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Insulin-like Growth Factor II (IGF2) Gene of Zebrafish and its Use as a Biogenetic Marker for the Essay of Epigenetic Toxins in Aquatic Environment

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Insulin-like Growth Factor-II (*IGF2*) Gene of Zebrafish and its Use as a Biogenetic Marker for the Assay of Epigenetic Toxin Exposure

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Your file Votre référence
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To my mother and father (God bless his soul)

who have given me unconditional love

and endless support

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ABSTRACT

Altered DNA methylation may play a key role in a variety of chemical induced toxicities. 5-azacytidine (5-azaC) and sodium arsenite ($NaAsO_2$) are examples of chemicals that cause alteration in epigenetic mechanisms such as DNA methylation. Epigenetic refers to modifications in gene expression that are controlled by heritable but potentially reversible changes in DNA methylation and/or chromatin structure. Processes such as DNA methylation and histone deacetylation influence the transcriptional activity of genes. The purpose of this research was to determine whether expression analysis of the *IGF2* gene in zebrafish, a gene whose transcription is known to be regulated by DNA methylation in mammals, can be used as a marker or indicator of DNA methylation due to toxin exposure in fish embryos. We examined the expression of *IGF2*, *IGF1*, and *IGFBP-1* in zebrafish embryos treated with sodium arsenite (thought to inhibit DNA methylation), nickel chloride (thought to cause DNA hypermethylation), trichostatin A (a histone deacetylase inhibitor), 5-azaC (thought to cause methyltransferase inhibition), cadmium chloride (thought to cause DNA hypermethylation) and mercury chloride (unknown). The treatment of zebrafish embryos with 5-azaC and sodium arsenite resulted in the transcriptional up-regulation of the IGF_2 gene in 8-hour zebrafish embryos, while *IGF1* and *IGFBP-1* expression

remained normal. Treatment of zebrafish embryos with $NiCl_2$, $CdCl_2$, TSA and $HgCl_2$ had no effect on the expression of all three genes. Thus, we can conclude that *IGF2* gene expression may provide a suitable marker for assaying the presence of a subset of epigenetic toxins in aquatic environments.

RÉSUMÉ

La méthylation modifieé d'ADN pourrait jouer un rôle-clé dans une variété de toxicités induites par des produits chimiques. Le 5-azacytidine et l'arsenite de sodium sont des exemples de produits chimiques qui causent un changement dans les mécanismes épigénétiques tels que la méthylation d'ADN. L'épigénétique se rapporte aux modifications dans l'expression de gène qui sont commandées par les changements héritables mais potentiellement réversibles de la méthylation d'ADN et/ou de la structure de chromatine. Les processus tels que la méthylation d'ADN et la déacétylation d'histone influencent l'activité de transcription des gènes. Le but de cette recherche était de déterminer si l'analyse d'expression du gène *IGF2* dans le poisson zèbre, un gène dont la transcription est connue pour être réglée par la méthylation d'ADN dans les mammifères, peut être employée comme marqueur ou indicateur des toxines de méthylation d'ADN dans les embryons de poissons. Nous avons examiné l'expression d'*IGF2*, d'*IGF1*, et d'*IGFBP-1* sur les embryons de poisson zèbre traités avec l'arsenite de sodium (empêche la méthylation d'ADN), le chlorure de nickel (cause l'hyperméthylation d'ADN), le Trichostatin A (un inhibiteur de déacétylase d'histone), le 5-azaC (cause l'inhibition de métase), le chlorure de cadmium (cause l'hyperméthylation d'ADN) et le chlorure de mercure (inconnu). Le

traitement des embryons de poisson zèbre avec le 5-azaC et l'arsenite de sodium a eu comme effet la haute-règulation de la transcription du gène *IGF2* dans les embryons de poisson zèbre agès de 8 heures, alors que les expressions *IGF1* et *IGFBP-1* demeuraient normales. Le traitement des embryons de poisson zèbre avec le chlorure de nickel, le chlorure de cadmium, le Trichostatin A et le chlorure de mercure n'a eu aucun effet sur l'expression de chacun des trois gènes. Ainsi, nous concluons que le gène *IGF2* peut fournir un marqueur approprié pour analyser la présence des toxines épigénétiques dans les environnements aquatiques.

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10T1/2	Mice embryonic cells
5-azaC	5-azacytidine
5-azac-Cdr	5-azac-2 deoxycytidine
A454 (9)	Lung cell lines
A549	Human lung cancer cell line
AP-1	Activating Protein-1
ATR-X Syndrome	X-linked mental retardation facial dysmorphism
bp	Base pairs
C57BL/6J	Wild type of mice
$CdCl_2$	Cadmium Chloride
cDNA	Complementary DNA
C-FOS	Proto-Oncogene
CH_3	Methyl group
Cmyc	Proto-Oncogene
CpG	Cytosine-Phosphate-Guanine
C-tun	Proto-Oncogene
DEPC	Diethylpyrocarbonate
Dnmts	DNA methyltransferases
dNTP	Deoxyribonucleotide Triphosphate
E	EcoR I
EDTA	Ethylenediamine Tetraacetic Acid
Egr-1	Early growth response 1
g	Gram
G12	G protein
Gadd 153	Growth Arrest and DNA damage inducible 153
Gadd 45	Growth Arrest and DNA damage inducible 45
gpt	Guanine phosphoribosyl transferase locus in transgenic Chinese hamster cell line
H	Hpa II
Ha-ras	Oncogenic gene
HDACs	Histone deacetylases
HEK293	Human embryonal kidney cell line
$HgCl_2$	Mercury Chloride
$HIPK_3$	Homeodomain-interacting protein kinase 3
hpf	Hour post fertilization
HSP70	Heat-shock protein

ICF Syndrome	Immunodeficiency centromere instability and facial anomalies
IGF1	Insulin like growth factor-1
IGF2	Insulin like growth factor-2
IGFBP-1	Insulin like growth factor binding protein-1
L	Litre
m	Milli
μ	Micro
M	Molarity
M	Msp I
M.SSI DNA MTase	Mammalian DNA methyltransferase
MBDs	Methyl-CPG binding domain proteins
$Mecp_2$	Methylcytosine binding protein
$MgCl_2$	Magnesium Chloride
MMLV-RT	Moloney murine leukemia virus-reverse transcriptase
MOPS	N-Morpholino Propane-Sulfonic acid
MT	Metallothionein
MTase	Methyltransferase
n	Nano
$NaAsO_2$	Sodium Arsenite
NF-KB	Nuclear factor Kappa B
$NiCl_2$	Nickel Chloride
P^{16}	Tumor suppressor gene
P^{53}	Tumor suppressor gene, crucial gene in human cancer (named for the molecular mass of its protein product)
PAGE	Polyacrylamide gel electrophoresis
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAM	S-adenosylmethionine
SDS	Sodium Dodecyle Sulfate
TA97	Salmonella typhimurium strain
Tris	Tris-hydoxymethylaminomethane
TRL 1215	Rat liver cells
TSA	Trichostatin A
UOK	Human kidney cell line
URA3	Telomeric marker gene
X $Dnmt_1$	**Xenopus DNA methyltransferase**

Insulin-like Growth Factor-II (*IGF2*) Gene of Zebrafish and its Use as a Biogenetic Marker for the Assay of Epigenetic Toxin Exposure

1.0 Introduction

Toxic metals are now known to contaminate air, water and soil ecosystems inhibiting growth and reproduction of microorganisms, plants and animals including humans. Concern has centered on commercially used metals. They are arsenic, cadmium, nickel, mercury, barium, chromium, lead, manganese, copper, zinc, beryllium, selenium, silver, and vanadium. Such metals are not degradable like most organic substances and accumulate in the environment. These metals are the so-called heavy metals. Conventional toxicity tests of such metals are well documented (reviewed in Buhler, 1973; Clark, 2001). Arsenic for example, has long been used as an instrument of murder and suicide. Lead, cadmium, mercury, arsenic, antimony and beryllium have all caused accidental deaths in industry (Buhler, 1973; Clark, 2001).

Concern over the metals has developed because of the following reasons (Buhler, 1973; Clark, 2001):

1) These elements are widely distributed throughout the environment.

2) They do not degrade and persist in nature for extended periods of time.
3) They are toxic to living organisms at low concentrations.
4) Some of them are biologically magnify or accumulate in plants and animals such as methyl mercury but some do not like copper.
5) Metals, such as mercury can be converted to the more toxic form of methyl mercury in the environment.
6) More recently, a wide variety of chemical agents (heavy metals) have been shown to have the potential to produce adverse affects by causing heritable changes to the genome, resulting in alterations in phenotype. For example, altered DNA methylation may play a key role in a variety of chemical induced toxicities (Watson and Goodman, 2002) expressed at the level of gene expression.

A number of compounds and heavy metals have been identified to affect normal DNA chromatin structure by altering DNA methylation or histone acetylation. Treatment with nickel, cadmium, mercury can affect epigenetic processes such as DNA methylation and histone acetylation and can lead to abnormal gene expression. In Chinese hamster embryo cells, exposure to nickel (Klein et al., 1997; Costa et al., 1999) can lead to decondensation of facultative heterochromatin and generates deletions that

are often as large as the entire long arm of the X-chromosome. Takiguchi et al., (2003) found to be an effective inhibitor of DNA Mtase when TRL 1215 rat liver cells and M.SSI DNA Mtase cell cultures were exposed to cadmium. In rodent cell tissue cultures exposed to mercury ($HgCl_2$), the metal ions were observed to concentrate in cell nuclei and to associate with chromatin (Rodgers et al., 2001). Likewise, 5-azacytidine (5-azaC) and sodium arsenite can cause alteration in epigenetic mechanisms such as DNA methylation (Gradecka et al., 2001; Jones, 1984). Furthermore, the *IGF2* gene in mammals is at least partially regulated by DNA methylation (Wutz et al., 1998). Thus, exposure of zebrafish embryos to epigenetic toxins may induce change in *IGF2* transcription.

The purpose of this study is to determine if the expression of genes *IGF2, IGF1* and *IGFBP-1* can be used as markers for toxins that affect DNA methylation in the zebrafish (*Danio rerio*). We exposed zebrafish embryos to $NaAsO_2$, $NiCl_2$, $HgCl_2$, and $CdCl_2$ as well as to chemicals known to alter DNA methylation and histone acetylation. These are 5-azaC and TSA (Jones, 1984; Yoshida et al., 1990). In this way we could test the hypothesis that treatment of zebrafish embryos with epigenetic toxins will cause abnormal expression of genes regulated by DNA methylation.

1.1 What is the DNA Methylation?

DNA methylation is the most ubiquitous form of DNA modification. It is clonally heritable, reversible, and thus satisfies the major requirements for an epigenetic mechanism that can control gene expression (Cedar, 1988). Epigenetic mechanisms are defined as heritable phenomena that regulate gene expression without involving changes to the DNA sequence (Pennisi, 2001). Abnormal DNA methylation can result in human diseases such as cancer as well as inherited disorders of DNA such as Rett syndrome that results from mutations in the methyl-cytosine binding protein-2 (*Mecp*2) (Amir et al., 1999). ICF syndrome (immunodeficiency centromere instability and facial anomalies) is caused by mutations in the gene DNA methyltransferase 3b (Maraschio et al., 1988). A third inherited condition associated with dysregulation of DNA methylation is the ATR-X syndrome. This syndrome is characterized by severe X-linked mental retardation and facial dysmorphism (Gibbons et al., 1995). Finally, fragile X-syndrome (common form of inherited mental retardation) is caused by epigenetic changes occurring on the X-chromosome of mammals (Verkerk et al., 1991).

DNA methylation has been found in many groups of evolutionary diverse organisms such as bacteria, plants, vertebrates, and a number of invertebrates. For instance, in bacteria, methylation occurs on both adenine

and cytosine residues and it primarily functions in the host's immune response against virus infections via the restriction modification system (Reviewed in Hubacek, 1992). Methylation of adenine is also involved in regulating DNA replication and targeting the correction of replication errors (Noyer-Weidner and Trautner, 1993). In plants, DNA methylation has been shown to be implicated in the development of the ovule. Furthermore, the genome of plants is even more methylated than in vertebrates. In vertebrates, approximately 60 % of all CpG dinucleotides are methylated (Jaenisch et al., 1995) and 5-methylcytosine represents approximately 1 % of nucleotide bases (Bestor and Verdine, 1994). In invertebrates like *Drosophila*, which was long thought to lack methylation, the genome has recently been shown to have low levels of 5-methylcytosine (Lyko, 2001).

In general, high levels of DNA methylation are associated with transcriptional repression. DNA methylation involves the addition of a methyl group to the 5-position of the cytosine base in DNA. DNA methyltransferase enzymes catalyze the transfer of the methyl group (CH_3) from S-adenosylmethionine (SAM) to deoxycytosine, producing S-deoxymethylcytosine and 5-adenosylhomocytosine (SAH) (Attwood et al., 2002). The repression of transcription by DNA methylation can occur through transcriptional repressive complexes, such as DNA

methyltransferase (*Dnmts*), methyl-CPG binding domain proteins (MBDs), and histone deacetylases (*HDACs*). *Dnmt1* has a principle function of maintaining already established methylation sites. *Dnmt3a* and *Dnmt3b* which is expressed at high levels in embryonic stem cells are believed to be responsible for *de novo* methylation (Attwood et al., 2002). Mutant mice that are deficient in DNA Mtase activity (*Dnmt1*) fail to develop to term and die at mid-gestation with a complex phenotype that includes mesodermal defects and high levels of apoptosis (Li et al., 1992). Loss of the maintenance methyltransferase *xDnmt1* triggers an apoptotic response during *Xenopus* development, which accounts for the loss of specific cell populations in the hypomethylated embryos (Stancheva et al., 2001).

The function of DNA methylation is permanent gene suppression mediated through the modification of CpG islands located in the promoter region of genes (Attwood et al., 2002). The methylation of cytosine can also form a mutation due to the hydrolytic deamination of m5C to thymine. The mutational rate of a methylated cytosine is 4 times greater than an unmethylated DNA (Duncan, 1980). There is also evidence of active demethylation. Thus, the pattern of methylation is maintained by a dynamic balance of methylation and demethylation activities and the local state of histone acetylation; therefore, this provides a simple mechanism to explain

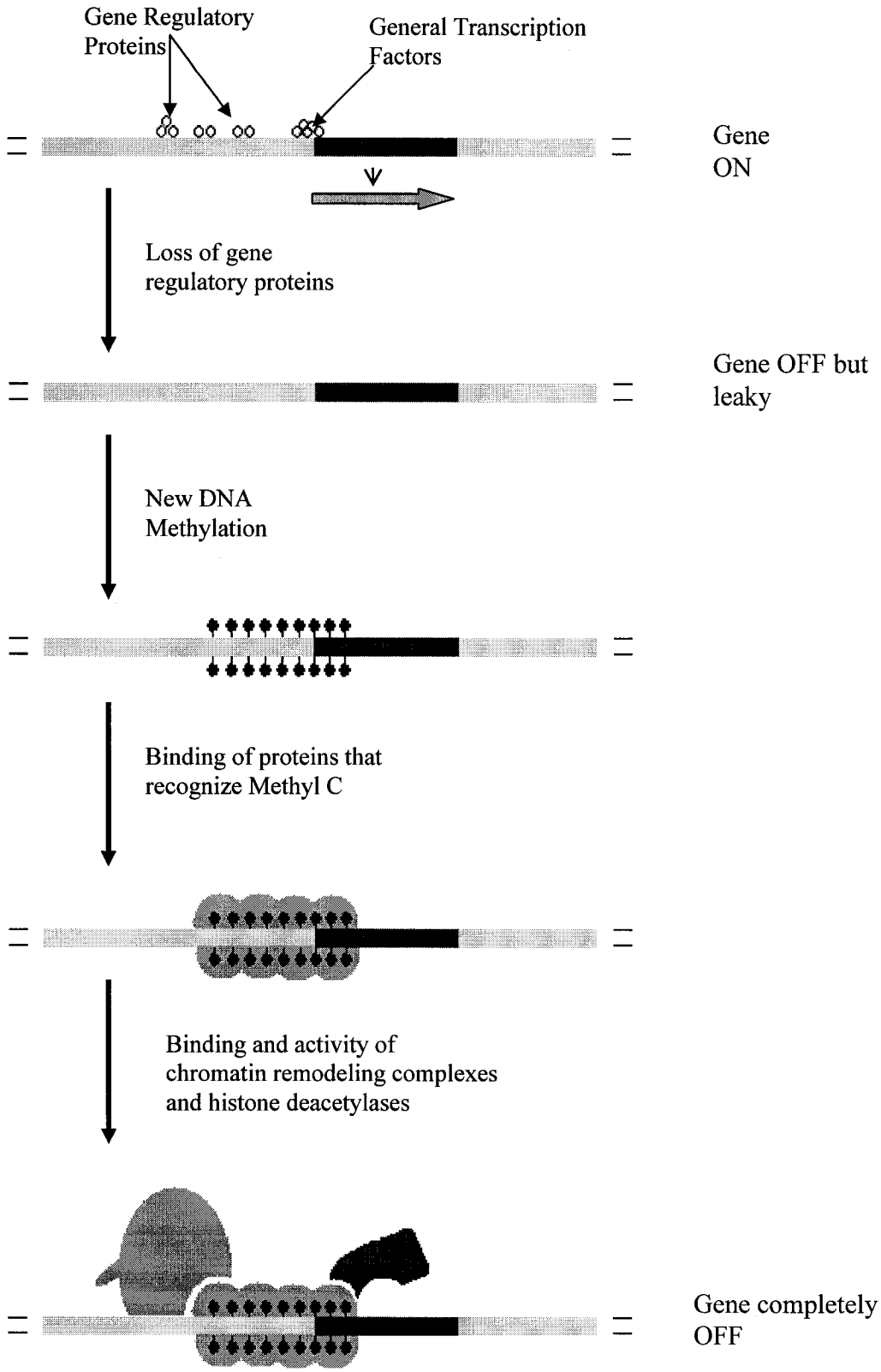
the reason active genes are not methylated (Cervoni and Szyf, 2001). In cancer cells, DNA hypomethylation is observed despite high levels of DNA Mtase. One possible explanation for this observation is that cancer cells express high levels of a demethylase that actively removes methyl groups from DNA (Szyf, 1994). For instance, Cervoni and Szyf, (2001) extracted DNA methylase activity from a human lung cancer cell line A549 (9) and showed that a human embryonal kidney cell line HEK293 processes active demethylase. Induction of DNA demethylase activity is required to accommodate the high levels of DNA Mtase, which override the normal growth inhibitory signals (Szyf, 1998).

The hypothesis that DNA methylation is involved in gene regulation was first proposed in the 1970s (Holliday, 1975). Many studies have shown a correlation between increased gene expression and decreased DNA methylation (Sullivan et al., 1989; Burbelo et al., 1990). Increases in DNA methylation are associated with many cases of gene silencing, and reductions in DNA methylation are often associated with gene activation (Cedar, 1988). DNA methylation contributes to the expression of a variety of genes including oncogenes (Compere et al., 1981) and *IGF2* (Wutz etal., 1998). There are two types of experimental approaches that have been used to test the effect of DNA methylation on gene activity in a cell. One

approach is to insert methylated DNA into animal cells either by DNA-mediated gene transfer or by injection techniques in order to observe the effect of the modification on the transcription. A second approach is to cause DNA hypomethylation by treatment of cells with 5-azacytidine (5-azaC) and to follow the effects of this treatment on specific gene expression. The results of both methods support the model that DNA methylation can inhibit gene expression (Razin, 1984). For instance, the promoter of most alleles on the inactive X-chromosome of mammals is methylated while the same sequences on the active X-chromosome are unmethylated (Rastan, 1994).

DNA methylation may suppress gene transcription in two general ways. First, methylated CG dinucleotides may directly interfere with the binding of transcription factors to their recognition sequences, presumably by protruding into the DNA's major groove (Attwood and Richardson, 2002). Second, regions of methylated DNA adjacent to the promoter sites of genes may recruit methylcytosine binding proteins that in turn associate with large protein complexes containing co-repressors and histone deacetylase (Figure 1). The binding of these complexes to DNA leads to a change in the

Figure 1: How DNA methylation regulates gene transcription.
The binding of gene regulatory proteins and the general transcription machinery near an active promoter may prevent DNA methylation by excluding *de novo* methylases. However, if most of these proteins dissociate from the DNA, as generally occurs when a cell no longer produces the required activator proteins, the DNA becomes methylated which enables other proteins to bind and these shut down the gene completely by further altering chromatin structure – (from Alberts et al., 2002).


Gene Regulatory Proteins
General Transcription Factors
Gene ON
Loss of gene regulatory proteins
Gene OFF but leaky
New DNA Methylation
Binding of proteins that recognize Methyl C
Binding and activity of chromatin remodeling complexes and histone deacetylases
Gene completely OFF

chromatin structure from an active to an inactive form. The best-characterized methylcytosine binding protein is Mecp2 (Lewis et al., 1992). Further studies were performed such as, "tissue-specific expression of chicken β globin" (McGhee et al., 1979), "Ovalbumin genes" (Mandel et al., 1979) as well as "The human β globin locus" (Vander Ploeg, 1980). The previous three studies demonstrated a positive correlation between hypomethylation and gene expression in different tissues.

1.2 DNA Methylation and Embryonic Development

DNA methylation has been shown to be essential for normal development in mice (Walsh and Bestor, 1999), zebrafish (Martin et al., 1999) and *Xenopus* (Stancheva and Meehan, 2000). The best understanding of DNA methylation during embryogenesis has been studied in mammals. In mammals, patterns of DNA methylation of cytosine residues are believed to have been established during gametogenesis and during early embryogenesis; they play an important role in gene regulation and normal embryonic development (Monk et al., 1987) and have also been implicated in genomic imprinting. Genomic imprinting is defined as an epigenetic change leading to differential expression of the two parental alleles in somatic cells (Li et al., 1993). DNA methylation in mammals modulates expression of many genes during development, causing major changes in or

important fine-tuning of expression (Ehrlich, 2003). Some of the most studied examples of these genes include *MyoD* (Brunk et al., 1996), *Xist* (Ariel et al., 1995), and the imprinted genes such as *IGF2*, *IGF2r* and *H19*, which are differentially methylated depending on parent-of-origin (Efstratiadis, 1994; Reik and Allen, 1994). DNA methylation is crucial for mammalian development because embryos that can not maintain normal methylation levels die after gastrulation (Jaenisch, 1997). Li et al. (1992) showed that mutant mice, deficient in *Dnmt1* activity fail to develop to term and die at mid-gestation with a complex phenotype that includes mesodermal defects and high levels of apoptosis. In mice, after fertilization, the male pronucleus is rapidly demethylated (Oswald et al., 2000; Mayer et al., 2000). In contrast, the maternal genome initially maintains its methylation status, then progressively drops until the 8-cell stage when the methylation content appears to be equivalent between the maternal and paternal genome. The level of methylation decreases to about 15 % in the blastocysts but returns to higher levels during implantation (Reik et al., 2001).

During the early development of placental mammals, a dramatic reduction in methylation levels occurs in the pre-implantation embryo (Monk et al., 1987). DNA methylation levels are high in mature gametes but

are eliminated during subsequent cleavage, and increased again after implantation. During cleavage, a wave of genome wide demethylation removes the epigenetic modification present in the zygote so that the DNA of the blastocyst is almost totally devoid of methylation (Monk et al., 1987; Kafri et al., 1992; Razin et al., 1995). The higher methylation level seen in the zygote is set by *de novo* methylation of the gametes DNA during gametogenesis. Thus, during development and gametogenesis, distinct phases of demethylation, *de novo* methylation and maintenance methylation alternate. Overall, the sperm genome is markedly more methylated than that of the oocyte (Jaenisch, 1997). Between implantation and gastrulation, a wave of global *de novo* methylation occurs. This is maintained throughout life in the somatic cells of the animal (Monk et al., 1987; Kafri et al., 1992; Razin et al., 1995). During differentiation of the somatic tissues, a subset of tissue-specific genes becomes demethylated (Soriano et al., 1986).

Stancheva and Meehan, (2000) injected anti-sense *XDnmt1* RNA into *Xenopus* embryos and they observed significant developmental abnormalities and increased levels of apoptosis in these embryos. This experiment helped to illustrate that maternal methyltranferase is very important for early development. In addition, there have been many studies in a number of animals that show the role of DNA methylation during

development. For example, some studies showed highest nuclear methyltransferase activity in the blastula stage of *Xenopus* and sea urchin (Adams et al., 1981). In chick embryos, the activity of methyltransferase is high in the early embryo while DNA methylation remains constant throughout development (Tanaka et al., 1980). DNA methylation patterns are also dynamic during amphibian development. In frogs, DNA methylation levels are initially high in fertilized embryos but drop by about 40 % in late blastula embryos and subsequently recover during later gastrula stages. As a result, the *Xenopus* genome has its lowest methylation content at the midblastula transition (Stancheva and Meehan, 2000; Stancheva et al., 2002). All of these examples illustrate that DNA methylation plays an important epigenetic role in the development of a wide range of organisms.

1.3 DNA Methylation in Zebrafish

The zebrafish, *Danio rerio*, is becoming increasingly popular as a model system for the analysis of vertebrate development and genetics. External fertilization, large reproductive potential and optically clear embryos give the zebrafish a number of distinct advantages over other organisms such as the mouse. The zebrafish embryos can be genetically modified through microinjection of nucleic acid or the biochemical

metabolism can be altered by the addition of chemicals to their water (Shin and Fishman, 2002). The establishment of genetic methods, for example large scale mutagenesis, has identified mutations that affect the development of zebrafish (Streisinger et al., 1981; Solnica et al., 1994). The fact that the genome sequence of zebrafish was recently released makes it a very useful model system for vertebrate genetics. Presently, there are zebrafish EST databases available that contain more than 90 000 cDNA clones (Shin and Fishman, 2002). The zebrafish genome is approximately 1700Mb in size, and has 25 chromosomes. The basis for all of the maps of the genome consists of 25 linkage groups on which the 25 centromeres have been placed (Johnson et al., 1996). Although DNA methylation has been documented for mammals, there is little known about methylation in the zebrafish. Virtually the only information in the literature concerning this important area of developmental research on these fish is the work on the effect of parental origin on the methylation of a transgene locus (Martin and McGowan 1995a, 1995b).

The loss of methylation from the mouse embryo may facilitate the early activation of zygotic transcription. However, in zebrafish there is a less pronounced genome demethylation occurring at the blastula stage. Thus, zebrafish may not require input from the zygotic genome until later and

instead rely on maternal factors derived from the oocyte. In the meantime, the silent embryonic genome remains methylated (Mcleod and Bird, 1999). The full-length cDNA sequence corresponding to the zebrafish DNA (cytosine-5) methyltransferase gene (*Dnmt1*) is 4907 base pairs long and has an open reading frame predicted to encode a 1499 amino acid protein. This is similar in size and sequence to a number of other methyltransferases identified in other organisms (Mhanni et al, 2001). DNA methyltransferase (Mtase) ubiquitously present at high levels in early embryos of zebrafish with slight decreases occurring during blastula stage (Martin et al, 1999).

The study of DNA methylation in this developmental model organism (zebrafish) may increase knowledge of the role of DNA methylation in other organisms. McGowan and Martin in 1997 examined the inheritance of a transgene locus in the zebrafish. They demonstrated that its methylation was affected by the sex of the parent contributing the allele. This parent-of-origin effect on the zebrafish transgene appears to be identical to imprinting in mammals. The identification of a parent-of-origin specific effect on the methylation of a transgene in fish which illustrates that genomic imprinting like phenomenon exists in zebrafish (McGowan and Martin, 1997). Medaka, a fish similar to zebrafish, has more than 90 % of its genomic DNA methylated at CCGG sites (Walter et al., 2002).

1.4 Putative Genetic Markers for Epigenetic Change

*IGF*s are very important molecules in virtually every tissue of mammals and in every physiological system. *IGF*s play an important role in regulating cell proliferation, differentiation, apoptosis, and transformation (Jones et al., 1995) *IGF*s including *IGF1* and *IGF2* are major fetal growth regulators. The importance of *IGF*s in fetal growth is underscored by the significantly reduced fetal size and birth weights of the *IGF1* and *IGF2* knockout mice (Barker et al., 1993). The biological effects of *IGF*s are dependent upon their interactions with specific receptors on the cell surface. In addition to the two types of *IGF* receptors, six secreted, high affinity *IGF* binding proteins (*IGFBPs*), designated *IGFBP-1* to *IGFBP-6* , have been isolated and characterized in humans and other mammals (Maures et al., 2002). In all mammal species studied to date, the expression of the *IGFBP-1* gene is highly tissue specific, being predominantly expressed in the liver (Lee et al., 1997). Recent studies suggest that *IGFBP-1* may play an important role in modulating fetal growth in response to altered nutritional and environmental conditions such as fetal hypoxia, a leading cause of human intrauterine growth restriction (Lee et al., 1997).

Up-regulation of the activity of *IGF2* promoter P_3 has previously shown to be tightly correlated with demethylation in hepatoblastoma (Therèse et al., 2001). Another feature of *IGF2* gene transcription is genomic imprinting in which the expression of a gene is associated with a particular parental allele, DNA methylation (Wutz et al., 1998), histone acetylation (Hu et al., 1998), and use of specific promoter sites (Vuth, 1994) i.e., that DNA methylation governs the genomic imprinting of *IGF2* (Hu et al., 1996). The genomic imprinting of *IGF2* is an epigenetic, reversible process (Hu et al., 1995). Differential DNA methylation has been proposed as the best candidate for epigenetic marking on the parental alleles. Studies in mice have demonstrated a dynamic pattern of DNA methylation during embryogenesis (Brandies et al., 1993). More direct and convincing evidence stems from studies that examined mice that are DNA methyltransferase-deficient (Li and Jaenisch, 1993). When the expression of *Dnmt1* was interrupted by gene targeting, there was no expression of *IGF2* from the paternal allele, indicating the importance of DNA methylation in maintaining *IGF2* expression and imprinting (Hu, 1996). One role for DNA methylation on *IGF2* expression during normal development is to function as an imprinting marker making the two parental alleles distinguishable from each other. This was shown in a mouse embryonic cell line, where *IGF2*

could be reactivated after treatment with the methylation inhibitor 5-azaC-2deoxycytidine (Eversole et al., 1993). It was subsequently shown that methyltransferase activity inhibition, through treatment of cells with 5-azaC, also led to biallelic expression of *IGF2* (Hu et al., 1996). Hu et al. (1996) treated two human astrocyte cells (HBR440 and HBR441) and mouse astrocyte cells with 5-azaC. They found that the DNA demethylation reagent resulted in the increased expression of *IGF2*. Altered DNA methylation has been linked to abnormal expression of *IGF2* in Wilm's tumors and Beckwith Wiedeman syndrome (Taniguchi et al., 1995). Therefore, *IGF2* may be a marker of abnormal changes in DNA methylation

1.5 Inhibitors of DNA Methylation and Histone Deacetylation

a) 5-Azacytidine

The nucleoside analog 5-azacytidine (5-azaC) was originally developed for use as a cancer chemotherapeutic agent, and it is still used in the treatment of certain types of acute myelogenous leukemia. However, recent interest in the drug has been directed towards its remarkable ability to induce the expression of repressed genes in eukaryotic cells and to act as an inhibitor of DNA methylation (Jones, 1984). The effect of 5-azaC occurs due to its incorporation into DNA

(Jones and Taylor, 1980) or by interfering with the action of the enzyme methyltranferase (Santi et al., 1983). The hypomethylation of DNA by 5-azaC has previously been demonstrated in mammals Hu et al. (1997); mice (Szyf et al., 1992; Walsh and Bestor, 1999); chicken (Zagris and Podimatas, 1994); zebrafish (Martin et al., 1999) and cell culture systems (Hu et al., 1997). In mammals, *IGF2* is regulated by DNA methylation and treatment of cultured primary human and mouse astrocytes with 5-azaC resulted in DNA hypomethylation and increased levels of *IGF2* expression (Hu et al., 1997; Cedar and Razin, 1990). In mice, embryonic 10T1/2 cells treated with 5-azaC differentiated in muscle cells and apidocytes. The DNA from 5-azaC treated cells was hypomethylated relative to DNA from untreated cells. Similar phenotypic changes and cell differentiation effects are observed in 10T1/2 cells that are transfected with plasmid constructs which express DNA cytosine-5-methyltransferase in the anti-sense orientation (Szyf et al., 1992). These treatments often resulted in cell differentiation and activation of a number of silent genes including genes located on the silent X-chromosome (Grant and Worton, 1989). In chicken, at blastoderm stage, chick embryos treated with 5-azaC developed with a short thickened primitive streak, and an abnormal neural plate (Zagris and Podimatas, 1994).

Many studies provide very strong correlative evidence that the effects of 5-azaC are linked to effects on methylation. For instance, embryonic mouse cells can be induced to change their phenotype after a 5-minute exposure to 5-azaC during the sensitive part of the S-phase (Taylor and Jones, 1982a). Moreover, the drug can alter the DNA structure of the X-chromosome DNA within the hybrids (Venolia et al., 1982). Experiments exposing embryos *in vivo* to 5-azaC have suggested that methylation plays a role in early development. Sea urchin embryos treated prior to the 16 cell stage with 5-azaC showed arrested and abnormal development (Branno et al., 1993). More recently, Martin et al., (1999) determined that hypomethylated zebrafish embryos through treatment with 5-azaC led to abnormal development of the notochord and somites. The axial defects seen in these embryos indicate that methylation is important for the development of proper axial and paraxial mesoderm (Martin et al., 1999).

Additional evidence has been obtained from chick embryos treated during the blastoderm stage with 5-azaC. They developed a short, thickened primitive streak, an abnormal neural plate and expressed larger numbers of polypeptides, than in control embryos (Zagris and Podimatas, 1994). In mammals, rat embryos derived from hypomethylated sperm (via 5-azaC injection of donor males) developed abnormally or did not develop

past the one cell stage while sperm derived from 6-azaC, injected donor males developed normally (Doerksen and Trasler, 1996). Pregnant mice and rats administrating 5-azaC showed a number of malformations including limb and digit abnormalities such as exencephaly, micrognathia, and rib defects (Takeuchi and Takeuchi, 1985; Rosen and House, 1990; Cummings, 1994). 5-azaC also prevented sporangia formation in slime mold *Physarum polycephalum.* This lead Hildebrandt (1986) to propose that methylation was required to repress some genes during that part of the slime mold's life cycle. More recently, another analog similar to 5-azaC, called 5-azaC-2 deoxycytidine (5-azaC-CdR), was used in several preclinical and clinical trials as a DNA methylation inhibitor in attempts to reactivate silenced genes in human cancer (Lubbert, 2000).

b) Trichostatin A

Trichostatin A (TSA), an antifungal antibiotic with cytostatic and differentiating properties in mammalian cell culture, is a potent and specific inhibitor of histone deacetylase (*HDAC*) activity (Yoshida et al., 1990). The TSA inhibition of histone deacetylation in *Xenopus* embryos delays gastrulation, leading to diminished midtrunk and defects in mesoderm formation (Almouzni et al., 1994). Low concentrations of TSA

inhibited starfish embryogenesis at the early gastrula stage before mesenchyme formation (Ikegami et al., 1993).

TSA is currently of major interest for its potential anticancer activity, largely because of its well-documented properties of inhibiting proliferation and inducing apoptosis of tumor cells (Williams, 2001). TSA inhibits hepatoma cell growth *in vitro* at submicromolar concentrations in human beings (Herold et al., 2002). TSA also has a potent antifibrogenic effect on hepatic stellate cells of rat. These cells are the major cellular sources of extracellular matrix proteins in fibrotic livers. Thus, TSA may be a promising compound in the treatment of fibroproliferative diseases (Niki et al., 1999). Collas et al., (1999) proposed that exposure of zebrafish embryos to histone deacetylation inhibitor TSA induces expression of most silent transgenes. Therefore, we treated zebrafish embryos with TSA to investigate alteration of gene expression.

1.6 Putative Epigenetic Toxins

a) Sodium Arsenite

In the environment, arsenic arises from weathering of rock, emissions from smelting of gold, silver, copper, zinc and lead ores, combustion of fossil fuels, and the use of arsenic compounds in agriculture as herbicides

and pesticides. Arsenic is taken up by plants. Arsenic also enters the water system through runoff and atmospheric fallout. Wells drilled through rocks containing arsenic will yield water high in arsenic. Exposure to arsenic has been associated with the occurrence of adverse health effects such as skin, bladder, liver, lung, and prostate tumors as well as peripheral vascular disease and neurological disorders. Rats exposed to arsenic during development present deficits in spontaneous locomotor activity and alterations in spatial learning tasks (Sommers and Mc Manus, 1953; Hu et al., 1988; Chiang et al., 1993; Chiou et al., 1995; Tsuda et al., 1995; Tseng et al., 1996; Smith et al., 1998; Chiou et al., 2001; Rodriguez et al., 2002). In addition, in the six arsenic affected districts of west Bengal, India about 175, 000 people are showing arsenical skin lesions, melanosis, conjunctivitis, keratosis and hyperkeratosis. Cases of gangrene in limbs and malignant neoplasms also have been observed (Das et al., 1995).

Arsenic is known as a human carcinogen (Chen et al., 1985; Wang et al., 1996). It causes the inhibition of DNA repair, disturbance in DNA methylation, oxidative stress induction, enhancement of cellular proliferation, and induction of apoptosis (Gradecka et al., 2001). Many studies have shown that arsenic is a major environmental contaminant in water, food, and air. The guidelines for Canadian drinking water quality

1978 listed the maximum acceptable concentration of arsenic in drinking water as 0.3 μM/L (Health and Welfare Canada 1979a). ACSH (American Council on Science and Health) reported that there is a detrimental health effect in humans from inorganic arsenic in drinking water at the current maximum contaminant level (MCL) of 50 μg/L or below, either in the United States or elsewhere (Brown et al., 2002). Arsenic contamination of water supplies that exceeds 50 μg/L has resulted in a very high incidence of skin lesions and cancers in exposed populations from Taiwan, China, Bangladesh, India, Thailand (Chen et al., 1999), and Central/South America (Smith et al., 2000). In addition, the U.S. Environmental protection Agency (EPA) estimates that over 350 000 people in the U.S. consume drinking water containing over 50 μg/L arsenic – the current USEPA standard (USEPA, 1987). Moreover, arsenic has been found to be above the permissible limit in seven districts of west Bengal, India with concentrations ranging from 200 – 600 μg/L. According to the latest reports, it has been regarded as the biggest arsenic calamity in the world (Das et al., 1995). Also, high concentrations of inorganic arsenic compounds of up to 4400 μg/L have been observed in artesian wells in some provinces of China–Chanxi and Xinjiang (Chen et al., 1999) and in Mongolia - from 1800 μg/L up (Ma et al., 1999). The USEPA (1980b) stated that short-term effect on embryos

and larvae of aquatic vertebrate species have been shown to occur at a concentration as low as 40 μg/L arsenic.

Inorganic arsenic can exist in drinking water in two major forms: a pentavalent form (arsenate) and trivalent form (arsenite), where arsenite is more toxic than arsenate (NRC, 1999). Both valence forms of arsenic undergo enzymatic methylation. Arsenic methylation requires S-adenosylmethionine (SAM) as a cofactor. SAM, as a methyl group donor, is an essential cofactor for a variety of Mtases, including DNA Mtases (Zhao et al., 1997). Arsenic is biotransformed and excreted via the liver by the methylation pathway. When arsenic is high, intracellular SAM reserves may be diverted from their normal intracellular functions such as maintaining methylation of DNA to the methylation of arsenic (Mass and Wang, 1997).

Recent genetic studies have focused on alterations in methylation patterns through the use of arsenic, suggesting that DNA hyper/hypomethylation may lead to altered gene expression, particularly of the P^{53} tumor suppressor gene (Mass and Wang, 1997; Zhao et al., 1997). Chai et al. (1997) has shown that high levels of immunoreactive P^{53} are expressed in Bowen's disease skin biopsies. Both hypomethylation and hypermethylation of DNA were associated with arsenite exposure in human kidney (UOK) and lung A454 (9) cell lines (Zhong and Mass, 2001). Other

studies have shown that arsenic can activate the expression of the genes *c-fos*, *c-myc* and *egr-1*, and of the stress genes *gadd 153* and *gadd 45* (Simeonova et al., 2000). Both acute and chronic exposure of human fibroblast cells to low dose of $NaAsO_2$ significantly affects activating protein-1 (AP-1) and nuclear factor Kappa B (*NF-KB*) DNA binding activity (Hu et al., 2002). In *Xenopus laevis*, Gornati (2002) examined the heat-shock protein (HSP70) following the exposure to $NaAsO_2$ by using the reverse transcriptase polymerase chain reaction (RT-PCR) and found the HSP70 mRNA is strongly induced by $NaAsO_2$. $NaAsO_2$ also induces HSP70 in zebrafish (P. Krone, personal communication). In addition, C57BL/6J male mice administered $NaAsO_2$ via drinking water showed altered hepatic tissue DNA methylation. $NaAsO_2$ increased genomic hypomethylation in a dose dependent manner, and reduced the frequency of methylation at several cytosine sites within the promoter region of the oncogenic gene *Ha-ras* (Okoji et al., 2002). All the above examples suggest that $NaAsO_2$ has the ability to alter gene expression whose regulation may be affected by epigenetic alterations such as DNA methylation. Hence, we will expose zebrafish embryos to $NaAsO_2$ to see if there are any changes in the gene expression.

b) Nickel Chloride

Nickel (Ni) is a potent carcinogen causing lung and nasal cancers in humans (IARC, 1990). The industrial usage of nickel compounds include nickel refining, electroplating, and the production of stainless steel and nickel-cadmium batteries. Furthermore, the combustion of fossil fuels and incineration of nickel-containing solid waste cause the spread of nickel-containing aerosols in the environment as well as in the workplace (IARC, 1990; Doll et al., 1970, 1977). In 1978a, the IJC (International Joint Commission) recommended that the concentration of total nickel in water should not exceed 0.1 μM/L. The effects of nickel, such as cancer of lung and nasal sinus, have occurred in people who have inhaled nickel dust while working in nickel refineries or processing plants of Norway (Anderson et al., 1996). Lung and nasal sinus cancers occurred when the workers were exposed to more than 1mg of nickel/m^3 in the air. These cancers were also found when workers were exposed to 10mg nickel/m^3, such as nickel compounds that were hard to dissolve such as nickel subsulfide (IARC, 1990). Moreover, in the electroplating field, there were 21 workers who drank nickel contaminated water (nickel concentration 1.63 g/L). One patient with known bronchial asthma developed an expiratory wheeze and

another patient with chronic obstructive airways disease developed cyanosis (Sunderman et al., 1988).

Nickel compounds may act as an epigenetic carcinogen by altering the expression of some genes via effects on DNA hypermethylation, histone deacetylation and chromatin condensation. The effects of nickel on altering the transcription of tumor suppressor and senescence genes may play an important role in the nickel's carcinogenecity (reviewed in Cangul et al., 2002). For instance, wild type C57BL/6J and mice heterozygous for P^{53} were implanted intramuscularly with nickel sulfide. All mice developed sarcomas at the site of implantation. In these tumors, hypermethylation in the promoter region of the tumor suppressor P^{16} was detected (Govindarajan et al., 2002). Exposure to soluble and insoluble nickel compounds results in morphological cell transformation *in vitro* (reviewed in Sutherland and Costa, 2003). Nickel subsulfide is more effective in inducing morphological transformation because it enters the cells by phagocytosis (reviewed in Sutherland and Costa, 2003).

Nickel has been found to alter the expression of a large number of genes. Exposure to nickel resulted in decondensation of the heterochromatin and large deletions in the long arm of the X-chromosome in nickel transformed male Chinese hamster embryo cells (Conway and Costa, 1989).

In G12 cells, exposure to nickel resulted in very high levels of *gpt* gene inactivation compared to other known mutagens (reviewed in Sutherland and Costa, 2003). Nickel also has been found to silence gene transcription by altering histone acetylation patterns. For example, in the yeast *Saccharomyces cerevisiae,* there is change in the histone acetylation patterns but not in the DNA methylation. Moreover, pretreatment of yeast cells with the histone deacetylase inhibitor Trichostalin A (TSA) resulted in reduced levels of *URA3* gene silencing following the nickel exposure (reviewed in Sutherland and Costa, 2003). Considering to these observation, we will expose zebrafish embryos to $NiCl_2$ to look at the gene expression changes.

c) Mercury Chloride

A dangerous environmental contamination problem with mercury developed gradually because of its extreme toxicity and its widespread use. Mercury occurs in three states in nature (WHO, 1990): elemental mercury vapor (Hg°), methylmercury (MeHg), and divalent mercury (Hg^{2+}). Hg° is present in certain workplaces where metallic Hg is used, emitted by dental amalgam tooth fillings and is present in the ambient atmosphere. MeHg is found principally in the tissues of fish eating mammals. MeHg represents a much larger concern and threat to public health because MeHg can

accumulate in animal tissues more easily than other types of mercury compounds and it can bind to body proteins and pass through the digestive tract wall (WPCD, 2003). However, Hg° becomes toxic when it is oxidized to divalent mercury (Hg^{2+}) in the body by the hydrogen peroxidase-catalase pathway and is primarily excreted via the kidneys (Clarkson, 1997). The guidelines for Canadian drinking water Quality 1978 give a maximum acceptable mercury concentration of 0.0037 μM/L (Health and Welfare Canada 1979a). One feature of mercury is its biological methylation into compounds which are more toxic than the mercuric ion itself. Also, mercury is a common aquatic pollutant that bioaccumulates in fish through dietary and water borne exposure (Wiener and Spry, 1996).

Mercury is used industrially in two predominant forms. The first form, metallic mercury - is used mainly for electrical apparatus such as switches, and thermostats. Also it used in the production of chlorine and sodium hydroxide. An organic mercury compound, phenylmercuric acetate, was once used in pulp and paper mills as an inhibitor of bacteria growth. It was also used to reduce the growth of fungus on golf course green. In addition, phenylmercuric acetate and methylmercury derivatives were used as fungicides (Buhler, 1973; WPCD, 2003).

The notion of environmental mercury appearing to be dangerous was discovered during the period of 1953 to 1960 in Japan. Forty four people out of one hundred and eleven fishermen and their families died from developing a mysterious neurological disease along the shores of Minamata Bay. A similar outbreak occurred in Niigata, Japan in 1964, with 26 cases and five deaths. The infected victims suffered from neurological symptoms and brain damage while other survivors were paralyzed for life. The source of the disease was eventually determined to be from methylmercury discharged by a plastic factory into Minamata Bay, where the methylmercury became concentrated in the fish and shellfish eaten by the local residents (Buhler, 1973; Clark, 2001). The marine products in Minimata Bay displayed high levels of mercury contamination - 5.61 to 35.7ppm (Harada, 1995). Likewise in Iraq, people received methylmercury in contaminated bread, resulting in the exposure to thousands of people where a large number of deaths were caused (Bakir et al., 1973). Therefore, methylmercury is considered to be the most common toxic form of mercury in the biological environment.

The effects of methylmercury on mammals may be neurotoxic, teratologic, embryotoxic, or genetic. The neurotoxic effects are the most widely studied and the most obvious. In mammals, mercury commonly

passes the blood-brain barrier, causing destruction to visual and hippocaurpal cells, and disrupts the neurons of the peripheral nervous system (Chang, 1979). Fathead minnows (*Pimephales Promelas*) were exposed to mercury and, compared with control fish and fish from the two highest exposure groups, revealed consistent performance deficits in foraging efficiency and capture speed (Grippo and Heath, 2003). Teratogenic and embryotoxic effects of mercury have been described in many studies. For example, embryo mortality, reduction in growth and developmental abnormalities have been documented in mallard eggs externally treated with methylmercury (Hoffman and Moore, 1979). In other studies, the effects of methylmercury exposure persisted beyond the embryo to affect behavior and survival, resulting in brain lesions of mallard ducklings (Heinz, 1974, 1979). In New England, common loon eggs' volume declined significantly as egg-mercury concentration increased (Evers et al., 2003). Mercury contamination in adult and juvenile fish has been shown to disrupt vital functions including reproduction, osmoregulation, orientation, food search, predator recognition and communication (Zillioux et al., 1993).

Genotoxic effects of mercury such as DNA break frequency were demonstrated recently on aquatic snail populations and found to be correlated to the whole-body total mercury concentration (mostly MeHg).

This data adds to the evidence supporting the use of DNA strand breakage as an indicator of chemical contamination by mercury (Benton et al., 2002). Mercury acts as a genotoxin, significantly altering the expression of genes that affect cell survival and apoptosis (Shenker et al., 2002). Possible effects in human populations are indicated by Skerfving et al. (1970), who claims that high exposure to methylmercury leads to a higher frequency of chromosome breakage in individuals. Mercury (Hg^{2+}) is converted to methylmercury by sulfate reducing bacteria using methyltransferase enzyme and S-adenosylmethionine as a donor. DNA methyltransferase also uses S-adenosylmethionine as a donor. Therefore there is the potential for it to affect DNA methylation (Siciliano and Lean, 2002). Methyltransferase pathway is one of the biochemical pathways that responsible for biological mercury methylation. The enzyme responsible for mercury methylation also methylate homocysteine, and in fact homomcysteine was a much better competitor for the methyl group than mercury (Siciliano and Lean, 2002). Considering to these all above observation, we will expose zebrafish embryos to $HgCl_2$ to see gene expression changes.

d) Cadmium Chloride

After arsenic, nickel and mercury, cadmium is considered next in importance as an environmental pollutant. The International Agency for Research on Cancer (IARC) has designated cadmium (Cd) as a poisonous transition metal that distributes widely in the environment as a human carcinogen (IARC, 1993). From 1940 to 1965, cadmium caused the deadly itai itai disease (Osteomalasia), which spread through Japan, in the Jinzu river region (Krenkel, 1975). According to the Geneva World Health Organization 1992, reported that the daily intake of cadmium in the most heavily contaminated area amounted to 600-2000 μg per day; in other less heavily contaminated area, daily intakes of 100-390 μg per day have been found. The highest value of cadmium content in well water was 0.225 ppm in Jinzu River, Japan (Frederick, 1978). Different routes of cadmium uptake via airborne particles, smoking, drinking water and food have been identified (Fels, 1999). Cadmium is absorbed by inhalation and ingestion. Therefore, low doses and exposure for long periods can still be a high risk factor. The Canadian drinking water quality guidelines 1978 give a maximum acceptable cadmium concentration of 0.027 μM/L (Health and Welfare Canada, 1979a).

The carcinogenecity of cadmium has been established in rodent models (IARC, 1976; Takenaka et al., 1983; Waalkes et al., 1992). The investigations by Schroeder (1965) have focused attention on this metal by linking hypertension to increased retention of cadmium in the kidneys. Cadmium levels in the air of 28 American cities have been closely correlated with the incidence of death from hypertension and arteriosclerotic heart disease (Carroll, 1969). Lung and bone are also the most critical organs in exposure to cadmium in humans (Piscator, 1985). In animal models, cadmium has been shown to be a developmental toxin that can induce defects in the developments of the lung, brain, testes, eye, palate, intrauterine growth retardation and fetal death (Ahokas et al., 1980; Daston, 1981, 1982; Holt and Webb, 1987; Levin and Miller, 1980; Parizek, 1964). In rats, injection of 1.25 mg Cd^{2+}/kg between 9 and 15 weeks of gestation resulted in a high incidence of hydrocephalus together with other malformations in the fetuses, while 1.35 mg Cd^{2+}/kg killed all the embryos. (Samarawickrama and Webb, 1979). There is evidence that cadmium may be indirectly genotoxic by compromising the cell's ability to accurately replicate DNA (Lohamnn et al., 1993). This may be done by perturbing apoptosis (Shimoda et al., 2001; Yuan et al., 2000) or by increasing background mutagenesis (Misra et al., 1998). Some researchers reported that

cadmium was mutagenic in *salmonella typhimurium* strain TA97, induced DNA single strand breaks and DNA-protein crosslink (Harris, 1991; Waalkes et al., 1992). Moreover, cadmium has been shown to induce expression of genes such as *c-myc*, *c-fos*, *c-jun* and *egr-1* proto-oncogenes and metallothionein (Matsuoka and Call, 1995; Wang and Templeton, 1998).

Cadmium is a metal that acts via epigenetic mechanisms and is an effective inhibitor of DNA Mtase. Initially, it induces DNA hypomethylation while prolonged exposure results in DNA hypermethylation and enhanced DNA Mtase activity in TRL 1215 rat liver cells on vitro (Takiguchi et al., 2003). Thus, considering to these previous observation, we will expose zebrafish embryos $CdCl_2$ to investigate the changes of gene expression.

In order to determine whether the expression of *IGF2* and other genes (*IGF1* and *IGFBP-1*) can be used as a marker or indicator of toxins that affect DNA methylation in the zebrafish (*Danio rerio*); we exposed zebrafish embryos to $NaAsO_2$, $NiCl_2$, $HgCl_2$, and $CdCl_2$. In addition, we exposed embryos to two chemicals known to alter DNA methylation and histone acetylation 5-azaC and TSA (Jones, 1984; Yoshida et al., 1990). Treatment of zebrafish embryos with epigenetic toxins may cause abnormal expression of genes that are regulated by DNA methylation, where DNA

methylation moderately regulates the *IGF2* gene in mammals (Wutz et al., 1998). Thus, exposure of zebrafish embryos to epigenetic toxins may induce change in *IGF2* transcription.

2.0 MATERIALS and METHODS

2.1 Zebrafish

The zebrafish were maintained in aquaria at 28° C on a 14 hour light/10 hour dark cycle and fed a standard diet such as frozen blood worms and dry flakes (Nutrafin). Prior to breeding, male and female zebrafish were placed in a spawning tank. The embryos were collected each morning and subsequently incubated at 28° C in the laboratory and staged according to hours post fertilization (hpf) (Kimmel et al., 1995). General zebrafish care was according to Westerfield (1995). A total of 10 treatment groups were used to determine mortality rates.

2.2 5-Azacytidine Treatment

5-Azacytidine (5-azaC) 0.024 g/L is dissolved in distilled water to produce a final stock concentration of 100 mM. Zebrafish embryos were sorted in 6 dishes containing 40 ml water. They were treated with 5-azaC at concentrations of 0 μM, 10 μM, 25 μM, 50 μM, 100 μM and 200 μM respectively. Embryos were examined at several time points during treatment to determine mortality rates.

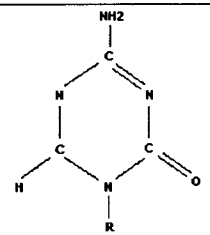
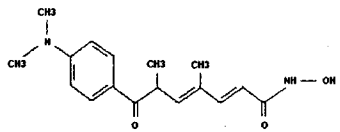
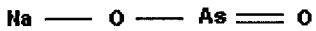
2.3 Trichostatin A Treatment

Trichostatin A (TSA) 0.0003 g/ml is dissolved in distilled water to produce a final stock concentration of 1 mM. Zebrafish embryos were placed in 6 dishes with 30 ml water and treated with 0 μM, 0.050 μM, 0.100 μM, 0.200 μM, 0.500 μM and 0.750 μM. Embryos were examined at 8 hpf and 24 hpf during treatment to determine mortality rate. The same steps as mentioned above were repeated while treating zebrafish embryos with $NaAsO_2$, $NiCl_2$, $CdCl_2$ and $HgCl_2$ (see Table 1 for concentration of treatments). Sodium arsenite is converted to sodium arsenate in the aqueous solution. For each chemical, experiment was carried out three times.

2.4 Northern Blot

Total RNA was extracted from zebrafish embryos using Trizol reagent (Invitrogen). Approximately 150 embryos were crushed in 1000 μl Trizol reagent using a Teflon dounce homogenizer. The resulting Trizol mixture was then processed according to the manufacturer's recommended protocol. A 5 μg sub-sample of total RNA from each sample was denatured in loading buffer solution (50 % formamide, 0.12 % bromophenol blue, 0.12

Table 1: The main functions & concentration of treatment for each chemical used in the experiments

Chemical form (Epigenetic toxins)	Chemical Structure	Stock solution	Concentration of treatments	Epigenetic effect
5-azacytidine (drug cancer)		100 mM	10μM – 200μM	DNA methyltransferase inhibition (Jones, 1984)
Trichostatin A (TSA)		1 mM	0.050μM – 0.750μM	Histone deacetylase inhibition (Yoshida et al., 1990)
Sodium Arsenite ($NaAsO_2$)		1 mM	1μM – 20μM	DNA Hypomethylation (Zhao et al., 1997)
Nickel Chloride ($NiCl_2$)		100 mM	50μM - 4000μM	DNA Hypermethylation (reviewed in Cangul et al., 2002)
Cadmium Chloride ($CdCl_2$)		1 mM	1.0μM – 40.0μM	DNA Hypermethylation (Takiguchi et al., 2003)
Mercury Chloride ($HgCl_2$)		37 mM	1μM – 32 μM	Unknown

% xylene cyanol final concentration) by heating at 70° C for five minutes. The RNA was subject to electrophoresis in an agarose gel containing 1.2 % agarose, 37 % formaldehyde and 10 x MOPS buffer (0.2 m MOPS pH 7.0, 10 mM EDTA pH 8.0, 20 mM sodium acetate). The gel was run at 2-3 V/cm for approximately 2-4 hours in 0.5 x MOPS buffer. A RNA size marker was also included on each gel. Following electrophoresis, the gel was stained using ethidium bromide and photographed. The RNA was transferred to nylon membrane (Hybond N^+, Amersham) and hybridized to ^{32}p labeled probes. A ^{32}P-labelled DNA probe was prepared by random primer labeling of gel purified DNA fragments corresponding to either full or partial length cDNAs of each gene assayed. Blots were hybridized overnight at 65° C in hybridization buffer (10 ml 1M Na Phosphate buffer pH 7.0, 7 ml 20 % SDS, 40 µl 0.5 M EDTA pH 8.0, 2.96 ml H_2O). The next day, blots were washed 5 x in wash buffer (20 ml 1M Na Phosphate buffer pH 7.0, 5 ml 10 % SDS, in total volume of 500 ml) at 65° C. Blots were put in an autoradiography cassette with X-ray film, or phosphorimager screen (Bio-Rad). X-ray film autoradiographs were stored at -80° C with intensifying screen for one day to one week. X-ray film was developed with GBX developer and fixer. Northern blots were subsequently reprobed with a β-

actin cDNA fragment, which provides a suitable control for RNA integrity. Phosphoimager screens were analyzed using Quantity One Software.

2.5 RT-PCR Assay

Embryos were ground in TRIZOL reagent (Invitrogen) and their total cellular RNA was extracted with chloroform and isopropyl alcohol. First strand synthesis was performed using an oligo-dT primer (ROCHE) and Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) (Invitrogen). The resulting cDNA was then used for PCR reactions using the following gene specific primers. The *IGF2* gene (AF250289): forward primer 5′- GAG GAA ACA CGA ACA ACG ATG CG - 3′, and the reverse primer 5′- TTG GAG TAG TTC ACA TTT ATG GTG TCC TTG - 3′. These primers were designed with the use of the zebrafish genomic project database in order to span 2 exons to insure no genomic amplification was possible. The *IGF1* gene (NM_131825) : forward primer 5′- TGT CTT TAA GTG TAC CAT GCG CTG TCT CCC - 3′, and the reverse primer 5′- TGT TTC CTC GGC TCG AGT TCT TCT G - 3′. The *IGFBP-1* gene (AJ299409): forward primer 5′- TTT CCA GCA GCA CCT GGT CAA GCT CCT - 3′, and the reverse primer 5′- GGA GAT GCG GAC GTA AAG

TGG ACA - 3′. The *β-actin* primers (AF057040) used in this experiment, as a control were the forward primer 5′- AGA AGA TCT GGC ATC ACA CC - 3′ and the reverse primer 5′- TCC ATA CCC AAG AAG GAT GG - 3′. These primers were also designed to span an exon to produce a different PCR product in the presence of genomic DNA. Both the *β-actin* and the *IGF2*, *IGF1*, *IGFBP-1* PCR mix reaction consisted of 1x standard PCR buffer (-$MgCl_2$), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 5 pmol forward and reverse primers, 2.5 units of Taq polymerase (Invitrogen), and 8 hpf zebrafish cDNA. PCR reactions produced an amplification product for *IGF2* of 350 bp, for *IGF1* 422 bp, and for *IGFBP-1* 269 bp. The cycling parameters were 5 minutes at 94° C followed by cycles of 30 seconds at 92° C, 30 seconds at 60° C, and 30 seconds at 72° C. The reaction was paused at predetermined cycles and held at 72° C to allow for the removal of 5 µl from each tube. The reaction was then continued for 2 cycles of 30 sec at 92° C, 30 sec at 60° C, and 30 sec at 72° C. Again, the samples were held at 72° C to facilitate the removal of 5 µL from each tube. This cycle stop and start was performed 3 times giving 4 different samples spanning 8 PCR cycles for each reaction mixture. The final extension was for 9 minutes at 72° C.

2.6 Radioactive Semi-Quantitative RT-PCR

Semi-quantitative RT-PCR was performed to determine the relative levels of gene expression between sample treatments. The *IGF2* primers used for this experiment were forward primer 5′-GAG GAA ACA CGA ACA ACG ATG CG-′3 and the reverse primer 5′-TTG GAG TAG TTC ACA TTT ATG GTG TCC TTG-′3 and produced a PCR product of 350 bp. The *β-actin* primers used in this experiment, as a control, were the forward primer 5′-AGA AGA TCT GGC ATC ACA CC-′3 and the reverse primer 5′-TCC ATA CCC AAG AAG GAT GG-′3 to produce a PCR product of 562 bp. Both the *β-actin* and *IGF2* PCR reactions contained 1x standard PCR buffer (-$MgCl_2$), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 5 pmol forward and reverse primers, 2.5 Units of Taq polymerase (Invitrogen), 5 uCi of (α^{32}P)-dCTP per reaction and 1.00 μg of zebrafish cDNA. Samples were taken from each reaction at PCR cycles corresponding to the exponential phase of amplification by producing a standard amplification curve across a wide range of cycles (data not shown). This type of sampling was conducted on all PCR reactions used in this study. As a result we could be certain that each reaction used for analysis was taken from a period of linear amplification. The samples were incubated at 94° C for 5 minutes in 50 %

STOP BUFFER (100 % formamide, 0.025 % bromophenol blue in 0.1 M Tris (pH 8.0), and 0.025 % xylene cyanol in 0.1 M Tris (pH 8.0). Then, 5 µl of each sample was run on a 6 % denaturing polyacrylamide gel and run in 1xTBE at 20 W for approximately 1 hour. The gel was then analyzed using autoradiography and the product yield was measured using a PhosphoImager (Bio-Rad).

2.7 Genomic DNA Isolation

Zebrafish embryos were treated with the following concentrations: 0 µM, 50 µM 5-azaC, 10 µM $CdCl_2$, 20 µM $NaAsO_2$, 200 µM $NiCl_2$ and 8 µM $HgCl_2$. DNA was extracted by placing 50 zebrafish embryos in 1.5 ml Eppendorf tube containing 50 µl of digest buffer (1% SDS, 100 mM NaCl, 100 mM EDTA, 50 mM Tris pH 8.0) and 0.5 µg/ml proteinase K (Sigma). The samples were incubated for 3-4 hours at 56° C in a water bath. The DNA was extracted from the digested embryos with phenol (saturated with 0.5 M Tris-HCl pH 8.0), PCI (25 ml phenol: 24 ml chloroform: 1 ml isoamyl alcohol) and CIA (24 ml chloroform: 1 ml isoamyl alcohol). The extracted DNA was precipitated with sodium acetate (0.2 M final concentration) and isopropanol (50 % final concentration). The resulting DNA pellet was washed in 85 % ethanol, and vacuum dried at room temperature. The DNA

pellet was redissolved in 50 µl milli Q water. The DNA concentration was determined spectro-photometerically. DNA preparations were digested 2 hours with restriction enzymes EcoRI, HpaII and MspI according to the manufactures instructions (Gibco/BRL,New England Biolabs). DNA fragments were separated by electrophoresis in a 0.8 % agarose gel. Hind III cut lambda DNA was added to a single well to act as a DNA fragment size marker. Following electrophoresis, the DNA within the gel was visualized by ethidium bromide staining and a photograph was taken using Quantity One Software. DNA fragments were transferred in 20X SSC by capillary action on to Hybond N^+ membrane (Amersham).

2.8 Southern Analysis

Southern blots containing 5 µg of EcoRI, EcoRI + HpaII, EcoRI + MspI digested DNA were prepared and hybridized according to Sapienza et al. (1987). Prior to Southern transfer of DNA to nylon membrane, the DNA fragments were denatured in 0.6 M NaCl and 0.2 M NaOH for 45 minutes. Blots were hybridized to a ^{32}P random primer labeled probe of the zebrafish interspersed repetitive element Mermaid/DANA (Izsvāk et al., 1996; Shimoda et al., 1996a; Shimoda et al., 1996b). Blots were hybridized overnight at 65° C in hybridization buffer (10 ml Na phosphate buffer pH 7.0, 7 ml 20 % SDS, 40 ml 0.5 EDTA pH 8.0, 2.96 ml H_2O). The next day,

blots were washed 5 x in wash buffer (20 ml Na phosphate buffer PH 7.0, % ml 10 % SDS, total volume of 500 ml) and blots were analyzed using a phospho-imager (Bio-Rad).

3.0 RESULTS

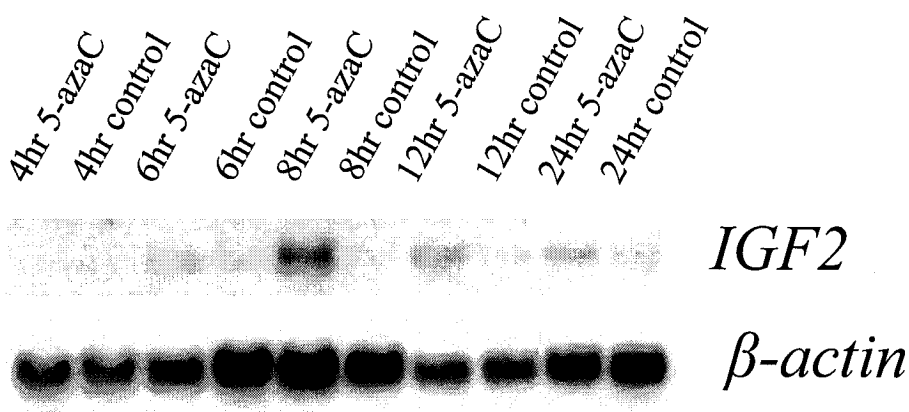
3.1 Treatment of zebrafish embryos with 5-azacytidine (5-azaC) during blastula causes up-regulation of *IGF2* expression

The purpose of this experiment is to investigate the effect of DNA hypomethylation on the transcriptional activity of *IGF2*, *IGF1* and *IGFBP-1* genes. We used 5-azaC, a drug known to inhibit DNA Mtase, to hypomethylate the DNA of zebrafish embryos.

If *IGF2* is regulated by DNA methylation in zebrafish embryos, then we would expect *IGF2* gene expression to be up-regulated upon treatment with 5-azaC. Martin et al. (1999) showed that treatment of zebrafish embryos with 100 μM 5-azaC caused developmental abnormalities of the body axis and DNA hypomethylation in 24 hpf treated embryos. Therefore, we initially treated zebrafish embryos for 1 hour – 24 hours with 100 μM 5-azaC. We collected total RNA from different embryo stages (dome stage-4 hpf, shield stage-6 hpf, epiboly stage –8 hpf, somite stage-12 hpf, tail stage-24 hpf) and used these for northern blot analysis to determine if *IGF2* expression was up-regulated during the course of these treatments (Figure 2). *IGF2* up-regulation was first observed at 6 hrs (epipoly stage). *IGF2* up-

Figure 2: Northern blot analysis showing that *IGF2* expression is up-regulated at 8 hpf of zebrafish embryos treated with 100 μM 5-azacytidine.

Northern blot of zebrafish embryos treated with 100 μM 5-azacytidine for different durations of development using *IGF2* as a probe. It shows that *IGF2* expression induction peaks at 8 hours of development, while at 12 hours and 24 hours, the *IGF2* up-regulation was reduced. The *β-actin* was used for RNA integrity.

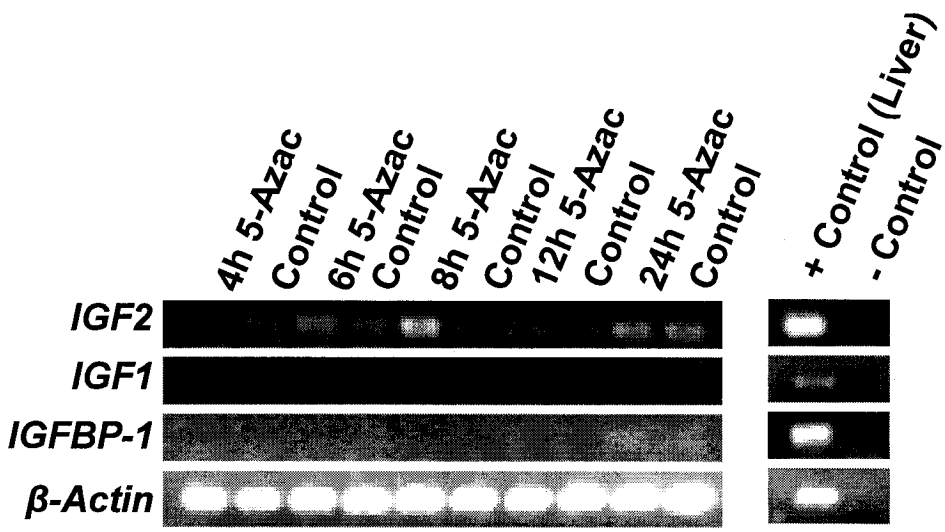

4hr 5-azaC
4hr control
6hr 5-azaC
6hr control
8hr 5-azaC
8hr control
12hr 5-azaC
12hr control
24hr 5-azaC
24hr control
IGF2
β-actin

regulation peaked at 8 hours while at the 6 hours (Shield stage) and 12 hours (Somite stage), the *IGF2* up-regulation was still observed but to a lesser extent. Normal expression of *IGF2*, as detected by northern blot, was not observed until 24 hpf (tail stage) (Figure 2). The *β-actin* was used as a control for RNA integrity and equal loading.

In order to confirm our result from northern blot, we used a more sensitive technique called Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Semi quantitative RT-PCR analysis was performed on cDNA generated from the total RNA of zebrafish embryos treated with various concentrations of 5-azaC and using primers specific for *IGF2, IGF1* and *IGFBP-1* genes. This experiment showed that there was no expression of *IGF1* and *IGFBP-1*, while *IGF2* showed similar results to the northern blot. In comparison to untreated control zebrafish embryos, *IGF2* was up-regulated at 8 hours of development. At 6 and 12 hours, the *IGF2* up-regulation was observed but induction was lower than that observed at 8 hpf. At 24 hours, normal endogenous *IGF2* gene expression was observed in treated and untreated zebrafish embryos. At 4 hours, there was no expression of *IGF2* (Figure 3). The adult zebrafish liver cDNA was used as a positive control for *IGF2*, *IGF1* and *IGFBP-1* because all of these genes are

Figure 3: Semi-quantitive RT-PCR analysis showing that *IGF2* expression is up-regulated at 8 hpf in zebrafish embryos treated with 100 μM 5-azacytidine.

Semi-quantitive RT-PCR analysis of *IGF2* expression in zebrafish embryos treated with 100 μM 5-azacytidine. (a) *IGF2* expression induction peaks at 8 hours of development, while 6 hours, 12 hours and 24 hours up-regulation was reduced. (b) No expression of IGF_1. (c) No expression of *IGFBP-1* at 4 hours, 6 hours, 8 hours and 12 hours. However, at 24 hours *IGFBP-1* expression seemed normal compared with control. (d) *β-actin* expression is relatively uniform in all sets. Liver cDNA and negative control were used as a control.


4h 5-Azac
Control
6h 5-Azac
Control
8h 5-Azac
Control
12h 5-Azac
Control
24h 5-Azac
Control
+ Control (Liver)
- Control
IGF2
IGF1
IGFBP-1
β-Actin

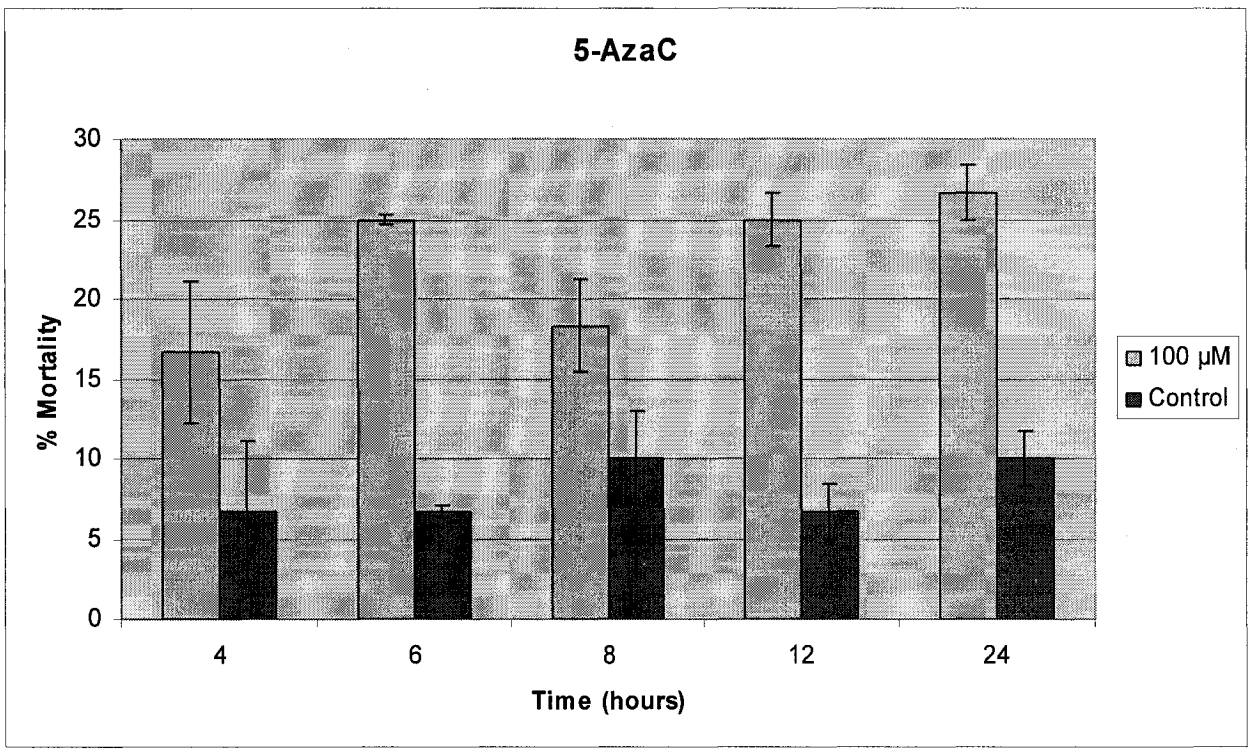
normally expressed in the liver. We used water as a negative control to confirm the absence of any contamination that might occur during the assembly of the reactions and result in an amplification product. *β-actin* was used to confirm equal loading and integrity of cDNA and it was relatively uniform in all samples.

In order to determine if 5-azaC causes death of zebrafish embryos, twenty embryos were placed in ten petridishes (n=10). Embryos were treated with a 100 μM concentration of 5-azaC. Treatment of zebrafish embryos with a 100 μM 5-azaC resulted in higher mortality rates at all developmental stages tested, compared to untreated control embryos. Overall cumulative mortality rates after 4 hpf was 17 %, 6 hpf was 25 %, 8 hpf was 19 %, 12 hpf was 25 % and 24 hpf was 27 %, whereas untreated control at all developmental periods produced less mortality. Mortality rates at 4 hpf, 6 hpf and 12 hpf were 7 %, while at 8 hpf and 24 hpf were 10 % (Figure 4).

Our previous experiment indicated that 5-azaC caused up-regulation of *IGF2* gene expression. The developmental stage where this up-regulation occurred was 8 hours (Epiboly stage). In order to determine if *IGF2* up-regulation is dependent on the concentration of 5-azaC treatment; we treated

Figure 4: The mortality rate of zebrafish embryos treated with 100 μM 5-azacytidine at different developmental stages.

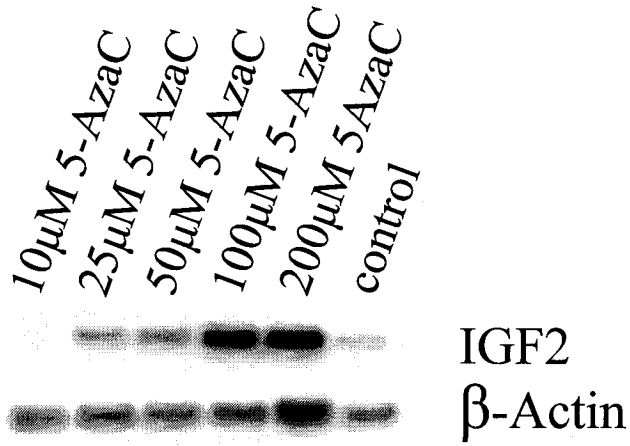
Cumulative mortality versus developmental time in embryos treated with 100 μM 5-azacytidine. Embryos were allowed to develop in an incubator at 28° C for 4 hours, 6 hours, 8 hours, 12 hours and 24 hours post-fertilization, n=10 (group of 20). Embryos treated with 100 μM show high mortality compared with untreated zebrafish embryos. Abnormal phenotype was observed just at 12 hours and 24 hours.


5-AzaC
% Mortality
30
25
20
15
10
5
0
4
6
8
12
24
Time (hours)
100 µM
Control

zebrafish embryos with 0 μM, 10 μM, 25 μM, 50 μM, 100 μM, 200 μM 5-azaC from 1 hpf to 8 hpf. Total RNA was collected from treated and control embryos at 8 hpf, and we performed northern blot analysis using a *IGF2* cDNA as a probe. Northern blots were stripped and re-probed with *β-actin*. We observed an increase in the abundance of *IGF2* mRNA in 5-azaC treated zebrafish embryos compared with that of control zebrafish embryos (Figure 5). The highest *IGF2* up-regulation was observed in embryos treated with 5-azaC at 100 μM and 200 μM concentration. Increasing the concentration of 5-azaC from 100 μM to 200 μM did not increase the level of *IGF2* up-regulation. The 25 μM and 50 μM treatment samples also showed up-regulation of *IGF2* gene expression while 10 μM showed no *IGF2* gene expression. Up-regulation of *IGF2* expression was lower in embryos that were treated with 25 μM and 50 μM 5-azaC compared to embryos that were exposed to 100 μM and 200 μM. To determine if 5-azaC treatment affects the gene expression of other *IGF* family members, we stripped and re-probed these blots with probes generated from insulin-like growth factor 1 (*IGF1*) cDNA and insulin- like growth factor binding protien-1 (*IGFBP-1)* cDNA. We did not observe any expression of these two genes in any of the samples tested using northern blot analysis (data not shown). To potentially provide a more quantitative analysis of the up-regulation of the *IGF2* gene in

Figure 5: Northern blot analysis showing that *IGF2* expression is up-regulated in zebrafish embryos treated with 100 μM and 200 μM 5-azacytidine.

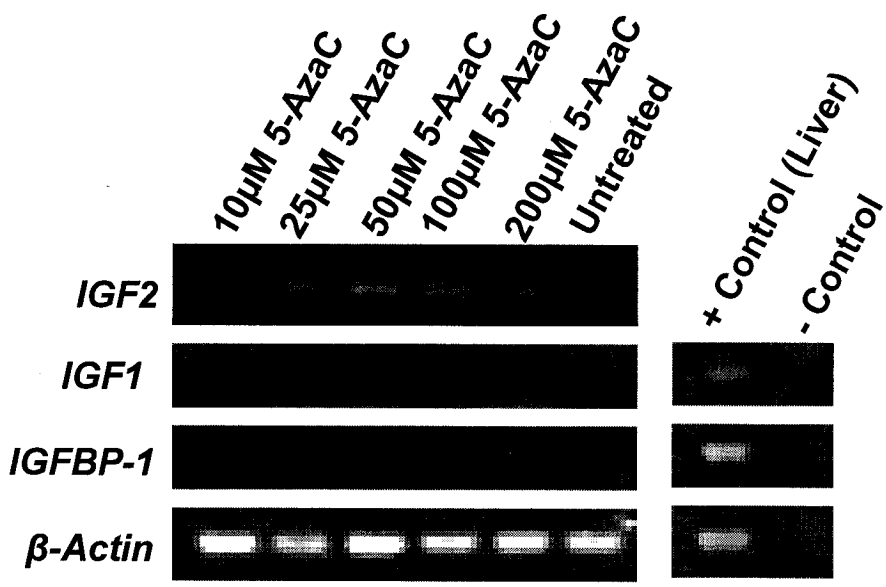
Northern blot of total RNA collected from zebrafish embryos treated for 8 hpf with 0 μM, 10 μM, 25 μM, 50 μM, 100 μM, 200 μM 5-azacytidine using *IGF2* as a probe. The highest *IGF2* expression was observed in embryos treated with 100 μM of 5-azacytidine. The *β-actin* was used to indicate RNA integrity.


10μM 5-AzaC
25μM 5-AzaC
50μM 5-AzaC
100μM 5-AzaC
200μM 5AzaC
control
IGF2
β-Actin

zebrafish exposed to 5-azaC, we subsequently conducted semi-quantitative RT-PCR analysis. RT-PCR is more sensitive than northern blot analysis and therefore might also allow us to identify the presence of genes expressed at lower levels (e.g. *IGF1* and *IGFBP-1*).

Semi quantitative RT-PCR analysis was performed on cDNA that was generated from the total RNA of zebrafish embryos collected at 8 hpf and treated with different concentrations of 5-azaC ranging from 0μM to 200μM. The results from the RT-PCR showed a similar result which was observed from the northern blot analysis (Figure 6). Up-regulation of *IGF2* was observed in embryos treated with 10 μM, 25 μM, 50 μM, 100 μM, and 200 μM 5-azaC. The highest levels of induction were observed at treatment concentrations of 50 μM and 100 μM. The RT-PCR showed that *IGF2* transcription was up-regulated by 5-azaC treatment while *IGF1* and *IGFBP-1* showed no expression or up-regulation compared to controls. The primers used for this RT-PCR analysis had previously been used by Maures et al. (2002). Liver cDNA was used as a positive control and water as a negative control. The *β-actin* amplification product was relatively uniform indicating that equal amounts of cDNA were used between samples. These results

Figure 6: Semi-quantitive RT-PCR analysis showing that *IGF2* gene expression is up-regulated following 5-azacytidine treatment.
Semi-quantitive RT-PCR analysis of *IGF2* expression in zebrafish embryos treated with 0 μM, 10 μM, 25 μM, 50 μM, 100 μM, 200 μM 5-azacytidine. (a) Highest *IGF2* expression was observed in embryos treated with 100 μM of 5-azacytidine. (b) and (c) No *IGF1* and *IGFBP-1* expression. (d) *β-actin* expression is relatively uniform in all sets. Liver cDNA and negative control were used as a control.


10μM 5-AzaC
25μM 5-AzaC
50μM 5-AzaC
100μM 5-AzaC
200μM 5-AzaC
Untreated
+ Control (Liver)
- Control
IGF2
IGF1
IGFBP-1
β-Actin

confirm our observations using the northern blot analysis that 5-azaC cause induction of *IGF2* expression.

To determine if 5-azaC affects survivability of zebrafish embryos, we exposed the embryos to 5-azaC at different concentrations and determined their total mortality at two time points. Twenty embryos were placed in each of the ten petridishes (n=10). Embryos were treated at 0 μM, 10 μM, 25 μM, 50 μM, 100 μM, 200 μM concentration of 5-azaC at 1 hpf. The mortality was determined at 8 and 24 hpf. Dead embryos were identified as those which showed either arrested development or appeared necrotic. In Figure 7 and 8, a 200 μM and 100 μM concentration of 5-azaC produced high mortality at 8 and 24 hpf whereas 10 μM, 25 μM and 50 μM resulted in less embryo mortality rate. After 8 hpf, 200 μM treatment produced a total mortality rate 16.5 %, 100 μM was 15%, 50 μM was 6 %, 25 μM was 4 % and 10 μM and 0 μM were 2.5 %. After 24 hpf, mortality rate at 200 μM was increased by 20 %, 100 μM was 18 %, 50 μM was 7 %, 25 μM was 7.5 %, 10 μM was 9 % same as untreated control. Thus, exposure to 5-azaC and the resulting DNA hypomethylation affects the survivability of fish embryos.

Figure 7: The mortality rate at 8 hpf of zebrafish embryos treated with various concentrations of 5-azacytidine.
Cumulative mortality versus concentration for zebrafish embryos were treated with 10 μM, 25 μM, 50 μM, 100 μM and 200 μM 5-azacytidine for 8 hpf. A 100 μM and 200 μM concentration produces high mortality, whereas the rest of the concentrations and the control resulted in less mortality.

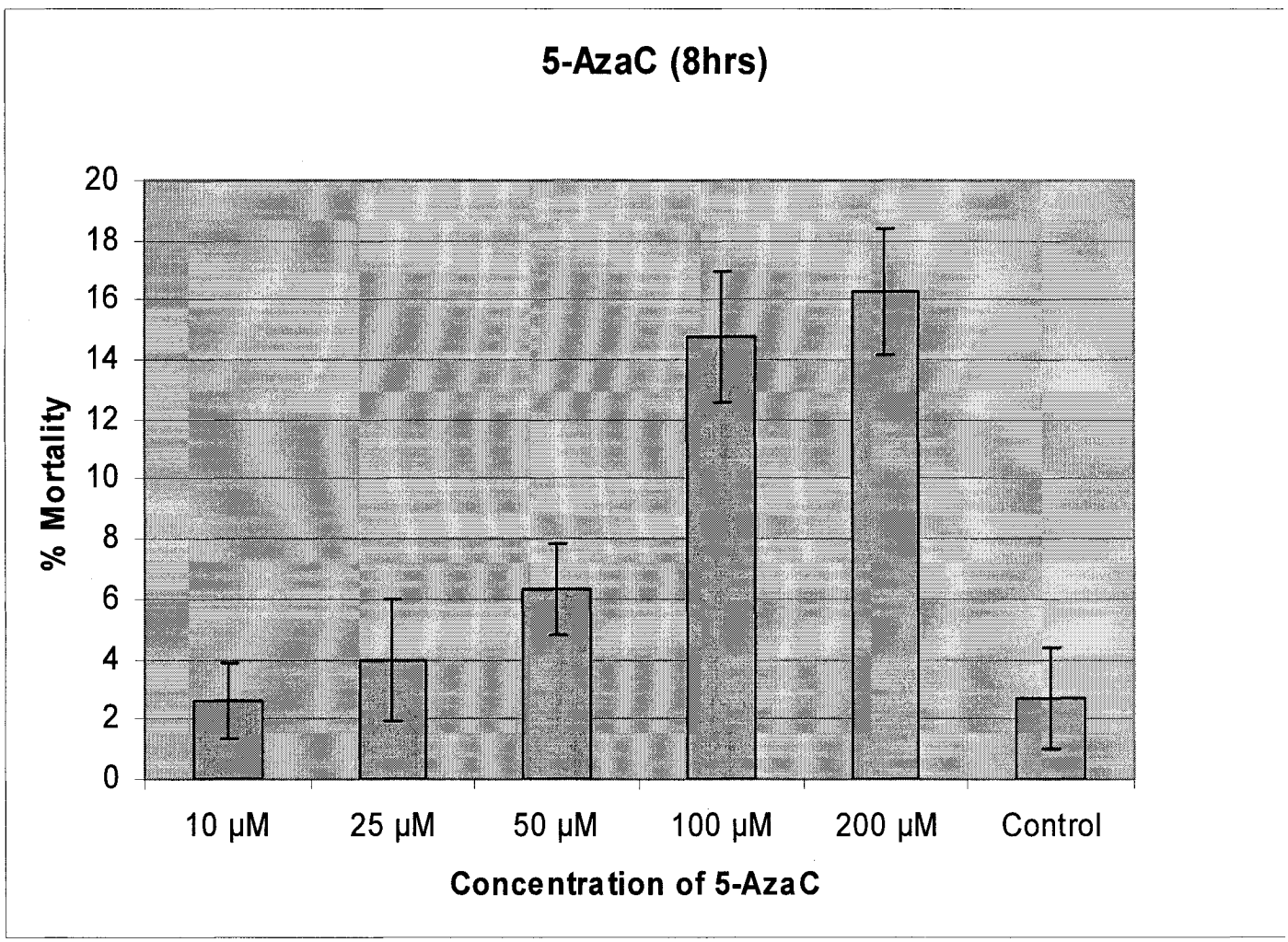
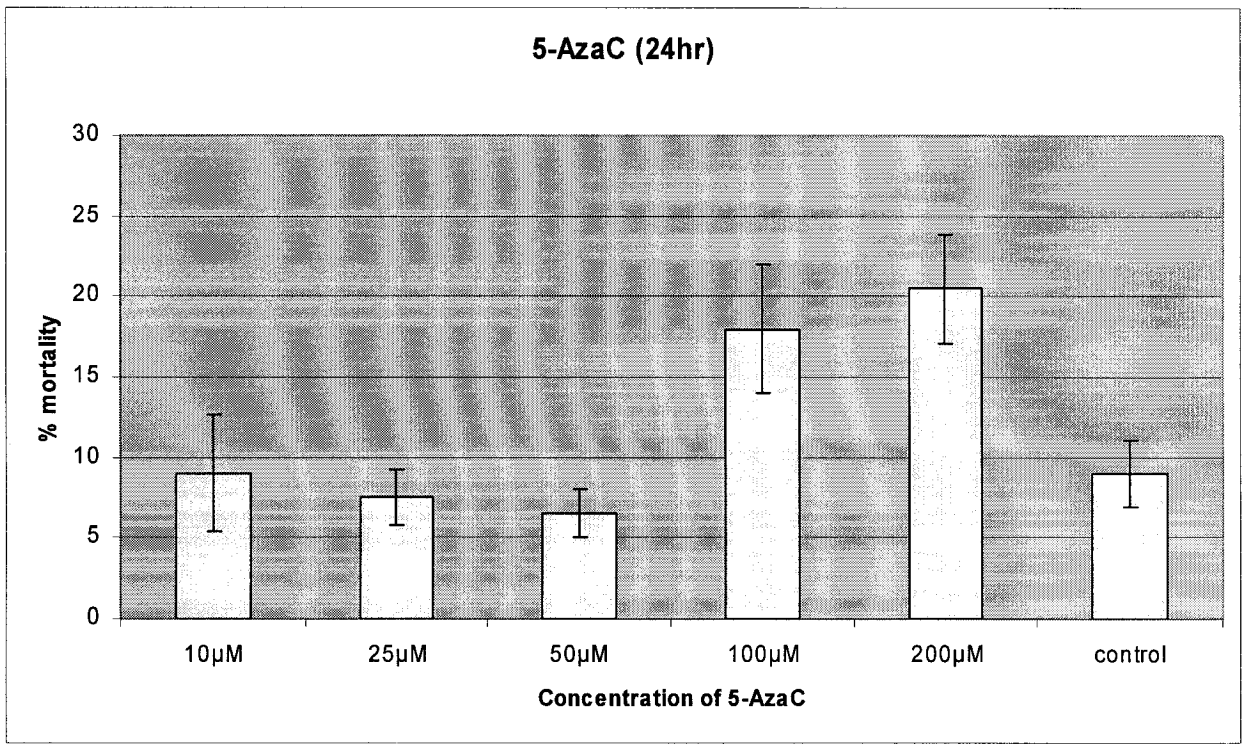

5-AzaC (8hrs)
% Mortality
0
2
4
6
8
10
12
14
16
18
20
10 µM
25 µM
50 µM
100 µM
200 µM
Control
Concentration of 5-AzaC

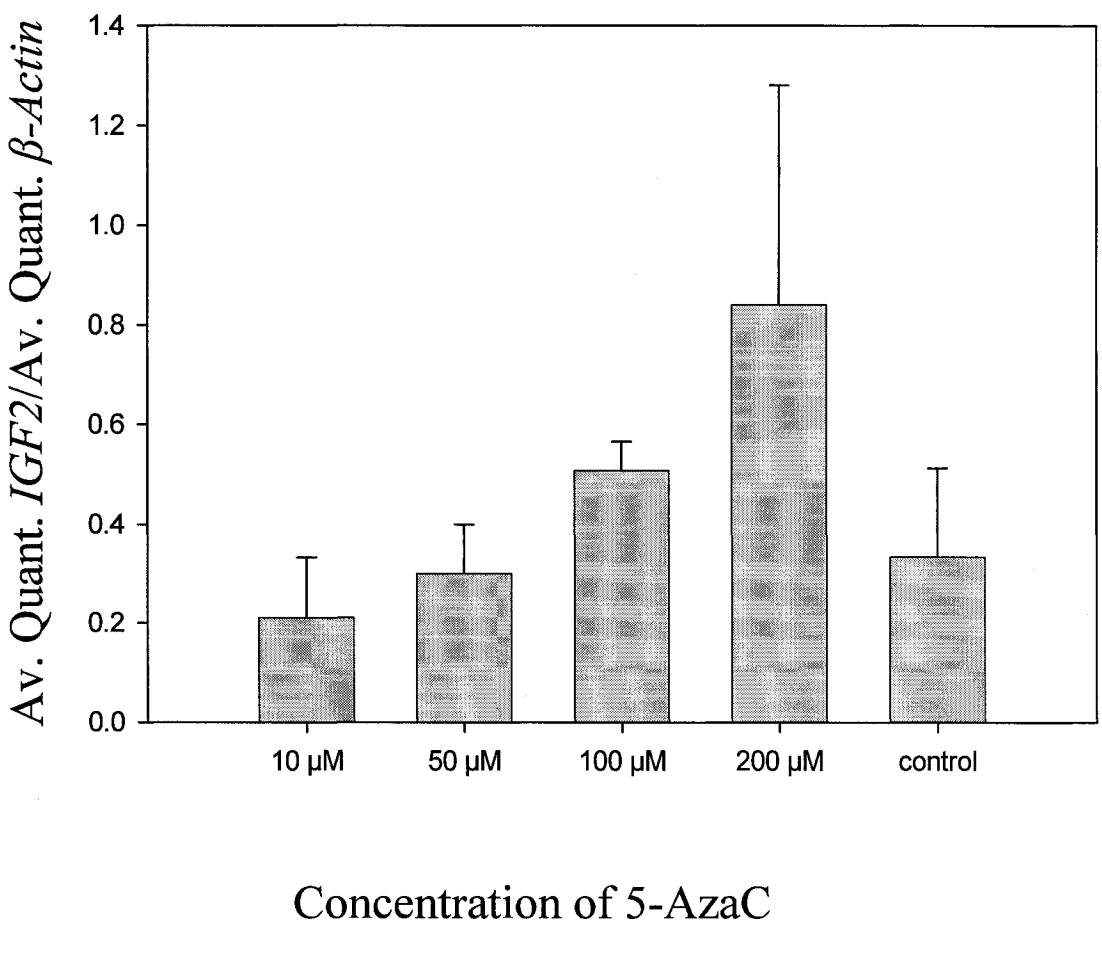
Figure 8: The mortality rate at 24 hpf of zebrafish embryos treated with various concentrations of 5-azacytidine.

Cumulative mortality versus concentration for zebrafish embryos were treated with 10 μM, 25 μM, 50 μM, 100 μM and 200 μM 5-azacytidine for 24 hours. Embryos were allowed to develop in an incubator at 28° C for 24 hours post-fertilization, n=10 (group of 20). A 100 μM and 200 μM concentration shows high mortality with abnormal phenotype. However, 10 μM, 25 μM, 50 μM and untreated zebrafish embryos produced normal phenotype.


5-AzaC (24hr)
% mortality
30
25
20
15
10
5
0
10μM
25μM
50μM
100μM
200μM
control
Concentration of 5-AzaC

In order to confirm the up-regulation of *IGF2* in zebrafish embryos exposed to 5-azaC, we repeated semi-quantitative RT-PCR in the presence of 5 μCi of α^{32}P-dCTP per reaction. The samples were taken at PCR cycles corresponding to the exponential phase of amplification as determined by producing a standard amplification curve (data not shown). They were run on a 6 % denaturing gel (PAGE) and the densities of the PCR product bands were analyzed through phospho-imager analysis and Quantity One Software. A graphical representation, shown in Figure 9, shows the average densities for *IGF2* / *β-actin* amplification products for five sets of embryonic cDNAs (25μM 5-azaC sample was not shown due to the degradation of total RNA for that sample). The graph shows high expression of *IGF2* at 100 μM and 200 μM concentration of 5-azaC similar to the RT-PCR result and the northern blot result. These results indicate that the up-regulation in *IGF2* gene expression by 5-azaC treatment can be used as a marker for DNA hypomethylation toxins. The ratio at 200 μM was approximately 3 x higher, and a 100 μM was about 2 x higher compared to untreated control.

Figure 9: A graphical representation of the average density ratio of *IGF2/β-actin* RT-PCR products for sets of cDNA from zebrafish embryos treated with different concentration of 5-azacytidine.
This graph identifies the high expression of *IGF2* at 100 μM and 200 μM, which is similar to the RT-PCR results, and low expression of *IGF2* in the other samples.


Av. Quant. IGF2/Av. Quant. β-Actin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10 μM
50 μM
100 μM
200 μM
control
Concentration of 5-AzaC

3.2 Treatment of zebrafish embryos with sodium arsenite ($NaAsO_2$) causes up-regulation of *IGF2* gene expression

One of the goals of these studies was to determine the effects of putative epigenetic toxins on *IGF2* expression. This was done by exposing embryos to environmentally relevant concentrations of toxins, and measuring the expression of the *IGF2* gene. Arsenic can act as a carcinogen by inducing DNA hypomethylation, which in turn facilitates aberrant gene expression (Zhao et al., 1997). For instance, in mammals, over-expression of several genes including oncogenes and genes regulating cell proliferation was detected in the arsenic-transformed rat liver TRL 1215 cells following chronic exposure to sodium arsenite (Chen et al., 2001). Thus, we hypothesize that $NaAsO_2$ will cause DNA hypomethylation in zebrafish, and result in *IGF2* up-regulation.

To determine whether zebrafish embryo exposure *in vivo* to $NaAsO_2$ causes up-regulation of *IGF2* gene expression, northern blot analysis was performed on total RNA of 8 hpf zebrafish embryos that were treated with various concentrations of $NaAsO_2$ (1 μM – 20 μM). Using an *IGF2* cDNA as a probe, we observed slight up-regulation of *IGF2* transcription in embryos treated with $NaAsO_2$ at 1 μM, 2 μM, 5 μM and 10 μM concentration of $NaAsO_2$. In comparison to the untreated control, in 20 μM

treatment there was little if any up-regulation of *IGF2* expression (Figure 10). We used 200 μM 5-azaC as a positive control because *IGF2* gene expression was up-regulated with this concentration of 5-azaC as shown in previous experiments. A *β-actin* probe was used to ensure equal loading of total RNA. In order to confirm this result, we conducted the same procedure of RT-PCR analysis because RT-PCR is more sensitive and potentially more quantitative than northern blot. RT-PCR analysis using *IGF2* primer pairs was done on cDNA generated from total RNA of zebrafish embryos collected at 8 hpf and treated with 1 μM – 20 μM concentration of $NaAsO_2$. This procedure was repeated using the *IGF1* and *IGFBP-1* primers. RT-PCR detected that *IGF2* gene expression was up-regulated in a similar fashion obtained from the result of the northern blot (Figure 11). Liver cDNA and *IGF2*, *IGF1* and *IGFBP-1* primers were used in RT-PCR reaction for a positive control due to their high expression in the liver. We observed expression of all three of these genes in the liver. Water was used as a negative control to detect the presence or absence of contamination and we did not observe any PCR product in these samples. The *β-actin* was used to confirm equal loading of cDNA in each reaction. The up-regulation of *IGF2* observed in embryos treated with 5-azaC was greater than the up-regulation of *IGF2* observed in embryos treated with $NaAsO_2$.

Figure 10: Northern blot analysis showing that *IGF2* gene expression is altered following $NaAsO_2$ treatment.

Northern blot of zebrafish embryos treated with 0 μM, 1 μM, 2 μM, 5 μM, 10 μM, 20 μM of $NaAsO_2$ until 8 hpf. High induction of IGF2 expression is observed at 1 μM, 2 μM, 5 μM and 10 μM $NaAsO_2$ treatments compared to untreated controls. A 200 μM 5-azacytidine treated sample was used as a positive control for *IGF2*. *β-actin* is relatively uniform.

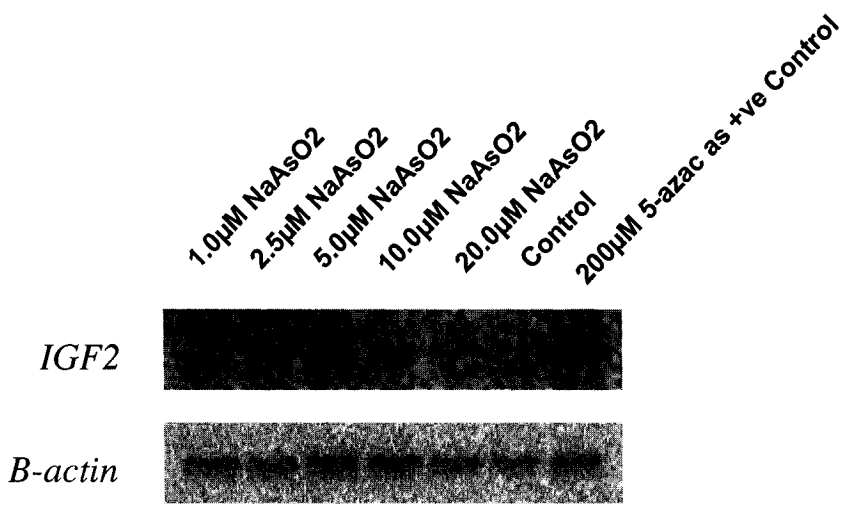
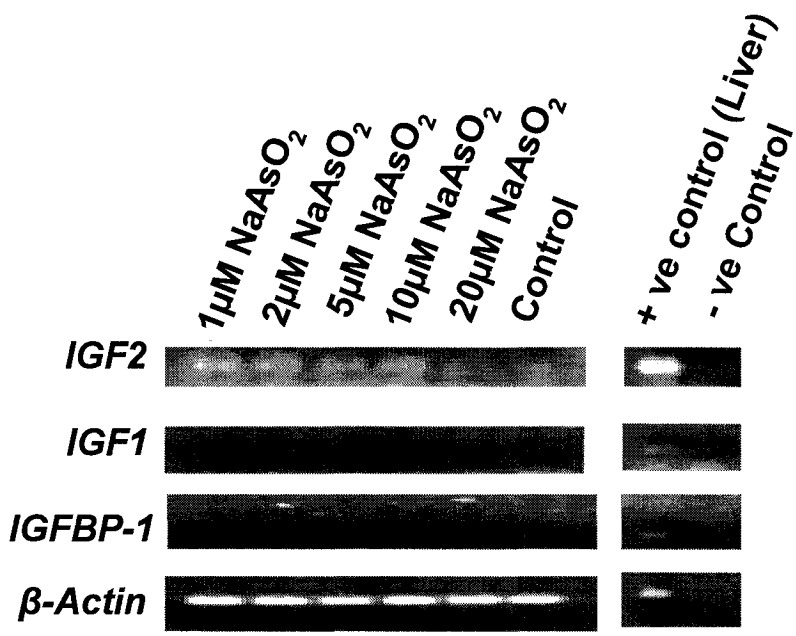

1.0μM NaAsO2
2.5μM NaAsO2
5.0μM NaAsO2
10.0μM NaAsO2
20.0μM NaAsO2
Control
200μM 5-azac as +ve Control
IGF2
B-actin

Figure 11: Semi-quantitive RT-PCR analysis showing that *IGF2* gene expression is altered following $NaAsO_2$ treatment.
Semi-quantitive RT-PCR analysis of *IGF2* expression in zebrafish embryos treated with 0 μM, 1 μM, 5 μM, 10 μM, 20 μM $NaAsO_2$ at 8 hpf. (a) High expression of *IGF2* observed in all sets (1 μM – 10 μM) but low expression of *IGF2* in 20 μM compared with control. (b) and (c) No expression of *IGF1* and *IGFBP-1*. (d) *β-actin* expression is relatively uniform in all sets. Liver cDNA and negative control were used as control.


1μM NaAsO2
2μM NaAsO2
5μM NaAsO2
10μM NaAsO2
20μM NaAsO2
Control
+ ve control (Liver)
- ve Control
IGF2
IGF1
IGFBP-1
β-Actin

In order to determine if $NaAsO_2$ can effect survivability of zebrafish embryos, twenty embryos were placed in each of the ten Petridishes (n=10). Treatment of zebrafish embryos with $NaAsO_2$ until 8 hpf and 24 hpf (1 μM – 20 μM) showed no significant changes in the mortality rate compared with zebrafish embryos that were not exposed to $NaAsO_2$ (Figure 12, 13). Potentially higher concentration might give higher mortality as well as inducing higher levels of $NaAsO_2$. DNA methylation has previously been shown to be affected by arsenic (Zhao et al., 1997), and a previous study indicated there was relationship between DNA methylation and *IGF2* (Wutz et al., 1998). Therefore, our observation suggests that *IGF2* can be used as a marker or indicator for toxins that cause DNA hypomethylation.

Figure 12: Cumulative mortality rate of zebrafish embryos at 8 hpf treated with $NaAsO_2$.

Embryos were allowed to develop in an incubator at 28° C for 8 hours post-fertilization, n=10 (group of 20). Treatment of zebrafish embryos with $NaAsO_2$ 0 μM, 1 μM, 5 μM, 10 μM and 20 μM shows insignificant mortality rate compared with the control zebrafish embryos.

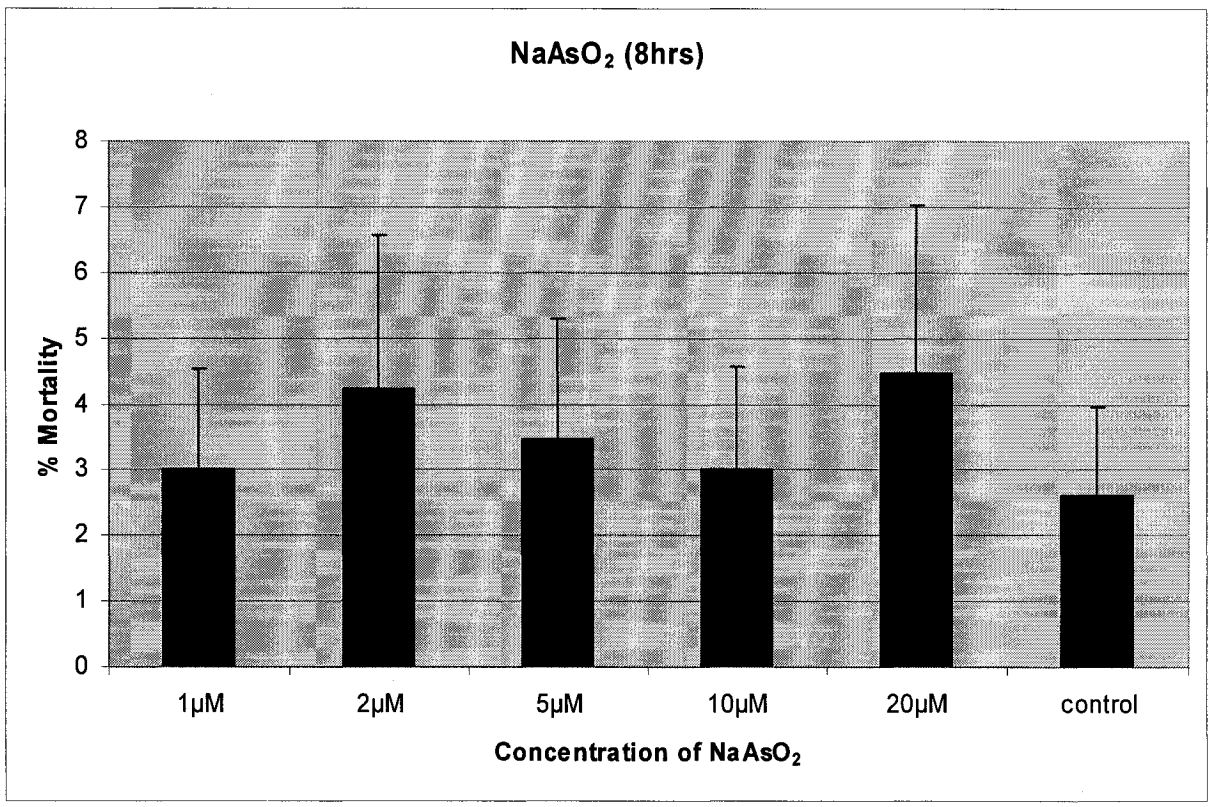
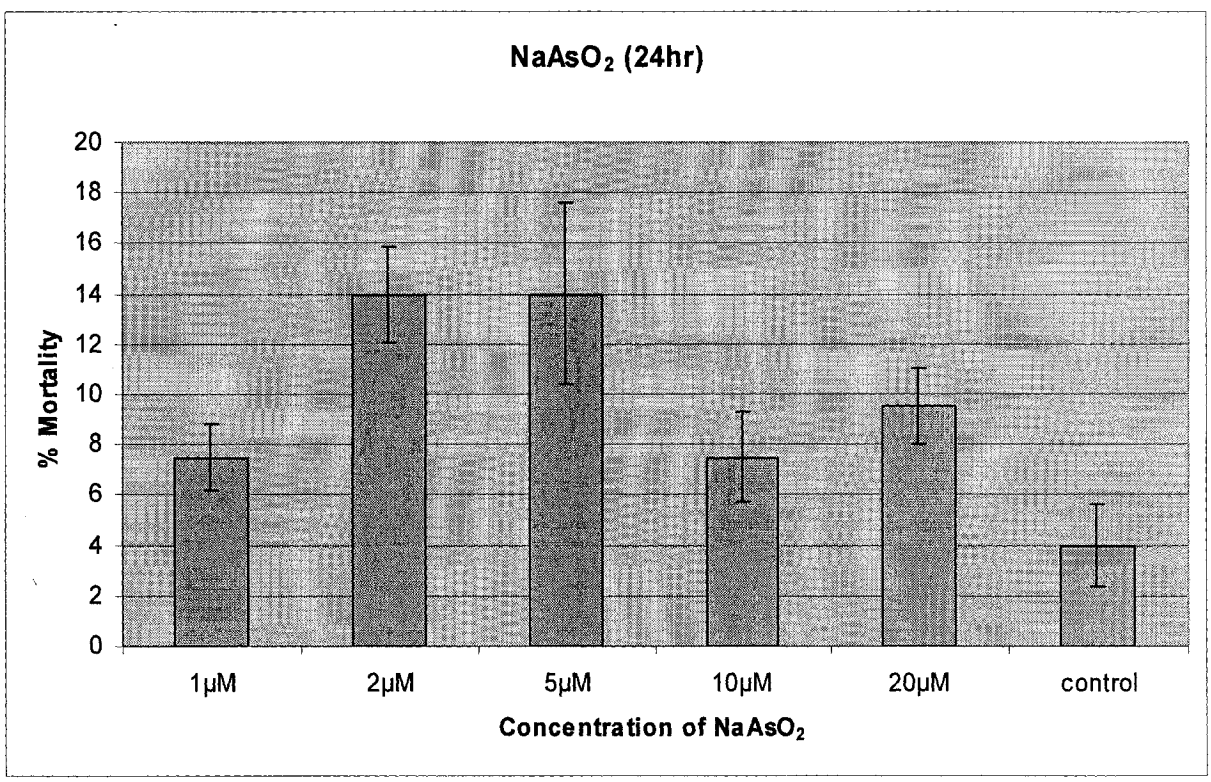

NaAsO2 (8hrs)
% Mortality
0
1
2
3
4
5
6
7
8
1μM
2μM
5μM
10μM
20μM
control
Concentration of NaAsO2

Figure 13: Cumulative mortality rate of zebrafish embryos at 24 hpf treated with $NaAsO_2$.

Embryos were allowed to develop in an incubator at 28° C for 24 hours post-fertilization, n=10 (group of 20). Treatment of zebrafish embryos with $NaAsO_2$ 0 μM, 1 μM, 5 μM, 10 μM and 20 μM shows insignificant mortality rate compared with the control zebrafish embryos.


NaAsO2 (24hr)
% Mortality
20
18
16
14
12
10
8
6
4
2
0
1μM
2μM
5μM
10μM
20μM
control
Concentration of NaAsO2

3.3 Treatment of zebrafish embryos with trichostatin A (TSA), nickel chloride ($NiCl_2$), mercury chloride ($HgCl_2$) and cadmium chloride ($CdCl_2$) does not result in abnormal expression of *IGF2, IGF1* and *IGFBP-1* genes

As previously shown, after treatment of zebrafish embryos with 5-azaC and $NaAsO_2$, there was an up-regulation of *IGF2* expression. On the other hand, other epigenetic toxins such as TSA, $NiCl_2$, $HgCl_2$ and $CdCl_2$ have shown no effect of *IGF2*, *IGF1* and *IGFBP-1*. Epigenetic effects of these metals are summarized in table 2. The same method of RT-PCR assay has been done on cDNA generated from total RNA zebrafish embryos collected at 8 hpf and treated with various concentration of TSA and $NiCl_2$. Northern blot analysis was used for $HgCl_2$ and $CdCl_2$ (table2). All of them, *IGF2*, *IGF1* and *IGFBP-1*, have shown no increase in expression levels (Figure 14, 15, 16, 17). To determine if the TSA affects survivability of zebrafish embryos, twenty embryos were placed in each of the ten petridishes (n=10). Treatment of zebrafish with TSA at 8 hours showed insignificant mortality rates compared to control zebrafish embryos (Figure 18). After 24 hours, the mortality rate increased at a higher concentration of 0.750 μm TSA 5.5% compared to 1 % control (Figure 19).

Table 2: Summary – Epigenetic toxins that may pose epigenetic effect to mankind

Epigenetical Toxins	sources	Effects on health	Water Quality*	Epigenetic effect	[] of treatment
$NaAsO_2$	Coal, Petroleum, detergent, mine tailing, pesticides (Rodriguez et al., 2002)	DNA repair inhibition, cancer, DNA methylation, disturbance, cellular proliferation, induction of apoptosis (Gradecka et al., 2001)	0.3 μM/L	DNA hypomethylation (cell culture) (Zhao et al., 1997)	1 μM – 20 μM
$NiCl_2$	Diesel oil, coal, tobacco smoke, steel and non ferrous alloys (IARC, 1990)	Lung and nasal cancer (IARC, 1990)	0.1 μM/L	DNA hypermethylation (cell culture) (reviewed in Cangul et al., 2002)	50 μM – 4000 μM
$HgCl_2$	Coal, electrical batteries, other industrials (Buhler, 1972)	Nerve damage and death (Chang, 1979)	0.0037 μM/L	Unknown	1 μM – 32 μM
$CdCl_2$	Coal, tobacco, water pipes (Fels, 1999)	Cancer, cardiovascular disease, hypertension (IARC, 1993)	0.027 μM/L	DNA hypermethylation (cell culture) (Takignchi et al., 2003)	1.0 μM – 40.0 μM
5-azac (cancer drug)				DNA methylatransferase inhibition (Jones, 1984)	10 μM – 200 μM
TSA Antifungal antibiotic				Histone deacetylase inhibition (Yoshida et al., 1990)	0.050 μM – 0.750 μM

* Guidelines for Canadian drinking water Quality (1978)

Figure 14: Semi-quantitive RT-PCR analysis using *IGF2*, *IGF1* and *IGFBP-1* primers and cDNA from embryos treated with 0 μM, 0.050 μM, 0.100 μM, 0.200 μM, 0.500 μM, 0.750 μM TSA.
(a) (b) and (c) show no expression of *IGF2*, *IGF1* and *IGFBP-1*. (d) *β-actin* expression is relatively uniform in all sets. Liver cDNA and negative control were used as controls.

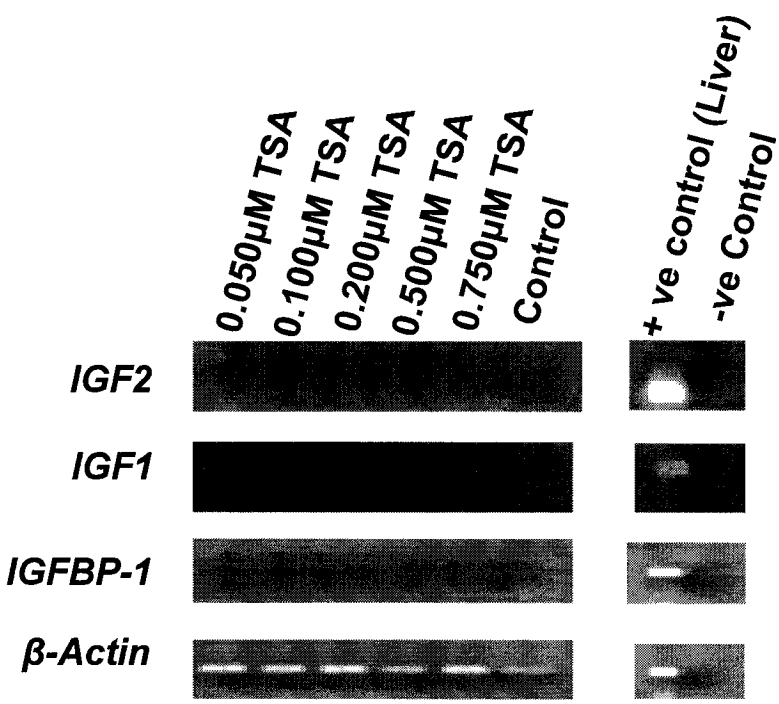

0.050µM TSA
0.100µM TSA
0.200µM TSA
0.500µM TSA
0.750µM TSA
Control
+ ve control (Liver)
-ve Control
IGF2
IGF1
IGFBP-1
β-Actin

Figure 15: Semi-quantitive RT-PCR analysis using *IGF2*, *IGF1* and *IGFBP-1* primers and cDNA from embryos treated with 0 μM, 50 μM, 100 μM, 200 μM, 500 μM, 1000 μM, 2000 μM, 4000 μM of $NiCl_2$. (a), (b) and (c) show no expression of *IGF2*, *IGF1* and *IGFBP-1*. (d) *β-actin* expression is relatively uniform in all sets. Liver cDNA and negative control were used as controls.

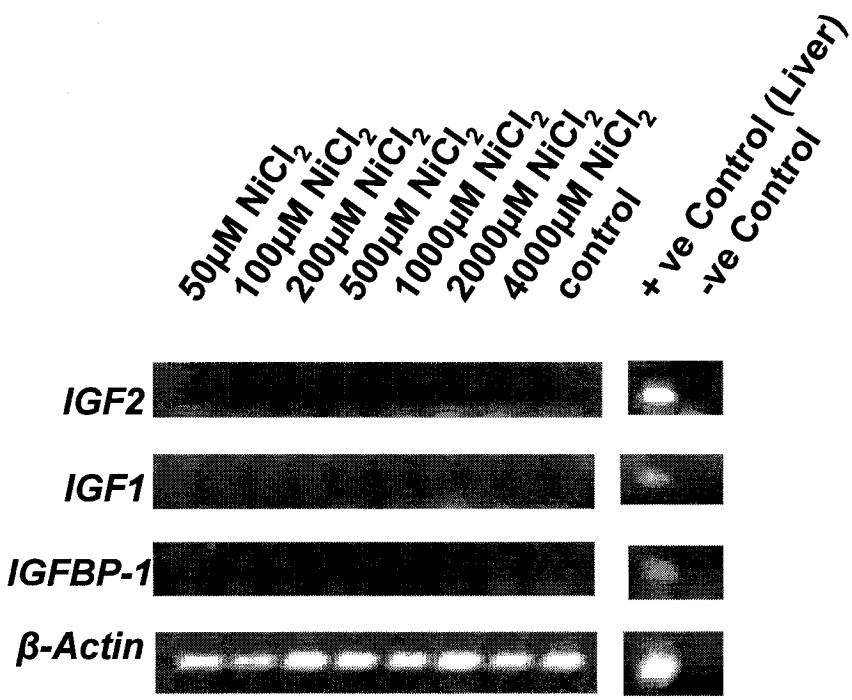

50μM NiCl2
100μM NiCl2
200μM NiCl2
500μM NiCl2
1000μM NiCl2
2000μM NiCl2
4000μM NiCl2
control
+ ve Control (Liver)
-ve Control
IGF2
IGF1
IGFBP-1
β-Actin

Figure 16: Northern blot analysis reveals that *IGF2* gene expression in zebrafish embryos was not affected by $CdCl_2$ treatment.
Northern blot of zebrafish embryos treated with 0 μM, 1.0 μM, 2.5 μM, 5.0 μM, 10.0 μM, 20.0 μM $CdCl_2$. 200 μM 5-azacytidine was used as a positive control. *β-actin* was relatively uniform.

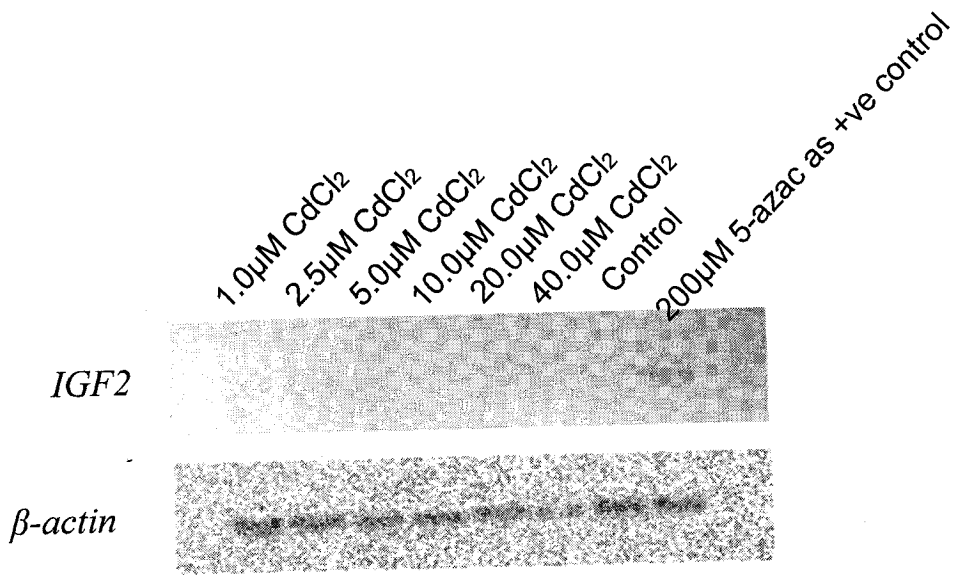

1.0μM CdCl2
2.5μM CdCl2
5.0μM CdCl2
10.0μM CdCl2
20.0μM CdCl2
40.0μM CdCl2
Control
200μM 5-azac as +ve control
IGF2
β-actin

Figure 17: Northern blot analysis reveals that *IGF2* gene expression in zebrafish embryos was not affected by $HgCl_2$ treatment.
Northern blot of zebrafish embryos treated with 0 μM, 1μM, 2 μM, 4 μM, 8 μM, 16 μM, 32 μM $HgCl_2$. 200 μM 5-azacytidine was used as a positive control. *β-actin* was relatively uniform.

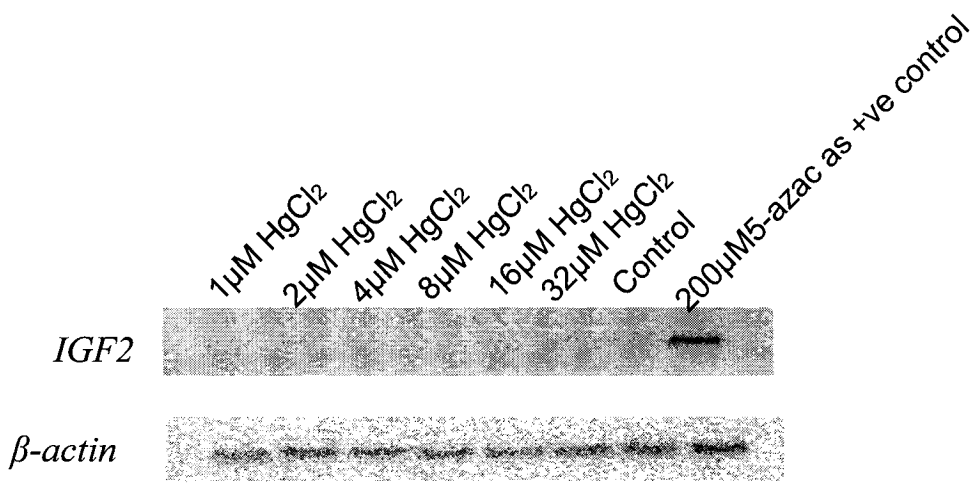

1μM HgCl2
2μM HgCl2
4μM HgCl2
8μM HgCl2
16μM HgCl2
32μM HgCl2
Control
200μM5-azac as +ve control
IGF2
β-actin

Figure 18: Cumulative mortality rate of zebrafish embryos at 8 hpf treated with TSA.

Embryos were allowed to develop in an incubator at 28°C for 8 hours post-fertilization, n=10 (group of 20). Treatment of zebrafish embryos with TSA 0 μM, 0.050 μM, 0.100 μM, 0.200 μM, 0.500 μM and 0.750 μM shows insignificant mortality rate compared with the control zebrafish embryos.

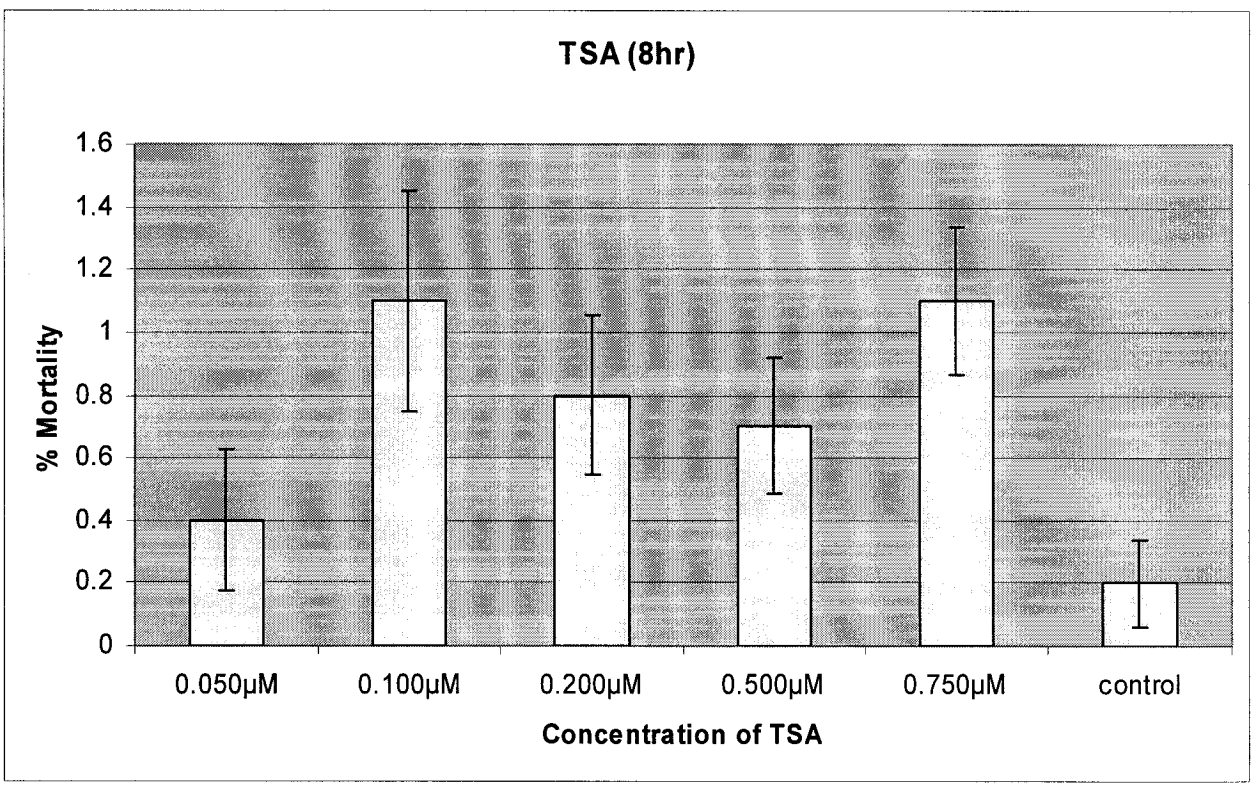
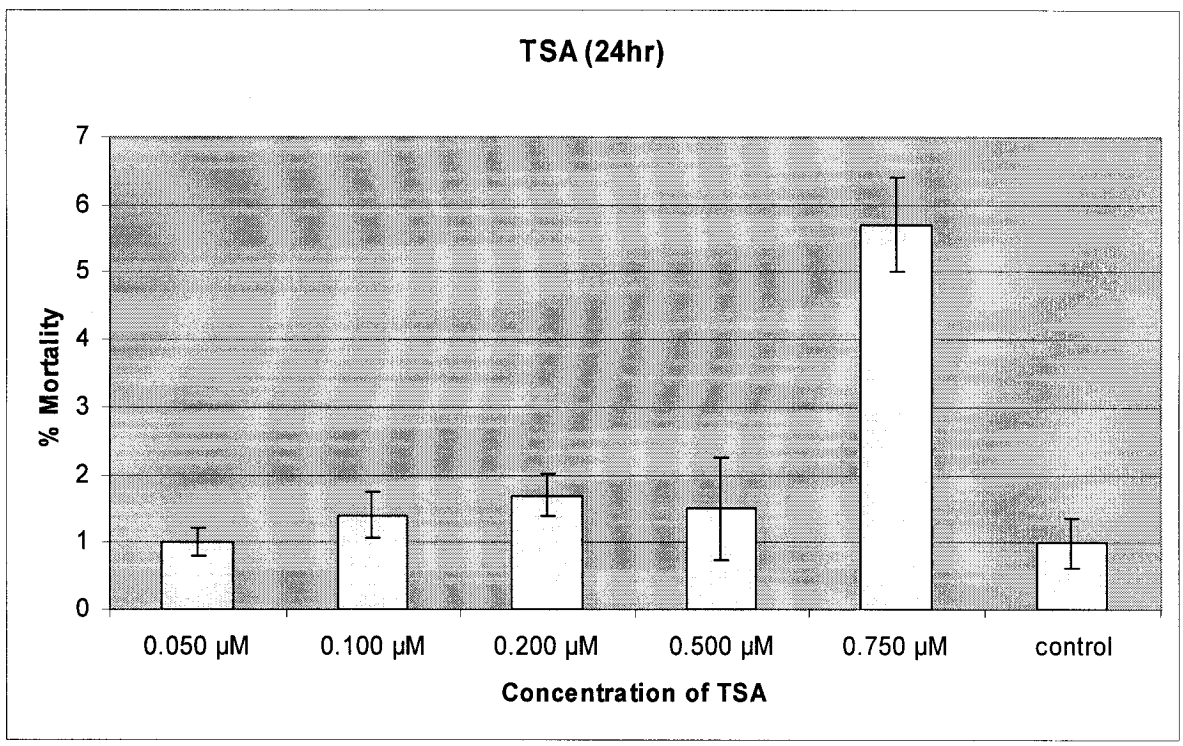

TSA (8hr)
% Mortality
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.050μM
0.100μM
0.200μM
0.500μM
0.750μM
control
Concentration of TSA

Figure 19: Cumulative mortality rate of zebrafish embryos at 24 hpf treated with TSA.

Embryos were allowed to develop in an incubator at 28° C for 24 hours post-fertilization, n=10 (group of 20). Treatment of zebrafish embryos with TSA 0.750 μM shows higher mortality rate than 0.050 μM, 0.100 μM, 0.200 μM, 0.500 μM compared with the control zebrafish embryos.


TSA (24hr)
% Mortality
7
6
5
4
3
2
1
0
0.050 µM
0.100 µM
0.200 µM
0.500 µM
0.750 µM
control
Concentration of TSA

Treatment of zebrafish with $NiCl_2$ at 8 hours and 24 hours also showed insignificant mortality rates (Figure 20, 21). There was no difference between control and treated zebrafish embryos, and no expression of *IGF2* was present. At this time we cannot exclude the possibility that $NiCl_2$ was not able to enter the cell (reviewed in Sutherland and Costa, 2003). Treatment of zebrafish with $HgCl_2$ for 8 hours showed higher mortality rate at 32 μM, which is approximately 50 % compared to other concentrations of $HgCl_2$ and untreated control (Figure 22). After 24 hours, there was 100 % mortality at 16 μM and 32 μM (Figure 23). Treatment of zebrafish with $CdCl_2$ for 8 hours and 24 hours has shown high mortality at 40 μM compared with untreated control (Figure 24, 25).

Riggio et al., 2003 determined that pre-treatment of zebrafish embryos at gastrula stage with 5-azaC-2-deoxycytidine, induces metallothionein (MT) synthesis following exposure to cadmium. They suggested that the DNA methylation is one of the factors regulating MT expression. Therefore, we pre-treated zebrafish embryos with 50 μM 5-azaC, 50 μM 5-azaC + 10 μM $CdCl_2$, 50 μM 5-azaC + 20 μM $NaAsO_2$, 50 μM 5-azaC + 200 μM $NiCl_2$, 50 μM 5-azaC + 8 μM $HgCl_2$, which resulted in the induction of IGF_2 expression at 8 hours compared with untreated control (Figure 26).

Figure 20: Cumulative mortality rate of zebrafish embryos at 8 hpf treated with $NiCl_2$.

Embryos were allowed to develop in an incubator at 28° C for 8 hours post-fertilization, n=10 (group of 20). Treatment of zebrafish embryos with $NiCl_2$ 0 μM, 50 μM, 100 μM, 200 μM, 500 μM, 1000 μM, 2000 μM and 4000 μM shows insignificant mortality rate compared with control zebrafish embryos.

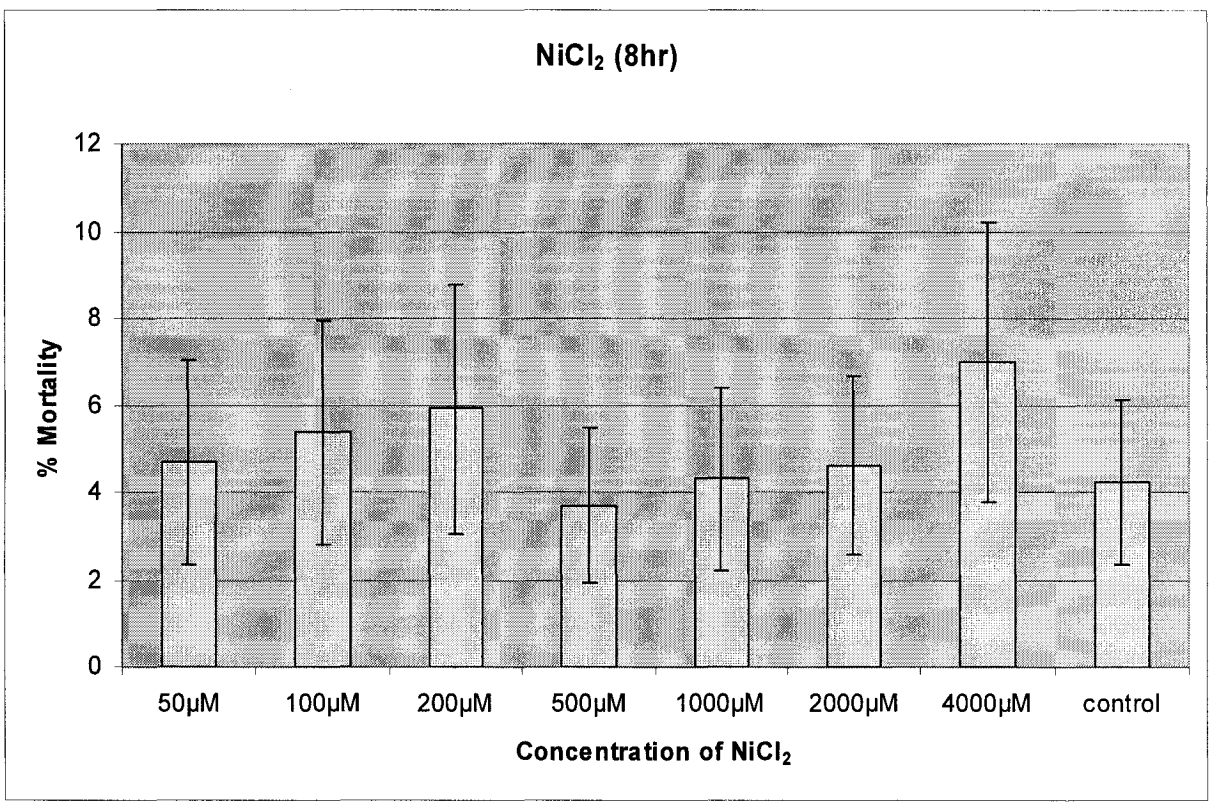

NiCl2 (8hr)
% Mortality
12
10
8
6
4
2
0
50μM
100μM
200μM
500μM
1000μM
2000μM
4000μM
control
Concentration of NiCl2

Figure 21: Cumulative mortality rate of zebrafish embryos at 24 hpf treated with $NiCl_2$.

Embryos were allowed to develop in an incubator at 28° C for 24 hours post-fertilization, n=10 (group of 20). Treatment of zebrafish embryos with $NiCl_2$ 0 μM, 50 μM, 100 μM, 200 μM, 500 μM, 1000 μM, 2000 μM and 4000 μM shows insignificant mortality rate.

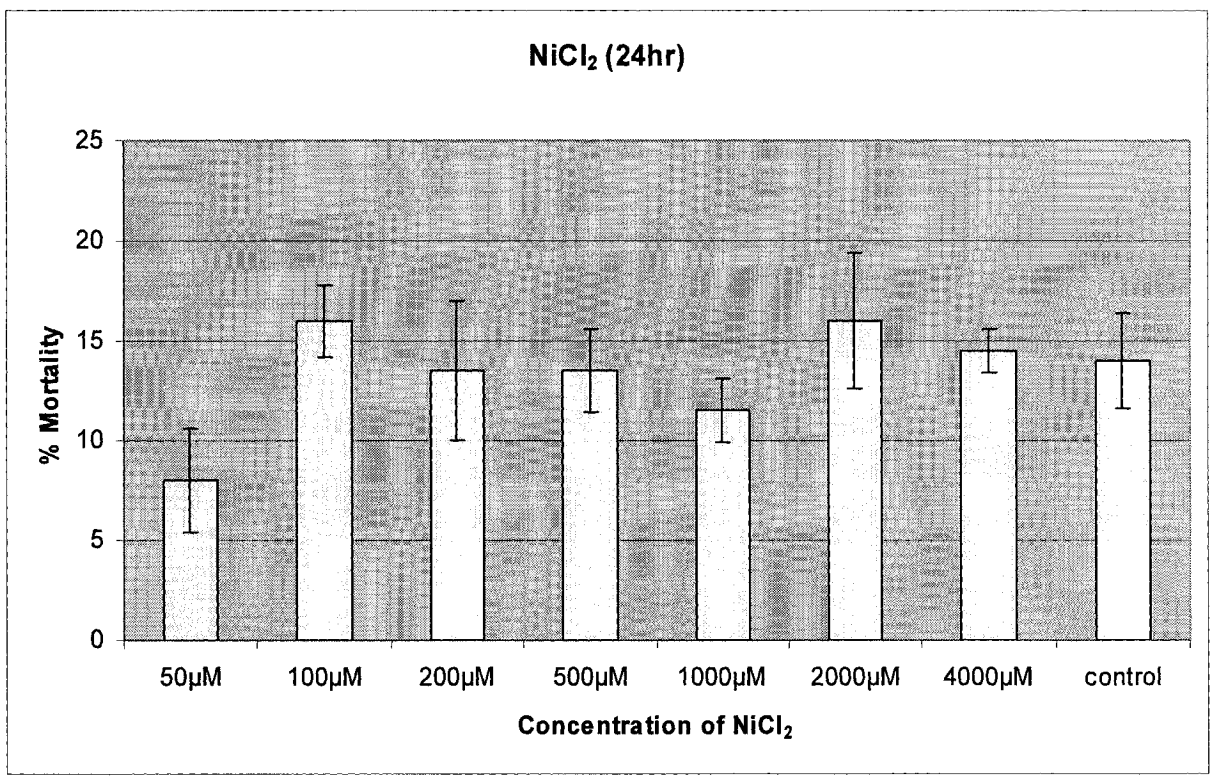

NiCl2 (24hr)
% Mortality
25
20
15
10
5
0
50µM
100µM
200µM
500µM
1000µM
2000µM
4000µM
control
Concentration of NiCl2

Figure 22: Cumulative mortality rate of zebrafish embryos at 8 hpf treated with $HgCl_2$.

Embryos were allowed to develop in an incubator at 28° C for 8 hours post-fertilization, n=10 (group of 20). Treatment of zebrafish embryos with $HgCl_2$ 32 µM showed 50 % over all mortality, 16 µM $HgCl_2$ showed 5 % mortality, 8 µM showed 3 % whereas 1µM, 2 µM and 4 µM showed no mortality; similar result was found with control.

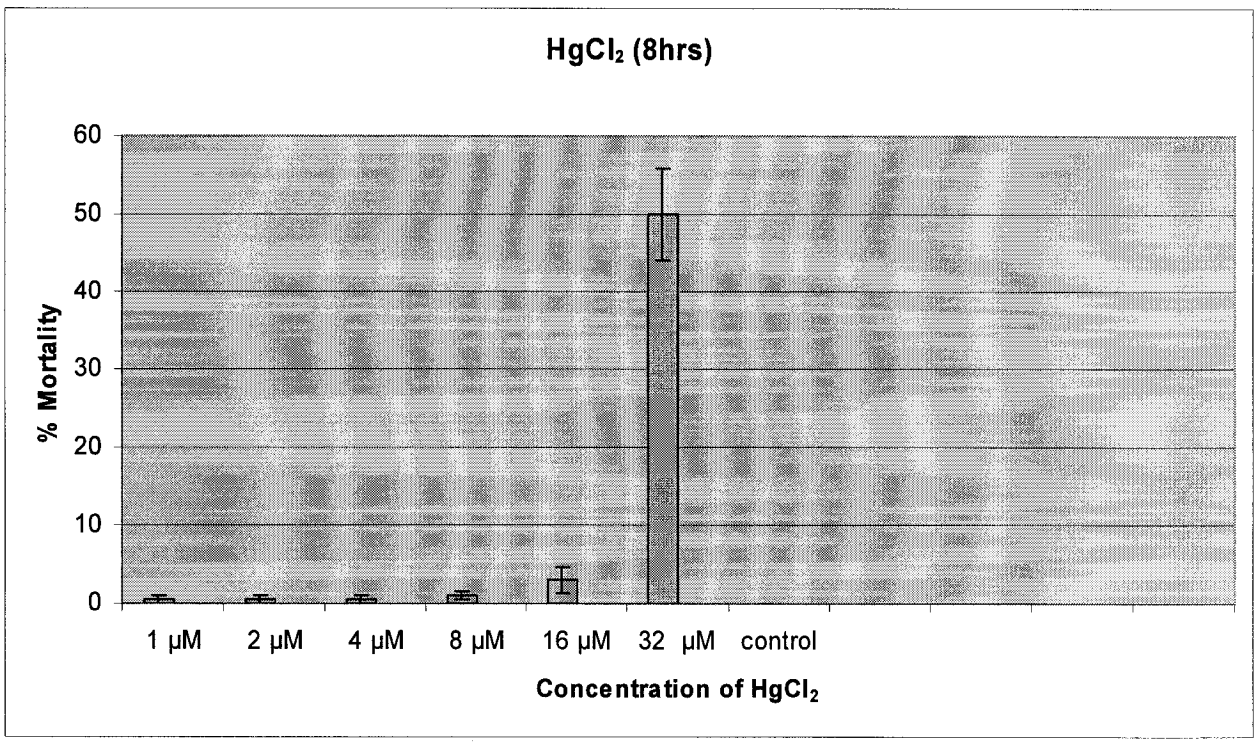

HgCl2 (8hrs)
% Mortality
60
50
40
30
20
10
0
1 µM
2 µM
4 µM
8 µM
16 µM
32 µM
control
Concentration of HgCl2

Figure 23: Cumulative mortality rate of zebrafish embryos at 24 hpf treated with $HgCl_2$.

Embryos were allowed to develop in an incubator at 28° C for 24 hours post-fertilization, n=10 (group of 20). Treatment of zebrafish embryos with 16 μM and 32 μM showed 100 % mortality. 2 μM and 4 μM showed 35 % mortality. 8 μM showed 63 % mortality while the control embryos showed less mortality.

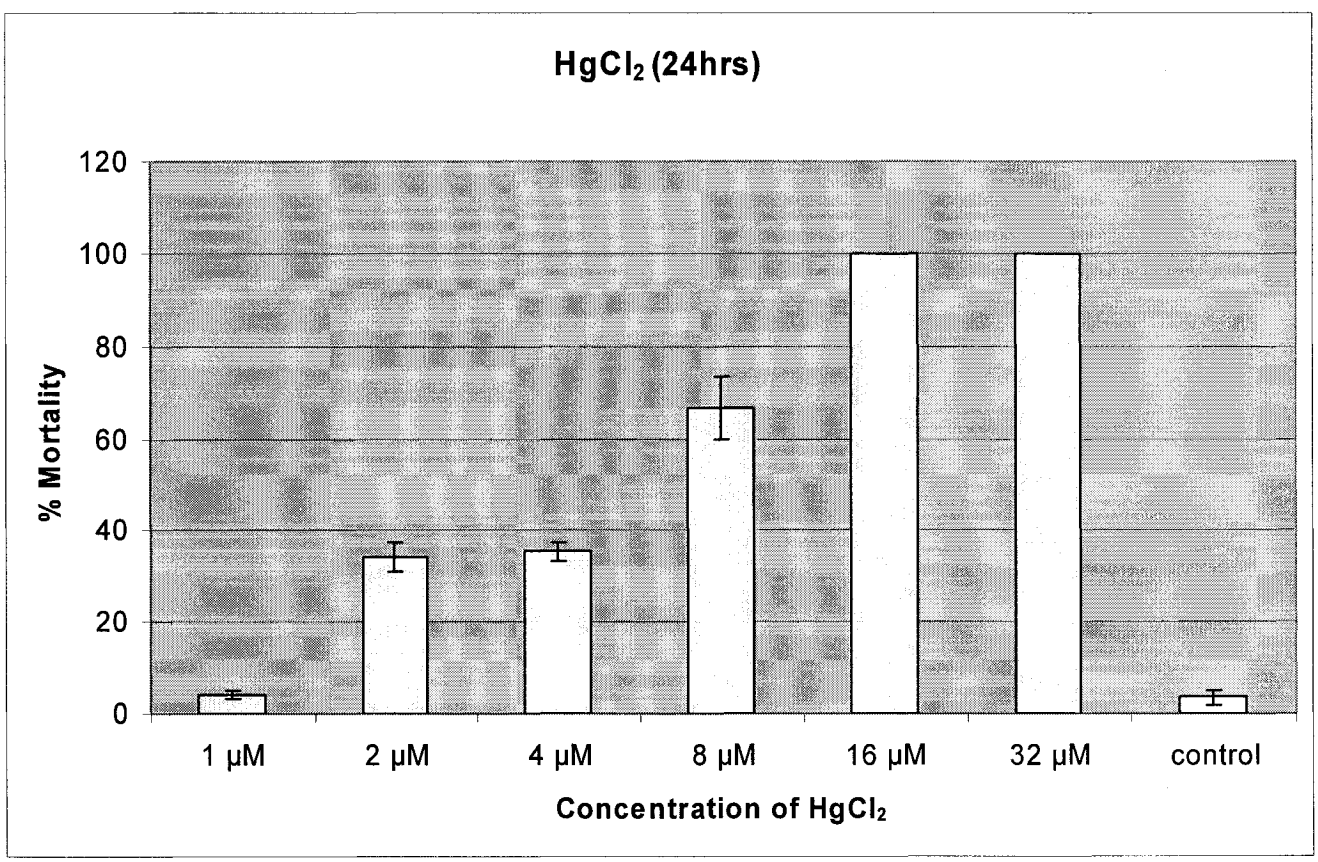

HgCl2 (24hrs)
120
100
80
60
40
20
0
% Mortality
1 µM
2 µM
4 µM
8 µM
16 µM
32 µM
control
Concentration of HgCl2

Figure 24: Cumulative mortality rate of zebrafish embryos at 8 hpf treated with $CdCl_2$.

Embryos were allowed to develop in an incubator at 28° C for 8 hours post-fertilization, n=10 (group of 20). Treatment of zebrafish embryos with 0 μM, 1.0 μM, 2.5 μM, 5 μM, 10 μM, 20 μM and 40 μM showed higher mortality than the rest of the concentrations compared with control zebrafish embryos.

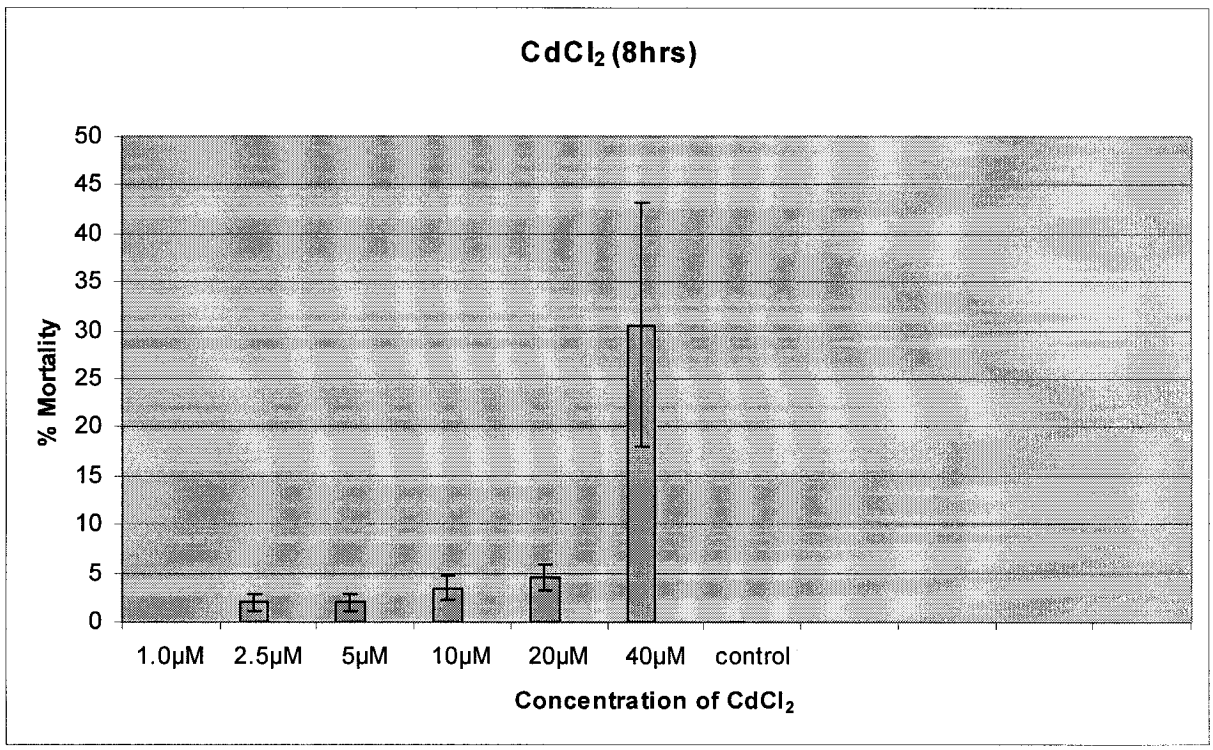

CdCl2 (8hrs)
% Mortality
50
45
40
35
30
25
20
15
10
5
0
1.0µM
2.5µM
5µM
10µM
20µM
40µM
control
Concentration of CdCl2

Figure 25: Cumulative mortality rate of zebrafish embryos at 24 hpf treated with $CdCl_2$.

Embryos were allowed to develop in an incubator at 28° C for 24 hours post-fertilization, n=10 (group of 20). Treatment of zebrafish embryos with 0 μM, 1.0 μM, 2.5 μM, 5 μM, 10 μM, 20 μM and 40 μM showed higher mortality than the rest of the concentrations.

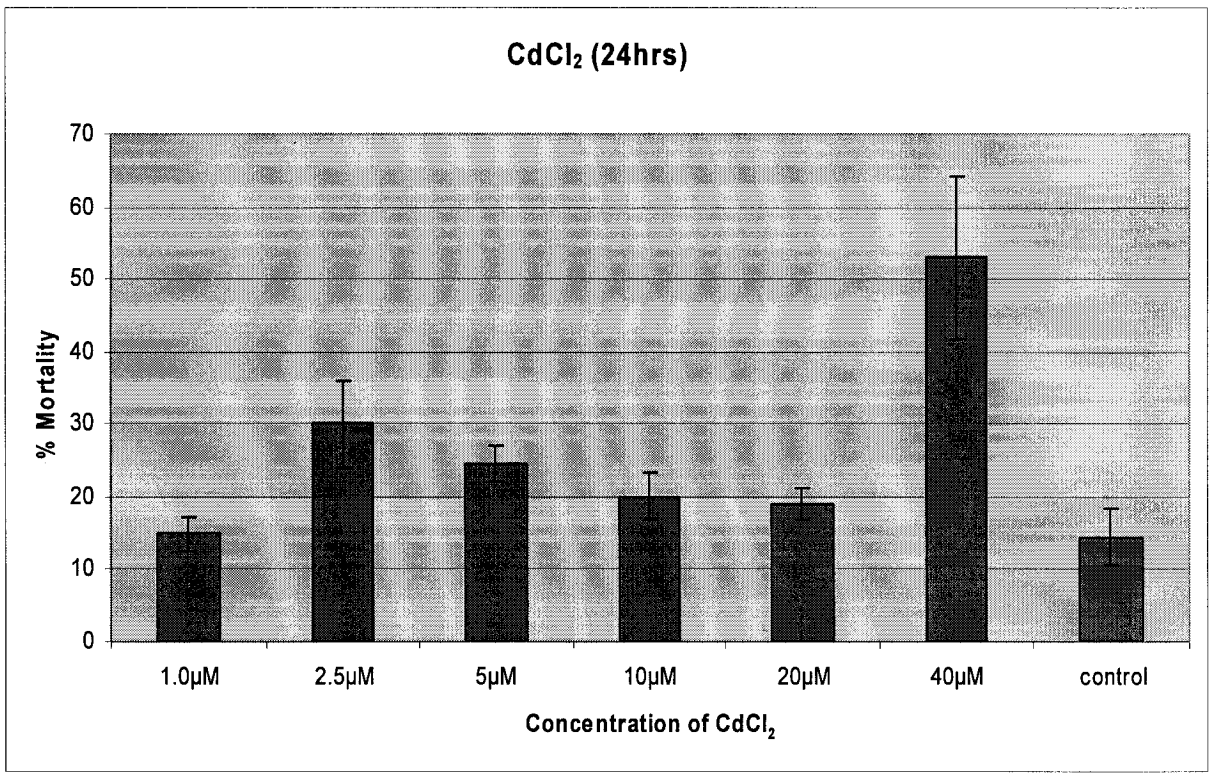
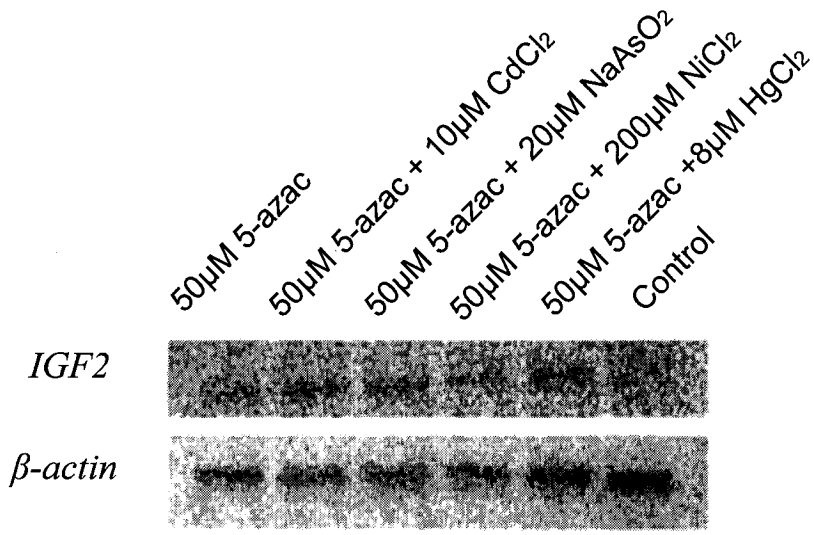

CdCl2 (24hrs)
% Mortality
70
60
50
40
30
20
10
0
1.0μM
2.5μM
5μM
10μM
20μM
40μM
control
Concentration of CdCl2

Figure 26: Northern blot analysis reveals that *IGF2* gene expression in zebrafish embryos was not affected pre-treated with 5-azacytidine following exposure to $CdCl_2$, $NaAsO_2$, $NiCl_2$ and HgCl2.

Northern blot of zebrafish embryos treated with 50 μM 5-azacytidine, 50 μM 5-azacytidine with 10 μM $CdCl_2$, 50 μM 5-azacytidine with 20 μM $NaAsO_2$, 50 μM 5-azacytidine with 200 μM $NiCl_2$, 50 μM 5-azacytidine with 8 μM $HgCl_2$ and untreated control for 8 hpf. 10 μM $CdCl_2$, 20 μM $NaAsO_2$, 200 μM $NiCl_2$, 8 μM $HgCl_2$ did not have a synergistic effect on up-regulation of *IGF2*. However, 5-azacytidine did affect *IGF2* gene expression. *β-actin* was relatively uniform.


50μM 5-azac
50μM 5-azac + 10μM $CdCl_2$
50μM 5-azac + 20μM $NaAsO_2$
50μM 5-azac + 200μM $NiCl_2$
50μM 5-azac +8μM $HgCl_2$
Control
IGF2
β-actin

The *β-actin* gene has a relatively uniform level of expression throughout development and serves to confirm equal loading of RNA and RNA integrity. These observations show that the stability in *IGF2* expression is not correlated to the $CdCl_2$, $NaAsO_2$, $NiCl_2$ and $HgCl_2$. However, they are correlated to the 5-azaC.

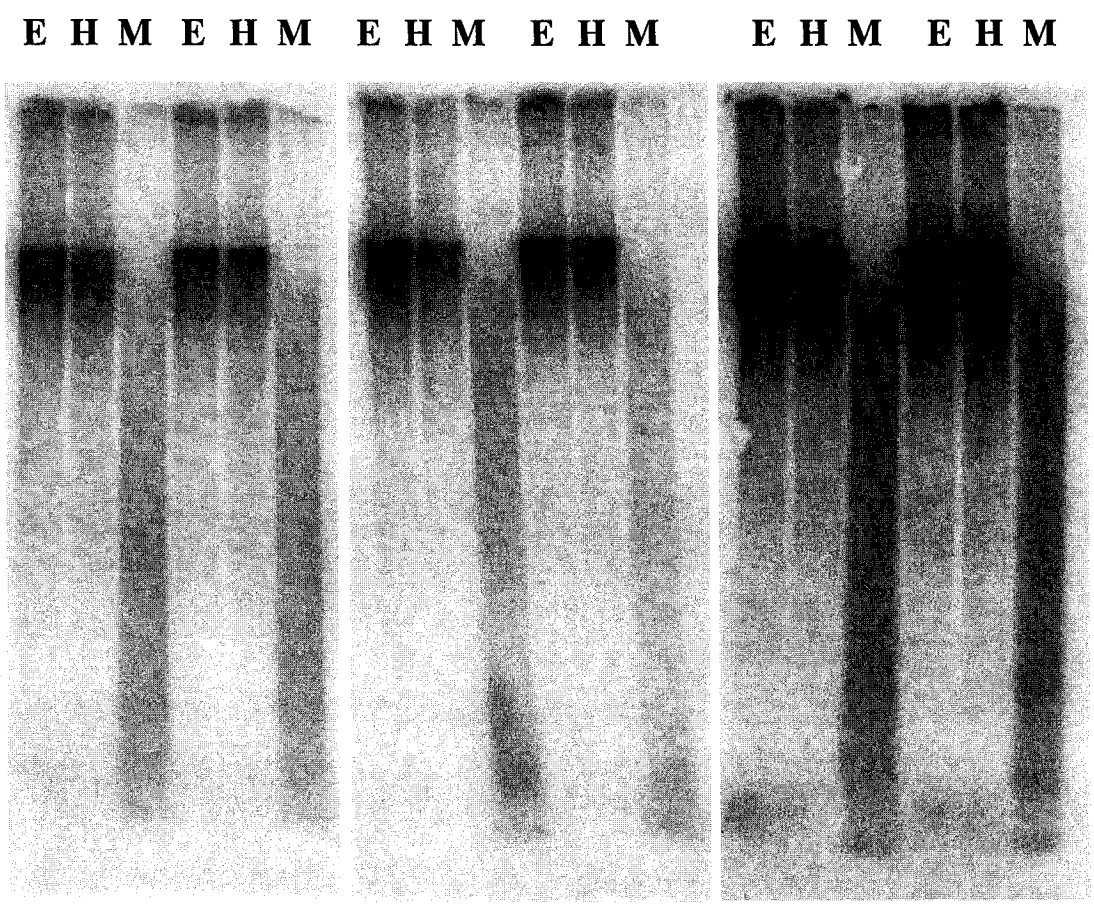
3.4 DNA Methylation of Zebrafish Genome

To evaluate the level of DNA methylation following treatment of zebrafish embryos with epigenetic toxins, DNA samples from zebrafish embryos treated with 50 μM 5-azaC, 10 μM $CdCl_2$, 20 μM $NaAsO_2$, 200 μM $NiCl_2$, 8 μM $HgCl_2$ and untreated control were digested with the restriction enzyme Hpa II or its isochizomer MspI both of which cut DNA at the sequence CCGG while EcoRI will cut DNA at the sequence GAATTC. MspI and HpaII are different in that MspI will cleave CCGG regardless of whether or not the cytosine residues are methylated while HpaII will only

cleave this sequence in the absence of methylation (Waalwijk and Flavell, 1978). The relative amount of DNA methylation can then be determined by comparing the relative proportion of high and low molecular weight DNA fragments. Southern blots were prepared from these digested DNAs and hybridized with the zebrafish repetitive sequence *Mermaid* (Shimoda et al., 1996a; Shimoda et al., 1996b). The results of this analysis presented in figure 27 showed no differences in the level of DNA methylation in 8 hpf embryos exposed to 5-azaC, $CdCl_2$, $NaAsO_2$, $NiCl_2$ and $HgCl_2$. Martin et al. (1999) used this technique and observed hypomethylation in 24 hpf embryos treated with 5-azaC. Therefore, this technique may not be sensitive enough to detect changes in methyl group (CH_3) at early stages of embryonic development or short term toxin exposure. Methods that utilize gene specific methylation analysis such as bisulfate sequencing may be required.

Figure 27: Southern blot analysis of DNA collected from zebrafish embryos treated with 5-azaC, $CdCl_2$, $NaAsO_2$, $NiCl_2$, $HgCl_2$ and untreated control digested with methylation sensitive restriction enzymes.

DNAs collected from zebrafish embryos at 8 hpf that were treated with 0 μM, 50 μM 5-azaC, 10 μM $CdCl_2$, 20 μM $NaAsO_2$, 200 μM $NiCl_2$ and 8 μM $HgCl_2$. Zebrafish embryos were digested with EcoRI, EcoRI/HpaII and EcoRI/MspI. Southern blots were hybridized to the zebrafish interspersed repetitive sequence *Mermaid*. Global DNA methylation changes were not detected in all zebrafish embryos treated with the above chemicals.


E H M E H M E H M E H M E H M E H M
50 uM 5-azaC
10 uM $CdCl_2$
20 uM $NaAsO_2$
200 uM $NiCl_2$
8 uM $HgCl_2$
control

4.0 DISCUSSION:

The main purpose of this study was to test whether the expression of *IGF2* and other genes (*IGF2* and *IGFBP-1*) can be used as a marker or indicator of epigenetic toxin exposure of an aquatic vertebrate, the zebrafish (*Danio rerio*). To examine the effect of DNA demethylation on the expression of *IGF2*, we treated the zebrafish embryos with 100 μM of 5-azaC. The increase of *IGF2* expression at 8 hpf that we observed in treated zebrafish embryos was confirmed using both RT-PCR and northern blot analysis. We observed precocious expression of the *IGF2* gene in embryos that are treated with 100 μM 5-azaC. Normally, *IGF2* is only expressed after 24 hpf. In order to determine if *IGF2* up-regulation is dependent on the concentration of 5-azaC treatment, we treated the zebrafish embryos with various concentrations of 5-azaC (0 μM -200 μM) from 1 hpf to 8 hpf. 5-azaC is an inhibitor of DNA methylation by inhibiting the DNA Mtase enzyme or by its incorporation into the DNA (Jones, 1984; Jones and Taylor, 1980). Therefore, the DNA hypomethylation in zebrafish embryos exposed to 5-azaC results in *IGF2* up-regulation. We also observed that 5-azaC treatments at any developmental stage or concentration did not cause up-regulation of *IGF1* or *IGFBP-1*. However, we observed normal *IGF1* and

IGFBP-1 expression after 24 hpf. This result was expected because research to date has shown that *IGF1* and *IGFBP-1* are not regulated by DNA methylation. Previous studies using mammalian systems support our observation that 5-azaC can alter *IGF2* expression. Eversole-Cire et al. (1993) examined *IGF2* expression in 5-azaC treated cells that were cultured from a mouse and they saw a 2-4 fold increase in *IGF2* expression from the imprinted allele. Jaenisch et al. (1985) also showed that 5-azaC treatment activated silent retroviral containing transgenes in postnatal mice, indicating that DNA demethylation affects the expression of a previously suppressed foreign gene *in vivo* as well. Hu et al. (1997) have also shown that 5-azaC leads to loss of *IGF2* imprinting in some tissues and allelic switching in other tissues. Taken together, these data confirm the importance of DNA methylation in the regulation of the imprinting process in mammals and demonstrates that the pharmacological agent 5-azaC can alter genomic imprinting *in vivo*. The evidence shows that DNA hypomethylation by treatments of zebrafish embryos with 100 μM 5-azaC causes transcriptional up-regulation of the *IGF2* gene at 8 hpf. Zebrafish embryos appear to be most sensitive to epigenetic insult during late blastula and gastrula stages of embryonic development. DNA methylation has previously been shown to be essential for normal development in zebrafish (Martin et al., 1999), in mice

(Walsh and Bestor, 1999) and *Xenopus* (Stancheva and Meehan, 2000). Interestingly, DNA methyltransferase (Mtase) is ubiquitously present at high levels in early embryos of zebrafish with overall levels decreasing slightly after the blastula stage (Martin et al., 1999). In addition, a similar phenomenon to genomic imprinting in mammals has been observed in zebrafish (McGowan and Martin, 1997). More specific evidence that *IGF2* expression is regulated by DNA methylation comes from studies using cultured primary human and mouse astrocytes. DNA demethylation induced by 5-azaC, dramatically increased the expression of *IGF2*. Fascinatingly, the increased expression of *IGF2* was primarily derived from the activation of imprinted maternal allele with normally expressed paternal allele also remaining active (Hu et al., 1996). I have shown in this study that, like mammals, the *IGF2* gene of zebrafish can also be affected *in vivo* by treatment with the DNA demethylation agent (5-azaC).

Normal levels of DNA methylation appear to be required for embryonic development of vertebrate organisms. In our study, the overall cumulative mortality of embryos treated with 100 μM 5-azaC until different developmental stages (4 hpf, 6 hpf, 8 hpf, 12 hpf and 24 hpf) showed relatively higher mortality rates compared to untreated controls (Figure 4). Comparison of mortality curves between embryos treated until 8 hpf and 24

hpf indicated that the majority of the mortalities are occurring after 8 hpf. We further observed that there is a correlation between concentration of 5-azaC, and presumably the corresponding level of DNA hypomethylation, and the induction of *IGF2* expression. This time period of highest mortality correlates with our observation of abnormal *IGF2* expression. We hypothesize that this increased mortality could be attributed at least in part to abnormal expression of *IGF2* and perhaps the mis-expression of other genes that have yet to be identified.

Thus, normal DNA methylation is required for successful embryonic development of aquatic vertebrates such as zebrafish (Martin et al., 1999). 5-azaC causes developmental abnormalities in zebrafish embryos such as abnormal development of the notochord and somites (Martin et al., 1999). Similar abnormalities are observed in frogs and mice whose genomes are hypomethylated. Stancheva et al. (2001) and Li et al. (1992) demonstrated that lossing of *Dnmt1* during development of mice and *Xenopus* can lead to high levels of apoptosis. This experiment helped to clarify that hypomethylation during early development can cause an increase in apoptosis and that *Dnmt1* is very important for early development. Our data, showed that hypomethylation causes induction of *IGF2* gene expression in zebrafish embryos at 8 hpf. The up-regulation of *IGF2* occurs shortly after

the sensitive period for 5-azaC treatments that results in axial defects (Martin et al., 1999). The *IGF2* system plays an important role in controlling animal development and growth such as regulating cell proliferation, differentiation, apoptosis and transformation (Jones et al., 1995). The biological actions of *IGF2*, mediated through their interactions with cell surface receptors and are modulated by *IGFBPs*. *IGFBPs* are crucial for normal animal growth and development (Maures et al., 2002). Therefore, abnormal expression of *IGF2* can cause abnormal growth and developmental of zebrafish embryos. This abnormal expression of *IGF2* may be contributing to the abnormal phenotype of 5-azaC treated zebrafish embryos that were observed by Martin et al. (1999). This work extends that of Martin et al., (1999).

To gain more insight into epigenetic toxins that might effect *IGF2* expression, we examined the effects of $NaAsO_2$ on *IGF2* expression levels in 8 hpf zebrafish embryos and detected increased expression of *IGF2* at treatment level of 1μM, 2 μM, 5 μM and 10 μM compared with untreated embryos. However, at 20μM treatment concentration we did not observe up-regulation of *IGF2* expression. This suggests that disturbances in the DNA methylation processes caused by 20 μM $NaAsO_2$, has lead to DNA hypermethylation rather than hypomethylation. Alternatively, treatment of

embryos at high concentration may simply cause cellular disfunction and metabolic shutdown. Gradecka et al., (2001) proposed that disturbances in the DNA methylation comes from a partial inhibition of SAM-dependent methyltransferase activity, which results in the increase of SAM pool in the cell and excessive methylation of cytosines in DNA. In a supporting study by Salazar et al. (1997), *in vitro* studies on human lung cancer cells A549 showed that exposure to sodium arsenite and arsenate (0.08-2 μM and 30-300 μM, respectively) leads to hypermethylation of cytosines in the promoter region of P^{53} gene. Cytosine hypermethylation may inhibit the transcription of the gene. As a result of P^{53} gene blocking, the concentration of P^{53} protein in the cell decreases, followed by disturbances in the cell cycle control, DNA repair control and apoptosis control. This result lead to the conclusion that arsenic may disturb protein P^{53} proteolysis and continuous overexpression of P^{53} genes results in shutting down its expression by hypermethylation of its promoter (Gradecka et al., 2001). This is similar to my result at 20 μM which showed that *IGF2* expression was not up-regulated. However, Zhong and Mass, (2001) also observed both hypermethylation and hypomethylation of DNA in human lung cells A549 after exposure to arsenite. These results indicate that both states (hypo- and hypermethylation) can exist in arsenite treated cells. Hypomethylation is

thought to occur by the consumption of methyl donor groups by the methylation of arsenic species within the cell. Hughes (2002) suggested that altered methylation within specific DNA sequences may be more important than the absolute level of DNA methylation. Furthermore, this finding also supports the view that $NaAsO_2$ may cause global hypomethylation of DNA and disturbances in the activities of methyltransferase. The exposure to inorganic arsenic compounds is associated with increased cellular proliferation and apoptosis (Zhao et al., 1997), Mitosis disturbances (Yih et al., 1999; Huang et al., 1998), aneuploidy (Yih et al., 1999), cytoskeleton disruption (Chou, 1989; Li et al., 1992), and apoptosis (wang et al., 1996; Gurr et al., 1999). Therefore, it is apparent that arsenic exposure causes pleiotropic effects and leads to chromosomal alterations (Yih et al., 2002). Since both DNA and arsenic methylation share the same methyl donor, S-adenosylmethionine, altered DNA methylation patterns, and either hypermethylation or hypomethylation, were observed in arsenite treated cells (Mass and Wang, 1997; Zhao et al., 1997; Zhong and Mass 2001). Therefore, Zhong and Mass proposed that DNA methylation imbalance could conceivably disrupt appropriate gene expression in arsenite exposed cells. In general, DNA methylation patterns are established during DNA replication. In other words, a methylated double stranded DNA needs two

sequential DNA replications to become a completely demethylated DNA. The arsenite-induced alteration in DNA methylation status occurred in cells chronically exposed to arsenite for 2-4 weeks (Mass and Wang, 1997; Zhao et al., 1997; Zhong and Mass, 2001). Whether the induction in zebrafish *IGF2* gene expression levels is due to the interference of DNA methylation by $NaAsO_2$ treatment of embryos remains to be clarified. A cumulative mortality relationship between the mortality level and $NaAsO_2$ exposure was not observed; in this case, there might be other risk factors interfering with gene expression. We also examined the effect of 5-azaC and $NaAsO_2$ on the *IGF1*, *IGFBP-1* and our results indicate there is no expression of *IGF1* and *IGFBP-1*. These findings suggest that the genes (*IGF1* and *IGFBP-1*) are not regulated by DNA methylation, whereas the *IGF2* is regulated by DNA methylation.

Our study shows that zebrafish embryos treated by 5-azaC and $NaAsO_2$ induced DNA hypomethylation and cause up-regulation of *IGF2* expression. In agreement with this hypothesis, it has been shown that the maternal allele of *IGF2* is hypermethylated in Wilm's tumors with normal *IGF2* imprinting, whereas it is hypomethylated in tumors that display a loss of imprinting of *IGF2* (Taniguchi et al., 1995). Abnormal *IGF2* expression has also been observed in a number of other cancerous tumors. Furthermore

a human disease called Beckwith-Wiedemann Syndrome (BWS) results from the dysregulation of *IGF2* imprinting. Similarly, transgenic mice that over-express *IGF2* also develop symptoms similar to BWS (Sun et al., 1997). In addition, this study provides evidence that significant imprinted genes are potential targets for dysregulation by epigenetic toxins that modify DNA methylation and histone acetylation (Susan et al., 2000). Clearly, DNA methylation is considered as an important factor in maintaining the differential allelic expression of *IGF2* gene (Hu et al., 1997), and altered DNA methylation may play a key of chemical induced toxicities (Watson and Goodman, 2002) such as 5-azaC $NaAsO_2$, TSA, $NiCl_2$, $CdCl_2$ and $HgCl_2$.

We have determined that Trichostatin A exposure did not effect *IGF2*, *IGF1* and *IGFBP-1* gene expression (Figure 14) in zebrafish embryos at 8 hpf. We treated the zebrafish embryos during blastula stage with various concentrations of TSA. Previous studies showed that TSA effectively inhibits histone deacetylation in mammalian cells and *Xenopus* embryos at concentrations as low as 30 nM (Yoshida et al., 1990). The inhibitions of histone deacetylation in *Xenopus* embryos by TSA delays gastrulation leading to diminished midtrunk and posterior formation consistent with

defects in mesoderm formation (Almouzni et al., 1994). Moreover, Selker (1998) has demonstrated that TSA selectively causes DNA hypomethylation of two DNA methylation sensitive genes (*hph* and *am*) in *Neurospora crassa.*

Ikegami and colleagues (1993) showed that low concentrations of TSA can lead to inhibited starfish embryogenesis at the early gastrula stage before mesenchyme formation. Thus, for both *Xenopus* and the starfish, regulated histone deacetylation appears to have an important role in executing differentiative programs during gastrulation (Almouzni et al., 1994). Hu et al. (1998) demonstrated that inhibition of histone deacetylation by Trichostatin A (TSA) induces the expression of the normally imprinted maternal *IGF2* gene, leading to biallelic expression in human and murine cells. Also, treatment of cultured fibroblasts of mice with the histone deacetylase inhibitor Trichostatin A induces partial relaxation of genomic imprinting as well as decreased DNA methylation of both *IGF2* sense and antisense promoters. These results demonstrate that increases in histone acetylation can lead to decreased DNA methylation (Hu et al., 2000). Histone acetylation/deacetylation plays a major role in gene activation and suppression (Alland et al., 1997; Heinzel et al., 1997). DNA methylation and histone acetylation, which represent two epigenetic modifications, are tightly

coupled in maintaining the chromatin structure of an imprinted locus, and thus the regulation and/or maintenance of genomic imprinting of *IGF2* (Hu et al., 1998).

Treatment of zebrafish embryos until 8 hpf with TSA caused insignificant mortality rate of zebrafish embryos while at 24 hpf, it caused a 5.5 % higher mortality rate at 0.750 μM concentration compared with untreated control zebrafish embryos. This result is similar to our observations of mortality in 5-azaC treated embryos – despite the fact that we did not observe any changes in gene expression. Pile and his colleagues (2001) examined the consequences of TSA on the development of *Drosophila melanogaster* by feeding them with TSA. They found TSA caused lethality and delayed development at concentrations as low as 5um. They suggest that TSA plays an important role in normal development progression and establishes parameters for genetic screens to dissect the role of deacetylases in development.

We also investigated whether *IGF2*, *IGF2* and *IGFBP-1* gene expression in zebrafish embryos at 8 hpf were affected by $NiCl_2$, $CdCl_2$ and $HgCl_2$ treatment. The data in figures 15, 16 and 17 show that epigenetic toxins such that $NiCl_2$, $CdCl_2$ and $HgCl_2$ do not affect *IGF2*, *IGF1* and *IGFBP-1* gene expression. First, IARC (1990) has reported that certain

nickel compounds including crystalline nickel sulfide and subsulfide were potent human and animal carcinogens. In addition, nickel also has been found to act as an epigenetic carcinogen by altering gene expression via effects on DNA hypermethylation, histone deacetylation and chromatin condensation in a heritable fashion (reviewed in Cangul et al., 2002). For example, Klein and colleagues (1991) proposed that in Chinese hamster embryo cells, an X-linked senescence gene was inactivated following nickel-induced DNA methylation. Further studies by Lee et al., (1995) had shown that nickel induced the inactivation of the *gpt*$^{+}$ Chinese hamster cell line, G12. Therefore, nickel may induce hypermethylation and DNA condensation in certain nickel responsive genome regions.

Treatment of zebrafish embryos with various concentrations of $NiCl_2$ at 8 hpf and 24 hpf showed insignificant mortality rates. Nickel ions (insoluble nickel) have the ability to enter cells and reach chromatin via phagocytic processes and accumulate inside the cell (reviewed in Sutherland and Costa, 2003). We treated zebrafish embryos with soluble nickel which has a lower ability to enter cells. $NiCl_2$ may not have entered the embryo and therefore we would not expect any different observations from the control.

Secondly, cadmium is considered as a human carcinogen, likely to act via epigenetic mechanisms (Takiguchi et al., 2003). Cadmium is often a

potent enzyme inhibitor (Goering et al., 1995). Takiguchi and colleagues (2003) showed that rat liver cells exposed to cadmium initially exhibited reduced DNA Mtase activity and decreased genomic DNA methylation. However, longer exposures of these cells showed increases in both DNA Mtase activity and DNA methylation. Cadmium has been shown to cause down regulation of several tumors suppressor genes, such as P^{53} and P^{27}, both *in vitro* and *in vivo* (Zhao et al., 1997; Xu et al., 1999; Fang et al., 2002). Takiguchi and colleagues (2003) demonstrated that basal expression of metallothionein (MT) in TRL 1215 cells chronically exposed to cadmium showed a clear reduction in basal MT expression that was correlated with DNA hypermethylation. Although not an oncogene, MT expression is clearly controlled by methylation, with increased methylation decreasing expression (Zhao et al., 1997; Compere et al., 1981).

We saw that treatment of zebrafish embryos until 8 hpf with various concentrations of $CdCl_2$ has no effect on *IGF2* gene expression. Thus, treatment of zebrafish embryos at 8 hpf with $CdCl_2$ may cause hypermethylation of the *IGF2* gene but we would not be able to detect this effect at the embryonic stages tested in our analysis. This is because *IGF2* is not normally expressed at 8 hpf and therefore the repressive effects of $CdCl_2$ can not be assayed.

Thirdly, mercury is a major issue in environmental health (Clarkson et al., 1993) as it can be biotransformed to methylmercury, accumulate into aquatic organisms, and enter the food chain (Wood et al., 1969). Methylmercury is recognized as being teratogenic (Shimai et al., 1985) and genotoxic (Shenker et al., 2002) in laboratory animals. Adverse effects observed in laboratory animals include malformation of the spinal cord and retarded growth of the cerebellum (Shimai et al., 1985). It acts as a genotoxin by altering gene expression that affects cell survival and apoptosis (Shenker et al., 2002). In addition, there are possible effects from high exposure to methylmercury which lead to higher frequency of chromosome breakage in humans (Skerfving et al., 1970). Monetti et al. (2002) demonstrated that methylmercury exposure to *Xenopus* embryos lead to the inhibition of the $HIPK_3$ expression. Although that mercury is recognized its toxicity, we demonstrated that the relative frequency of death in zebrafish embryos same as *Xenopus laeuis* embryos (Prati et al., 2002) is reduced by very low concentrations of $HgCl_2$ (In Figure 22 and 23). However, at a high concentration, 8 hpf, zebrafish had a 50 % mortality rate, and a 24 hpf 100 % mortality was observed compared with untreated control.

Riggio et al., (2003) determined that pre-treatment of zebrafish embryos at the gastrula stage with 5-azaC-2-deoxycytidine followed by

cadmium treatment causes up-regulation of MT synthesis. Their observations showed that changes in metals and 5-azaC can have a synergistic affect. Therefore, we pre-treated zebrafish embryos with 50 μM 5-azaC, 50 μM 5-azaC + 10 μM $CdCl_2$, 50 μM 5-azaC + 20 μM $NaAsO_2$, 50 μM 5-azaC + 200 μM $NiCl_2$, 50 μM 5-azaC + 2.5 μg $HgCl_2$, all of which resulted in the induction of *IGF2* expression at 8 hours compared with untreated control (Figure 26). The *β-actin* gene has a relatively uniform level of expression throughout development and serves to confirm equal loading of RNA and RNA integrity. These observations showed that the increases in *IGF2* expression are not further increased by the presence of $CdCl_2$, $NaAsO_2$, $NiCl_2$ or $HgCl_2$. In fact, 5-azaC caused the induction of *IGF2* with all metals in zebrafish embryos at gastrula stage compared with untreated control.

Finally, we investigated the level of DNA methylation during treatment of zebrafish embryos with epigenetic toxins at these following concentrations: 0 μM, 50 μM 5-azaC, 10 μM $CdCl_2$, 20 μM $NaAsO_2$, 200 μM $NiCl_2$, and 2 μM $HgCl_2$ at 8 hpf. DNA from control and treated zebrafish embryos was digested with the restriction enzyme EcoRI, EcoRI/HpaII, and EcoRI/MspI. Southern blots were prepared from the digested DNAs and hybridized with the zebrafish repetitive sequence

Mermaid (Shimoda et al., 1996a; Shimoda et al., 1996b). The Southern blot analysis showed no differences in the level of DNA methylation at 8 hpf in zebrafish embryos exposed to any of the chemical toxins mentioned above. Actually, this technique showed low sensitivity for determining the level of DNA methylation during treatment of zebrafish embryos with epigenetic toxins. Perhaps later at 24 hpf we could observe methylation changes or using a technique such as bisulfate sequencing that looks at individual genes instead of the whole genome. Using the aforementioned technique, Martin et al. (1999) observed hypomethylation at 24 hpf zebrafish embryos treated with 5-azaC.

In conclusion, we determined that *IGF2* may provide a suitable marker for assaying epigenetic toxins in aquatic environments. This study has also introduced new directions for the detailed investigation of the changes in gene and protein expressions in the cells that have undergone chemical exposures. Moreover, this study opens a door to identify target genes such as *IGF2* whose regulation may be affected by epigenetic alterations.

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