpha* (*Rara*) also showed 1.5-fold decrease in mRNA expression (data not shown). Retinoic acid receptors (RARs) are known to bind all-trans and 9-cis retinoic acid (RA), to form heterodimers with retinoic X receptors (RXRs) and to play critical roles in embryonic development, including closure of the hindbrain and antero-posterior patterning of the somatic mesoderm and hindbrain neuroectoderm (Chambon, 1996; Leid et al., 1992; Mark et al., 2006). Although *Rara*- and *Rarg*-null mutant mice are viable, possibly reflecting the existence of functional redundancies between RARs, *Rara/g*-null embryos result in NTDs (Ang and Duester, 1997; Lohnes et al., 1994). Together, our results indicate that decreased expression of these genes may well be involved in the NTDs observed in *p53* -/- embryos.

In addition, our microarray analysis identified three key axon guidance-related genes, i.e. *ephrin A2 (Efna2), ephrin B1 (Efnb1),* and *ephrin receptor B4 (Ephb4*), that are significantly down-regulated in *p53* -/- embryos. Published studies show that the deletion of *ephrin A5 (Efna5)* or *ephrin receptor A7 (Epha7)* leads to NTDs (Holmberg et al., 2000), suggesting the important role of ephrins and ephrin receptors in the etiology of NTDs. In the nervous system of developing embryos, a variety of attractive and repulsive signals have been identified that guide axonal processes as part of their

complex migration pathways (Goldshmit et al., 2006). Ephrin receptors and their ligands are required for repulsive interactions necessary to form the anterior commissure, to select spinal cord motor neuron pathways, and to facilitate migration of neural crest cells.

Finally, our analyses have identified two key ATM-related genes, *H2AX (H2afx)* and *53BP1 (Trp53bp1),* that are significantly down-regulated in *p53* -/- embryos. *53BP1* is a p53 binding protein that binds to the central DNA-binding domain of p53 (Derbyshire et al., 2002). In response to DNA insult, ataxia telangiectasia-mutated (ATM) in mammalian cells phosphorylates the C-terminal tail of *H2AX* protein (Celeste et al., 2002; Paull et al., 2000), which then recruits BRCA1 C-terminal (BRCT) domain-containing proteins including *53BP1* (Wang et al., 2002; Ward et al., 2003). *53BP1* then recruits p53 to the site of the DNA damage for the transcriptional activation of p53 target genes. Although statistically significant down-regulation in the expression of *H2AX* or *53BP1* was observed, neither gene is a putative target gene of p53. *H2AX* and *53BP1* are clearly involved in the ATM-dependent DNA damage responses and the cellular function of p53; however, no study has shown that these genes may be direct p53 target genes or that transcription of these genes is somehow regulated by p53. Further experiments are needed to determine whether any of these cell cycle-related, retinoid-related, axon guidance-related, and ATM-related genes, in fact, play a causal role in neural tube closure.

To investigate whether miRNAs are expressed in early organogenesis-stage mouse embryos, whether the expression of miRNAs is altered by deletion of *p53* alleles, and whether these changes could contribute to NTDs observed in *p53* -/- embryos, we quantified the expression levels of 368 miRNAs. In day 8.5 mouse embryos, 111 mouse-specific miRNAs were expressed, whereas 29 miRNAs were not detected, suggesting that the expression of miRNAs is strictly controlled at specific stages of mammalian embryonic development. Our data also showed that three miRNAs, *miR-1, miR-142-3p,* and *miR-301* showed statistically significant decrease, and two miRNAs, *miR-30e-3p* and *miR-331,* showed statistically significant increase in their expression in

p53 -/- embryos compared to *p53* +/+ embryos, indicating that these miRNAs might be regulated by *p53*. Precedent for p53-mediated regulation of miRNAs comes from studies showing that transcription factors can regulate miRNA expression. For example, p53 has been shown to bind and regulate expression of *miR-34* family (Corney et al., 2007; He et al., 2007) and NFI-A can bind to the promoter of *miR-223* and regulate its transcription (Fazi et al., 2005).

The target gene analysis using both our stepwise regression and two publicly available computational tools, PicTar and miRBase, further identified putative target genes of these miRNAs that are supported by two completely independent approaches. R*etinoic acid receptor gamma* (*Rarg*), which is a known direct putative target gene of *p53* that was significantly down-regulated in *p53* -/- embryos, was also shown to be regulated by *miR-331*, which showed increased expression in *p53* -/- embryos. This indicates that *p53* may synergistically regulate *Rarg* with *miR-331*, where the deletion of *p53* alleles directly up-regulates the expression of *miR-331* and down-regulates *Rarg*, as well as the up-regulation of *miR-331* contributing further down-regulation of *Rarg*. Similarly, the increased expression of *miR-30e* was correlated with the down-regulation of its putative target genes, *guanine nucleotide binding protein alpha inhibiting 2* (*Gnai2*), *v-maf musculoaponeurotic fibrosarcoma oncogene family protein g* (*Mafg*), and *ras homolog enriched in brain like 1* (*Rhebl1*). In addition, the decreased expression of *miR-1* with the up-regulation of its putative target gene, *Fyn proto-oncogene* (*Fyn*, different RefSeq from the one seen in Table 3.2), as well as the decreased expression of *miR-142* with the increased expression of its putative target gene, *aryl hydrocarbon receptor nuclear translocator-like* (*Arntl*) were observed. These results follow the conventional understanding of miRNA function in that miRNAs negatively regulate gene expression by repressing translation or directing sequence-specific degradation of target mRNAs (Meister and Tuschl, 2004; Pillai et al., 2007). The identification of these putative target genes of the selected miRNAs using two completely independent methods, computational algorithms and the stepwise regression model of the experimental data provides strong support that these genes might be actual miRNA

target genes; however, further research is necessary to determine whether any of these target genes are directly regulated by these miRNAs.

In addition to the well-studied function of miRNAs, recent studies have revealed another potential function of miRNA in regulating gene expression. Place et al. (2008) provided data showing that *miR-373* induces *E-cadherin* and *cold-shock domain-containing protein C2* (*CSDC2*), both possessing a putative *miR-373* target site within their promoter. Furthermore, two other studies showed that synthetic dsRNAs also induce gene expression, referred to as RNA activation (RNAa) (Janowski et al., 2007; Li et al., 2006). In our analysis, decreased expression of *miR-1* was correlated with the down-regulation of its putative target gene, *REV1 homolog* (*Rev1*), as well as the increased expression of *miR-30e-3p* and *miR-331* with the up-regulation of their putative target genes, suggesting the possible induction of gene expression by these miRNAs. Further study and a better understanding of the function of miRNAs and how they regulate genes are necessary to understand the roles of miRNAs in the etiology of NTDs and development.

4. THE ROLES OF P53 AND P21 IN NORMAL DEVELOPMENT AND HYPERTHERMIA-INDUCED MALFORMATIONS

4.1. OVERVIEW

Hyperthermia (HS) is a well studied teratogen that induces serious malformations, including neural tube defects (NTDs). Our previous studies have shown that HS induces apoptosis by activating the mitochondrial apoptotic pathway. Prior to activation of the mitochondrial apoptotic pathway, HS also activates p53. Activation of p53, in turn, leads to the expression of p53 target genes, e.g., p21. In the present study, we determine whether p53 and/or p21 play a role as teratogen suppressors or inducers of HS-induced malformations. Pregnant mice carrying all three *p53* or *p21* genotype embryos were exposed to HS in a 43 °C water bath for 10 min on day 8.5. Subsequently, fetuses were collected on day 15.5 and genotyped. For p53 fetuses the sex of each fetus was also determined using DNA isolated from the accompanying yolk sac. In addition to genotype, (sex for p53 fetuses), we also determined the number of resorptions and dead fetuses as well as the number and types of external malformations. In the absence of HS exposure, fetuses exhibiting exencephaly, spina bifida, and preaxial polydactyly were observed in approximately 11% of *p53* -/- fetuses, whereas no malformations were observed among *p21* -/- fetuses. A female predominance in exencephaly and spina bifida, and a male preference in preaxial polydactyly were also observed in control *p53* fetuses. Exposure to HS resulted in an increase in exencephaly and preaxial polydactyly in fetuses of all three *p53* genotypes. In addition, the incidence of these malformations was statistically significantly higher in *p53* -/- compared to *p53* +/- and *p53* +/+ fetuses. Interestingly, no fetuses with spina bifida were observed among any of the *p53* genotypes exposed to HS. Exencephaly was the only malformation

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observed in *p21* fetuses exposed to HS, with an approximately 2-fold increase in exencephaly among *p21* +/- and a 3-fold increase among *p21* -/- compared to *p21* +/+ fetuses. Our study confirms that p53 plays a role in normal development and has shown, for the first time, that p53 and p21 function to suppress HS-induced malformations.

4.2. INTRODUCTION

Each year approximately 3% of babies born in the United States have birth defects that are life threatening, require major surgery, or present a significant disability (Shepard, 1986). One of the most common human birth defects, neural tube defects (NTDs), represent a group of serious congenital malformations resulting from failure of neural tube closure during early development, with a prevalence of approximately 1 in 1000 live births in the United States (Copp et al., 2003). Of these, the most common NTDs are anencephaly/exencephaly and spina bifida resulting from the failure of neural tube closure in the cranial and spinal region, respectively. The etiology of NTDs is complex and is known to involve both genetic and environmental factors. In humans, genetic factors, for example, chromosomal abnormalities and single-gene disorders, including Fraser syndrome and Waardenburg syndrome, are associated with NTDs (Padmanabhan, 2006). Although no single gene mutation has been shown to be solely responsible for human NTDs, animal studies have identified more than 190 genes that cause NTDs when deleted (Harris and Juriloff, 2007). Examples of environmental factors that contribute to NTDs include geographic and temporal variations as well as physical and chemical agents, such as maternal hyperthermia, x-irradiation, antiepileptic drugs, thalidomide, and folate antagonists (Mitchell, 2005; Padmanabhan, 2006).

Hyperthermia (HS) is a well recognized teratogen that induces a variety of malformations, including NTDs during critical periods of embryo development (Edwards, 2006). The teratogenicity of HS was first recognized in laboratory animals (Edwards, 1966), and subsequent epidemiological and clinical studies have shown that HS is also a suspected teratogen in humans (Moretti et al., 2005). The sources of HS in humans include febrile illnesses, sauna bathing, and hot tub use (Moretti et al., 2005).

Although the central nervous system is especially susceptible to HS-induced damage (Edwards et al., 2003; Kline et al., 1985; Shiota, 1982), the mechanisms of HS-induced NTDs are not well understood.

Previously, we showed that HS induces apoptosis in early postimplantation rodent embryos by activating the mitochondrial apoptotic pathway, which is characterized by the release of cytochrome c and the subsequent activation of caspases, cleavage of poly ADP-ribose polymerase, and DNA fragmentation (Little et al., 2003; Little and Mirkes, 2002; Mirkes and Little, 1998, 2000). Subsequent studies, using DNA microarray gene expression profiling, identified five candidate "apoptosis-related" genes (Mikheeva et al., 2004), three of which were p53-regulated genes. These data implicated p53 in both HS-induced apoptosis and HS-induced malformations. Our research has further shown that HS induced the accumulation and phosphorylation of p53 at serine 15 as part of the activation of this transcription factor (Hosako et al., 2007). Called "the guardian of the genome", p53 is a transcription factor that is essential for preventing inappropriate cell proliferation, removing damaged cells by apoptosis, and for maintaining genomic integrity. Activation of p53 can lead to apoptosis, cell cycle arrest, DNA repair, senescence, or differentiation following a variety of stresses (Hofseth et al., 2004; Lane, 1992; Levine et al., 2006).

Studies using transgenic mice have shown that p53 plays a role in the etiology of NTDs (Harris and Juriloff, 2007). Early studies (Armstrong et al., 1995; Sah et al., 1995) reported that, although mice lacking *p53* are viable, 2-6% of *p53* -/- newborn mice exhibit abnormal neural tube closure resulting in exencephaly as well as a significant increase in mortality *in utero* or soon after birth. In addition, these studies revealed that the *p53* -/- mice exhibiting NTDs are predominantly females, which is also found in human exencephaly cases as well as in other mouse mutants, e.g., *1/Hipk2, Efna5, Marcks, Nif1,* and *xn* mutants (Harris and Juriloff, 2007; Seller, 1987, 1995). In mouse embryos, both p53 mRNA and protein are ubiquitously expressed until mid gestation (Louis et al., 1988; Rogel et al., 1985; Schmid et al., 1991). At later organogenesis stages, however, the expression levels decrease rapidly and only specific differentiating

tissues show the expression of p53. Although p53 is expressed in developing embryos, most p53 protein in embryos is relatively inactive, at least with respect to some intracellular pathways regulated by p53 (Gottlieb et al., 1997; Komarova et al., 1997; MacCallum et al., 1996). Given the importance of p53 in processes critical to normal development, one of the objectives of the present study was to determine whether p53 functions as an enhancer or suppressor of HS-induced malformations.

Previous studies have also shown that HS induces the expression of p21 mRNA and protein, suggesting a role for p53 in teratogen-induced cell cycle arrest via the up-regulation of p21 (Hosako et al., 2007). More recent studies, using p53 deficient mice, have confirmed that the expression of p21 is regulated by p53 in day 8.5 mouse embryos (section 5). p21 has the ability to induce cell cycle arrest at both the G1 and G2 checkpoints. p21 binds and inactivates CDK4, 6/Cyclin D and CDK2/Cyclin E complexes, resulting in pRB hypophosphorylation and cell cycle arrest at G1 phase (Stewart and Pietenpol, 2001). p21 arrests cells at G2 checkpoint by inhibiting CDK1/Cyclin B1 (Flatt et al., 2000; Innocente et al., 1999). Given the importance of cell proliferation in normal development and the important role of p21 in regulating cell division, another objective of the present study was to determine whether p21 acts as an enhancer or suppressor of HS-induced malformations.

4.3. MATERIALS AND METHODS

Experimental Animals: All animals were maintained in accordance with animal use protocol approved by the Institutional Animal Care and Use Committee at Texas A&M University. A pair of *p53* +/- mice (a gift of Lawrence A. Donehower, Baylor College of Medicine) with C57BL6-Tyrc-Brd background (Zheng et al., 2002) and highly inbred *p21* -/- mice of 129S2 strain from Jackson Laboratory were used for breeding. *p21* hybrids were generated by crossing *p21* -/- to wild-type males of C57BL6 strain obtained from a local supplier. Mice were kept with freely available pellet food and water at 21-23 ˚C and 12-h light/dark cycle (6:00-18:00). Virgin *p53* +/- and *p21* -/- females of 6-17 weeks old were separately cohabited with *p53* +/- and *p21* +/+ males

and checked the following morning for the vaginal plug. Upon detecting a plug, 12 a.m. was designated day 0.

Hyperthermia Treatment and Fetal Collections: On gestation day 8.5, when the embryos are at the most sensitive period for the induction of NTDs (Finnell, 1981), pregnant dams were exposed to HS for 10 min at 43 ˚C (or 38 ˚C for a control) in a water bath equipped with a thermolegulated heater. For the HS treatment, each pregnant dam was restrained in a 50-ml plastic centrifuge tube with holes drilled on the sides for the adequate water circulation and immersed in water up to the lower two-thirds of the body as described by Finnell (1986). Rectal temperature was monitored at 1-second intervals by Mini-logger and Mini-mitter reader with a small rectal probe (Respironics, Bend, OR) so that the rise in rectal temperatures occurred at approximately the same rate for all animals (38 °C at min 0, 39 °C at min 1, 40 °C at min 2, 41 °C at min 3, 42 °C at min 4, 42.5 °C at min 5, and 42.8 °C at min 6, and 43 °C for min 7-10). After exposure to HS, treated dams were removed from the centrifuge tube, quickly dried with paper towels, and placed in an incubator set at 38 °C to minimize the recovery differences among different mice. Temperature was continually monitored until the baseline temperature was reestablished. The dams were then returned to their cage where they remained until day15.5.

PCR for Genotyping and Sex Identification, and Fetal Examinations: On day 15.5, fetuses were removed from the uterus and the yolk sac of each conceptus was collected in ice-cold Hanks' balanced salt solution. Genomic DNA was isolated from each yolk sac using PureLink™ Genomic DNA kits (Invitrogen, Carlsbad, CA), and the isolated sample was used for *p53* or *p21* genotyping of its matching fetus. Primers used for *p53* PCR genotyping were: p53 forward primer (in the intron 1): 5'-TTTCCCACCCTCGCATAAGTTTCC-3', p53 reverse primer (in the intron 2): 5'-CGCAGGATTTACAGACACCCAACA-3' (558 bp), puro forward primer: 5'-ATGACCGAGTACAAGCCCAC-3', and the puro reverse primer: 5'-GCGTGAGGAAGAGTTCTTGC-3' (166 bp). Primers used for *p21* PCR genotyping were: 5'-AAGCCTTGATTCTGATGTGGGC-3', 5'-

TGACGAAGTCAAAGTTCCACC-3', and 5'-GCTATCAGGACATAGCGTTGGC-3' (872 bp for *p21* and 700 bp for *neo*, The Jackson Laboratory). The PCR program for *p53* genotyping consisted of one cycle of 2 min at 95 ˚C, 40 cycles of 40 s at 95 ˚C, 40 s at 65 ˚C, and 60 s at 72 ˚C, followed by one cycle of 7 min at 72 ˚C. The PCR program for *p21* genotyping consisted of one cycle of 3 min at 94 ˚C, 35 cycles of 30 s at 94 ˚C, 60 s at 64 ˚C, and 60 s at 72 ˚C, followed by one cycle of 7 min at 72 ˚C. DNA isolated from each *p53* fetus was also used for sex determination as described by Sah et al (1995). All samples were visualized on a 1.5% agarose electrophoresis gel after staining with ethidium bromide.

Examination of Fetuses: Day 15.5 fetuses were examined under a microscope for neural tube defects as well as other external malformations, and their body length was carefully measured using Nikon Digital Sight DS-L (Nikon, Melville, NY). The number of implantation sites, resorptions, and dead fetuses were also recorded. Dead fetuses that showed any degree of degeneration were considered resorptions whereas fetuses that retained their integrity but exhibited no blood circulation were counted as dead fetuses. The yolk sac from dead fetuses was used for genotyping. We were unable to genotype resorptions because we were unable to isolate the yolk sac from resorbed conceptuses. For the statistical analyses, we used the Chi-square test to analyze the Mendelian distribution ratio of *p53* and *p21* fetuses. Statistical significance of malformations observed in each genotype or/and sex were evaluated by Fisher's exact test, and the t-test was utilized to evaluate the statistical significance of body length of each genotype or/and sex ($p<0.05$). A total of at least 40 litters per treatment were used to collect at least 65-80 fetuses for each genotype.

Examination of Neonatal p53 Mice: To examine the distribution of genotypes among neonatal mice born from *p53* heterozygous mice, *p53* +/- females were separately cohabited with *p53* +/- males, and their pups were evaluated for their genotype and gender by visual examination (Zheng et al., 2002) upon weaning at 21 days after birth. A total of 395 neonatal mice from 86 litters were evaluated.

4.4. RESULTS

p53 and Normal Growth and Development: In control litters, the number of resorptions and dead fetuses on day 15.5 were 16.2% and 1.3%, respectively (Table 4.1). Gross examination of the dead fetuses revealed that 20% (1 of 5 fetuses) were exencephalic, *p53* -/- , and female. The distribution of genotypes among the collected day 15.5 fetuses (+/+: +/-: -/-) approximated the expected 1:2:1 Mendelian ratio (26%:50%:24%); however, at weaning the distribution of genotypes was significantly skewed (29%: 57%:14%, p=0.0001). In addition, the number of male and female fetuses of each genotype at gestation day 15.5 were equivalent. Although the ratio of male and female pups at weaning was equivalent, 89% of the *p53* -/- neonates were male (Table 4.1 and Table 4.2). Finally, no statistically significant differences in body size among genotypes were observed, although there was a slight but consistent decrease observed in both male and female *p53* -/- fetuses (Table 4.3).

External malformations observed in control *p53* fetuses include exencephaly (1.3%), spina bifida (1.3%), preaxial polydactyly (1.0%), cleft lip (0.3%), and microphthalmia (0.3%) (Table 4.1). As expected, all fetuses with exencephaly were *p53* -/- and female (11%) (Table 4.2). In addition, all fetuses with spina bifida were also identified as *p53* -/- and female (11%). All cases of preaxial polydactyly involved thumb duplications and were only observed in males. All of polydactylous fetuses were *p53* -/- and involved the hind limb except for one fetus, which had a *p53* +/+ genotype and involved the fore limb. Finally, one *p53* +/+ female showed cleft lip and microphthalmia.

p53 and HS-induced Malformations: Although no statistically significant increase in the number of resorption was observed in the HS-treated group compared to control, the number of dead fetuses in the HS-treated group showed a statistically significant increase compared to that in control (Table 4.1). Gross examination of the dead fetuses revealed that 20% (3 of 15 fetuses) were exencephalic, *p53* -/-, and female.

Table 4.1
Evaluation of *p53* Fetuses

	CT				HS			
	+/+	+/-	-/-	Total	+/+	+/-	-/-	Total
Collected litters				41				40
Resorptions				61 (16.2)				71 (18.7)
Dead fetuses				5 (1.3)				15 (3.9)*
Collected fetuses	80 (26)	156 (50)	74 (24)	310(100)	69 (24)	161 (54)	64 (22)	294 (100)
Newborns	116 (29)	224 (57)	55 (14)	395 (100)				
Malformations								
Exencephaly			4 (5.4)	4 (1.3)	12 (17.4)*	26 (16.1)*	30 (47)*†	68 (23.1)*
Spina Bifida			4 (5.4)	4 (1.3)				
Polydactyly	1 (1.3)		2 (2.7)	3 (1.0)		2 (1.2)	10 (16)*†	12 (4.1)*
Cleft lip	1 (1.1)			1 (0.3)	1 (1.4)			1 (0.3)
Micropthalmia	1 (1.1)			1 (0.3)		1 (0.6)		1 (0.3)
Hamartomas					3 (4.3)	4 (2.5)	2 (3.1)	9 (3.1)*

The number of litters or fetuses collected or observed is shown. * indicates that the incidence rate presented showed a statistically significant increase compared to the same genotype group in the absence of HS treatment. † indicates that the incidence rate presented showed a statistically significant increase compared to *p53* +/+ fetuses exposed to HS. $p<0.05$.

Table 4.2
Birth Defects Evaluation of *p53* Fetuses Except for Exencephaly (%)

	CT								HS							
	+/+		+/-		-/-		Total		+/+		+/-		-/-		Total	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Collectedfetuses	40	40	78	78	39	35	157	153	35	34	77	84	32	32	144	150
Newborns	57	59	108	116	49	6	214	181								
Malformations																
Exencephaly						4		4	3	9*	9*	17*	8*	22*†‡	20*	48*‡
						(11)		(2.6)	(8.6)	(27)	(12)	(20)	(25)	(69)	(14)	(32)
Spina Bifida						4		4								
						(11)		(2.6)								
Polydactyly	1				2		3				1	1	5*†	5*†	6	6*
	(2.5)				(5.1)		(1.9)				(1.3)	(1.2)	(16)	(16)	(4.2)	(4.0)
Cleft lip		1						1		1						1
		(2.5)						(0.7)		(2.9)						(0.7)
Micropthalmia		1						1				1				1
		(2.5)						(0.7)				(1.2)				(0.7)
Hamartomas									1	2	1	3	1	1	3	6*
									(2.9)	(5.9)	(1.3)	(3.6)	(3.1)	(3.1)	(2.1)	(4.0)

The number of litters or fetuses collected or observed are shown. * indicates that the incidence rate presented showed a statistically significant increase compared to the same genotype group in the absence of HS treatment. † indicates that the incidence rate presented showed a statistically significant increase compared to *p53* +/+ fetuses exposed to HS. ‡ indicates that the incidence rate presented showed a statistically significant increase compared to the different sex group of the same genotype/treatment. $p<0.05$.

The ratio of genotypes among HS-treated fetuses on day 15.5 (+/+: +/-: -/-) was the expected Mendelian ratio of 1:2:1 (24%:54%:22%) and the number of male and female fetuses were equivalent among all genotypes. Finally, no statistically significant difference in body size between control and HS-treated fetuses was observed (Table 4.3).

An increased number of malformed fetuses was also observed in the HS-treated group. Malformations observed included exencephaly (23.1%), preaxial polydactyly (4.1%), cleft lip (0.3%), micropthalmia (0.3%), and hamartomas along the sagittal suture of the skull (3.1%). Fetuses with exencephaly were observed in all genotypes following HS treatment, comprising a total of 23.1% of the fetuses collected. The increase in the number of exencephalic fetuses after HS treatment was statistically significant compared to control in all three genotypes (indicated as *) and in those with a *p53* -/- genotype, almost half of the fetuses (46.9%) showed exencephaly. In addition, the increase in the number of exencephalic fetuses in HS-treated *p53* -/- group was statistically significant compared to the number of exencephalic fetuses observed in HS-treated *p53* +/+ and *p53* +/- groups (indicated as†). The exencephalic fetuses observed after HS treatment included both males and females, yet in all genotypes more females were affected, and the number of exencephalic females was statistically higher than that of males in *p53* -/- fetuses as well as in total *p53* fetuses collected (indicated as‡).

Interestingly, spina bifida was observed in control *p53* -/- fetuses; however, no spina bifida fetuses were observed among HS-treated fetuses. In addition, the number of fetuses exhibiting preaxial polydactyly was statistically significantly increased among HS-treated *p53* -/- fetuses compared to control. The increase of preaxial polydactyly in HS-treated *p53* -/- fetuses was also statistically significant compared to that of *p53* +/+ fetuses following HS treatment. Although both males and females exhibit preaxial polydactyly after HS exposure, only the increase in HS-treated female is statistically significant compared to control.

Table 4.3
Body Size (mm) of *p53* and *p21* Fetuses on GD 15.5

	CT			HS		
	+/+	+/-	-/-	+/+	+/-	-/-
p53 ♂ fetuses	14.4±1.4	14.5±1.1	14.2±1.2	14.3±1.2	14.3±1.1	13.9±1.0
p53 ♀ fetuses	14.7±1.2	14.3±1.0	13.4±1.1	14.2±1.0	14.1±1.0	13.8±1.2
p21 fetuses	15.6±0.8	15.5±0.7	15.4±0.8	15.2±0.9	15.2±0.8	15.1±1.0

Table 4.4
Evaluation of *p21* Fetuses

	CT				HS			
	+/+	+/-	-/-	Total	+/+	+/-	-/-	Total
Collected litters				45				43
Resorptions				30 (6.6)				17 (4.1)
Dead fetuses				1 (0.2)				6 (1.5)
Collected fetuses	103 (24)	206 (49)	112 (27)	421(100)	91 (23.4)	207 (53.2)	91 (23.4)	389 (100)
Malformations								
Exencephaly					3 (3.3)	15 (7.2)*	9 (9.9)*	27 (6.9)*
Polydactyly						1 (0.5)		

The number of litters or fetuses collected or observed is shown. * indicates that the incidence rate presented showed a statistically significant increase compared to the same genotype group in the absence of HS treatment. $p<0.05$.

Interestingly, 42% of the preaxial polydactyly fetuses after HS treatment also showed exencephaly, and again all of the polydactyly cases were thumb duplication on their hind limb. In addition, majority of control and HS-treated fetuses exhibited right limb preaxial polydactyly. There was one *p53* +/+ female showing cleft lip and one *p53* +/- female with microthalmia following HS treatment. Finally, we observed 9 cases of hamartomas along sagittal suture of the skull in HS-treated fetuses. These cases were found in all three genotypes and both genders. The increase in hamartomas in total HS-treated fetuses as well as HS-treated females was statistically significant compared to those in control.

p21 and Normal Growth and Development: No malformations were observed among control *p21* fetuses, and the distribution of genotypes among day 15.5 fetuses (+/+: +/-: -/-) was the expected 1:2:1 Mendelian ratio (24%:49%:27% for control fetuses) (Table 4.4). In addition, there was no significant difference in body size among genotypes in control fetuses.

p21 and HS-induced Malformations: No statistically significant differences in the numbers of resorptions and dead fetuses were observed in HS-treated fetuses compared to control fetuses (Table 4.4). Likewise, the distribution of genotypes among the day 15.5 fetuses (+/+: +/-: -/-) was the expected 1:2:1 Mendelian ratio (23.4%: 53.2%: 23.4% for HS-treated fetuses). In addition, there was no significant difference in body size among each genotype of HS-treated fetuses and no significant difference in body size between control and HS-treated fetuses.

The malformations observed in HS-treated *p21* fetuses were exencephaly and one case of preaxial polydactyly (Table 4.4). A statistically significant increase in the number of exencephalic fetuses after HS treatment compared to control was observed. Fetuses with exencephaly were observed in all genotypes, and the increases in the number of *p21* -/- exencephalic fetuses were most prominent; however, the increase in *p21* -/- exencephalic fetuses was not statistically significant compared to *p21* +/+ exencephalic fetuses. Finally, one case of preaxial polydactyly (duplication of the thumb) was observed on the right hind limb of a *p21* +/- fetus after HS treatment.

4.5. DISCUSSION

Although we observed the expected Mendelian distribution of *p53* genotypes (1+/+:2+/-:1-/-) and the expected distribution of males and females (1:1) among control day 15.5 fetuses, at weaning p53 null and female pups were underrepresented. These differences are, in part, the result of the increased fetal death in *p53* -/- female fetuses sometime after organogenesis and before birth. We observed no differences in neonatal death among genotypes (data not shown). Although we hypothesize that increased resorptions also contribute to this difference, we were unable to confirm this due to an inability to extract embryonic DNA from resorptions for genotyping. Nonetheless, our results showing increased prenatal death among p53 null fetuses confirm the findings of Armstrong et al. (1995).

Several published studies have examined malformations in *p53*-deficient mice, and the observed malformations included exencephaly, fusion of the upper incisors, ocular abnormalities, and preaxial polydactyly of the hind limb (Armstrong et al., 1995; Kaufman et al., 1997 ; Pani et al., 2002; Sah et al., 1995). Our study, in which we examined a greater number of litters than in previous studies, basically confirmed the spectrum of previously reported malformations. In addition, we observed fetuses with spina bifida, a malformation not reported in previous studies. Although the percentage of fetuses with exencephaly observed in our study was slightly lower compared to that of early studies (1.3% compared to 6.2% and 2.1%), this is most likely due to the strain differences (Finnell et al., 1986, 2000; Lundberg et al., 2003; Sah et al., 1995).

The previously described predominance of fetuses with exencephaly in females (Armstrong et al., 1995; Sah et al., 1995) was also confirmed by our study. In addition, we showed the female predominance of fetuses with spina bifida. The biological origin of the predominance of NTDs in females, which has also been observed in humans, has been debated for decades. Although the gonads are not yet developed during the neural tube closure, possible causes of the female predominance have been investigated, which include growth differences and DNA methylation for X-chromosome inactivation (Juriloff and Harris, 2000; Seller, 1995). Research studies have shown that female

embryos have a slower embryonic growth rate compared to males (Brook et al., 1994; Golding, 1982; Renwick, 1972), although whether the growth rate difference is causally linked to the female predisposition to NTDs is not shown (Brook et al., 1994). X chromosome inactivation is a developmental process by which one of the two copies of the X chromosome in females is transcriptionally silenced by methylation, thus equalizing the expression of X-linked genes between the sexes (Lyon, 1961). Methylation regulates X chromosome inactivation as well other processes such as embryonic development and genome integrity (Turek-Plewa and Jagodzinski, 2005). Methylation of mammalian genomic DNA is mediated by DNA methyltransferases (DNMTs). Mammalian DNMTs, which include DNMT1, DNMT3A, DNMT3B, and DNMT3L, are important for methylation pattern acquisition during embryogenesis and are divided into two groups according to their functions (Turek-Plewa and Jagodzinski, 2005). *De novo* DNMTs, including DNMT3A and DNMT3B, methylate C to m^5C post-replicatively in unmethylated DNA, whereas maintenance DNMTs such as DNMT1 attaches a methyl group to DNA during replication (Bestor, 2000; Margot et al., 2003). Because an X chromosome must be methylated in every new cell in female embryos during X chromosome inactivation, this may lead to a "reduced methylation status" in female embryos undergoing rapid embryonic growth (methylation of DNA, protein, and lipids (Juriloff and Harris, 2000). A recent study has shown that the number of copies of X chromosomes is associated with the sex differences in neural tube defects observed in *p53*-deficient embryos (Chen et al., 2008). Our previous microarray study, using the same strain of *p53* mice, identified 388 genes that were disregulated in *p53* -/- day 8.5 embryos (submitted). One of these genes was *Dnmt1*, whose activity is critical for the maintenance of DNA methylation and the appropriate histone H3 modification (Turek-Plewa and Jagodzinski, 2005). Another study has shown that the deletion of *Dnmt3b*, which is expressed in cranial neural folds, leads to exencephaly (Okano et al., 1999). Taken together, these studies suggest that X chromosome inactivation by DNMTs may explain the female predominance of exencephaly and spina bifida observed in p53-deficient embryos.

We have observed that *p53* -/- fetuses showed increased incidence of NTDs whereas no malformations were observed in *p21* -/- fetuses in the absence of stress. Our previous study has shown that in the absence of cellular stress, p53 target gene, *p21*, is downregulated in *p53* -/- embryos compared to that in *p53* +/+ embryos, suggesting the p53-dependent expression of *p21* and a possible contribution of p21 in the induction of NTDs observed in *p53* -/- embryos. However, deletion of p21 gene did not lead to exencephaly, suggesting that the decreased expression of p21 in *p53* -/- embryos is not crucial to the induction of exencephaly and spina bifida observed in *p53* -/- embryos.

Interestingly, all the *p53* fetuses exhibiting preaxial polydactyly involved duplication of the thumb, referred to as preaxial polydactyly type I. In our study preaxial polydactyly type I was observed on the hind limb. In addition, all of the fetuses with this defect were males and *p53* -/- except for one *p53* +/+ fetus. Although no previous studies have shown the correlation of preaxial polydactyly type I and p53, an extra digit on the hind limb of an exencephalic *p53* -/- fetus has been also been reported previously (Armstrong et al., 1995), suggesting some causal relationship between preaxial polydactyly type I and p53. In humans, the prevalence of preaxial polydactyly type I is approximately 0.8-2.5/10,000 (Firth et al., 2005). The etiology of preaxial polydactyly type I is complex, and most cases are sporadic. Males are more often affected than females, at least in certain populations (Bingle and Niswander, 1975; Orioli and Castilla, 1999). In our study, all the polydactyly fetuses observed exhibited hind limb preaxial polydactyly type I; therefore, p53 deficient mice might be a useful model to study the mechanisms of this specific defect.

Following the HS exposure, we observed an increased incidence of exencephaly in all p53 genotypes and in both genders; however, females in all genotypes and *p53* -/- fetuses were especially susceptible (47% vs. 16% in +/- and 17% in +/+ fetuses). These results suggest that p53 functions as a suppressor of HS-induced exencephaly. Whether p53 plays a role as a teratogen suppressor or inducer has been controversial, although the role of p53 as a teratological suppressor has been more favored based on the protective role of p53 following a variety of environmental stresses (Choi and Donehower, 1999).

However, recent studies have shown that p53 can function as a teratological inducer (Boreham et al., 2002; Narai et al., 2006; Wang, 2001), as well as as a teratogen suppressor (Baatout et al., 2002; Bekaert et al., 2005; Moallem and Hales, 1998). For example, it has been shown that the loss of *p53* alleles increase the incidence of fetal resorption and developmental anomalies in mouse embryos exposed to benzo[a]pyrene (Nicol et al., 1995). It was also shown that *p53* -/- embryos showed higher incidence of structural anomalies and fetal death and a fewer number of apoptotic cells than that of *p53* +/+ embryos exposed to radiation (Baatout et al., 2002; Norimura et al., 1996). Others have also shown that the absence of p53 resulted in the increased limb defects but no signs of apoptosis (Moallem and Hales, 1998). These studies suggest that p53 acts as a teratological suppressor. In contrast, eye defects in mouse embryos exposed to 2-chloro-2'-deoxyadenosine (2CdA) was mediated by p53-dependent apoptosis, suggesting that p53 acts as a teratogen inducer (Wubah et al., 1996). Other studies have also shown that the lack of p53-dependent apoptosis resulted in reduced the incident of limb defects and cleft palate (Boreham et al., 2002; Narai et al., 2006; Wang, 2001). Close examination of the literature suggests that whether p53 acts as a suppressor or inducer is determined by the type of teratogen as well as the type of malformations evaluated. Studies that have identified p53 as a teratological inducer have investigated specific malformations such as eye defects, limb malformations, and cleft palate (Boreham et al., 2002; Narai et al., 2006; Wang, 2001; Wubah et al., 1996), whereas most studies that have identified p53 as a teratogen suppressor have examined multiple types of malformations as well as resorptions (Baatout et al., 2002; Bekaert et al., 2005; Nicol et al., 1995; Norimura et al., 1996). In addition, p53 was a teratological inducer of limb malformations following irradiation (Boreham et al., 2002; Wang, 2001), whereas it was a teratological suppressor of limb malformations following exposure to 4-hydroperoxycyclophosphamide (Moallem and Hales, 1998).

The evaluation of *p21* fetuses has revealed that *p21* -/- mice are developmentally normal and no statistically significant increase in the incidence of exencephaly was observed following the exposure to HS. Our previous study has shown that the

expression of both p21 mRNA and protein is upregulated in HS-treated day 8.5 embryos, suggesting that cell cycle arrest induced by p21 may play an important role in HS-treated embryos (Hosako et al., 2007). Although we observed that there was a slight but consistent increase in NTDs in *p21* -/- embryos compared to *p21* +/+ embryos, the increase was not statistically significant. Therefore, it suggests that other biological pathways and proteins may be heavily involved in the induction of NTDs observed in *p53* -/- embryos. Taken together, our results indicate that although the statistically significant increase in NTDs was not observed, its consistent increase suggest that *p21* may act as a very weak teratogen suppressor.

Overall, these studies showed that the deletion of both *p53* alleles in normal embryos leads to NTDs. In contrast, although *p21* is regulated and controlled by p53 during the normal embryonic development, the regulation of p21 is not causally linked to the induction of NTDs. In the presence of HS exposure, p53 functions as a teratogen suppressor. Following the deletion of *p21* alleles, the incidence of exencephaly slightly but consistently increased, suggesting that p21 may be a very weak teratogen suppressor, although we did not show that the increase was statistically significant.

5. THE REGULATION OF APOPTOSIS AND P53 TARGET GENES IN P53-DEFICIENT DAY 8.5 MOUSE EMBRYOS EXPOSED TO HYPERTHERMIA

5.1. OVERVIEW

HS activates the mitochondrial apoptotic pathway and its upstream regulator, p53, in day 9 mouse embryos. The activation of p53 results in the upregulation of p21 mRNA and protein, inducing cell cycle arrest. The p53 protein also upregulates *Noxa* and *Puma* mRNAs, but not their proteins. Although whether these genes play a role in the induction of the mitochondrial apoptotic pathway in embryos exposed to HS is not clear, these studies suggest that the induction of apoptosis and cell cycle arrest may play a critical role in HS-induced NTDs. Using *p53*-deficient mice, our previous study showed that the deletion of p53 alleles induces the sensitivity to NTDs, therefore p53 acting as a teratogen suppressor. To explore the role of p53 in the regulation of apoptosis and NTDs, we assessed the induction of apoptosis and p53 target genes in HS-treated mouse embryos lacking *p53* allele(s).

HS induced a significant increase in *p21, Puma*, and *Noxa* mRNAs, transcripts of p53 target genes. In contrast, HS induced no significant increases in these mRNAs in *p53*-deficient embryos, suggesting that the upregulation of *p21*, *Puma*, and *Noxa* in embryos exposed to HS are p53-dependent. Very preliminary results showed that HS induced a higher level of caspase-3 activity assay in *p53* +/+ embryos compared to *p53* +/- embryos (*p53* -/- embryos not available), indicating that the activation of apoptosis in embryos exposed to HS is also p53-dependent. Together with our previous study that *p53* +/- and *p53* -/- embryos exposed to HS exhibited a higher incidence of NTDs, our results suggest that the regulation of the p53 target genes as well as the apoptosis is p53-dependent, although the induction of apoptosis does not causally link to the NTDs observed in embryos exposed to HS.

5.2. INTRODUCTION

Previously, we showed that HS induces apoptosis in early postimplantation rodent embryos by activating the mitochondrial apoptotic pathway. Activation of this pathway is characterized by the release of cytochrome c and the subsequent activation of caspases, cleavage of poly ADP-ribose polymerase, and DNA fragmentation (Little et al., 2003; Little and Mirkes, 2002; Mirkes and Little, 1998, 2000). To begin to identify proteins and signaling pathways that regulate cytochrome c release, we compared gene expression patterns in HS-treated and –untreated mouse embryos before and during the activation of the mitochondrial apoptotic pathway, using DNA microarray gene expression profiling. Our studies identified five candidate ‘‘apoptosis-related’’ genes (Mikheeva et al., 2004). Three of these genes, *Mdm2, Gtse1*, and *Cyclin G*, are coordinately upregulated by HS during the first 5 hr after embryos are exposed to these teratogens. Because these three genes are all p53-regulated genes, our microarray data suggested that HS activates p53. The first study of this thesis (section 2) then showed that HS induced the accumulation of p53 and phosphorylation of p53 at ser-15, two hallmarks of p53 activation. HS also induced an increase in *Noxa* and *Puma* mRNAs, transcripts of two known proapoptotic p53 target genes; however, these two teratogens did not induce significant increases in NOXA and PUMA proteins, suggesting that p53 does not activate the mitochondrial apoptotic pathway by transcriptionally upregulating the expression of NOXA and PUMA proteins. HS also induced the expression of p21 mRNA and protein, suggesting a role for p53 in teratogen-induced cell cycle arrest. In the third study (section 4), we determined whether p53 and/or p21 play a role as teratogen suppressors or inducers of HS-induced malformations. The study showed that p53 plays a role in normal development and that p53 and p21 function to suppress HS-induced malformations. However, these studies did not examine whether the regulation of p53 target genes is p53-dependent or p53-independent and whether there is a causal relationship among the activation of p53, induction of apoptosis and cell cycle arrest, and the induction of serious malformations including NTDs in embryos exposed to HS. Therefore, the objective of the current studies were (1) to assess the p53-dependence of

p53 target genes in HS-treated embryos and (2) to determine whether the activation of p53, induction of apoptosis and cell cycle arrest, and the induction of NTDs are causally linked with each other using *p53*-deficient mouse embryos. Our preliminary data show that the activation of *p21*, *Noxa*, and *Puma* in embryos exposed to HS is p53-dependent. In addition, we show that the induction of apoptosis is, at least from our preliminary data, p53-dependent; however, the induction of apoptosis was not causally linked to NTDs observed after HS treatment.

5.3. MATERIALS AND METHODS

Experimental Animals: All animals were maintained in accordance with animal use protocol approved by the Institutional Animal Care and Use Committee at Texas A&M University. A pair of *p53* +/- mice (a gift of Lawrence A. Donehower, Baylor College of Medicine) with C57BL6-Tyrc-Brd background (Zheng et al., 2002) was used for breeding. Mice were kept with freely available pellet food and water at 21-23 ˚C and 12-h light/dark cycle (6:00-18:00). Virgin *p53* +/- females were cohabited with *p53* +/- males and checked the following morning for the vaginal plug. Upon detecting a plug, 12 a.m. was designated GD 0.

Hyperthermia Treatment and Fetal Collections: On gestation day 8.5, when the embryos are at the most sensitive period to the induction of NTDs (Finnell, 1981), pregnant dams were exposed to HS for 10 min at 43 ˚C (or 38 ˚C for a control) in a water bath equipped with a thermolegulated heater. For the HS treatment, each pregnant dam was restrained in a 50-ml plastic centrifuge tube with holes drilled on the sides for the adequate water circulation and immersed in water up to the lower two-thirds of the body as described by Finnell et al. (1986). A rectal temperature was monitored at 1-second intervals by Mini-logger and Mini-mitter reader with a small rectal probe (Respironics, Bend, OR) so that the rise in rectal temperatures is approximately the same rate for all animals (38 °C at min 0, 39 °C at min 1, 40 °C at min 2, 41 °C at min 3, 42 °C at min 4, 42.5 °C at min 5, and 42.8 °C at min 6, and 43 °C for min 7-10). The treated dams were removed from the centrifuge tube, quickly dried with paper towels,

and placed in an incubator set at 38 °C to minimize the recovery differences of individuals. Their temperature was continually monitored until the baseline temperature was reestablished.

Embryo Collections and Genotyping PCR: Embryos were explanted 5 hr after the exposure, and their yolk sac was separated in ice-cold Hanks' balanced salt solution on GD 8.5. Each embryo was placed in a separate PCR tube with RNAlater (Ambion, Austin, TX) for RT-PCR or quickly flash frozen using liquid N_2 for caspase-3 activity assay and stored at -80 °C until use. The yolk sac dissected from each embryo was put in a PCR tube with 7.5 µl of tail lysis buffer (25 mM NaOH and 0.2 mM EDTA), and heated at 95 °C for 20 min. The same volume of Tris-HCl (40 mM) was added to the tube, and 3 µl of the yolk sac sample was used for *p53* genotyping of its matching embryo. Primers used for PCR genotyping were: *p53* forward primer (in the intron 1): 5'-GTGTGTGAAATGGTGGATGG-3', *p53* reverse primer (in the intron 3/4): 5'-AGGTGATGGCTGTGGATG-3', *puro* forward primer: 5'-ATGACCGAGTACAAGCCCAC-3', and the *puro* reverse primer: 5'-GCGTGAGGAAGAGTTCTTGC-3'. The expected length of each band was 1147 bp for *p53* and 166 bp for *puro* gene. The PCR program consisted of one cycle of 2 min at 95 ˚C, 40 cycles of 40 s at 95 ˚C, 40 s at 65 ˚C, and 60 s at 72 ˚C, followed by one cycle of 7 min at 72 ˚C. Samples were visualized on a 1.5% agarose electrophoresis gel after staining with ethidium bromide.

Total RNA Preparation and Quantitative Real-Time PCR: Based on the PCR results, 3-7 embryos of similar genotype from multiple litters were pooled in RNAlater to obtain quadruplicate samples of each of three genotypes. Total RNA was extracted from each sample using mirVana™ miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. Concentration and quality of the total RNA were assessed using a Nanodrop (Nanodrop, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), respectively. The RNA samples were stored at -80 ˚C until analyzed. Total RNA (1-10 µg) was reverse-transcribed using 25 ng/µl oligo(dT)15 primer and 9.6 µM random hexamers by heating at 65 °C for 5 min,

followed by cooling on ice. The mRNA was then copied into cDNA by 300 U SuperScript II RNase H^- reverse transcriptase, 1^{st} strand buffer, 10 mM DTT (Invitrogen, Carlsbad, CA), and 30 U RNase block (Qiagen, Valencia, CA) and incubated at 37 °C for 1 hr. The reaction was terminated by placing at 90 °C for 5 min and then brought to 4 °C and stored at -80 °C until ready to use. Subsequently, 1 µl cDNA was added to 96-well fast plate with master mix (Applied Biosystems, Foster City, CA) and TaqMan probe to carry out fluorogenic 5' nuclease assays (TaqMan) using 7500 Fast Real-Time PCR System (Applied Biosystems). Pre-designed probes of the selected genes were purchased from Applied Biosystems, and relative quantification was performed using ΔΔCt method.

Preparation of Samples, BCA Protein Assay, and Caspase-3 Activity Assay: Ice-cold cell lysis buffer (Biomol, Plymouth Meeting, PA) was added to each embryo frozen (10-30 µg per 10 µl buffer) and the tissue was broken up using a Teflon pestle. The sample was kept on ice for 5 min and centrifuged 10 min at 10,000 rpm at 4 °C. The supernatant was transferred to a new tube. The protein concentration was measured using BCA™ Protein Assay kit (Pierce, Rockford, IL), and Caspase-3 Cellular Activity Assay kit plus (Biomol) was used to quantitate caspase-3 activity of each embryo. The caspase-3 activity was measured every 10 min for 8 hr by a plate reader.

5.4. RESULTS

Examination of Known p53 Target Genes: To determine whether the upregulation of known p53 target genes, *p21*, *Noxa*, and *Puma* are p53-dependent, the mRNA expression level of these genes were examined using TaqMan quantitative real-time PCR method. Figure 5.1 shows that HS induces a significant increase in *p21* mRNA of *p53* +/+ embryos. In addition, it shows that HS did not induce *p21* mRNA expression in *p53* -/- embryos. Figure 5.2 shows that significant increases in *Noxa* and *Puma* mRNA levels after the exposure of *p53* +/+ embryos to HS were also observed. In addition, it shows that HS did not induce *Noxa* and *Puma* mRNA expression in *p53* -/- embryos.

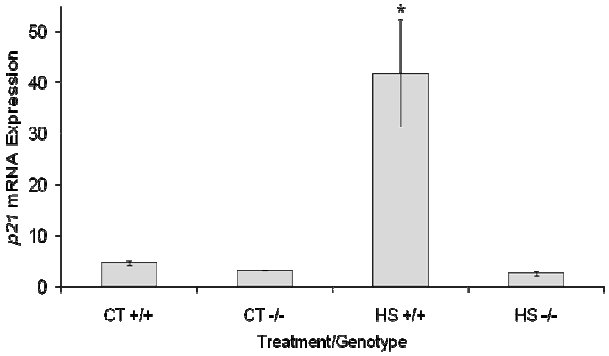

Figure 5.1. p21 mRNA expression in day 8.5 *p53* +/+ or *p53* -/- mouse embryos receiving no treatment (CT) or HS (43 °C for 15 min). * Indicates a significant difference from CT or/and *p53* -/- ($p<0.05$).

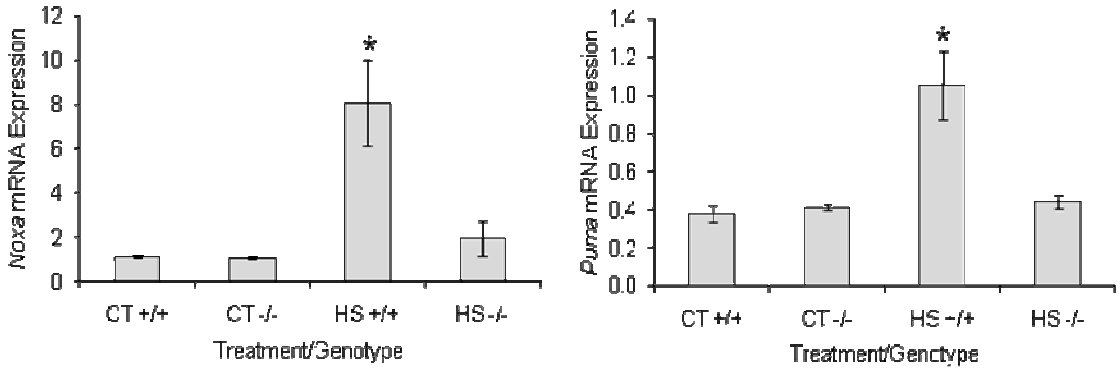

Figure 5.2. *Noxa* and *Puma* mRNA expression in day 8.5 *p53* +/+ or *p53* -/- mouse embryos receiving no treatment (CT) or HS (43 °C for 15 min). * Indicates a significant difference from CT or/and *p53* -/- ($p<0.05$).

Examination of Apoptosis: Because of the time constraint and sample availability, very limited results are shown in Figure 5.3. Caspase-3 activity assay from 3 *p53* +/+ and 2 *p53* +/- embryos showed that increase of apoptosis in *p53* +/+ embryos exposed to HS compared to *p53* +/- embryos exposed to HS.

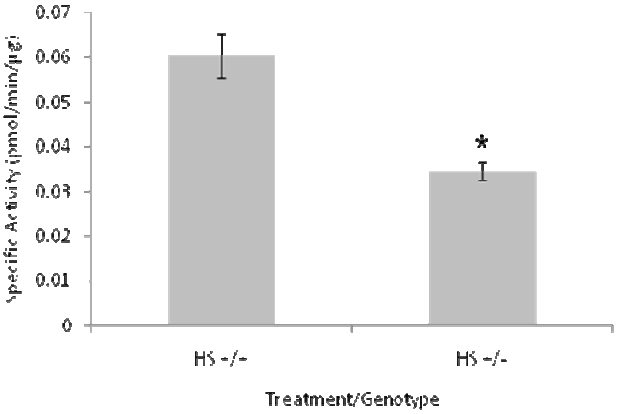

Figure 5.3. Caspase-3 activity assay in day 8.5 *p53* +/+ and *p53* +/- mouse embryos receiving no HS (43 °C for 15 min). * Indicates a significant difference from *p53* +/+ embryos ($p<0.05$).

5.5. DISCUSSION

The cell cycle related gene, p21, and proapoptotic genes, Noxa and Puma, are known target genes of p53. These target genes are regulated and induced by p53 in response to a variety of cellular stresses. However, these p53 target genes are also regulated by other transcription factors, suggesting that they may be upregulated both in p53-dependent and –independent manner. For example, the induction of p21, independent of p53, was observed following the exposure of human hepatocarcinoma cells to ceramide (Kim et al., 2000). Another study showed that p53-independent induction of p21 contributes to the activation of caspases in mycophenolic acid-induced apoptosis in insulin-secreting cells (Huo et al., 2004). In response to ischemia the induction of Puma, which mediates apoptosis, was observed independent of p53 (Wu et al., 2007). In our study, the expression level of *p21*, *Puma*, and *Noxa* were not altered between *p53* -/- embryos in the absence and presence of HS, suggesting that the induction of these genes are p53-depenent. Although the p53-dependent upregulation of *p21* leads to cell cycle arrest in embryos exposed to HS, whether the p53-dependent upregulation of *Puma* and *Noxa* are associated with the induction of apoptosis is not clear.

Our very preliminary data have indicated that a decreased level of apoptosis was observed in *p53* +/- embryos compared to that in *p53* +/+ embryos after HS treatment. These preliminary results suggest that although the induction of teratogen-induced apoptosis is p53-dependent, the induction of teratogen-induced apoptosis appears not to be causally linked to the increased incidence of NTDs.

6. CONCLUSION

Over 1,200 chemical and physical agents are known to cause structural and/or functional congenital anomalies in experimental animals (Shepard, 2001). One of the most common birth defects, NTDs, represents a group of serious congenital anomalies resulting from failure of neural tube closure during early development. The etiology of NTDs as well as other birth defects is complex and is known to involve both genetic and environmental factors. Although the mechanisms by which teratogens disrupt normal development are often not well understood, recent studies have shown that many teratogens induce cell death in tissues that subsequently develop abnormally and give rise to structural malformations (Knudsen, 1997; Scott, 1977). In addition, teratogens often induce cell death in areas of normal PCD such as CNS, suggesting a mechanistic link between PCD and teratogen-induced cell death (Menkes et al., 1970; Milaire and Rooze, 1983; Sulik et al., 1988). Mouse mutants, in which genes known to play a role in apoptosis have been deleted, also exhibit abnormal levels of apoptosis associated with abnormal development often culminating in structural birth defects (Boya and de la Rosa, 2005). Taken together, these studies indicate that the dysregulation of apoptosis is consistently correlated with embryo/fetal lethality and/or birth defects.

Previously, we showed that HS and 4CP, known animal teratogens, induce apoptosis in early postimplantation rodent embryos by activating the mitochondrial apoptotic pathway. Activation of this pathway is characterized by the release of cytochrome c and the subsequent activation of caspases, cleavage of PARP, and DNA fragmentation (Little et al., 2003; Little and Mirkes, 2002; Mirkes and Little, 1998, 2000). Using vital dyes and TUNEL staining, we have also shown that teratogen-induced cell death is cell specific, that is, some cells in the mouse embryo die, particularly in areas of normal PCD, while other cells, often neighboring cells, survive (Mirkes and Little, 1998; Umpierre et al., 2001). For example, neuroepithelial cells are particularly sensitive to teratogen-induced cell death, whereas mesenchymal cells surrounding the neuroepithelium are less sensitive (Umpierre et al., 2001). In contrast,

cells of the embryonic heart are resistant to cell death induced by a variety of teratogens (Gao et al., 1994; Umpierre et al., 2001). Although teratogens activate the apoptotic pathway in sensitive cells, these hallmarks of apoptosis are not activated in cells of the heart (Mirkes and Little, 1998, 2000; Umpierre et al., 2001). These results indicate that the mitochondrial apoptotic pathway is blocked in heart cells at the level of the cytochrome c release from mitochondria or at some point upstream of cytochrome c release.

The rapid induction of the mitochondrial apoptotic pathway in teratogen-sensitive neuroepithelial cells and the failure to activate this pathway in teratogen-resistant heart cells suggest that the embryo must possess factors that regulate the efflux of cytochrome c and thereby the activation of the mitochondrial apoptotic pathway. To begin to identify proteins and signaling pathways that regulate cytochrome c release, we previously compared gene expression patterns in HS- or 4CP-treated and –untreated mouse embryos before and during the activation of the mitochondrial apoptotic pathway, using DNA microarray gene expression profiling. Three genes, *Mdm2*, *Gtse1*, and *Cyclin G*, were coordinately upregulated by both HS and 4CP during the first 5 hr after embryos were exposed to these teratogens. Because these three genes are all p53-regulated genes, our microarray data suggested that HS and 4CP both activate p53.

p53 is essential for preventing inappropriate cell proliferation and maintaining genomic integrity following a variety of stresses (Harris and Levine, 2005). The level of p53 is maintained at a low level in unstressed conditions. Following a cellular damage, p53 undergoes posttranslational modifications, which result in the accumulation and translocation of p53, enhanced binding to DNA, and transcriptional activation of its target genes. The protein products of p53 target genes subsequently regulate a number of cellular processes, the most well studied being cell cycle arrest and apoptosis. To investigate the roles of p53 in normal development, teratogen-induced apoptosis, and birth defects, we conducted several studies, which are described in this dissertation.

First, we have shown that HS and 4CP activate p53, in part at least, by the phosphorylation of p53 at ser-15 between 1 and 2.5 hr after exposure to HS or 4CP.

Phosphorylation of p53 at serine-15 is correlated with the stabilization and subsequent accumulation of p53. These results indicated that p53 is activated before HS- and 4CP-induced release of mitochondrial cytochrome c and activation of the caspase cascade, which occur between 2.5 and 5 hr after exposure to these two teratogens (Little and Mirkes, 2002; Mirkes and Little, 1998, 2000).

Published data consistently show that teratogens induce apoptosis in some cells of the embryos and not others (Gao et al., 1994; Mirkes, 1985; Mirkes et al., 1985, 1991; Thayer and Mirkes, 1995). In the present studies, we now show that heart cell resistance is associated with significant attenuation of the activation of p53 in heart cells. Despite the attenuated activation of p53 in heart cells in response to teratogenic exposures, our data indicate that both HS and 4CP induce increased levels of p21 in heart cells. These results suggest that p53 is activated in the heart and when activated subsequently upregulates the expression of p21, thereby arresting heart cells.

To further investigate the mechanisms by which p53 activates the mitochondrial apoptotic pathway in the day 9 mouse embryo, we have examined the protein and mRNA expression of p53 target genes. Our results indicate that HS and 4CP both induce increased expression of *Noxa* and *Puma* mRNAs, transcripts of proapoptotic genes belonging to the Bcl-2 family; however, the increased expression of these mRNAs was not coupled with an increased expression of these proteins. Thus, our data suggest that p53-mediated upregulation of NOXA and PUMA proteins is not involved in HS- and 4CP-induced activation of the mitochondrial apoptotic pathway. We speculated that 1) should Noxa and Puma play a role in the activation of mitochondrial apoptotic pathway, these proteins may be sequestered in the absence of an apoptotic stimulus and then activated after an appropriate cell death signal, although we are not aware of such published data 2) should NOXA and PUMA proteins not play a role in activating the mitochondrial apoptotic pathway in teratogen-exposed mouse embryos, other proapoptotic target proteins of p53, for example, BAX, p53AIP, and PIGs may be upregulated 3) alternatively, p53 may play a transcription-independent role in teratogen-induced apoptosis in day 9 mouse embryos exposed to HS or 4CP.

We have also examined the mRNA and protein expression of cyclin-dependent kinase p21. Our results have shown that HS and 4CP induce the upregulation of both p21 mRNA and protein. Because p21 is a known p53 target that plays a central role in arresting the cell cycle after various genotoxic stresses (Harris and Levine, 2005; Taylor and Stark, 2001), our results suggest that cells of the day 9 mouse embryo have activated the cell cycle arrest arm of the p53 pathway in response to teratogenic exposures. Although we have not shown that HS induces cell cycle arrest in early postimplantation rodent embryos, we have shown that phosphoramide mustard, the major teratogenic metabolite of 4CP, induces alterations in the cell cycle in postimplantation rat embryos (Little and Mirkes, 1992; Mirkes et al., 1989). Together, these results demonstrate that 4CP induces alterations in the cell cycle in early postimplantation mouse embryos exposed in vitro or in vivo.

To examine the role of p53 in normal and abnormal embryo at the molecular level, we next identified genes and miRNAs whose expressions were modified by deletion of *p53* in day 8.5 mouse embryos. Our data show that deletion of both *p53* alleles results in a significant alteration in the mRNA expression of 388 genes. Among the significantly altered genes during the neural tube closure, the majority (77%) are down-regulated in the absence of *p53* and only 11% of the disregulated genes contain putative p53 DNA-binding sites. Thus, our data suggest that not only the loss of *p53* gene itself and the alteration of expression of p53 target genes but also alteration of "downstream" genes could well contribute to the mechanisms of NTDs.

Interestingly, in *p53* -/- embryos the expression of several known p53 target genes, for example, *Mdm2, Noxa*, and *Puma*, was not altered, whereas a few p53 target genes such as *p21* and *cyclin G1* showed decreased expression, indicating that even in normal day 8.5 developing embryos *p21* and *cyclin G1* are activated and regulated by p53. This also suggests that even in day 8.5 embryos without any stress, p53 is activated to regulate specific target genes. Published research has shown that although a high level of inactive p53 protein can be observed during embryogenesis, active p53 protein is observed in the developing nervous system (Gottlieb et al., 1997; Komarova et al.,

1997; MacCallum et al., 1996). Taken together, it is possible that these cell cycle arrest related genes, *p21* and *cyclin G1*, are activated by p53 mainly in the nervous system, where cell cycle arrest is important for the control of cell numbers at the time of neural tube closure (Copp et al., 2003). In contrast, expression of pro-apoptotic genes such as *Noxa* and *Puma* are unchanged, yet these genes are expressed in *p53* -/- embryos, suggesting that at this embryonic stage, these p53 target genes are not regulated by p53 but could be regulated by transcription factors other than p53. Our data collectively suggest that at the time of neural tube closure, p53 tightly controls its specific transcriptional target genes to activate particular pathways.

To assess the possible contribution of the altered genes to the induction of NTDs , we identified by microarray expression profiling six genes (*Csk, Itga3, Jarid2, Prkaca, Rarg, and Sall4*) known to cause NTDs when they are deleted, that were significantly down-regulated in *p53* -/- embryos. RT-PCR analysis to confirm the expression levels of these six genes showed decreases in expression; however, these differences were not statistically significant. Nonetheless, our microarray and RT-PCR analysis suggest that these six genes may contribute to the NTDs observed in *p53* -/- embryos. Additional research is necessary to determine whether any or all of these genes play a causal role in the etiology of NTDs in *p53* -/- embryos. Our microarray analysis also identified other genes that may contribute to NTDs in *p53* -/- embryos including cell cycle-related genes, retinoid-related genes, axon guidance-related genes, and ATM-related genes.

Although it is well known that mRNA transcription and post-transcriptional processing are key determinants in the regulation of gene expression, miRNAs have also been shown to regulate gene expression in mammals, with about 800 miRNAs currently predicted to exist in humans (Bartel, 2004; Bentwich et al., 2005). Recent studies have suggested that miRNAs play important regulatory roles in developmental timing and patterning, cellular differentiation, proliferation, organogenesis, and apoptosis (Alvarez-Garcia and Miska, 2005; Ambros, 2004). In addition, studies have identified p53 as one of the gene that regulates particular miRNAs, *miR-34* family in response to DNA damage and oncogenic stress (Bommer et al., 2007; Chang et al., 2007; Raver-Shapira et

al., 2007; Tarasov et al., 2007). To investigate whether miRNAs are expressed in early organogenesis-stage mouse embryos, whether the expression of miRNAs is altered by deletion of *p53* alleles, and whether these changes could contribute to NTDs observed in *p53* -/- embryos, we quantified the expression levels of 368 miRNAs. Our data showed that in day 8.5 mouse embryos three miRNAs, *miR-1, miR-142-3p,* and *miR-301* showed statistically significant decrease, and two miRNAs, *miR-30e-3p* and *miR-331,* showed statistically significant increase in their expression in *p53* -/- embryos compared to *p53* +/+ embryos, indicating that these miRNAs might be regulated by *p53*. The target gene analysis using both our stepwise regression and two publicly available computational tools, PicTar and miRBase, further identified putative target genes of these miRNAs that are supported by two completely independent approaches. Further study and a better understanding of the function of miRNAs and how they regulate genes are necessary to understand the roles of miRNAs in the etiology of NTDs and development.

The target gene analysis using both our stepwise regression and two publicly available computational tools, PicTar and miRBase, further identified putative target genes of these miRNAs that are supported by two completely independent approaches. R*etinoic acid receptor gamma* (*Rarg*), which is a known direct putative target gene of *p53* that was significantly down-regulated in *p53* -/- embryos, was also shown to be regulated by *miR-331*, which showed increased expression in *p53* -/- embryos. This indicates that *p53* may synergistically regulate *Rarg* with *miR-331*, where the deletion of *p53* alleles directly up-regulates the expression of *miR-331* and down-regulates *Rarg*, as well as the up-regulation of *miR-331* contributing further down-regulation of *Rarg*.

Finally, to further investigate the role of p53 and p21 in teratogen-induced birth defects, the incidence of NTDs was investigated in *p53* and *p21* null mice exposed to HS. In the absence of HS, we observed that 11% of *p53* -/- develop NTDs, i.e., exencephaly or spina bifida. This correlates with our earlier finding that deletion of both *p53* alleles results in a significant alteration of embryonic gene expression. Following the HS exposure, we observed an increased incidence of exencephaly in all p53 genotypes and in both genders; however, females in all genotypes and *p53* -/- fetuses

were especially susceptible (47% vs. 16% in +/- and 17% in +/+ fetuses). The predominance of fetuses with exencephaly in females has been previously reported, although the biological origins of the predominance of NTDs in females are unclear (Armstrong et al., 1995; Sah et al., 1995). Interestingly, all of the fetuses with spina bifida cases were also females. Together, our results suggest that p53 functions as a suppressor of HS-induced exencephaly**.** Whether p53 plays a role as a teratogen suppressor or inducer has been controversial, although the role of p53 as a suppressor has been more favored based on the protective role of p53 following a variety of environmental stresses (Choi and Donehower, 1999). Recent studies have shown that p53 functions as a teratological inducer (Boreham et al., 2002; Narai et al., 2006; Wang, 2001), as well as a teratogen suppressor (Baatout et al., 2002; Bekaert et al., 2005; Moallem and Hales, 1998). Examination of this literature suggests that the role of p53 differs by the type of teratogen as well as the type of malformations evaluated. Studies that have identified p53 as a teratological inducer have investigated specific malformations such as eye defects, limb malformations, and cleft palate (Boreham et al., 2002; Narai et al., 2006; Wang, 2001; Wubah et al., 1996), whereas most studies that have identified p53 as a teratogen suppressor have examined multiple types of malformations as well as resorptions (Baatout et al., 2002; Bekaert et al., 2005; Nicol et al., 1995; Norimura et al., 1996). In addition, p53 was a teratological inducer of limb malformations following irradiation (Boreham et al., 2002; Wang, 2001), whereas it was a teratological suppressor of limb malformations following exposure to 4CP (Moallem and Hales, 1998).

To investigate whether the deletion of p53 leads to the alteration of apoptosis in embryos exposed to HS, we examined the induction of apoptosis using caspase-3 activity assay in *p53* +/- mouse embryos exposed to HS. Our very preliminary data have indicated that a decreased level of apoptosis was observed in p53 +/- embryos compared to that in p53 +/+ embryos after HS treatment. These preliminary results suggest that although the induction of teratogen-induced apoptosis is p53-dependent, the

induction of teratogen-induced apoptosis appears not to be causally linked to the increased incidence of NTDs.

Although *p21* -/- mice develop normally, an increased incidence of exencephaly is observed following the exposure to HS. Our earlier findings have shown that the expression of both p21 mRNA and protein are upregulated in HS-treated day 8.5 embryos, suggesting that cell cycle arrest induced by p21 may play an important role in the etiology of HS-induced malformations. Taken together, our results indicate that p21 also acts as a teratogen suppressor and that the activation of cell cycle arrest induced by p21 may partially account for the HS-induced exencephaly observed in *p53* -/- fetuses.

Overall, the studies presented in this thesis showed that in normal embryo development p53 tightly regulates and controls cell cycle-related genes at the time of neural tube closure. In embryos exposed to teratogens, p53 activates both cell cycle arrest and apoptosis and acts as a teratogen suppressor. However, our results also suggested that the activation of mitochondrial apoptotic pathway may not be causally linked to the mechanisms of teratogen-induced NTDs. Published studies and the studies presented here using *p53* and *p21* null mice suggest that both too much or too little apoptosis and cell cycle arrest lead to abnormalities and that precise control of these biological pathways are critical for normal neural tube development and the prevention of teratogen-induced NTDs.

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