DNA REPAIR PATHWAYS IN THE ORGANOGENESIS-STAGE
RAT CONCEPTUS

by

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This thesis is dedicated to my parents, Robin and Gloria Vinson.

They ensured that I could fulfill my ambitions of higher education without worry, by providing encouragement and support, sometimes at their own expense. It is only through their belief in my abilities, as well as their dedication and sacrifice, that I am ultimately able to present this thesis.
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...no one with an unbiased mind can study any living creature, however humble, without being struck with enthusiasm at its marvellous structure and properties.

Charles Darwin, 1874
Abstract

Birth defects seen after teratogen exposure depend on the nature of the insult and the stage of development of the embryo. Many teratogens induce DNA damage in the organogenesis-stage conceptus; the consequences of this insult depend on the extent of the damage and on the ability of the embryo to repair DNA. The hypothesis for this thesis is that DNA repair capacity plays a role in the response of the conceptus to DNA-damaging teratogens. The first objective of this thesis was to elucidate the expression profile of DNA repair pathway genes during mid-organogenesis (gestational days 10-12) in the rat, a period during which the conceptus is most susceptible to the malformations induced by genotoxic teratogens. Four main repair pathways were examined: the nucleotide excision repair, base excision repair, mismatch repair, and recombination repair pathways. The genotoxic stress-activated cell cycle checkpoint pathway was also examined. Genes from these pathways showed developmental time-specific alterations in transcript and protein levels, as well as activity. Differences in expression between yolk sac and embryo proper were observed at the transcript, protein, and activity levels.

The second goal of this thesis was to determine whether DNA repair machinery expression and activity are modified in the conceptus following exposure to teratogens. Changes in gene transcript, protein, and activity levels were observed following embryo culture with three known teratogens (cyclophosphamide, oxidative stress, methotrexate). While one base excision repair gene increased in expression, the remaining repair genes examined either did not change transcript levels or decreased in expression following teratogen
exposure, indicating a near complete inability to induce a DNA repair response following genotoxic stress. These results demonstrate that differential expression and regulation of DNA repair genes and gene products exist in the mid-organogenesis stage rat conceptus, and that the conceptus has a limited capacity to alter DNA repair responses at the transcript, protein, or activity level following genotoxic teratogen exposure.
Abrégé

Les malformations congénitales observées après une exposition à des tératogènes sont fonction de la nature des agressions et du stade de développement de l'embryon auquel celles-ci se produisent. Plusieurs tératogènes provoquent des dommages au niveau de l'ADN durant l'organogénèse; les conséquences de ces agressions dépendent de l'étendue des dommages et de la capacité de l'embryon à réparer son ADN. L'hypothèse testée dans cette thèse est que la capacité de réparer l'ADN joue un rôle dans la réponse de l'embryon à des tératogènes qui vont endommager l'ADN. Le premier objectif de cette thèse était de révéler le profil d'expression des gènes impliqués dans les processus de réparation de l'ADN durant une période clé de l'organogénèse chez le rat (jours 10 à 12 de la gestation), période durant laquelle l'embryon est le plus susceptible aux malformations induites par des tératogènes génotoxiques. Quatre voies de réparations ont été examinées: "nucleotide excision repair", "base excision repair", "mismatch repair" et "recombination repair". Les voies de contrôle du cycle cellulaire activées par un stress génotoxique ont aussi été étudiées. Les gènes impliqués dans ces voies présentent des modifications spécifiques reliées au stade du développement, aux niveaux de la transcription et des protéines de même qu'au niveau de leur activité.

Le second objectif de cette thèse était de déterminer si l'expression et l'activité des mécanismes de réparation de l'ADN sont modifiées chez l'embryon après exposition à des tératogènes. Des changements au niveau de la transcription des gènes, des protéines et des niveaux d'activités ont été observés
après avoir cultivé des embryons en présence de trois tératogènes connus: la cyclophosphamide, le stress oxidatif et le methotrexate. Alors que l'expression de l'un des gènes de la voie "base excision repair" augmente, les autres gènes de réparation examinés restent inchangés en terme de leur transcription ou leur expression diminue après exposition aux tératogènes. Ceci semble indiquer une totale incapacité à induire une réponse des voies de réparation de l'ADN à la suite d'un stress génotoxique. Ces résultats démontrent qu'il existe différentes régulations et expressions des gènes de réparation de l'ADN chez l'embryon durant certaines périodes clés de l'organogénèse et que l'embryon a des capacités limitées pour modifier les réponses des voies de réparation de l'ADN tant au niveau de la transcription qu'à celui des protéines et de leur activité à la suite d'exposition à des tératogènes génotoxiques.
# TABLE OF CONTENTS

Abstract ........................................................................................................ vi
Abrégé ........................................................................................................... viii
List of Figures .................................................................................................. xiii
List of Tables .................................................................................................... xiv
Glossary ........................................................................................................... xv
Acknowledgements .......................................................................................... xviii
Preface ............................................................................................................. xix

## CHAPTER ONE: Introduction

1.1 Genotoxic Stress and DNA Damage ....................................................... 1
   1.1.1 Examples of Genotoxic Agents ...................................................... 2
1.2 DNA Repair Pathways ............................................................................ 4
   1.2.1 Nucleotide Excision Repair ......................................................... 5
   1.2.2 Base Excision Repair ................................................................. 8
      i. UNG: Uracil DNA Glycosylase .................................................... 11
   1.2.3 Mismatch Repair ....................................................................... 13
   1.2.4 Recombination Repair .............................................................. 17
   1.2.5 DNA Repair During Development ........................................... 20
1.3 Cell Cycle Checkpoints ......................................................................... 22
   1.3.1 Genotoxic Stress-Induced Cell Cycle Checkpoints ..................... 22
   1.3.2 Genotoxic Stress Response/Checkpoint Machinery .................... 24
      i. ATM: Ataxia-Telangiectasia Mutated ..................................... 25
      ii. ATR: ATM and Rad3-related ................................................. 28
      iii. DNA-PK: DNA-Dependent Protein Kinase ......................... 29
   1.3.3 Downstream Targets of PI3K Family Members ......................... 30
      i. p53 ......................................................................................... 31
      ii. GADD45 .............................................................................. 32
      iii. p21 ..................................................................................... 32
   1.3.4 Genotoxic Stress Checkpoints During Development .................. 33
1.4 Teratogens and Teratology ................................................................... 34
   1.4.1 Genotoxic Teratogens ............................................................... 36
   1.4.2 Teratogenicity of Alkylating Agents ......................................... 37
      i. Cyclopsphamid ....................................................................... 39
      ii. Nucleotide Excision Repair and Cyclopsphamid .................... 41
   1.4.3 Teratogenicity of Folate Deficiency .......................................... 41
      i. Effects of Thymidine Depletion Due to Folic Acid Deficiency .... 45
      ii. Methotrexate ........................................................................ 47
      iii. Uracil DNA Glycosylase and Folate Deficiency ................. 49
   1.4.4 Oxidative Stress as a Teratogen .................................................. 49
      i. Base Excision Repair and Oxidative Stress ............................. 50
      ii. ATM and Oxidative Stress Checkpoints .................................. 51
   1.4.5 DNA Repair/Cell Cycle Gene Expression During Development .... 51
   1.4.6 DNA Repair Gene Null Mutant Animals and Teratogenesis ....... 52
   1.4.7 DNA Repair Pathways and Cell Cycle Checkpoints in Mechanisms
       of Teratogen Action .................................................................. 53
1.5 Rationale and Purpose of the Investigation ....................................... 54
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6 Hypothesis of the Thesis</td>
<td>55</td>
</tr>
<tr>
<td>1.7 Objectives of the Thesis</td>
<td>56</td>
</tr>
<tr>
<td>Connecting Text</td>
<td>57</td>
</tr>
<tr>
<td>CHAPTER TWO: Nucleotide Excision Repair Gene Expression in the Rat Conceptus During Organogenesis</td>
<td>58</td>
</tr>
<tr>
<td>2.1 Abstract</td>
<td>59</td>
</tr>
<tr>
<td>2.2 Introduction</td>
<td>60</td>
</tr>
<tr>
<td>2.3 Materials and Methods</td>
<td>62</td>
</tr>
<tr>
<td>2.4 Results</td>
<td>69</td>
</tr>
<tr>
<td>2.5 Discussion</td>
<td>77</td>
</tr>
<tr>
<td>2.6 Acknowledgements</td>
<td>81</td>
</tr>
<tr>
<td>2.7 Appendix 1: aRNA Control Experiments</td>
<td>82</td>
</tr>
<tr>
<td>Connecting Text</td>
<td>89</td>
</tr>
<tr>
<td>CHAPTER THREE: Expression of Base Excision, Mismatch, and Recombination Repair Genes in the Organogenesis-Stage Rat Conceptus and Effects of Exposure to a Genotoxic Teratogen, 4-Hydroperoxycyclophosphamide</td>
<td>90</td>
</tr>
<tr>
<td>3.1 Abstract</td>
<td>91</td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>92</td>
</tr>
<tr>
<td>3.3 Materials and Methods</td>
<td>95</td>
</tr>
<tr>
<td>3.4 Results</td>
<td>99</td>
</tr>
<tr>
<td>3.5 Discussion</td>
<td>106</td>
</tr>
<tr>
<td>3.6 Acknowledgements</td>
<td>113</td>
</tr>
<tr>
<td>Connecting Text</td>
<td>114</td>
</tr>
<tr>
<td>CHAPTER FOUR: Expression and Activity of the DNA Repair Enzyme Uracil DNA Glycosylase During Organogenesis in the Rat Conceptus and Following Methotrexate Exposure In Vitro</td>
<td>115</td>
</tr>
<tr>
<td>4.1 Abstract</td>
<td>116</td>
</tr>
<tr>
<td>4.2 Introduction</td>
<td>118</td>
</tr>
<tr>
<td>4.3 Materials and Methods</td>
<td>121</td>
</tr>
<tr>
<td>4.4 Results</td>
<td>126</td>
</tr>
<tr>
<td>4.5 Discussion</td>
<td>139</td>
</tr>
<tr>
<td>4.6 Acknowledgements</td>
<td>144</td>
</tr>
<tr>
<td>Connecting Text</td>
<td>145</td>
</tr>
<tr>
<td>CHAPTER FIVE: Genotoxic Stress Response Gene Expression in the Mid-Organogenesis Rat Conceptus</td>
<td>146</td>
</tr>
<tr>
<td>5.1 Abstract</td>
<td>147</td>
</tr>
<tr>
<td>5.2 Introduction</td>
<td>149</td>
</tr>
<tr>
<td>5.3 Materials and Methods</td>
<td>151</td>
</tr>
<tr>
<td>5.4 Results</td>
<td>155</td>
</tr>
<tr>
<td>5.5 Discussion</td>
<td>162</td>
</tr>
<tr>
<td>5.6 Acknowledgements</td>
<td>168</td>
</tr>
<tr>
<td>CHAPTER SIX: General Discussion</td>
<td>169</td>
</tr>
<tr>
<td>6.1 The Consequences of Genotoxic Teratogen Exposure</td>
<td>169</td>
</tr>
<tr>
<td>6.2 Summary of Thesis</td>
<td>172</td>
</tr>
<tr>
<td>6.2.1 Expression of DNA Repair Pathways in the Organogenesis-Stage Rat Conceptus</td>
<td>173</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1.1. The nucleotide excision repair (NER) pathway.......................... 6
Figure 1.2. The base excision repair (BER) pathway.................................. 10
Figure 1.3. The mismatch repair (MMR) pathway......................................... 15
Figure 1.4. The recombination repair (RCR) pathway.................................. 19
Figure 1.5. DNA damage-induced cell cycle checkpoints................................ 26
Figure 1.6. Cyclophosphamide metabolism and alkylation adducts................... 40
Figure 1.7. Folate metabolism........................................................................ 44
Figure 1.8. Structure of folic acid and methotrexate..................................... 48

Figure 2.1. Representative aRNA blots......................................................... 65
Figure 2.2. NER gene expression in vivo......................................................... 70
Figure 2.3. Western blot analysis of NER proteins in vivo............................... 72
Figure 2.4. Quantification of NER protein expression in vivo........................... 73
Figure 2.5. Photographs of embryos cultured with 4-OOHCPA....................... 75
Figure 2.6. NER gene expression following exposure to 4-OOHCPA..................... 76
Figure 2.7. The aRNA technique..................................................................... 83
Figure 2.8. Test of aRNA amplification reaction............................................. 86
Figure 2.9. aRNA blot reproducibility............................................................. 87
Figure 2.10. Comparison of aRNA standards................................................ 88

Figure 3.1. BER gene expression in vivo......................................................... 100
Figure 3.2. MMR gene expression in vivo....................................................... 101
Figure 3.3. RCR gene expression in vivo........................................................ 103
Figure 3.4. BER, MMR, and RCR gene expression following 4-OOHCPA exposure................................................................. 105

Figure 4.1. UNG gene expression in vivo......................................................... 127
Figure 4.2. UNG protein expression in vivo...................................................... 128
Figure 4.3. UNG enzyme activity in vivo......................................................... 130
Figure 4.4. Photographs of embryos cultured with MTX.................................. 132
Figure 4.5. Quantification of developmental parameters following exposure to MTX...................................................................................... 133
Figure 4.6. UNG gene expression following exposure to MTX........................... 135
Figure 4.7. UNG protein expression following exposure to MTX....................... 136
Figure 4.8. UNG enzyme activity following exposure to MTX........................... 138

Figure 5.1. PI3K pathway gene expression in vivo.......................................... 156
Figure 5.2. PI3K pathway gene expression following short-term culture............ 158
Figure 5.3. ATM pathway gene expression following long-term culture............ 159
Figure 5.4. PI3K pathway gene expression following exposure to 4-OOHCPA 161

Figure 6.1. DNA repair and teratogenesis....................................................... 171
Figure 6.2. DNA repair pathways during organogenesis................................... 180
List of Tables

Table 1.1. DNA repair during development. .................................................. 21
Table 1.2. Genotoxic teratogens. ................................................................. 38
Glossary

4-OHCPA, 4-hydroperoxycyclophosphamide
5,10-MTHF, 5,10-methylene tetrahydrofolate
AP, apurinic/apyrimidinic
APE, AP endonuclease
APE/Ref-1, apurinic/apyrimidinic endonuclease/redox factor-1
APNG, alkylpurine-DNA-N-glycosylase
A-T, Ataxia-Telangiectasia
ATA, aurintricarboxylic acid
ATM, Ataxia-Telangiectasia Mutated
ATR, ATM and Rad3-related
BCNU, N,N'-bis(2-chloroethyl)-N-nitrosourea
BER, base excision repair
BSA, bovine serum albumin
Cdk, cyclin-dependent kinase
CPA, cyclophosphamide
CS, Cockayne Syndrome
DHFR, dihydrofolate reductase
DNA-PK, DNA-dependent protein kinase
DNA-PKcs, DNA-dependent protein kinase catalytic subunit
DRPase, DNA deoxyribosephosphatase
DSB, double strand DNA break
dTMP, deoxythymidine monophosphate
DTT, dithiothreitol
dTTP, deoxythymidine triphosphate
dUMP, deoxyuridine monophosphate
dUTP, deoxyuridine triphosphate
E. coli, Escherichia coli
ERCC1, excision repair cross-complementing gene 1
GADD45, Growth Arrest and DNA Damage-Inducible gene 45
GD, gestational day
GGG, global genome repair
H$_2$O$_2$, hydrogen peroxide
HR, homologous recombination
ICL, interstrand cross-link
IR, ionizing radiation
JNK, c-Jun N-terminal kinase
Ligl, DNA ligase I
LigIII, DNA ligase III
LigIV, DNA ligase IV
MAPK, mitogen-activated protein kinase
MGMT, $O^6$-methylguanine DNA methyltransferase
MLH1, MutL homologue 1
MMR, mismatch repair
MMS, methylmethane sulfonate
MNNG, N-methyl-N'-nitro-N-nitrosoguanidine
MRE11, meiotic recombination gene 11
MSH2, MutS homologue 2
MTHFR, 5,10-methylene tetrahydrofolate reductase
MTX, methotrexate
NER, nucleotide excision repair
NHEJ, non-homologous end joining
NTD(s), neural tube defect(s)
O$_2^-$, superoxide radical
O$_6$MeG, O$_6$-methylguanine
·OH, hydroxyl radical
PAR, poly (ADP-ribose) glycohydrolase
PARP, poly (ADP-ribose) polymerase
PCNA, proliferating cell nuclear antigen
PI3K, phosphatidylinositol 3-kinase
PMS1, postmeiotic segregation increased 1
PMS2, postmeiotic segregation increased 2
PolB, DNA polymerase β
PolD, DNA polymerase δ
PolE, DNA polymerase ε
RAD50, radiation sensitivity gene 50
RAD51, radiation sensitivity gene 51
RAD52, radiation sensitivity gene 52
RAD54, radiation sensitivity gene 54
RAD57, radiation sensitivity gene 57
RCR, recombination repair
RFC, replication factor C
RNR, ribonucleotide reductase
ROS, reactive oxygen species
RPB, replication protein A
SAM, S-adenosylmethionine
SCID, severe combined immunodeficiency
SDS, sodium dodecyl sulphate
SSA, single strand annealing
SSB, single strand DNA break
SSC, sodium chloride citrate
TBE, Tris-buffered EDTA
TBS, Tris-buffered saline
TCR, transcription-coupled repair
TFIIF, transcription factor IIH
THF, tetrahydrofolate
TTD, trichothiodystrophy
UDG, uracil DNA glycosylase base excision repair group
UGI, uracil DNA glycosylase inhibitor
UNG, uracil DNA glycosylase
uracil-diBTMBz, N1,N3-(3,5-bis(trifluoromethyl)benzyl)uracil
UV(R), ultraviolet (radiation)
XP, Xeroderma Pigmentosum
XRCC1, X-ray repair cross-complementing gene 1
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xviii
Preface

Format of the Thesis

This thesis consists of an Introduction Chapter (Chapter One), followed by four data Chapters (Chapters Two-Five), and finally a Summary Chapter (Chapter Six). Chapter One contains an overview of DNA repair pathways, teratology research, and genotoxic teratogens, as well as the hypothesis and objectives of this thesis. Chapter Two is as published in Mutation Research: DNA repair (486: 113-123, 2001) and Chapter Three in Teratology (in press at time of submission). Chapters Four and Five are manuscripts in preparation. Chapter Six includes a general discussion of the results of the thesis work, a summary of the major findings, and a List of Original Contributions.

Note on Gene and Protein Nomenclature

The classification scheme used for genes and gene products in this thesis is as follows: mouse genes are italicized, with the first letter capitalized (e.g. Atm); mouse gene products (i.e. proteins/enzymes) are not italicized (e.g. Atm). Human and rat genes are both italicized and capitalized (e.g. ATM), with their product not italicized (e.g. ATM). Genes and gene products from other species are listed as per usual conventions.

Contribution of Authors

This thesis conforms to the “Guidelines for Submitting a Doctoral or a Master's Thesis” from the Faculty of Graduate Studies and Research, McGill University. The candidate performed all experiments presented in this thesis.
CHAPTER ONE

Introduction

1.1 Genotoxic Stress and DNA Damage

All cellular life is exposed to stresses that cause lesions to its genetic material. It is estimated that each human cell experiences $2 \times 10^4$ lesions per day due to normal cellular metabolism (Ames and Shigenaga, 1992). Endogenous metabolites, such as lipid peroxides (Burcham, 1988) and reactive nitrogen species (Wink et al., 1994) create as broad a spectrum of genotoxic lesions as do external agents (for review see Burcham, 1999). A lifetime of such exposure may disrupt cellular pathways, resulting in disorders such as cancer, and the exacerbation of normal organismal endpoints such as aging (reviewed in: Kowald, 2001 and Ronen and Glickman, 2001). Increased expression of enzymes necessary for repairing genotoxic damage can lengthen the lifespan of lower organisms such as *Drosophila melanogaster* (Orr and Sohal, 1994) and *Caenorhabditis elegans* (Larsen, 1993).

Genotoxic agents give rise to a wide variety of lesions in DNA. Depending on the agent involved, DNA may be damaged via strand breaks (both single-strand breaks (SSBs) and double-strand breaks (DSBs)), covalent adduct formation, DNA crosslinks (intra- and interstrand crosslinks (ICL)), as well as DNA-protein crosslinks.
1.1.1 Examples of Genotoxic Agents

It is currently impossible to determine how many chemical compounds, both naturally occurring and man-made, are genotoxic. Recent studies show that many such exogenous genotoxic agents are encountered on a daily basis by most of the world’s population. Solar UV radiation (UVR; Griffiths et al., 1998), cigarette smoke (Pryor, 1997), and air pollution (Courtois et al., 1988; Hsiao et al., 2000; Don Porto Carero et al., 2001), as well as nitrates (Rubenchik et al., 1990; Routledge et al., 1994; Wang et al., 1995), dyes (Lakdawalla and Netrawali, 1988; Moller et al., 1998; Tsuda et al., 2001), and heterocyclic amines (reviewed in Sugimura, 2000) found in human diets, are just a few of the agents shown to cause significant genotoxic damage at exposures commonly encountered during a person’s lifetime. Humans are also exposed to genotoxic agents due to occupational exposure (reviewed in Keshava and Ong, 1999) or during their use as treatments for certain diseases, such as cancer.

The four main sources of genotoxic stress in mammalian organisms include oxidative stress, UVR, ionizing radiation (IR), and alkylating agents. For example, reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$) and superoxide radicals (O$_2^-$), formed from endogenous oxidative metabolism (Chance et al., 1979), IR (Ward, 1994), UVR (Tyrrell and Keyse, 1990) and metabolism of xenobiotic agents (Smith et al., 1991; Seidegard and Ekstrom, 1997), can create strand breaks, base modifications, and crosslinks (Burcham, 1999).
Different tissues within an organism can be exposed to different genotoxic stresses. For instance, neural tissue, owing to the formation of $\text{H}_2\text{O}_2$ by monoamine oxidases during neurotransmitter breakdown, may incur higher levels of oxidative genotoxic stress than other tissues (Hauptmann et al., 1996; Wei et al., 1996). As well, different tissues within the same organism can have differing repair capabilities, *e.g.* in rat (Coudore et al., 1997) and mouse (Washington et al., 1988). As well, differences can exist in the levels of damage and repair of nuclear *versus* mitochondrial DNA lesions. Mitochondrial DNA is affected by oxidative stress to a greater degree than genomic DNA due to its proximity to the source of ROS, the mitochondrial oxidative respiration pathways (Papa and Skulachev, 1997). Genetic information, wherever it may be located within the cell, must be protected from genotoxic stress by repair mechanisms. Recently, specific DNA repair pathways have been found in mitochondria (Bohr and Anson, 1999; Croteau et al., 1999). While mitochondria have the ability to remove small lesions from DNA, they do not appear to have the enzymes required to remove bulky adducts (reviewed in Croteau et al., 1999 and Bogenhagen et al., 2001).

Genotoxic agents are not only detrimental factors acting on modern day man: genotoxic stressors have been interacting with cellular life since the origins of life itself. In fact, certain genotoxic stressors have been implicated in the evolution of new species (Boulikas, 1992). Apart from this evolutionary by-product, DNA damage is usually damaging to an organism. As such, cellular organisms have evolved multiple biochemical pathways to recognize, respond, and repair genotoxic stress when it arises.
1.2 DNA Repair Pathways

To cope with the onslaught of genotoxic stress caused both by endogenous and exogenous agents, organisms have evolved cellular pathways that recognize and repair specific types of damage. Even the most primitive organisms, such as Archaea, have the capability to repair DNA (DiRuggiero et al., 1999). DNA repair enzymes are found in all organisms examined to date (Brendel et al., 1997). In fact, organisms that are not truly defined as “alive” possess DNA repair activity. For example, the Human Immunodeficiency Virus (Mansky et al., 2000), as well as the lambda (reviewed in Kuzimov, 1999), T4 (Stohr and Kreuzer, 2001) and T7 bacteriophages (Lai and Masker, 2000) all possess either their own DNA repair machinery or take over host cell DNA repair enzymes to ensure the integrity of their own genomes. Conservation of DNA repair pathways from the most primitive unicellular to the most complex multicellular organisms indicates the absolute necessity for specific DNA repair pathways in all organisms (reviewed in Taylor and Lehmann, 1998).

There are four main DNA repair pathways in mammalian cells: nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), and recombination repair (RCR) (reviewed in: Modrich, 1996, Wood, 1996, Norbury and Hickson, 2001, and Ronan and Glickman, 2001). All of these pathways are required for a cell, as well as the complete organism, to remain viable. Many null-mutant animals lacking specific DNA repair enzymes have compromised cellular functions and problems with cell and tissue growth, causing embryolethality (reviewed in Friedberg and Meira, 2000).
1.2.1 **Nucleotide Excision Repair**

The NER pathway is the major mammalian DNA repair system, repairing a broad spectrum of lesions caused by agents such as UVR and alkylating agents (Sancar, 1996). The major types of lesions removed via the NER pathway are bulky, helix distorting adducts or DNA modifications. The NER pathway is also involved in the initial repair of ICLs (Bessho *et al.*, 1997; De Silva *et al.*, 2000; Wang *et al.*, 2001) which are the primary lesions caused by nitrogen mustards (Povirk and Shuker, 1994).

NER is an active process requiring the concerted step-wise effort of 20-30 different polypeptides, and can be divided into three main biochemical stages: recognition of the lesion, incision around the lesion, and nascent DNA synthesis/ligation (de Laat *et al.*, 1999; Volker *et al.*, 2001) (**Figure 1.1**). Most members of the NER pathway are named after the Xeroderma Pigmentosum (XP) disease sub-groups that they complemented, in order to restore DNA repair capability in cells derived from those patients (reviewed in Cleaver *et al.*, 1999).

The NER pathway has two mechanisms of damage recognition which are tied to the transcriptional status of the damaged DNA. In global genome repair (GGR), a complex of XPC-hHR23B binds to DNA lesions, in particular DNA helix distortions (Sugasawa *et al.*, 2001), irrespective of transcription. The transcription-coupled repair (TCR) pathway repairs lesions in actively transcribed DNA (Leadon, 1999) through recognition by Cockayne Syndrome (CS) proteins CSA and CSB (Venema *et al.*, 1990) interacting with RNA polymerase II. XPA
Figure 1.1. The nucleotide excision repair (NER) pathway. Repair can occur initially through either the global genome repair (GGR) or transcription coupled repair (TCR) pathways; subsequent steps are the same for either pathway.
may also recognize and bind to damaged DNA and interact with other damage recognition proteins to organize them around the lesion (Jones and Wood, 1993). XPE, also known as UV-DDB, is primarily involved in recognizing and binding to UV-damaged DNA (Feldberg and Grossman, 1976; Chu and Chang, 1988), but also binds lesions caused by nitrogen mustards and cisplatin (Payne and Chu, 1994).

After recognition, the DNA is unwound around the lesion in an ATP-dependent fashion by the transcription factor IIH (TFIIH) (Evans et al., 1997). TFIIH contains two helicases, XPB and XPD, which are required for opening the DNA around the lesion in 3'→5' and 5'→3' directions, respectively (Schaeffer et al., 1993, 1994). XPA and replication protein A (RPA) are also required for complete opening of the DNA, and interact with several proteins in the repair complex (Aboussekhra et al., 1995). Two endonucleases cleave the DNA on either side of the lesion: first XPG on the 3' side (O'Donovan et al., 1994), then a complex of the excision repair cross complementing gene product 1 (ERCC1) and XPF on the 5' side (Mu et al., 1996). Repair of the DNA strand proceeds by DNA polymerase δ (PolD) or ε (PolE), replication factor C (RFC), proliferating cell nuclear antigen (PCNA) and DNA ligase I (LigI).

All core NER polypeptides (Mu et al., 1995), as well as several associated proteins, are necessary for full functionality of this pathway; mutations in NER genes result in the human disorders XP, CS, and trichothiodystrophy (TTD) (Cleaver et al., 1999). XP patients exhibit neurological abnormalities and a 1000-fold increased risk of developing skin cancer (reviewed in Bootsma et al., 1998).
CS patients exhibit developmental and neurological abnormalities and impaired physical development, but do not have increased cancer susceptibility (Nance and Berry, 1992), while patients with TTD have neurodevelopmental abnormalities and sulfur-deficient brittle hair (reviewed in Bootsma et al., 1998). Null mutations in murine NER gene homologues result in either a predisposition to cancer (in the case of Xpa, Xpb, and Xpc), reduced life span (for Ercc1, Xpg, Csa, Csb and Rad23B), or early embryonic lethality (for Xpd) (reviewed in Friedberg and Meira, 2000). The myriad of symptoms seen in human NER syndromes demonstrates the requirement of NER components in a variety of cellular processes, indicating the importance of NER gene expression both in counteracting exposure to genotoxic agents and for normal growth and development.

1.2.2 Base Excision Repair

The BER pathway is a collection of single enzymes called glycosylases, which recognize and remove bases with specific lesions, along with enzymes that replace the damaged base (reviewed in Krokan et al., 1997; Memisoglu and Samson, 2000). Some glycosylases, such as uracil DNA glycosylase (UNG), only recognize a single lesion, in this case, misincorporated uracil (Krokan et al., 2000). Others, such as 3-methyl adenine DNA glycosylase, recognize a multitude of lesions (Krokan et al., 1997). Ten human and five murine DNA glycosylases have been found to date (Krokan et al., 2000), each removing a
specific damaged base. Several of these remove various forms of uracil, demonstrating the importance of removing this RNA base from DNA.

BER is initiated by a structure-specific glycosylase, which removes the damaged base via cleavage of the glycosydic bond between the base and the sugar phosphate backbone (Figure 1.2A), creating an apurinic/apyrimidinic (AP) site. BER may then proceed via either short-patch repair, which replaces only the damaged base (Figure 1.2B; left) or long-patch repair, which replaces 2-13 nucleotides around the site of damage (Figure 1.2B; right). The AP sites are themselves a form of genotoxic stress, recognized and repaired by downstream members of the BER pathway (Mol et al., 2000).

Following the initial removal of the damaged base, incision of the sugar backbone occurs either by one of a subset of DNA glycosylases with intrinsic AP lyase activity in the case of short-patch repair, or by an AP endonuclease in the case of long-patch repair (Matsumoto et al., 1999; Pascucci et al., 1999) (Figure 1.2B). The major mammalian AP endonuclease is the AP endonuclease/redox factor-1 (APE/Ref-1) (reviewed in Evans et al., 2000). DNA polymerase β (Polβ), as well as some DNA glycosylases, possess DNA deoxyribosephosphatase (dRPase) activity which removes the sugar at the AP site, leaving a 5' phosphate group. The exonuclease FEN1 is involved in removing overhanging DNA strands that develop via long-patch repair. The gapped DNA is then filled by DNA Polβ (Wilson, 1998), DNA LigI or ligase III (LigIII) (Tomkinson and Mackey, 1998), and the product of the X-ray repair cross-complementing gene 1 (XRCC1) (Thompson and West, 2000) (Figure 1.2C).
Figure 1.2. The base excision repair (BER) pathway. (A) Removal of damaged base by a glycosylase; (B) incision of the DNA backbone and filling in of gapped DNA; (C) ligation of DNA backbone. *Italicized* terms indicate general enzyme activity. Figure adapted from Memisoglu and Samson, 2000.
Repair intermediates formed by the BER pathway, such as AP sites, can progress into DNA strand breaks if BER enzymes are in limited supply; an imbalanced BER pathway can also lead to sensitization to genotoxic agents (Coquerelle et al., 1995). Murine deficiencies in Ape/Ref-1 (Xanthoudakis et al., 1996; Ludwig et al., 1998), DNA polβ (Gu et al., 1994), or Xrcc1 (Tebbs et al., 1999) are embryo-lethal, signifying the importance of this pathway during normal development.

i. **UNG: Uracil DNA Glycosylase**

UNG was the first BER enzyme discovered (Lindahl, 1974). It belongs to a sizeable group of enzymes that remove uracil from DNA, commonly termed uracil DNA glycosylases (UDG) (Krokan et al., 2000). Uracil can arise in DNA either by deamination of cytosine, which occurs between 100 to 500 times per cell per day (Krokan et al., 2000), or due to incorporation of dUTP by DNA polymerases in place of dTTP (Lindahl, 1993). The resulting U:G or U:A mispairs are promutagenic, leading to C:G→T:A transitions in the case of U:G mispairs (Duncan and Weiss, 1982; Impellizzeri et al., 1991). Either type of mispair can cause mutation fixation and altered gene function, which could affect the cell adversely.

The mammalian UNG gene encodes two isoforms of the enzyme via different promoter sites and alternate splicing (Nilsen et al., 1997, 2000b): UNG1, which localizes to mitochondria, and UNG2, which is either completely nuclear in humans (Haug et al., 1996; Nilsen et al., 1997) and rats (Domena and
Mosbaugh, 1985) or both mitochondrial and nuclear in mice (Nilsen et al., 2000b). The UNG gene products account for the majority of UDG activity in cells (Slupphaug et al., 1995). The significance of having two UNG isoforms has not been studied extensively, although genetic material, whether nuclear or mitochondrial, can be a target for uracil misincorporation and therefore for mutation fixation.

Both human UNG1 and UNG2 are expressed highly in proliferative tissues such as testis, colon, and thymus (Haug et al., 1998). Transcripts for both gene products are also cell cycle-regulated, with higher transcript levels in late G1/early S phase, with a corresponding increase in enzymatic activity (Haug et al., 1998). Murine homologues Ung1 and Ung2 are also expressed highly in proliferative tissues such as spleen and testis (Nilsen et al., 2000b). Both homologues, in particular Ung2, are induced in murine embryos during mid-gestation; it has been postulated that this induction is due to transcription factors that are highly expressed during this developmental period (Nilsen et al., 2000b). In the rat, expression and activity of UNG peak around birth and are highest in cells with active DNA replication and cell division (Weng and Sirover, 1993). The ability to alter UNG expression during development may reflect a mechanism to ensure genetic integrity is maintained during a highly proliferative state of an organism.

E. coli and S. cerevisiae ung− mutants show greatly increased rates of C:G→T:A transition mutations, demonstrating a clear necessity for removal of deaminated cytosine in vivo (Duncan and Weiss, 1982; Impellizzeri et al., 1991). Ung-deficient mice, on the other hand, have only a slight increase in mutation frequency, but do exhibit four-fold higher levels of uracil in DNA compared to
normal cells (Nilsen et al., 2000a). In mammals it appears that a novel uracil-removing enzyme, SMUG1, is responsible for removing deaminated cytosine residues from the genome (Nilsen et al., 2001). The major role for mammalian UNG therefore, is the removal of uracil due to misincorporation of dUTP during DNA synthesis. This role for UNG in DNA replication is further evidenced by the interaction of UNG2 with PCNA and RPA at replication foci (Otterlei et al., 1999).

### 1.2.3 Mismatch Repair

The MMR pathway is responsible for removing mismatches that occur during replication and recombination, as well as those arising from the spontaneous deamination of the naturally occurring DNA base 5-methylcytosine (Modrich, 1991). The mammalian MMR system is similar to that found in *E. coli* (Modrich, 1991). While the complete *E. coli* MMR pathway has been elucidated, MMR is still not fully understood in mammals. In *E. coli*, the products of three genes, *mutS*, *mutL*, and *mutH*, are responsible for removing mispaired bases. There are five mammalian homologues of *mutS*: *MSH2* (Reenan and Kolodner, 1992), *MSH3* (New et al., 1993), *MSH4* (Paquis-Flucklinger et al., 1997), *MSH5* (Winand et al., 1998) and *MSH6* (Marsischky et al., 1996). Four homologues of *mutL* exist: *MLH1* (Prolla et al., 1994), *MLH3* (Lipkin et al., 2000), *PMS1* (Kramer et al., 1989) and *PMS2* (Horii et al., 1994). *E. coli* MutH senses the methylation status of DNA thereby targeting repair to the newly synthesized strand. Mammalian MMR does not appear to be methylation-directed, with MutH homologues dispensable for MMR. The human MED1 protein has been
suggested to be a MutH homologue, but may be involved in the BER pathway (Bellacosa et al., 1999).

Mammalian MMR recognizes DNA base pair mismatches via two separate heterodimeric protein complexes: MutSα and MutSβ (Figure 1.3). MutSα contains MSH2 and MSH6 (also known as GTBP), while MutSβ is a complex of MSH2 and MSH3. These two MMR complexes have different substrate specificities: MutSα binds to single base mismatches and extrahelical bases (Marsischky et al., 1996), while MutSβ binds to larger insertion/deletion mismatches (Sia et al., 1997). The other MutS homologues, MSH4 and MSH5, function in non-MMR DNA processes such as recombination (Kolodner and Marsischky, 1999).

Once MutSα or MutSβ have bound to mismatched DNA, they interact with a complex of MutL homologues MLH1 and PMS2, termed MutLα (Räschle et al., 1999), leading to the excision of the mismatched region. MutLβ is a heterodimer of MLH1 and PMS1 (Räschle et al., 1999); the role of MutLβ in mammalian MMR is not currently understood, nor is the involvement of another heterocomplex of MLH1/MLH3. The excision step is also unclear in mammalian organisms. Evidence suggests that mammalian MutL homologues may have the same functions as their E. coli counterparts, namely ATP hydrolysis and activation of other repair components (Ban and Yang, 1998). MutL homologues may also enhance the detection of mismatched bases (Habraken et al., 1997). In the final stage of MMR, DNA synthesis and ligation occur using RPA (Lin et al., 1998), PCNA (Umar et al., 1996), DNA polδ (Longley et al., 1997), and possibly RFC
Figure 1.3. The mismatch repair (MMR) pathway.
(Xie et al., 1999). Other factors involved in MMR include the exonucleases FEN1 (Tishkoff et al., 1997) and Exonuclease 1 (Schmutte et al., 1998), although their precise roles remain unclear.

In addition, the MMR pathway has been implicated in repair of specific forms of alkylation damage. MutSα can bind to $O^6$-methylguanine ($O^6$MeG) lesions caused by the alkylating agent $N$-methyl-$N'$-nitro-$N$-nitrosoguanidine (MNNG) (Ceccotti et al., 1996; Duckett et al., 1996), which are normally repaired by the BER enzyme $O^6$-methylguanine DNA methyltransferase (MGMT) (Kaina et al., 1991). Other roles for MMR proteins include the induction of apoptosis via MSH2 and MSH3 by alkylating agents such as $O^6$MeG (Hickman and Samson, 1999). Overlaps exist between the various DNA repair pathways as well as with cell cycle and apoptotic pathways.

Deficiencies in MMR genes produce a range of effects implicating them in the maintenance of chromosomal stability. Mutations in human MMR genes result in cancer predisposition syndromes exemplified by hereditary nonpolyposis colorectal cancer (Jiricny and Nystom-Lahti, 2000; Peltomaki, 2001) and Muir-Torre syndrome (Cohen et al., 1995). Mice deficient in $Mlh1$ or $Pms2$ are sterile due to meiotic arrest, and have chromosomal abnormalities such as microsatellite instability (Baker et al., 1995, 1996). $Pms2$- and $Msh2$-null mice have increased predisposition to cancers (Baker et al., 1995; de Wind et al., 1995). As well, mutations in human MMR genes create defects in the TCR pathway of NER (Mellon et al., 1996).
1.2.4 Recombination Repair

Damage due to genotoxic stress can be divided into two major types of lesions. The first involves small, local lesions that are confined to a single DNA strand, or a minute portion of the chromosome; the first three DNA repair pathways described are involved in the repair of these types of lesions. The second family of genotoxic lesions involve large-scale chromosomal structural abnormalities, mainly strand breaks, which are more readily translated into genomic instability. It is these lesions which are repaired by the RCR pathway.

The RCR pathway was first elucidated in bacteria (reviewed in Modrich and Lahue, 1996), then in yeast (reviewed in Paques and Faber, 1999); the pathways in these lower organisms are well conserved. The RCR pathway is mainly involved in repair of ICLs and DSBs; DSBs can occur due to exogenous agents such as IR, the breakdown of the DNA replication fork, or via endonucleases as intermediates of other forms of DNA repair (reviewed in: Haber, 2000 and Karran, 2000). A single DSB can lead to cell death, demonstrating the importance of repair of these lesions.

The mammalian RCR pathway is still largely unresolved; members of the mammalian RCR pathway are identified solely by their functional similarity to bacterial proteins such as RecA (reviewed in Morrison and Takeda, 2000). The involvement of RCR gene products with chromosome processes has mainly been demonstrated by the range of effects produced by deficiencies in radiation sensitivity (RAD) genes (Morrison and Takeda, 2000).
the mammalian RAD gene family is in homologous recombination (HR), repairing DSBs.

Once strand breaks are formed, two separate RCR pathways may be used (Figure 1.4). The first is HR, which involves sister chromatid exchange (Kadyk and Hartwell, 1993) (Figure 1.4A). This involves the RAD52 gene family protein products Rad51, Rad52, and Rad54 (Karran, 2000). Rad51 catalyses the homologous pairing of chromosomes at a DSB; Rad52 further stimulates this activity (Liu and Maizels, 2000). DNA is then resected back to create 3' single-stranded ends; in yeast the Rad50/Mre11/Xrs2 complex allows the formation of Holliday junctions by Rad51 which, along with Rad54, allow for strand invasion, exchange, and branch migration (Mazin et al., 2000; Van Komen et al., 2000). Mammalian homologues of Rad50 and Mre11 exist (Dolganov et al., 1996); nibrin (Nbs1) is similar to yeast Xrs2 (Carney et al., 1998), and its mutation results in a DNA damage disorder, Nijmegen breakage syndrome (reviewed in Digweed et al., 1999). Yeast Rad52, Rad54, and the Rad55/Rad57 heterodimer all participate in HR by enhancing the efficiency of Rad51 (Clever et al., 1997; Sung, 1997; Petukhova et al., 1998). Mammalian homologues, including XRCC2 and XRCC3 (Thacker, 1999), also appear to function in RCR. The Holliday junction is then resolved, leading to the formation of DNA that now contains a portion of the other sister chromatid. The tumour suppressor genes BRCA1 and BRCA2 also play a role in HR (Venkitaraman, 1999). HR is highly accurate; no loss of genetic material occurs.

DSB repair via HR has an additional sub-pathway, termed single-strand annealing (SSA) (Figure 1.4B), which is also dependent on the RAD52 gene
Figure 1.4. Recombination repair (RCR) pathway. (A) Homologous recombination (HR); (B) single strand annealing (SSA); and (C) non-homologous end joining (NHEJ) pathways. Only those proteins with defined stage-specific activities are shown.
family (Sugawara and Haber, 1992). This form of RCR occurs when a break forms in a repeated homologous sequence, and does not lead to the creation of a Holliday junction. The DNA strands are resected, homologous sequences aligned, and the non-homologous sequence removed, creating a shorter DNA element.

The other method by which DSBs can be resolved is via the non-homologous end joining (NHEJ) pathway (Moore and Haber, 1996). The NHEJ pathway involves the DNA-dependent protein kinase (DNA-PK), Ku70/80, and a complex of DNA LigIV/XRCC4 (Figure 1.4C), which rejoins DNA ends regardless of sequence. This may lead to small deletions near the breakage site, which can alter gene function. The Mre11/Rad50/Nbs1 complex can also play a role in NHEJ (reviewed in Haber, 1998).

1.2.5 DNA Repair During Development

The ability to repair DNA damage during development has been examined in the mouse conceptus at a variety of developmental timepoints and following exposure to a variety of genotoxic agents (Table 1.1). The majority of these studies examined very similar alkylation type damage repaired by the NER pathway. While the activity of some BER enzymes has been examined at specific timepoints during development, for instance UNG (Weng and Sirover, 1993), no research to date has examined the repair ability in the rat conceptus, a major model for drug toxicology and teratogenicity testing.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Tissue examined</th>
<th>Genotoxic teratogen used</th>
<th>Developmental age (GD)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Whole conceptus</td>
<td>X-ray, N-methyl-N-nitrosourea, methylmethane sulfonate</td>
<td>10.5-12.5</td>
<td>Jirakulsomchok and Yielding, 1984</td>
</tr>
<tr>
<td>Mouse</td>
<td>Whole conceptus</td>
<td>Phenytoin</td>
<td>12</td>
<td>Liu and Wells, 1995</td>
</tr>
<tr>
<td>Mouse</td>
<td>Limb bud</td>
<td>N-methyl-N-nitrosourea</td>
<td>10-11</td>
<td>Loch-Caruso and Baxter, 1984</td>
</tr>
<tr>
<td>Mouse</td>
<td>Yolk sac primary cell cultures</td>
<td>UVR</td>
<td>11.5-12.5</td>
<td>Latimer et al., 1996</td>
</tr>
</tbody>
</table>

**Table 1.1.** Studies examining DNA repair ability following genotoxic teratogen exposure during development.
1.3 **Cell Cycle Checkpoints**

The familiar sequential cell cycle progression ($G_1 \rightarrow S \rightarrow G_2 \rightarrow M$) has been studied since 1953, when Howard and Pelc demonstrated that a discrete period exists between mitoses during which cells synthesize DNA (Howard and Pelc, 1953). At the boundaries between each stage of the cell cycle exists a molecular stop sign, or checkpoint, which can halt the cycle in response to specific stimuli. As well, intra-stage checkpoints exist which halt the cell cycle within a stage. These checkpoints are signal-transduction pathways that specifically recognize problems with genomic integrity (Hartwell and Weinert, 1989; Kaufmann and Paules, 1996).

Co-ordination of the cell cycle is a key aspect in the response to DNA damage, allowing DNA repair to take place before DNA synthesis and cell division occur. There are two main cell cycle checkpoints involved in the response to DNA damage: the $G_1/S$ checkpoint, in which cells are halted before replicating their genome; and the $G_2/M$ checkpoint, which allows the cell to repair damage before cell division occurs (reviewed in Mercer, 1998).

1.3.1 **Genotoxic Stress-Induced Cell Cycle Checkpoints**

DNA damage can activate specific cell cycle checkpoints that respond to genotoxic stress by activating and recruiting DNA repair and cell cycle control proteins. These checkpoint mechanisms, as well as the components thereof, are conserved from yeast to mammals (reviewed in Lowndes and Murguia, 2000).
The ability of a cell to slow the replication of damaged DNA is vital for survival of the daughter cells to be produced.

The cell cycle checkpoint machinery is composed of various protein complexes which contain a regulatory subunit (cyclin) and a protein kinase subunit (cyclin-dependent kinase; Cdk) (reviewed in Johnson and Walker, 1999). The specificity of a cell cycle checkpoint for the boundaries between two cell cycle stages is determined by the composition of these protein complexes (reviewed in Niggs, 1995). Progression from G₁ to S phase is controlled by the cyclin D/E family, up to a critical restriction point after which the cell is committed to continuing on to S phase. Activation of Cdc25A by the protein kinase Chk2 leads to a loss of function of the G₁/S phase specific Cdk2/CyclinE complex and checkpoint arrest (reviewed in Bartek and Lukas, 2001).

The cell cycle transition from G₂ to M is mediated by Cdc2 complexed with the G₂-phase specific kinase cyclin B1. In order for progression through to the M phase, Cdc2 must be dephosphorylated by the phosphatase Cdc25C. The phosphorylation of Cdc25C by the kinases Chk1 or Chk2 inactivates it, preventing the dephosphorylation of Cdc2, thus leading to the G₂/M checkpoint arrest. These two mechanisms ensure that the DNA repair machinery has an opportunity to repair the damage, and prevent further damage from occurring due to replication of damaged DNA.
1.3.2 Genotoxic Stress Response/Checkpoint Machinery

The ability to sense and signal within the cell that DNA damage has occurred is one of the most important cellular defences against genotoxic agents. Activation of DNA damage checkpoints involves three components: sensors, transducers, and effectors. The sensor either recognizes specific forms of DNA lesions, or recognizes DNA repair enzymes already bound to genotoxic lesions, and activates transducers. These transducers amplify the signal, activating specific effectors that cause cell cycle arrest, induction of DNA repair, transcription, and/or cell death via apoptosis. Members of the phosphatidylinositol 3-kinase (PI3K) superfamily are the main DNA damage sensors implicated in responding to genotoxic stressors. This family includes the genes Ataxia-Telangiectasia Mutated (ATM; reviewed in Rotman and Shiloh, 1999), ATM and Rad3-related (ATR; Tibbetts et al., 1999), and DNA-PK (reviewed in Smith and Jackson, 1999). All three genes encode for serine/threonine kinases which are activated by as yet unknown mechanisms following DNA damage, and in turn activate several downstream targets that result in cell cycle arrest, DNA repair, and apoptosis (reviewed in: Durocher and Jackson, 2001 and Shiloh, 2001). They are involved in invoking DNA repair responses as well as in signalling the cell to halt DNA synthesis until repair can take place (reviewed in Lowndes and Murguia, 2000). The main downstream transducer is p53 (reviewed in Levine, 1997), while main effectors include p21.
and GADD45 (Chan et al., 2000). Many of these components are directly involved in regulating DNA repair pathways (Weinert, 1998).

i. ATM: Ataxia-Telangiectasia Mutated

One of the key integrators of DNA damage checkpoint control is ATM (Savitsky et al., 1995), whose gene is mutated in Ataxia-Telangiectasia (A-T), a human autosomal recessive disorder characterized by cerebellar ataxia, infertility, immunodeficiency, and most notably a predisposition to cancer and hypersensitivity to IR due to lack of DNA damage-induced cell cycle arrest (Sedgwick and Boder, 1991). Cells from A-T patients fail to activate either G1/S or G2/M checkpoints, and are unable to downregulate DNA synthesis following IR or radiomimetic drugs, but not UVR, exposure (Canman and Lim, 1998; Rotman and Shiloh, 1999), indicating that ATM is activated following DSB formation.

ATM is a serine/threonine specific kinase; in response to DNA damage, ATM phosphorylates and activates Chk2 (Matsuoka et al., 1998) which then phosphorylates and activates p53 (Nakagawa et al., 1999; Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000) (Figure 1.5). Chk2 also phosphorylates and inactivates the phosphatase Cdc25C, which is required for the progression through the G2 phase of the cell cycle via activation of Cdc2, leading to G2/M checkpoint arrest (Peng et al., 1997; Sanchez et al., 1997).

ATM phosphorylation of p53 is rapid in response to DSB formation, occurring within minutes following genotoxic insult (Banin et al., 1998). ATM can also modify p53 stability by phosphorylating its inhibitor, MDM2 (Khosravi et al.,
Figure 1.5. Genotoxic stress checkpoint pathways. The DNA-PK pathway activates apoptosis, not checkpoint arrest.
1999), and by activating a phosphatase that dephosphorylates p53 (Waterman et al., 1998). ATM is therefore able to activate p53 at multiple levels, stabilizing it and leading to its accumulation within the cell.

ATM can activate other proteins involved in damage response and cell cycle control, notably Brca1 (Cortez et al., 1999; Gatei et al., 2000), Rad51 (Chen G. et al., 1999) and Nbs (Wu et al., 2000; Zhao et al., 2000). These interactions can recruit DNA repair machinery around a damage site, allowing ATM not only to arrest the cell cycle in response to genotoxic stress, but also to activate specific repair pathways required to repair the lesion.

ATM regulation is complex and can occur at the transcript, protein, and activity level. Classically, ATM is regulated at the level of kinase activity; in response to IR, ATM is phosphorylated, activating its kinase activity (Banin et al., 1998). Studies using IR or IR mimetics showed no increase in ATM gene expression following exposure (Brown et al., 1997). Following epidermal growth factor exposure of human fibroblasts and lymphoblasts, however, ATM is transcriptionally down-regulated, increasing cellular radiosensitivity (Gueven et al., 2001). Non-proliferating breast myoepithelial cells and quiescent lymphocytes both induce ATM when converted to a more proliferative status (Clarke et al., 1998; Fukao et al., 1999). This indicates complex regulation of ATM at the transcript, protein, and activity levels, particularly in rapidly proliferating or differentiating cells.

Atm-null mice are viable, but exhibit growth retardation, chromosomal instability, and an increased susceptibility to develop cancer (Elson et al., 1996). However, deficiency in both Atm and other members of recombination and/or
DNA damage sensor pathways is embryolethal, *e.g.* *Atm* null in *Dna-Pk* null-mutant backgrounds (Gurley and Kemp, 2001; Sekiguchi *et al.*, 2001). While some checkpoint functions may overlap between the PI3K family members, loss of more than one member is not compatible with life.

**ii. ATR: ATM and Rad3-related**

ATR is also a member of the PI-3 kinase superfamily (Cimprich *et al.*, 1996), and, like ATM, is activated by an unknown mechanism in the presence of damaged DNA (Hall-Jackson *et al.*, 1999). Activated ATR phosphorylates cellular targets such as p53 inducing cell cycle arrest (**Figure 1.5**). ATR is related to *RAD3*, a yeast gene which, when mutated in *Schizosaccharomyces pombe*, results in loss of checkpoint function in the presence of damaged DNA (Jimenez *et al.*, 1992). Similarly, expression of a kinase-inactive ATR in human cells also causes defective *G*₂ checkpoint control (Cliby *et al.*, 1998; Wright *et al.*, 1998).

ATR has a pattern of activation distinct from ATM, in that it is activated in response primarily to UVR (Tibbetts *et al.*, 1999) and alkylating agents such as cisplatin (Damia *et al.*, 2001). In particular, ATR phosphorylates Chk1, activating it and leading to *G*₂/*M* arrest via p53 and Cdc25C (Liu *et al.*, 2000). The induction of ATR activity may be due to its localization to discrete nuclear foci, along with its downstream kinase targets (Tibbetts *et al.*, 2000). ATR can complement the defective *G*₁/*S*-phase checkpoint that is defective in cells from A-T patients, indicating a partial overlap in ATM and ATR pathways (Cliby *et al.*, 1998).
1998). ATR induces a late-phase phosphorylation of p53 in response to IR (Tibbetts et al., 1999), thus prolonging the activation of p53 by ATM phosphorylation. ATR and Brca1 colocalize to nuclear foci in response to DNA damage (Tibbetts et al., 2000); ATR can phosphorylate Brca1, both on sites unique from and overlapping with ATM (Gatei et al., 2001a).

Unlike ATM, no human syndrome is associated with ATR mutations; null-mutant mice lacking Atr die in early development, with massive apoptotic cell death due to chromosome fragmentation (Brown and Baltimore, 2000; de Klein et al., 2000), indicating that Atr is crucial for developmental processes. In particular, Atr may be required in rapidly proliferating cells for coordination of the entry into the M phase of the cell cycle, which when lacking could lead to chromosomal aberrations.

iii. DNA-PK: DNA-Dependent Protein Kinase

DNA-PK has distinct functional differences with respect to the other PI3K family members. DNA-PK is activated following association with DNA, and activates pathways primarily involved in inducing apoptosis (reviewed in Smith and Jackson, 1999). As well as being involved in the NHEJ pathway of recombination repair (see Section 1.2.4), DNA-PK is involved in V(D)J recombination in the immune system (see below).

DNA-PK is composed of three subunits: Ku70, Ku80, and the catalytic subunit, DNA-PKcs (reviewed in Smith and Jackson, 1999). All three subunits are present at high levels in human cells, while rodent cells have much lower
levels of the three subunits (Anderson and Lees-Miller, 1992; Finnie et al., 1995). DNA-PK protein levels do not appear to be modulated following genotoxic exposure (Lee S.E. et al., 1997), although the effect of genotoxic agents on transcript levels is not known.

No mutations for human DNA-PK exist. However, severe combined immunodeficiency (SCID) mice harbour mutations in Dna-Pkcs (Blunt et al., 1995; Kirchgessner et al., 1995; Peterson et al., 1995). The major defect in SCID mice is a complete lack of an immune system due to lack of V(D)J recombination (Lieber et al., 1988), which is necessary to create the variety of immunoglobulin and T-cell receptors required for proper immune defences. SCID mice are hypersensitive to IR (Fulop and Phillips, 1990; Biedermann et al., 1991; Hendrickson et al., 1991), indicating impaired response to DSBs. DNA-PK acts upstream of p53 in activating the apoptotic machinery, but not cell cycle arrest, in response to IR-generated genotoxic stress (Jhappan et al., 2000; Wang et al., 2000).

1.3.3 Downstream Targets of PI3K Family Members

In order to arrest the cell cycle at specific phase boundaries, the members of the PI3K superfamily interact with several downstream targets. The three major targets following genotoxic stress are p53, GADD45, and p21.
i. **p53**


While *p53*-null mice are viable, they exhibit increased tumour susceptibility and cannot activate cell cycle checkpoints in response to genotoxic stress (reviewed in Levine, 1997 and Vogenstein *et al.*, 2000). Mice lacking *Mdm2*, the negative regulator of p53, are embryolethal (Montes de Oca Luna *et al.*, 1995). Thus the ability to regulate p53 activity is required for proper development and protection from genotoxic stress-induced disease.
GADD45

GADD45, a member of the Growth Arrest and DNA Damage-inducible family of genes (Fornace et al., 1989a), is transcriptionally upregulated by p53 (Wang et al., 1999) following UVR, hypoxia, IR, and alkylating agents (Papathanasiou et al., 1991). GADD45 expression is also increased by nutrient withdrawal (Papathanasiou et al., 1991), and hyperoxia (O'Reilly et al., 2000). Increased GADD45 expression causes p53-dependent growth inhibition (Wang et al., 1999) by interacting with p21\textsuperscript{WAF/CIP1} (Kearsey et al., 1995) and PCNA (Smith et al., 1994), as well as Cdc2 (Zhan et al., 1999). Inhibition of Cdc2 allows for G\textsubscript{2}/M cell cycle arrest (Zhan et al., 1999) (Figure 1.5).

Animals deficient in Gadd45 display chromosomal aberrations and increased risk of cancer following IR (Hollander et al., 1999) and dimethylbenzantracene, an agent that creates bulky DNA adducts (Hollander et al., 2001). GADD45 may also have a role in the NER pathway (Smith et al., 1994), and in apoptosis (reviewed in Sheikh et al., 2000); GADD45 expression is increased following exposure to genotoxic agents, such as UVR, which are known to induce apoptosis (Hollander et al., 1993). Although the exact role of GADD45 in apoptosis is unclear, it is nonetheless required for proper signalling to and activation of the apoptotic machinery.

p21

p21, also known as p21\textsuperscript{Cip1/WAF1/SDI1/CAP20/Plc1/mda-6}, is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors (reviewed in Sherr and
Induction of p21 by p53 causes G₁/S cell cycle arrest through interaction with Cdk2/CyclinE complex. p21 may also activate the apoptotic machinery in response to genotoxic stress due to its interactions with upstream apoptosis signalling molecules (reviewed in Dotto, 2000). Animals deficient in p21 develop normally, although they exhibit defective G₁/S arrest following genotoxic stress (Deng et al., 1995).

1.3.4 Genotoxic Stress Checkpoints During Development

Rapid cellular division during mammalian development requires the ability to precisely regulate cell division. The cell cycle is much shorter in embryonic cells compared to adult cells: gastrulation-stage (gestational day (GD) 8.5-9.5) rat embryonic cells divide every 3-7.5 hours (MacAuley et al., 1993). Checkpoint arrest following genotoxic stress has been examined in early Drosophila (Su and Jaklevic, 2001), Xenopus (Finkielstein et al., 2001), and Zebrafish embryos (Ikegami et al., 1999), however no studies have examined these checkpoints during mid-organogenesis in mammalian organisms.

Mouse embryos are extremely sensitive to low levels of IR. Cells of embryonic origin undergo Atm- and p53-dependent apoptosis, with no activation of cell cycle checkpoints following IR, whereas extraembryonic cells do not die (Heyer et al., 2000). The window of sensitivity corresponds to the start of gastrulation (GD5.5) through to the beginning of neurulation (GD8.5), indicating that a specific period of development is dependent on Atm activity to cope with
genotoxic stress. Atm may be required to eliminate damaged cells before they have time to divide and cause abnormal tissue development.

Although the specific activity of cell cycle checkpoints has not been examined during mammalian organogenesis, null mutant mice lacking Atm (de Klein et al., 2000) or Chk1 (Takai et al., 2000) do not survive to birth.

1.4 Teratogens and Teratology

A teratogen is an agent that induces malformations when administered during susceptible stages of development. The word "teratogen" is derived from the Latin word "teratos", meaning monster, relating to the gross malformations that can be caused by teratogen exposure. Teratogens can be found in nature, such as heavy metals (reviewed in Leonard et al., 1983; Leonard and Jacquet, 1984; Agarwal et al., 1989; Leonard and Gerber, 1997) and alkaloid cyclopamine derivatives (Keeler, 1973, 1978). They can be man-made medications, such as valproic acid (reviewed in Lammer et al., 1987 and Cotariu and Zaidman, 1991), retinoic acid (reviewed in Collins and Mao, 1999), and thalidomide (reviewed in Lenz, 1988), or natural products consumed by humans, such as ethanol (reviewed in Spagnolo, 1993). They can also be by-products of human consumption and industry, such as methylmercury (reviewed in Myers et al., 1998).

Humans are exposed to a wide variety of teratogens in daily life. Unfortunately, the consequences of those exposures are not well understood. Current estimates are that 3300 chemicals have been tested for teratogenicity, with 37% of these demonstrating some teratogenic activity (Schardien, 1993).
This represents only a fraction of known chemicals to which humans may be exposed; unknown teratogenic agents pose a great risk to human foetal health.

The aim of research in teratology is to understand the mechanisms underlying the adverse effects of chemical or drug exposure during development. Six original principles of teratology were put forth in 1973, following a report to the Teratology Subcommittee of the Commission on Drug Safety, from the Federation of American Societies for Experimental Biology in 1964 (Wilson, 1973). These principles, in effect, encompass the underlying theme of research in teratology and have held up to this day. They are:

1. Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which this interacts with adverse environmental factors.
2. Susceptibility to teratogenesis varies with the developmental stage at the time of exposure to an adverse influence.
3. Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate sequences of abnormal developmental events (pathogenesis).
4. The access of adverse influences to developing tissues depends on the nature of the influence (agents).
5. The four manifestations of deviant development are death, malformation, growth retardation, and functional deficit.
6. Manifestations of deviant development increase in frequency and degree as dosage increases from the no-effect to the totally lethal level.
These principles govern the way scientists examine the teratogenic effects of compounds, and are crucial to ensure that the causes of birth defects are examined at a mechanistic level. Research into the mechanisms underlying teratogen action is increasingly focused on molecular aspects, such as the effects on metabolic activity, energy utilization, and changes in gene expression following teratogen exposure.

1.4.1 **Genotoxic Teratogens**

Classical teratogens, whose mechanisms' of action have been elusive for decades, are now being examined for their potential to induce genotoxic stress. 25% of known human teratogens are mutagenic due to direct genotoxic stress (reviewed in Bishop *et al.*, 1997). For example, ethanol induces H$_2$O$_2$ and O$_2^-$ production; both are potent ROS that create oxidative damage to DNA (Henderson *et al.*, 1999). Other studies have implicated ROS-induced genotoxic stress as a factor in the actions of other well-studied teratogens such as phenytoin (Liu and Wells, 1995) and thalidomide (Parman *et al.*, 1999).

It is not surprising that exposure to genotoxic agents during development causes adverse outcomes. The process of creating a complete, multicellular organism from a single, fertilized egg requires a complex, interwoven pattern of gene expression and regulation. Any conditions that interfere with gene function could lead to dramatic consequences in the developing embryo, where cell division times and windows of gene expression are rapid and transitory. It is estimated that 25-30% of all birth defects are due to gene mutations and
chromosomal abnormalities (Beckman and Brent, 1984), both of which can be induced by genotoxic stress.

Genotoxic teratogens can be categorized as either directly modifying DNA (e.g. creating alkyl group adducts on DNA in the case of nitrogen mustards), or those that induce ROS formation leading to oxidative genotoxic damage (e.g. phenytoin). To date, nine genotoxic teratogens have been examined in experimental models (Table 1.2); this number will undoubtedly continue to grow as new techniques to examine genotoxic stress during development become available.

1.4.2 Teratogenicity of Alkylating Agents

An alkylating agent is a chemical that causes covalent modification to another molecule by adding a carbon chain backbone. Alkylating agents may create simple adducts, such as methyl group addition in the case of MNNG (Gichner and Veleminsky, 1982), or more complex moieties like chlorambucil (N,N-Bis-(2-chloroethyl)-p-aminophenylbutyric acid), which creates N-(7-guaninylethyl)-N-hydroxyethyl-p-aminophenylbutyric acid adducts (Haapala et al., 2001). One major group of alkylating agents are the nitrogen mustards. These agents have been used as chemotherapeutic drugs since the 1950s, and are paradoxically amongst the most potent carcinogenic chemicals in humans (Kaldor et al., 1988). Nitrogen mustards include mechlorethamine, melphalan, and cyclophosphamide (CPA) (reviewed in Povirk and Shuker, 1994).
<table>
<thead>
<tr>
<th>Genotoxic teratogen</th>
<th>Developmental age (GD)</th>
<th>Organism</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzo[a]pyrene</td>
<td>9.5</td>
<td>Mouse</td>
<td>Winn and Wells, 1997</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>9</td>
<td>Mouse</td>
<td>Murthy, 1973</td>
</tr>
<tr>
<td>Dioxin</td>
<td>12</td>
<td>Mouse</td>
<td>Hassoun et al., 1995</td>
</tr>
<tr>
<td>Ethylmethane sulfonate</td>
<td>9-12</td>
<td>Mouse</td>
<td>Platzek et al., 1982</td>
</tr>
<tr>
<td>Methylmethane sulfonate</td>
<td>10.5-12.5</td>
<td>Mouse</td>
<td>Jirakulsomchok and Yielding, 1984</td>
</tr>
</tbody>
</table>
| N-methyl-N-nitrosourea | 10-11  
10.5-12.5            | Mouse    | Loch-Caruso and Baxter, 1984     |
|                        |                        |          | Jirakulsomchok and Yielding, 1984|
|                        |                        |          | Bochert et al., 1991             |
| Phenytoin              | 12                     | Mouse    | Liu and Wells, 1995              |
| Pyrimethamine          | 13                     | Mouse    | Tsuda et al., 1998               |
| Thalidomide            | 8-12                   | Rabbit   | Parman et al., 1999              |

**Table 1.2.** Studies demonstrating the genotoxic effects of teratogens during development.
Bifunctional nitrogen mustards first form a complex with DNA, yielding a covalently bound monoadduct. This monoadduct can then react with (i) a solvent or protein molecule, creating a single adduct or DNA-protein crosslink; (ii) a base on the same DNA strand, yielding an intrastrand crosslink; or (iii) a base on the opposite strand of DNA, to create an ICL (Hopkins et al., 1991). These last two lesions are amongst the most toxic forms of genotoxic stress.

i. **Cyclophosphamide**

The nitrogen mustard CPA, one of the best-studied teratogens, is one of the most widely used chemotherapeutic agents for malignant tumours (reviewed in Colvin and Hilton, 1981). CPA is bioactivated by liver cytochrome P450 enzymes into its two active metabolites, phosphoramide mustard and acrolein (Figure 1.6) (reviewed in Sladek, 1988). Phosphoramide mustard alkylates DNA creating both mono- and crosslinked DNA adducts, including nor-G, nor-G-OH and G-nor-G lesions, while acrolein alkylates DNA, RNA, and protein (reviewed in Hemminki, 1994). It is believed that phosphoramide mustard is the major alkylating species formed, and is responsible for the antitumor properties of CPA (Wrabetz et al., 1980).

The teratogenicity of CPA has been studied since the 1960s (von Kreybig, 1965), both *in vivo* and *in vitro*. CPA produces central nervous system (*e.g.* exencephaly, microcephaly, and cleft palate/lip) and skeletal abnormalities (*e.g.* syndactyly and ectrodactyly) in mice, rats, rabbits, monkeys, and humans (for review, see Mirkes, 1985). As CPA is bioactivated *in vivo, in vitro* studies use
Figure 1.6. Activation and metabolism of cyclophosphamide and 4-hydroperoxycyclophosphamide, and the alkylation adducts formed.
4-hydroperoxycyclophosphamide (4-OHCPA), an activated analog of CPA that breaks down spontaneously to 4-hydroxycyclophosphamide in solution (Low et al., 1982; Slott and Hales, 1988). Postimplantation rat embryos exposed to 4-OHCPA in vitro at teratogenic doses exhibit crosslinks, strand breaks and alkali-labile sites (Little and Mirkes, 1987). Acrolein itself is teratogenic at much higher doses than phosphoramide mustard (Slott and Hales, 1986), thus the primary DNA damaging effects of CPA are due to the alkylating effect of phosphoramide mustard (reviewed in Mirkes, 1985).

ii. **Nucleotide Excision Repair and Cyclophosphamide**

DNA lesions induced by CPA and other nitrogen mustards are repaired primarily by the NER pathway. It would therefore be valuable to know the expression profile of this DNA repair pathway during stages of development when the conceptus is susceptible to CPA. However, as with the expression of many DNA repair genes, little is known about NER expression during development. NER genes determine the sensitivity of cultured cells to CPA (Andersson et al., 1996), as cells deficient in several NER genes are hypersensitive to the alkylating drug. No such studies have been performed with embryonic cells or tissues.

1.4.3 **Teratogenicity of Folate Deficiency**

One well-known teratogen is in fact not a chemical, but the lack of a specific compound: folic acid. Reduced folic acid (pteroylmonoglutamic acid) is a water-soluble B vitamin which acts as a methyl donor in several biochemical
processes and, along with its metabolites, is required for the one-carbon metabolic pathway (Lucock, 2000). The large numbers of chemically similar folic acid derivatives are commonly referred to as folates; these cannot be synthesized de novo in human cells, and are thus essential nutrients (Bailey and Gregory, 1999).

Folic acid deficiency during pregnancy is a known pro-teratogenic condition (for review, see Hall and Solehdin, 1998; Smithells et al., 1981b, 1983; Wald et al., 1991), although there is still some uncertainty about the correlation between teratogenicity and low maternal serum folate levels (Molloy et al., 1985; Mills et al., 1992). Birth defects attributed to folic acid deficiency include neural tube defects (NTDs), congenital heart defects, renal and urinary tract defects, limb malformations, and facial clefts (palate and lip). NTDs, such as spina bifida and anencephaly, occur with a frequency of one per 1000 births in the United States, and are among the most common forms of birth defects (Campbell et al., 1986) and perinatal mortality observed (Hall and Solehdin, 1998). In the United Kingdom, 15% of perinatal deaths are attributed to NTDs (Copp et al., 1990). Folic acid deficiency in mothers was first shown to be associated with increased incidence of NTD-affected infants in 1976 (Smithells et al., 1976). Since then, there have been many studies showing that folic acid supplementation reduces both the occurrence (Mulinare et al., 1988; Czeizel and Dudás, 1992; Werler et al., 1993) and recurrence (MRC Vitamin Study Research Group, 1991; Van Allen et al., 1993) of NTDs. Up to 70% of human NTDs can be prevented by supplementation of folic acid in the diet of pregnant women (Smithells et al., 1981a).
Dietary folic acid deficiency results in thymidylate deficiency, as folic acid is a required cofactor for dihydrofolate reductase (DHFR) (Gready, 1980). DHFR catalyzes the formation of tetrahydrofolate (THF) from dihydrofolate; THF is an essential cofactor for de novo synthesis of purine bases and the pyrimidine base thymidine (Figure 1.7). THF is converted to 5,10-methylene tetrahydrofolate (5,10-MTHF), and used by the enzyme thymidylate synthetase to form deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP). Folic acid is also essential for the conversion of serine to glycine, the catabolism of histidine, regulating homocysteine levels, and regenerating methionine from homocysteine (reviewed in Lucock, 2000).

The benefits of folic acid supplementation vary in different population subgroups, arguing for a genetic component to NTD risk (Shaw et al., 1995). Embryos from splotch mice, which are prone to NTDs, have been shown to be deficient in 5,10-MTHF for de novo pyrimidine biosynthesis, as exogenous folic acid added during embryo culture prevented NTDs (Fleming and Copp, 1998). Interestingly, the gene mutated in splotch mice, Pax3 (Epstein et al., 1991), is also mutated in human genetic disorders causing a predisposition for NTDs (Read and Newton, 1997). It has been suggested that altered folate uptake and metabolism in the conceptus may relate to NTDs (reviewed in Barber et al., 1999). There exists no clear correlation, however, between mutations in genes encoding folate receptors (Trembath et al., 1996; Barber et al., 1998; Trembath et al., 1999) or methionine synthetase (Shaw et al., 1999) and increased NTDs. A small increase in risk for infants born with NTDs may exist in mothers with a C→T mutation (C677T) in the gene encoding MTHFR (Papapetrou et al., 1996; Shaw
Figure 1.7. Cellular pathways dependent on folic acid. Shown above are some key metabolic pathways in the synthesis of purine and pyrimidine nucleotides, methionine and glycine synthesis, and homocysteine degradation.
et al., 1998), which decreases its enzymatic activity. A deficiency of vitamin B12, a cofactor required for THF synthesis, has also been implicated in increased incidence of NTDs (Kirke et al., 1993; Steen et al., 1997). Therefore, while the cause of a small proportion of NTDs can be accounted for, current studies demonstrate that either single mutations in critical genes of the folate metabolic pathway or decreased activity of these enzymes do not account for the majority of NTDs in infants born from folate-deficient mothers. Therefore, an understanding of the downstream pathways that utilize folate one-carbon pathway products may shed light on the aetiology of NTDs.

i. Effects of Thymidine Depletion Due to Folic Acid Deficiency

Thymidine depletion due to the lack of folic acid leads to an increase in cellular dUMP, as a result of the inhibition of the enzyme thymidylate synthetase. While folic acid is used in purine as well as thymidine synthesis, it is the shunting of dUMP, the precursor of deoxythymidine triphosphate (dTTP), to form deoxyuridine triphosphate (dUTP) rather than dTTP that leads to MTX-induced genotoxic stress (Figure 1.7). DNA polymerases preferentially utilize dTTP, however if cellular dUTP pools grow larger than dTTP pools, dUTP is incorporated due to a lack of substrate specificity in the polymerase nucleotide binding pocket (Kunkel and Loeb, 1981; Focher et al., 1990). This leads to incorporation of dUTP into DNA (Goulian et al., 1980a).

Thymidine depletion leads to inhibition of DNA synthesis, and a futile cycle of excision of uracil from DNA by UNG (Goulian et al., 1980a,b), and
reincorporation of dUTP, leading to DNA fragmentation and cell death (Duthie and Hawdon, 1998). The loss of cell viability following dramatic decreases in thymidine levels is termed “thymidineless death” (Seno et al., 1985). Concomitant loss of DNA repair capacity occurs due to lack of substrates (Borchers et al., 1990). In this instance, the DNA repair activity of UNG is detrimental to the cell, as it exacerbates the genotoxic stress of thymidine depletion. The cause of thymidineless death is due to dUTP incorporation into DNA, leading to SSBs, DSBs, and DNA fragmentation (Lorico et al., 1988), rather than the lowering of dTTP levels per se. Artificially increasing dUTP levels without significant decreases in dTTP levels will also lead to cell death (Ingraham et al., 1986). Proof that UNG mediates the toxicity of thymidine depletion comes from bacteria harbouring a mutation in the gene encoding ung, which have diminished lethality when exogenous thymidine is added to the culture (Makino and Munakata, 1978).

Folic acid deficiency has also been implicated in birth defects induced by a variety of other teratogens, including heat shock (Shin and Shiota, 1999), methanol (Sakanashi et al., 1996), and arsenic exposure (Ruan et al., 2000). Another method of thymidine limitation occurs following administration of folic acid analogues, or antimetabolites, which are used clinically. Examples include tomudex, trimethoprim, and methotrexate.
ii. **Methotrexate**

Methotrexate (8-amino-10-methyl-pteroylglutamic acid; aminopterin; MTX) is a structural analog of folic acid (Figure 1.8). MTX lowers cellular thymidine levels by inhibiting the action of DHFR, increasing uracil misincorporation into DNA (Blount and Ames, 1994). MTX is primarily used as a chemotherapeutic agent for the treatment of lymphoblastic leukemia (Gokbuget and Hoelzer, 1996) and several female reproductive tract and breast cancers (Fleisher, 1993). MTX is also used in the treatment of autoimmune disorders, such as rheumatoid arthritis and systemic lupus erythematosus (reviewed in Wilke, 1997), because of its secondary cellular effect of modulating cytokine production (Dayan et al., 1997), and is also used as an abortifacient (Christin-Maitre et al., 2000).

MTX is a potent teratogen. The teratogenic effects of MTX are seen in rats (Berry, 1971), mice (Darab et al., 1997), rabbits (Jordan et al., 1977), cats (Khera, 1976), and humans (Milunsky et al., 1968; Warkany, 1978). Malformations include limb reduction abnormalities, craniofacial abnormalities (cleft lip and palate), NTDs, congenital heart defects, and renal and urinary tract defects. MTX also causes intrauterine death. Many of these abnormalities are identical to those seen with folic acid deficiency. The teratogenic action of MTX has been demonstrated to be mainly due to the inhibition of DHFR; transgenic animals expressing a methotrexate-resistant form of DHFR have decreased incidences of malformations upon MTX exposure (Sutton et al., 1998).
Figure 1.8. Structure of (A) folic acid and (B) methotrexate.
iii. Uracil DNA Glycosylase and Folate Deficiency

Removal of dUTP from DNA is critical for normal genetic functioning. Decreased cellular thymidine levels require the activity of UNG, particularly during cell proliferation or development. However, the role of UNG in folic acid deficiency-induced birth defects has not been explored. Its role in preventing uracil incorporation into DNA during development is evident as both murine Ung homologues are expressed during organogenesis (Nilsen et al., 2000b). However, UNG expression and activity during mid-organogenesis, when organ systems are rapidly forming, as well as in different tissues in the conceptus, have not been examined.

1.4.4 Oxidative Stress as a Teratogen

Oxidative stress has been implicated in a wide variety of disease states, including cancer (reviewed in Marnett, 2000), Alzheimer's disease (reviewed in Smith et al., 2000), and aging (reviewed in Ames and Shigenaga, 1992). ROS such as $O_2^-$, $H_2O_2$, and the hydroxyl free radical (‘OH) are just some of the reactive molecules that induce oxidative genotoxic stress. The involvement of oxidative stress in teratogenesis has been studied for some time, and several disease states are known to be associated with increased oxidative stress in the conceptus. Diabetic rodents have an increased incidence of foetal malformations that are reduced upon supplementation of the maternal diet with a variety of antioxidants (Eriksson and Simán, 1996; Viana et al., 1996; Simán and Eriksson, 1997; Wiznitzer et al., 1999). Embryos from diabetic rats have increased
oxidative stress loads (Lee et al., 1995), demonstrating a link between a teratogenic disease state and genotoxic oxidative stress. As well, several chemical teratogens may exert some of their effects on the conceptus via oxidative stress, including thalidomide (Parman et al., 1999) and phenytoin (Liu and Wells, 1995). Better understanding of the cellular responses to oxidative stress can give insight into the sensitivity of a conceptus to ROS-mediated malformations.

i. **Base Excision Repair and Oxidative Stress**

Specific members of the BER pathway repair the genotoxic damage caused by ROS. The enzyme 8-oxoguanine DNA glycosylase, present in bacteria, yeast, and mammals, repairs the mutagenic oxidative lesion 7,8-dihydro-8-oxoguanine from DNA (Nash et al., 1996; van der Kemp et al., 1996; Radicella et al., 1997). Oxidative stress can also lead to thymine and cytosine glycols, which are repaired in mammalian cells by homologues of the *E. coli* endonuclease III family (Aspinwall et al., 1997; Hilbert et al., 1997). The BER AP endonuclease, APE/Ref-1, is involved in redox regulation of the transcription factors AP-1 (Xanthoudakis and Curran, 1992) and p53 (Jayaraman et al., 1997), and is itself regulated by oxidative stress (reviewed in Evans et al., 2000); yeast strains lacking the homologous *APN1* gene are hypersensitive to oxidative stress (Ramotar et al., 1991).
ii. **ATM and Oxidative Stress Checkpoints**

Like other genotoxic agents, oxidative stress can elicit a cell checkpoint response. ATM has been implicated in sensing DNA damage due to IR or oxidative stress (for review, see Rotman and Shiloh, 1997a,b). Strengthening this hypothesis is the fact that IR forms oxygen free radicals (Skov, 1984). Increased lipid peroxidation and decreased antioxidant enzyme activity is found in cells from A-T patients (Lee et al., 1990; Watters et al., 1999). *Atm* null-mutant mice have a phenotype similar to that of A-T patients (Elson et al., 1996; Xu et al., 1996) and exhibit high levels of oxidative damage in cells and tissues that develop pathological changes in A-T (Barlow et al., 1999; Gatei et al., 2001b). *Atm*-null mice also exhibit altered tissue redox status (Kamsler et al., 2001), and altered antioxidant enzyme levels and activity, consistent with an increased oxidant load. Null-mutant *Atm* mice that over-express superoxide dismutase, an enzyme which generates H$_2$O$_2$ and OH radicals, have further enhancement of the deleterious effects of A-T indicating a relationship between oxidative radical formation and the disease state (Peter et al., 2001). These studies indicate that ATM may play a role in sensing oxidative stress and in regulating cellular antioxidant defences, which when perturbed may underlie some of the characteristics of A-T.

1.4.5 **DNA Repair/Cell Cycle Gene Expression During Development**

Examining the expression profiles of multiple genes following genotoxic exposure has only been done a few times (Keyse, 1993; Amundson et al., 1999;
Fewer studies have specifically examined the expression of DNA repair genes in different embryonic tissues during development (Hubank and Mayne, 1994; Kim et al., 2000). The coordinate examination of several DNA repair genes during development is also rare; this has only been done for members of the Growth Arrest and DNA Damage inducible family (Ou et al., 1997; Rees et al., 1999).

1.4.6 DNA Repair Gene Null Mutant Animals and Teratogenesis

A large majority of animals lacking DNA repair enzymes are not viable, with pre-implantation death being a common result (reviewed in Friedberg and Meira, 2000). The majority of embryonic loss occurs around implantation, demonstrating the absolute requirement for repair ability when the embryonic cells begin to proliferate rapidly, forming specific tissues. Null-mutant animals which lack DNA repair or genotoxic stress response genes but are still viable are beginning to be used to elucidate the mechanisms of teratogenesis. *p53* null mice have been used to examine the mechanism of action of several teratogens (Nicol et al., 1995; Norimura et al., 1996; Wubah et al., 1996; Moallem and Hales, 1998; Frenkel et al., 1999; Wang, 2001); this is the extent to which DNA repair gene null mutant mice have been used.
1.4.7 **DNA Repair Pathways and Cell Cycle Checkpoints in Mechanisms of Teratogen Action**

All four major mammalian DNA repair pathways (NER, BER, MMR, and RCR) as well as genotoxic stress sensors, interact with components of the cell checkpoint machinery. These interactions are mainly to slow cell cycle progression or cell division until the damage is repaired. However, some DNA damage sensors elicit a cell checkpoint that may then activate the apoptotic cell death pathway. While the roles of cell cycle components that are regulated in response to genotoxic stress have been extensively studied in cancer models, little is known of their regulation or activity during development. Some studies have examined the expression of genotoxic stress-activated proteins that interact with the cell cycle machinery, either during normal development or after teratogen exposure. *GADD45* expression was found to be high in various tissues in the rat embryo during late stages of development (Rees *et al.*, 1999), while Ou *et al.* (1997) determined that the teratogen methylmercury increased *GADD45* mRNA levels at doses that are toxic to the developing nervous system. Chen and Lee (1996) examined *Atm* expression during mouse development, and found ubiquitous expression with particularly high mRNA levels on GD10.5 and 13.5 in the nervous system and lung. Soares *et al.* (1998) determined that the expression pattern of *Atm* in GD10-16.5 mouse embryos was consistent with a role for *Atm* in genome maintenance during cell division, as the highest levels of expression were found in areas undergoing rapid cell division. Therefore, links
exist between DNA repair machinery, cell cycle checkpoints, and teratogen action.

1.5 **Rationale and Purpose of the Investigation**

Congenital defects are the most common cause of perinatal and infant mortality in the United States (CDC, 1996), accounting for greater than 25% of infant deaths (Lynberg and Khoury, 1990). Five percent of individuals have some type of congenital defect (Baird et al., 1988), with only half of affected individuals recognized at birth. Congenital abnormalities may be so inconsequential as to remain undetectable throughout the life of the individual affected, or can lead to gross physical and mental deficiencies, developmental retardation, and peri- and postnatal lethality. A great deal of effort has been put into determining the basis for these defects, and consequently, the field of teratology has flourished. However, a great number of unanswered questions remain, chief among them are the underlying genetic factors for congenital defects: over 60% of birth defects are of unknown aetiology.

Prevention of congenital birth defects would save an estimated $8 billion (US) per year in medical expenses (CDC, 1995), and remains a major medical opportunity. Programs to educate and counsel expecting mothers are already part of regular medical practice, as is dietary supplementation with nutrients whose deficiency during pregnancy leads to birth defects. While these combined efforts have decreased preventable birth defects, it is the unwitting exposure to teratogens of unknown origin that continue to elude modern medical testing. Only detailed research into the cellular mechanisms of a broad spectrum of
teratogenic agents will allow us to understand new, potential teratogens, before they become a health issue.

The importance of cellular pathways that recognize and respond to genotoxic stress is obvious for the above-mentioned reasons. Examining the involvement of DNA repair and cell cycle checkpoint pathways during development should give insights into the mechanisms of action of genotoxic teratogens.

1.6 Hypothesis of the Thesis

While the DNA repair field has, for the most part, elucidated the role of the major repair pathways in maintaining genomic integrity during normal cell growth, as well as determined their roles in disease states, there is a dearth of information with regard to the involvement of these pathways during development. More and more agents, either man-made or naturally occurring, induce genotoxic stress. For these reasons, I determined that a specific investigation of DNA repair pathways during development would help understand, and perhaps prevent, teratogenic incidences following maternal or foetal exposure to genotoxic stressors. Therefore, the hypothesis of this thesis is that DNA repair capacity plays a role in the response of the conceptus to DNA-damaging teratogens.
1.7 **Objectives of the Thesis**

The objectives of this thesis were to:

1. Determine the *in vivo* expression pattern of DNA repair genes, their protein products, and their activity in the yolk sac and embryo proper in the mid-organogenesis stage rat conceptus.

2. Determine the effect of teratogen exposure on DNA repair gene expression and activity in the rat conceptus.
The first objective of this thesis was to examine the DNA repair gene expression profile of the mid-organogenesis stage rat conceptus. As a result, we decided to first examine the NER pathway as a separate entity, and determine whether the conceptus could modulate NER gene expression when exposed to 4-OOHCPA, the activated form of the teratogen CPA, whose lesions are predominantly repaired via NER.
CHAPTER TWO

Nucleotide Excision Repair Gene Expression in the Rat Conceptus During Organogenesis

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2.1 **Abstract**

DNA repair may be a determinant of the susceptibility of the conceptus to DNA damaging teratogens. The nucleotide excision repair (NER) pathway repairs a substantial amount of chemically induced DNA damage. The goals of this study were to assess the coordinate expression of NER genes in the midorganogenesis-stage rat conceptus and determine the consequences of exposure to the genotoxic teratogen, 4-hydroperoxycyclophosphamide (4-OOHCPA), on NER gene expression. Most NER genes were expressed at low levels in both yolk sac and embryo on gestational day (GD) 10, with the exception of *XPD*, *XPE* and *PCNA*. No significant alterations in gene expression occurred between GD10 and 11; in the yolk sac *XPB* expression increased on GD12 compared to either GD10 or 11. In the embryo, *XPE* expression increased between GD10 and 12, while *hHR23B*, *XPB*, *ERCC1*, and DNA polymerase ε expression increased on GD12 relative to both GD10 and 11. Contrary to gene expression data, XPB protein was found at high levels and XPD at low levels in GD10-12 embryos and yolk sacs. Mirroring gene expression, high levels of PCNA protein were found in both tissues; XPA protein levels were minimal in yolk sac from GD10 to 12 but increased in the embryo from moderate on GD10 to high on GD12. Therefore, NER gene expression during organogenesis was regulated in a developmental stage- and tissue-specific manner. Exposure of the conceptus to a teratogen, 4-OOHCPA, induced malformations without affecting NER transcript levels. Thus, NER gene expression in the conceptus was unresponsive to regulation by DNA alkylation.
2.2 Introduction

The birth defects seen after teratogen exposure during susceptible stages of development depend on the nature of the insult and the stage of development of the embryo. Teratogens such as ethylmethanesulfonate (Platzek et al., 1982), methylnitrosourea (Jirakulsomchok and Yielding, 1984), and oxidative stress (Liu and Wells, 1995) induce DNA damage in the organogenesis-stage conceptus. In order to maintain cellular integrity, genotoxic damage must be repaired before cell division occurs, as these lesions can interfere with cellular functions of nascent daughter cells. Lesions created by genotoxic agents require specific groups of gene products to effect repair (Wood, 1996).

The nucleotide excision repair (NER) pathway is the major mammalian DNA repair system, repairing a broad spectrum of lesions caused by agents such as UVR and chemical mutagens (Sancar, 1996). NER is an active cellular process requiring the concerted effort of over 16 different polypeptides; NER can be divided into three main biochemical stages: recognition of the lesion, incision around the lesion, and nascent DNA synthesis/ligation (de Laat et al., 1999). In addition, the transcription-coupled repair (TCR) pathway repairs lesions in actively transcribed DNA (Leadon, 1999). All core NER polypeptides (Mu et al., 1995), as well as several associated proteins, are necessary for full functionality of this pathway; mutations in NER genes result in the human disorders Xeroderma Pigmentosum (XP), Cockayne Syndrome (CS), and trichothiodystrophy (TTD) (Cleaver et al., 1999). In addition, null mutations in murine NER homologues may result in either early embryonic lethality or reduced life span (Friedberg and Meira, 2000), demonstrating the importance of NER
gene expression both in counteracting exposure to genotoxic agents and for viability during normal development.

We hypothesize that differential expression of NER genes during development may be a critical factor in determining sensitivity to DNA-damaging teratogens. However, little is known about the expression of many NER genes in the conceptus. The present study was undertaken to elucidate a profile of the NER pathway during midorganogenesis (GDs 10-12) in the rat; this is the period during which the conceptus is most susceptible to the malformations induced by genotoxic teratogens. The conceptus responds to genotoxic insult with a stress response characterized by alterations in the expression and activity of genes such as the transcription factor activator protein-1 (AP-1), composed of the Fos and Jun families of protooncogenes (Ozolins and Hales, 1997). Cyclophosphamide (CAS 6055-19-2), a well-studied antineoplastic agent and genotoxic teratogen (Mirkes, 1985), induces specific limb and neural tube malformations (Greenway et al., 1982), apoptosis (Chen et al., 1994; Mirkes and Little, 1998), and alterations in cell cycle kinetics (Little and Mirkes, 1992) when administered during organogenesis. Exposure to cyclophosphamide induces genotoxic DNA lesions which are repaired by the NER pathway (Little and Mirkes, 1987). Thus, a second goal of this study was to determine whether NER transcript levels in the conceptus are modified following exposure to 4-OOHCPA, an activated analog of cyclophosphamide.
2.3 Materials and Methods

Tissue preparation. Timed-pregnant female Sprague-Dawley rats (200-225g) were obtained from Charles River Canada (St. Constant, Québec) and housed in the McIntyre Animal Resource Centre. Food and water were provided ad libitum, and animals were exposed to a 14hr light: 10hr dark cycle. All treatments were in accordance with a protocol approved by the Animal Care Committee of McGill University. Gestational day (GD) 0 was defined as the morning following mating. On GD10, 11, and 12, uteri were removed and embryo and yolk sac tissues were dissected immediately and either frozen individually in liquid nitrogen for gene expression analysis or pooled for protein analysis, and stored at -80°C.

Embryo culture. To determine the effect of 4-OHCPA on development and on gene expression, rat conceptuses were explanted from timed-pregnant dams on GD10 and cultured using established techniques (New, 1978). The whole embryo culture model removes any confounding maternal effects that may occur following drug exposure. In culture, 4-OHCPA breaks down spontaneously in solution to its active metabolites, phosphoramid mustard and acrolein (Slott and Hales, 1988). Conceptuses with an intact yolk sac and ectoplacental cone were cultured in the presence of vehicle (sterile water) or 10µM 4-OHCPA (a gift from M. Colvin) for either 24 or 44hr at 37°C in 90% heat-inactivated rat serum supplemented with penicillin and streptomycin. Following culture, embryos were removed and dissected as described above.
Antisense RNA (aRNA) technique. The aRNA technique was used to examine the NER gene expression profile on a per embryo basis. This technique, essentially a "reverse Northern blot", allows for the simultaneous examination of multiple gene transcripts from a single tissue sample (van Gelder et al., 1990; Taylor et al., 1995), and was performed as previously described (Harrouk et al., 2000). Briefly, individual embryos and yolk sacs were sonicated, and mRNA was reverse-transcribed in the presence of an oligo(dT) primer attached to a T7 RNA polymerase promoter. Double-stranded cDNA was formed by a combination of T4 DNA polymerase (Gibco BRL) and Klenow fragment (New England Biolabs, Mississauga, Ontario). Antisense RNA was then created from this cDNA pool by T7 RNA polymerase (New England Biolabs) and simultaneously radiolabelled with $\alpha^{32P}$ CTP (10mCi/mol; Amersham Pharmacia Biotech, Baie d’Urfé, Québec). The linearity and reproducibility of this amplification reaction was determined by trichloroacetic acid precipitation of $\alpha^{32P}$ CTP incorporated into the acid-insoluble fraction (data not shown).

Gene array membranes and hybridization. To examine the expression of multiple genes from a single tissue sample, gene expression arrays were created, each with 16 NER genes: XPA, XPB, XPC, XPD, XPE, XPF, XPG, ERCC1, CSA, CSB, hHR23B, DNA ligase I (ligI), PCNA, RPA (14kDa subunit), and DNA polymerases $\delta$ (PolD-catalytic subunit) and $\epsilon$ (PolE). Nylon membranes (Zeta-Probe GT, Bio-Rad, Mississauga, Ontario) were slot-blotted with equimolar amounts of cDNAs encoding the genes of interest, as per the manufacturer’s
protocol (Bio-Rad). Wherever possible, rodent cDNA probes were used. When rodent probes were not available, human probes were chosen for the genes of interest. The homologies of these human probes to rodent cDNAs were: XPE (96% compared to rat), XPF (86% compared to mouse; no rat sequence available), XPG (79% compared to mouse; no rat sequence available), PolE (85% compared to mouse; no rat sequence available), RPA (87% compared to rat), hHR23B (87% compared to mouse; no rat sequence available), CSA (91% compared to rat), and CSB (no rodent clone). It is possible, despite the high homology of most of these probes to the rodent genes, that the use of cross-species cDNA probes in these gene arrays may have an effect on the assessment of absolute NER transcript levels. Gene expression arrays were pre-hybridized in hybridization buffer, and heat denatured radiolabelled aRNA probes originating from a single individual embryo or yolk sac were hybridized overnight to arrays. Then the arrays were washed in solutions of decreasing stringency and exposed to phosphorimager plates overnight. Arrays were stripped of probe by boiling in stripping buffer and reused a maximum of five times.

**Quantification and analysis of aRNA data.** Images of the gene expression arrays were obtained using a STORM phosphorimager (Molecular Dynamics, Sunnyvale, CA). Representative images from GD12 yolk sac and embryo arrays are shown in **Figure 2.1.** Quantification of gene expression was done using ImageQuant 5.0 software (Molecular Dynamics). Gene expression intensity values were normalised relative to the expression of vimentin, a structural protein, from the same membrane, as its expression did not vary by more than
**Figure 2.1.** Antisense RNA blots illustrating NER gene expression profiles in the rat conceptus during organogenesis. Phosphorimager images of gene expression arrays hybridized overnight with GD12 yolk sac (A) and embryo (B) aRNA samples. These data are representative of results obtained from five to seven samples, each from a single embryo or yolk sac from separate litters, hybridized to different membranes; arrays were exposed for 24hr to a phosphorimager plate.
2-4% of total blot intensity between the different time points and tissues. To account for non-specific hybridization, the intensity value from pUC18 plasmid DNA blotted onto each array was subtracted from each gene intensity value. Data are from five to seven separate GD10-12 yolk sac and embryo samples for \textit{in vivo} data, and from four to seven separate samples for culture alone or 4-OHHCPA treatment, each obtained from separate litters.

**Nuclear protein extraction.** Embryos or yolk sacs from a single litter were combined for GD10 samples; two to three yolk sacs or embryos were combined for GD11 and 12 samples. Tissues were sonicated on ice (Vibracell, Sonics & Materials, Danbury, CT) in buffer A (10mM Tris pH8.0, 1.5mM MgCl$_2$, 5mM KCl, 0.5mM DTT, 0.5% Nonidet P-40) in the presence of a protease inhibitor cocktail and kept on ice for 10min. Samples were centrifuged at 400x $g$ for 15min at 4°C, the pellet was resuspended and then centrifuged at 400x $g$ for 10min at 4°C and further resuspended in buffer B (20mM Tris pH8.0, 25% glycerol, 1.5mM MgCl$_2$, 0.2mM EDTA, 0.5mM DTT, 0.4mM NaCl, 0.5mM PMSF) with protease inhibitors. Samples were kept on ice 15min, then centrifuged at 21,000x $g$ for 30min at 4°C. Supernatants were transferred to new tubes, and pellets resuspended in buffer B and centrifuged as before. The supernatants were combined and protein concentrations determined by the Bio-Rad Protein Assay (Bio-Rad). Samples were frozen at -80°C until used.
Western blot analysis. Nuclear protein (10 µg) was mixed with loading buffer and electrophoresed on a 7% polyacrylamide gel for 2.5 hr at 75 V. Samples were transferred to Hybond-C Super nitrocellulose membranes (Amersham) at 125 V for 2 hr at 4°C, incubated with TBS-0.1% Tween-20 (TBS-T) supplemented with 10% non-fat dry milk and 1% Bovine Serum Albumin (BSA) for 1 hr to block non-specific antibody binding, then incubated with the primary antibody in TBS-T with 5% non-fat dry milk and 1% BSA overnight at room temperature. Anti-XPA (1:2000 dilution), XPB (1:4000), and XPD (1:1000) antibodies were obtained from Santa Cruz (Santa Cruz, CA); anti-PCNA antibody (1:1000) was obtained from Sigma (St. Louis, MO). Secondary antibodies (1:2500) conjugated to horseradish peroxidase (HRP) were used to detect specific antibody interactions, and were visualized with the ECL-Plus Kit (Amersham) and BioMax MR film (Kodak, Rochester, NY). Molecular weight determination was done by visualization of prestained and biotinylated protein standards (Bio-Rad) with Streptavidin-HRP conjugate (1:5000; Amersham). HeLa cell and MM-142 nuclear extracts (Santa Cruz) were used as positive controls. Quantification of western blot data was done using a ChemiImager 4000 imaging system (Alpha Innotech, San Leandro, CA) with AlphaEase 3.3b software. Histone H1 protein expression was used as a loading control, using anti-histone H1 antibody (1:1000 dilution; Santa Cruz).
Statistical analysis. Statistical analysis was done on an individual gene basis using one-way ANOVA followed by a Tukey post-hoc test with SigmaStat version 2.03 software (SPSS, Chicago, IL).
2.4 Results

Developmental-stage specific regulation of NER gene expression during organogenesis. Specific banding patterns were observed on the NER gene expression arrays probed with yolk sac and embryo samples (Figure 2.1A,B; GD12 samples shown). A quantitative analysis of the expression profiles of NER genes in the yolk sac (Figure 2.2A) and the embryo (Figure 2.2B) on days 10, 11 and 12 of gestation is presented in Figure 2.2. Genes were grouped according to their role in the three stages of NER: recognition of DNA damage, incision around the damage, and nascent DNA synthesis and ligation. With three notable exceptions, namely *XPD, XPE,* and *PCNA,* the expression of NER genes on GD10 was low in both embryo and yolk sac tissue. *XPD, XPE,* and *PCNA* were highly expressed in both yolk sac and embryo in an age-independent manner. No significant increases in expression of the NER genes were observed between GD10 and GD11 in either the yolk sac or embryo.

Interestingly, an age-dependent increase in *XPB* gene expression did occur in yolk sac tissue between GD10 to 11 and GD12 (3-fold, p<0.05; Figure 2.2A). Between GD10 and GD12 there were age-dependent increases in the expression of a number of genes in the embryo (Figure 2.2B); steady-state concentrations of *hHR23B, XPB, ERCC1,* and *PolE* transcripts increased significantly on GD12 compared to either GD10 (25, 23, 31, and 12-fold respectively; p<0.05) or GD11 values (~7, ~8, 4, 5-fold respectively, p<0.05; Figure 2.2B). As well, the expression of *XPE* increased significantly between GD10 and 12 (2-fold; p<0.05).
Figure 2.2. Quantitative analysis of NER gene expression profiles in the rat conceptus during organogenesis. NER gene expression was analyzed from arrays probed with aRNA samples from yolk sac (A) or embryo (B) tissues on GD10 (shaded bars), GD11 (dark cross-hatched bars) or GD12 (light cross-hatched bars). Data are expressed as the mean % of vimentin expression ± SEM of tissue from five to seven separate GD10-12 yolk sac and embryo samples obtained from different litters. *, expression significantly different from GD10 by one-way ANOVA and Tukey Test, p<0.05. †, expression significantly different from both GD10 and GD11 by one-way ANOVA and Tukey Test, p<0.05. TCR, Transcription-Coupled Repair.
Tissue-specific variations in NER gene expression. Dramatic differences in gene expression between tissues were observed for several NER genes. While no differences were observed in gene expression between yolk sac and embryo proper on GD10, by GD11 XPD expression was significantly higher in the embryo compared to yolk sac (p<0.05). On GD12 the expression of hHR23B, ERCC1, and PolE was significantly higher in the embryo (~4, 6, and ~4-fold respectively; p<0.05) than in the yolk sac; many other genes showed a trend towards increased expression in the embryo.

Analysis of NER proteins during organogenesis. XPA, XPB, XPD, and PCNA were chosen for the analysis of NER proteins in order to establish how changes observed in NER gene expression correlated with changes in protein levels. Figure 2.3 shows representative western blots of GD10, 11 and 12 yolk sac and embryo nuclear protein samples probed with antibodies against XPA, XPB, XPD, and PCNA. The nuclear protein histone H1 was used as a loading control to quantify protein expression levels. XPA and histone H1 are both found as two bands on SDS/PAGE gels; both bands were combined for quantification analysis.

Immunoreactive XPA was found in the embryo on GD10, 11 and 12 (Figure 2.3); however, there was minimal expression in the yolk sac at these times. Furthermore, XPA protein concentrations increased in a developmental stage-specific manner from GD10 to 12 in the embryo (Figure 2.4), mirroring a similar trend in gene expression in the embryo. In contrast, XPB immunoreactivity was present at similar high levels on GDs 10, 11 and 12 in both the yolk sac and embryo. This is contrary to XPB gene transcript concentrations,
Figure 2.3. NER protein patterns during organogenesis in the rat conceptus. Representative western blots of XPA, XPB, XPD, PCNA, and histone H1 in GD10-12 yolk sac and embryo samples (XPA and histone H1 are both found as 2 bands on SDS/PAGE gels). MM-142 and HeLa cell nuclear extracts were used as positive controls. Histone H1 was used as a loading control. This experiment was repeated using three different sets of tissue samples from embryos from separate litters.
Figure 2.4. Quantification of NER protein patterns during organogenesis in the rat yolk sac (black bars) and embryo proper (grey bars). These data represent a single blot and are expressed relative to histone H1.
which increased significantly between GD10 and 12 in both tissues. XPD immunoreactivity was relatively low at all timepoints in both tissues, in contrast with the high XPD gene transcript levels observed. PCNA immunoreactivity was found at similarly high levels in both yolk sac and embryo from GD10 to 12, reflecting high gene transcript steady state concentrations levels in both tissues. These data suggest that NER genes are subject to gene, stage and tissue-specific post-transcriptional regulation.

**Effect of teratogen exposure on NER gene expression.** To determine whether NER transcript levels in the conceptus are modified following exposure to a DNA damaging teratogen, embryos were cultured in the absence (control; Figure 2.5A) and presence of 10μM 4-OOHCPA (Figure 2.5B). 4-OOHCPA-treated embryos exhibited characteristic growth retardation and microprosencephaly (Slott and Hales, 1988).

While NER gene expression in control embryos cultured for 24 or 44hr had a high degree of variability, expression patterns were similar between in vivo and in vitro cultured embryos. Only XPC expression changed, decreasing following 24hr culture (Figure 2.6) compared to GD11 in vivo values in the embryo (Figure 2.2B). Culture with 4-OOHCPA did not induce significant alterations in NER gene expression in either yolk sac or embryo (Figure 2.6A,B), although a trend towards decreased gene expression was observed for several NER genes, particularly in the embryo following 44hr culture (Figure 2.6B).
Figure 2.5. Embryos cultured for 44 hr in the absence (A) or presence (B) of 10\(\mu\)M 4-OHCPA. Bar, 1 mm.
Figure 2.6. Expression profile of NER genes following 4-00HCPA exposure in vitro. Yolk sac (A) or embryo (B) tissue gene expression profile following 24hr culture without (dark bars), or with (light cross-hatched bars) 10μM 4-00HCPA; and after 44hr without (dark double cross-hatched bars) or with (light reverse cross-hatched bars) 10μM 4-00HCPA. Data are expressed as the mean % of vimentin expression ± SEM of tissue from four to seven separate yolk sac and embryo samples obtained from different litters. TCR, Transcription-Coupled Repair.
2.5 Discussion

Responses to genotoxic stress include alterations in gene expression, repair of the damaged genetic material, cell cycle arrest, and cell death. These responses can occur alone or in combination. The alkylating teratogen cyclophosphamide has been shown to alter cell cycle parameters in susceptible tissues in the rat conceptus (Little and Mirkes, 1992), as well as to induce apoptosis (Chen et al., 1994; Mirkes and Little, 1998). However, little is known about the ability of the conceptus to repair specific forms of genotoxic damage. Regulation of the NER pathway in the conceptus during organogenesis is of particular interest because of the major role this pathway plays in the repair of chemically induced lesions and because a functional NER repair response requires the coordinate regulation of the expression of a large family of genes. Although NER genes are expressed in tissues from all organisms tested to date, there are few examples in which the coordinate expression of several NER genes has been examined in any one tissue or cell type (Wei et al., 1995; Damia et al., 1998; Cheng et al., 1999; Vogel et al., 2000). To the best of our knowledge, this is the first study to elucidate the expression profile of all major NER genes in any tissue. We found that most NER genes were expressed in the conceptus during midorganogenesis, albeit at low levels. Moreover, the expression profiles of NER genes were similar in both the yolk sac and embryo. Dramatic increases were observed in the embryo on GD12, demonstrating an age-depandant regulation in gene transcription. The increases in gene expression were found throughout all three stages of NER (recognition of DNA damage, incision, and synthesis/ligation); this may indicate a general increase in NER capability,
exclusively in the embryo, on GD12. Of note are the relatively low levels of expression of CSA and CSB in the conceptus on GD10 and 11; as both genes are part of the TCR pathway, their low expression may suggest that this pathway is not active until GD12.

We hypothesized that genotoxic stress produced by 4-OOHCPA may alter NER expression in the conceptus, and thus the ability of the conceptus to respond to this stress. Following exposure to 4-OOHCPA during embryo culture, no alterations in NER gene expression were observed in either the embryo or yolk sac. While the ability of an organism to directly regulate genes in the NER pathway is still not fully understood, studies to date with non-mammalian species (Lee S.-K. et al., 1997), and cancer cell lines (Li et al., 1998) have shown increased NER gene expression following genotoxic stress. Andersson et al. (1996) showed that mutations in two NER genes, ERCC1 and XPF, determined the sensitivity of hamster CHO cells to 4-OOHCPA; this was the first link between a genotoxic teratogen and specific DNA repair genes of the NER pathway. Vogel et al. (2000) found that transcript levels of ERCC1, along with XPD, correlated with repair activity, but were not limiting factors. Both ERCC1 and XPF were expressed at very low levels in the embryo and yolk sac on GDs 10 and 11, which may provide a basis for the susceptibility of the conceptus to insult at these developmental timepoints. Increased in vivo expression of these genes on GD12 in the embryo may suggest an increased capability of the conceptus to protect itself against this teratogen at this time during development.

Variability in the expression of individual NER genes in the conceptus may also be due to their involvement in other cellular pathways. The potential role of
NER genes in alternate pathways is evidenced by the pleiotropic conditions seen in XP and CS patients (Jaspers, 1996). Several NER genes are involved in other facets of DNA metabolism, such as transcription (Tomkinson and Mackey, 1998) and replication (Burgers, 1998), whereas others are involved in other DNA repair pathways (Tomkinson and Mackey, 1998).

The data presented in this study represent the first co-ordinate elucidation of NER gene and protein expression patterns during development. Gene and protein expression profiles matched for PCNA and XPD; however, transcript levels for XPA and XPB did not match with their protein levels. XPB transcript levels were low in both tissues until GD12. Previous studies have shown XPB to be expressed at low levels during development in the mouse (Weeda et al., 1991; Hubank and Mayne, 1994). While XPB transcript concentrations were increased on GD12 in the embryo, XPB protein levels remained relatively constant. It is interesting to note that XPB protein concentrations in the conceptus are high relative to those in HeLa cells. XPB and XPD are key components of transcription factor IIH (TFIIH), and mutations in either gene affect both NER and DNA transcription (Coin et al., 1999). Both are found in a 1:1 stoichiometric ratio in TFIIH. In contrast to XPB, XPD protein concentrations in the conceptus were similar to those in HeLa cells. This may be due to differential stability of gene transcripts, and/or utilization of XPB in alternate cellular pathways. XPB is a component of the p53-mediated apoptotic pathway, and its expression level may be regulated by p53-dependent apoptosis during development (Wang et al., 1996; Wallingford et al., 1997).
XPA transcripts were present at very low levels in both yolk sac and embryo on all three days of gestation. This is in agreement with a study that reported very low levels of XPA transcripts in human and mouse cell lines (Layher and Cleaver, 1997); these authors hypothesized that XPA may be rate limiting for NER in mice and humans. Interestingly, XPA protein levels differed dramatically between tissues; if XPA is indeed a limiting factor in NER, there may be a major difference in NER capability between the embryo and yolk sac during this period of development. These data suggest that there is a need for post-translational processes in determining the ultimate levels of NER proteins in the conceptus.

Genes involved in responses that are downstream to DNA damage (e.g. p53) are a determinant in chemical-induced teratogenesis (Nicol et al., 1995; Moallem et al., 1998). If these genes, or their homologues, are required for successful cell proliferation and division, it is likely that genes encoding DNA repair enzymes are required also when faced with genotoxic insult. Despite the observation that many known teratogens induce genotoxic stress (Bishop et al., 1997), little is known about the role of DNA damage and repair in teratogenesis. We postulate that, as with tumour sensitivity to genotoxic agents (Dabholkar et al., 1994; Li et al., 1998), there is a link between the expression of DNA repair genes and progeny outcome after exposure to a stressor. The expression of DNA repair enzymes may be the fundamental difference between cellular survival and cell death following genotoxic insult during development.
2.6 **Acknowledgements**

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2.7 Appendix 1: aRNA Control Experiments

The aRNA technique is used to examine gene expression profiles on a per embryo basis. This technique is essentially a “reverse Northern blot”, allowing for the simultaneous examination of multiple gene transcripts from a single tissue sample (Van Gelder et al., 1990; Taylor et al., 1995). The aRNA technique derives its name from the fact that tissue mRNA is converted first into cDNA, then amplified into RNA, which is complementary (“antisense”) to the original mRNA molecules. This antisense RNA is then hybridized to gene array membranes to examine gene transcript levels. Individual embryos and yolk sacs are sonicated on ice in lysis buffer, then reverse-transcribed in the presence of an oligo(dT) primer attached to a T7 RNA polymerase promoter, which recognizes and binds to the poly(A) tail of mRNA in the tissue sample (Figure 2.7). Self-priming of the resultant single-stranded cDNAs by hairpin loop formation, with subsequent addition of S1 nuclease to excise the hairpin, allows the formation of double-stranded cDNA. Antisense RNA is created from the double-stranded cDNA pool and simultaneously radiolabelled.

The aRNA technique takes into account potential differences in starting material by describing gene transcript levels as a percent of another transcript from that same tissue sample (equivalent to an internal control). The larger GD12 embryo has equally greater amounts of the transcripts for the gene of interest and the reference gene. The strength of the aRNA technique is that gene expression can be examined on an individual embryo basis, or even in a single cell (Harrouk et al., 2000).
GD10, 11, and 12 rat embryos

mRNA

TTTTT-T7 primer
Reverse Transcriptase
dNTPs

mRNA-cDNA
hybrid

Denaturation @ 95°C

ss cDNA

Klenow T4 DNA Polymerase
dNTPs

Equimolar amounts of cDNAs encoding genes of interest

Klenow
S1 nuclease
Klenow
dNTPs

ds cDNA

T7 RNA Polymerase
α^{32}P-CTP
NTPs

Amplified antisense RNA (aRNA)

Hybridize
Wash
Expose

Band intensity is directly proportional to amount of mRNA in tissue sample

Quantitate by image analysis software

Figure 2.7. The antisense RNA (aRNA) technique.
In order to determine the linearity of the aRNA amplification step, the amount of α\(^{32}\)P-CTP incorporated into new DNA molecules was analyzed by trichloroacetic acid precipitation. Incorporation of α\(^{32}\)P CTP into the acid-insoluble fraction over time (Figure 2.8) indicates that the new DNA molecules are being radiolabelled, and that the rate of synthesis of DNA molecules is linear.

To determine the reproducibility of the aRNA technique, a single sample was amplified twice, and probed to the same membrane that had been stripped of the previous probe (Figure 2.9). Identical banding patterns are observed in both instances, demonstrating the reproducibility of the overall technique.

Quantification of gene expression is done by expressing gene expression intensity values relative to the expression of a control gene from the same membrane. The data were represented relative to vimentin as its expression did not vary by more than 2-4% of total blot intensity between the different timepoints and tissues; thus, indicating its suitability as a stable reference for gene expression between tissues and timepoints during mid-organogenesis. Other candidate control genes, namely GCS50 and DNA methyltransferase 1 (DNMT1), did vary between tissues when compared to each other (Figure 2.10). To account for non-specific RNA-DNA hybridization, the intensity value from pUC18 plasmid DNA blotted onto each array is subtracted from each gene intensity value.

84
Together, these control experiments demonstrate the validity of using the aRNA technique, as well as the analysis of the gene expression determined by this technique.
Figure 2.8. Incorporation of $^{32}$P-CTP into aRNA amplification products, expressed as a percent of total radioactivity. Closed circles, yolk sac; open circles, embryo. Data are mean +/- SEM of three separate reactions.
Figure 2.9. Reproducibility of the antisense RNA (aRNA) technique, as exemplified by amplifying a single sample on two separate occasions, and hybridizing to the same membrane. In this instance, a GD12 embryo sample was used.
Figure 2.10. Expression of candidate aRNA control genes, relative to each other, from GD10, 11, and 12 embryo samples. Data are the mean % of gene expression +/- SEM of five to seven different tissue samples.
Examination of gene expression levels for the NER pathway in the embryo in the previous Chapter indicated that, at least for alkylation stress, the embryo does not upregulate gene expression following genotoxic teratogen exposure. We next examined three other main mammalian DNA repair pathways *in vivo*: the BER, MMR, and RCR pathways. None of the genes from these three pathways have been examined with respect to their response to genotoxic or teratogenic agents during development. We therefore examined whether the BER, MMR, and RCR gene expression profiles were altered following exposure to the genotoxic teratogen 4-OOHCPA.
CHAPTER THREE

Expression of Base Excision, Mismatch, and Recombination Repair Genes in the Organogenesis-Stage Rat Conceptus and Effects of Exposure to a Genotoxic Teratogen, 4-Hydroperoxycyclophosphamide

Robert K. Vinson and Barbara F. Hales

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3.1 **Abstract**

DNA repair capability may influence the outcome of genotoxic teratogen exposure. The goals of this study were to assess the expression of base excision repair (BER), mismatch repair (MMR), and recombination repair (RCR) genes in the mid-organogenesis rat conceptus and to determine the effects on expression of exposure to the genotoxic teratogen, 4-hydroperoxycyclophosphamide (4-OHCPA). The expression of 17 BER, MMR, and RCR genes was examined in gestational day (GD) 10-12 rat conceptuses using the antisense RNA (aRNA) technique. Embryos were cultured with 10 µM 4-OHCPA to examine effects on gene expression. Yolk sacs and embryos had similar gene expression patterns for all three DNA repair pathways from GD10-12. Transcripts for APNG, PMS1, and RAD54 were present at high concentrations in both tissues. The remainder of the genes were expressed at low levels in yolk sac, with a few not detected on GDs 10 and 11. In the embryo, transcripts for most genes were low on GD10 and 11; several increased by GD12. After exposure to 4-OHCPA for 24 hr, XRCC1 and RAD57 expression decreased in yolk sac, whereas only RAD51 transcripts decreased in the embryo. By 44 hr, transcripts for all BER genes decreased in yolk sac; in the embryo, most BER, MMR, and RCR genes decreased, many below the level of detection. The expression of DNA repair genes in the mid-organogenesis rat conceptus is varied and subject to down-regulation by 4-OHCPA. DNA repair gene expression may determine the consequences of genotoxicant exposure during development.
3.2 Introduction

The integrity of genetic material in the cell is crucial for proper cell growth and function. In order to maintain this integrity, genotoxic damage must be repaired before cell division occurs as it has the ability to modify gene and/or chromosome structure, leading to gene expression changes, altered cell functioning, and abnormal cell differentiation and growth in nascent daughter cells. Repairing these alterations is particularly important during the periods of cellular proliferation that occur during development, as aberrant cell growth or death can lead to malformations (Sulik et al., 1988; Wolpert, 1999). Birth defects observed following teratogen exposure depend on the nature of the insult and the stage of development of the embryo. Many known teratogens are genotoxic (Bishop et al., 1997), including thalidomide (Parman et al., 1999), dioxin (Shertzer et al., 1998), pyrimethamine (Tsuda et al., 1998), and phenytoin (Liu and Wells, 1995). While the link between genetic instability and teratogenesis is not fully established (for review, see Ferguson and Ford, 1997), the role of DNA damage in abnormal cell growth, death, and functioning points to a causal role in the etiology of malformations (Snow, 1997; Wells et al., 1997).

Genotoxic lesions require specific groups of gene products to effect repair (Wood, 1996). Of the major mammalian DNA repair pathways, the BER, MMR, and RCR pathways recognize and remove a wide spectrum of lesions, including single base damage (Memisoglu and Samson, 2000), mismatched bases and extrahelical loops (Harfe and Jinks-Robertson, 2000), and DNA cross-links and strand breaks (Liang et al., 1998; De Silva et al., 2000), respectively. While some null mutant animals lacking BER, MMR, and RCR genes do not display an overt
phenotype due to lack of DNA repair function (APNG, Pms1, Rad52), null mutations in other murine homologues result in either stunted growth (MGMT), increased risk of cancer (Msh2, Pms2), infertility and/or chromosomal aberrations (Ku86, Mlh1, Msh2, Pms2, and Rad54), or early embryonic lethality (APE1/Ref-1, PolB, Rad50, Rad51, Xrcc1) (for references, see Friedberg and Meira, 2000), demonstrating the importance of the expression of these genes during normal development.

We hypothesize that differential expression of DNA repair genes during development may be a critical factor in determining sensitivity to DNA-damaging teratogens. Little is known about the expression of many DNA repair genes in the conceptus, hence this study was undertaken to elucidate a profile of the expression of genes in the BER, MMR, and RCR pathways during mid-organogenesis (GDs 10-12) in the rat, when the conceptus is most vulnerable to malformations induced by genotoxic teratogens.

A second goal of this study was to determine whether DNA repair gene transcript concentrations in the conceptus are modified following exposure to the alkylating agent 4-0OHCPA, an activated analog of cyclophosphamide (CPA), a nitrogen mustard teratogen (Slott and Hales, 1988). Cyclophosphamide creates DNA adducts, strand breaks (single (SSB) and double-strand (DSB)) and cross-links (both intra- and interstrand (ICL)) (reviewed in Povirk and Shuker, 1994); these lesions are seen when CPA is administered at teratogenic doses (Mirkes et al., 1992). The nucleotide excision repair (NER) pathway is involved in repair of bulky adduct lesions formed by CPA (Sancar, 1996). Homologous recombination via RCR is required to repair the DSBs formed by nitrogen mustards (De Silva et
al., 2000). Members of the BER pathway are involved in the repair of monoadduct lesions formed by CPA (Cai et al., 1999). Thus, we determined the consequences of exposure of the mid-organogenesis conceptus to CPA on the gene expression profiles of major DNA repair gene families.
3.3 **Materials and Methods**

**Tissue preparation.** Timed-pregnant female Sprague-Dawley rats (225-250g) were obtained from Charles River Canada (St. Constant, Québec) and housed in the McIntyre Animal Resource Centre (McGill University). Rat Chow (Purina, St. Louis, MO) and water were provided *ad libitum*, and animals were exposed to a 14hr light: 10 hr dark cycle. All treatments were in accordance with a protocol approved by the Animal Care Committee of McGill University. Gestational day (GD) zero was defined as the morning following mating. On GD10, 11, and 12, uteri were removed and embryo and yolk sac tissues were dissected immediately, frozen individually and stored at -80°C in liquid nitrogen for gene expression analysis.

**Embryo culture.** To determine the effect of 4-OOHCPA on gene expression, rat conceptuses were explanted from timed-pregnant dams on GD10 and cultured using established techniques (New, 1978). In culture, 4-OOHCPA breaks down spontaneously in solution to its active metabolites, phosphoramidon mustard and acrolein (Low *et al.*, 1982; Slott and Hales, 1988). Conceptuses with an intact yolk sac and ectoplacental cone were cultured in the presence of vehicle (sterile water) or 10μM 4-OOHCPA (a gift from M. Colvin) for either 24 or 44hr at 37°C in 90% heat-inactivated rat serum supplemented with penicillin and streptomycin. Following culture, embryos were removed and dissected as described above.

**Antisense RNA (aRNA) technique.** The aRNA technique was used to examine the DNA repair gene expression profile on a per embryo basis, as previously
described (Eberwine et al., 1992; Vinson and Hales, 2001a). This technique is essentially a "reverse Northern blot", allowing for the simultaneous examination of multiple gene transcripts from a single tissue sample (van Gelder et al., 1990; Taylor et al., 1995). Individual embryos and yolk sacs were sonicated on ice in lysis buffer (1 mg/ml digitonin, 5 mM DTT, 50 mM Tris pH 8.3, 6 mM MgCl₂, 0.12 mM KCl), and reverse-transcribed for 2hr at 37°C in the presence of an oligo(dT) primer attached to a T7 RNA polymerase promoter, which recognises and binds to the poly(A) tail of mRNA in the tissue sample. Self-priming allowed the formation of double-stranded cDNA by a combination of T4 DNA polymerase (Gibco BRL, Burlington, Ontario) and Klenow fragment (New England Biolabs, Mississauga, Ontario). Antisense RNA was created from the cDNA pool by T7 RNA polymerase (New England Biolabs) and simultaneously radiolabelled with α³²P-CTP (10 mCi/mol; Amersham Pharmacia Biotech, Baie d'Urfe, Quebec) for 4hr at 37°C. The linearity and reproducibility of this amplification reaction were determined by trichloroacetic acid precipitation of α³²P-CTP incorporated into the acid-insoluble fraction (data not shown).

Gene array membranes and hybridization. Gene expression arrays were created to examine the expression of genes from the BER, MMR and RCR DNA repair pathways. The genes examined were: base excision repair genes alkylpurine-DNA-N-glycosylase (APNG), methylguanine-DNA methyltransferase (MGMT), apurinic/apyrimidinic endonuclease/redox factor-1 (APE/Ref-1), DNA ligase III (LigIII), DNA polymerase β (PolB), and X-ray repair cross-
complementing gene 1 (XRCC1); mismatch repair genes MutL homologue 1 (MLH1), MutS homologue 2 (MSH2), postmeiotic segregation increased 1 (PMS1) and postmeiotic segregation increased 2 (PMS2); and recombination repair genes DNA-dependent protein kinase Ku 86kDa subunit (Ku86), meiotic recombination gene 11 (MRE11), and radiation sensitivity genes 50 (RAD50), 51 (RAD51), 52 (RAD52), 54 (RAD54), and 57 (RAD57). To create these arrays, nylon membranes (Zeta-Probe GT, Bio-Rad, Mississauga, Ontario) were slot-blotted (Bio-Dot SF, Bio-Rad) with equimolar amounts of cDNAs, as per the manufacturer's protocol. Wherever possible, rodent cDNA probes were used. Non-rodent probes were chosen based on their high homology to rodent cDNAs for the genes of interest. Gene expression arrays were pre-hybridized for 30min in hybridization buffer (7% SDS, 0.12M Na2HPO4 pH7.2, 0.25M NaCl, 50% formamide). Heat denatured radiolabelled aRNA probes were hybridized overnight to arrays at 42°C.

Following hybridization, the arrays were washed in solutions of decreasing stringency (from 2X SSC/0.1% SDS to 0.1X SSC/0.1% SDS) at 42°C for 20min each, and exposed to phosphorimager plates overnight. Arrays were stripped of probe by boiling in 0.1X SSC/0.5% SDS twice for 20min each. Stripping efficiency was determined by exposing membranes to phosphorimager plates overnight. Arrays were reused a maximum of five times, at which point no appreciable degradation in signal was observed (data not shown).

Quantification and analysis of aRNA data. Images of the gene expression arrays were obtained using a STORM phosphorimager (Molecular Dynamics,
Sunnyvale, CA), and gene expression quantified using ImageQuant 5.0 software for Windows NT (Molecular Dynamics). Gene expression intensity values on each membrane were normalized relative to the expression of vimentin, a structural protein; vimentin was chosen because its expression remained constant between the different time points and tissues. To account for the slight variation in vimentin expression, the degree to which its expression varied (4.7%) was subtracted from each gene intensity value. The intensity value from pUC18 plasmid DNA blotted onto each array (non-specific hybridization) was also subtracted from each gene intensity value. In order to ensure the consistency and reliability of the data (Lee et al., 2000), several replicates for each tissue and treatment were performed, each from separate litters. *In vivo* data were from five to seven separate GD10-12 yolk sac and corresponding embryo samples; data for culture alone or treatment with 4-OOHCPA were from four to seven separate samples. Differences in expression as a consequence of exposure to 4-OOHCPA compared to control are reported when they are least two-fold; a two-fold change is equivalent to an increase by 100% or a decrease by 50%.
3.4 Results

Base excision repair gene expression. Hybridization of cDNA blots with radiolabelled aRNA gave characteristic banding patterns for the different DNA repair genes. With the exception of MGMT on GD10 in both tissues and APE/Ref-1 in the GD10 and 11 yolk sac, transcripts for all BER genes studied were present in the mid-organogenesis rat conceptus (Figure 3.1). Quantification of gene intensities showed that 3-methyladenine DNA glycosylase (APNG) was the highest expressed BER gene in the yolk sac at all three developmental time points (Figure 3.1A). LigIII expression decreased in the yolk sac between GDs 10 and 12; in contrast, the expression of APE/Ref-1 increased from GD10 to 12. In the embryo, APNG expression was high on GDs 10 and 11 and decreased on GD12 (Figure 3.1B). With the exception of APNG, the expression of all BER genes in the embryo proper increased on GD12 compared to GDs 10 and 11; transcript levels for LigIII, MGMT, APE/Ref-1, and XRCC1 were all over two-fold higher in the embryo than in the yolk sac on GD12.

Mismatch repair gene expression. Quantification of MMR gene expression (Figure 3.2) showed that PMS1 was the highest expressed MMR gene on GDs 10-12 in both yolk sac (Figure 3.2A) and embryo proper (Figure 3.2B); expression was 60-75% of vimentin levels. In both tissues, the other MMR genes (MLH1, MSH2, and PMS2) were expressed at low levels on GDs 10 and 11, with a decrease on GD11 in MLH1 expression to near undetectable levels. However, the expression of all three genes increased in the embryo on GD12; the
Figure 3.1. Base excision repair gene expression in the (A) yolk sac and (B) embryo on GD10 (black bars), GD11 (light grey bars), and GD12 (dark grey bars). Values represent the mean transcript concentrations +/- SEM relative to vimentin from five to seven replicates.
Figure 3.2. Mismatch repair gene expression in the (A) yolk sac and (B) embryo on GD10 (black bars), GD11 (light grey bars), and GD12 (dark grey bars). Values represent the mean transcript concentrations +/- SEM relative to vimentin from five to seven replicates.
expression of MLH1, MSH2, and PMS2 was higher in the embryo than in the yolk sac on GD12.

**Recombination repair gene expression.** Most of the RCR genes examined were expressed at very low levels in the yolk sac on GDs 10-12 (Figure 3.3A); RAD52 transcript levels were undetectable or near the threshold of detection on GDs 10 and 11, as were Ku86 and RAD57 levels. Only RAD50 and RAD52 expression increased from GDs 10-11 to GD12. At 70-100% of vimentin levels, RAD54 was the highest expressed RCR gene in both tissues at all gestational ages. While transcript levels for most of the other RCR genes were low in the embryo on GDs 10 and 11, steady state concentrations increased by GD12 for Ku86, MRE11, RAD51, RAD52, and RAD57 (Figure 3.3B); transcript levels for Ku86, MRE11, RAD51, and RAD57 were over two-fold higher in the embryo than in the yolk sac.

**Expression of BER, MMR, and RCR genes following exposure to 4-OOHCPA.** To determine whether DNA repair gene transcript steady state concentrations in the conceptus were affected by exposure to a DNA damaging teratogen, embryos were cultured in the absence or presence of 10µM 4-OOHCPA. 4-OOHCPA-treated embryos exhibited characteristic growth retardation and microprosencephaly (data not shown) (Slott and Hales, 1988). Changes in gene expression as a result of 4-OOHCPA treatment are shown in Figure 3.4. Following culture with 4-OOHCPA for 24hr, two genes decreased in expression in yolk sac: the BER gene XRCC1 (Figure 3.4A) and the RCR gene
Figure 3.3. Recombination repair gene expression in the (A) yolk sac and (B) embryo on GD10 (black bars), GD11 (light grey bars), and GD12 (dark grey bars). Values represent the mean transcript concentrations +/- SEM relative to vimentin from five to seven replicates.
RAD57 (Figure 3.4C). In the embryo, expression of the RCR gene RAD51 decreased (Figure 3.4F). The expression of APEIRef-1, a BER gene, increased in the embryo following 24 hr culture with 4-OOHCPA (Figure 3.4D, asterisk), but only to ~1% of vimentin expression.

Following culture with 4-OOHCPA for 44 hr, all the BER genes in the yolk sac and embryo decreased, most to levels below the limit of detection (Figures 3.4A, D); APNG levels in the yolk sac decreased, but below the two-fold threshold. In the embryo, the expression of three of the four MMR genes (Figure 3.4E) and five of the seven RCR genes decreased (Figure 3.4F); the decreases in gene transcript levels were mostly down to levels below the limit of detection.
Figure 3.4. Changes in DNA repair gene expression after exposure to 10μM 4-OHCPA compared to control: 24hr culture (black bars) or 44hr culture (grey bars). (A, D) Base excision repair; (B, E) mismatch repair; (C, F) recombination repair. Data are expressed as a ratio of expression (four to seven replicates) in the presence of 4-OHCPA compared to culture alone. Differences in expression are at least two-fold; a bar at 0% indicates a change in expression that was less than two-fold. A two-fold change is equivalent to an increase by 100% or a decrease by 50%; a decrease of 100% (-100%) corresponds to a decrease to levels below the limit of detection following 4-OHCPA exposure. *, increase in expression to just above detection limit cut-off.
3.5 Discussion

Understanding the genotoxic and mutagenic effects of teratogens is a necessity in elucidating their mechanism of action. Bishop et al. (1997) concluded that 25% of the known human teratogens are mutagenic. Although this data set is small due to the stringent criteria established for the identification of human teratogens (Shepard, 1998), this observation argues for a distinct role for DNA repair in counteracting the action of teratogens. This is the first study to examine the expression profile of multiple BER, MMR, and RCR genes simultaneously in the same embryonic tissue during development, yielding a more global view of the potential capability for DNA repair in the conceptus. Interestingly, transcript concentrations for genes in the same pathway differed dramatically, suggesting specific requirements for certain DNA repair enzymes during development. While alterations in gene expression do not necessarily imply that there is a change in DNA repair capability, this is one of the key mechanisms by which DNA repair activity can be modified. During organogenesis there were also widely varying transcript concentrations for members of the NER pathway (Vinson and Hales, 2001a); in particular, transcript levels for a number of the NER pathway genes were both tissue-specific and age-dependant. There may be different requirements for members of each pathway in the conceptus. In addition, many DNA repair enzymes function in other DNA metabolism pathways that may be crucial during development.

Three patterns of gene expression were seen in all three DNA repair pathways: age-independent low expression, age-independent high expression, and age-dependent increased expression; the latter pattern was found
predominantly in the embryo proper. A number of DNA repair genes were not expressed on GD10 in the conceptus; these genes were the same in both tissues. As well, the genes that were strongly expressed were identical in both tissues, indicating a tight association between yolk sac and embryo proper with respect to the regulation of expression of DNA repair genes. Finally, the age-dependent increases in expression on GD12 occurred in the embryo in almost all genes in all three pathways examined. This raises the possibility that there is an increased requirement for DNA repair on GD12 in the embryo; this was suggested previously for the NER pathway (Vinson and Hales, 2001a) and would coincide with major changes of oxidative metabolism. Alternatively, the differential induction of gene expression of DNA repair pathway genes may be due to their use in other cellular pathways.

**Base excision repair during organogenesis**

The BER family is made up of a collection of single enzymes that each recognize specific, small DNA base lesions. BER is initiated by structure-specific glycosylases that remove the damaged base, leaving an abasic site. Following the initial removal of the damaged base, incision of the sugar backbone occurs by an apurinic/apyrimidinic (AP) endonuclease (Matsumoto *et al.*, 1999; Pascucci *et al.*, 1999). Of the glycosylases examined, APNG was highly expressed. APNG null mutant mice exhibited a mild phenotype, but were more susceptible to alkylating agents such as methylmethane sulfonate (MMS) (*Elder et al.*, 1998), \(N,N'\)-bis(2-chloroethyl)-\(N\)-nitrosourea (BCNU), and mitomycin C (*Engelward et al.*, 1996), all of which are teratogenic (*Thompson *et al.*, 1974; *Bochert et al.*, 1974).
1978; Nagao et al., 2000). As well, APNG removes hypoxanthine, which is created naturally from deamination of adenine, from DNA (Saparbaev and Laval, 1994). Therefore, the high levels of APNG in the conceptus may enable the embryo to cope with genotoxic stress from alkylating agents and from normal cellular metabolism during organogenesis.

The major mammalian AP endonuclease involved in the second phase of BER is APE/Ref-1 (reviewed in Evans et al., 2000). APE/Ref-1 is involved also in other cellular pathways, most notably redox regulation of the transcription factors AP-1 (Xanthoudakis and Curran, 1992) and p53 (Jayaraman et al., 1997). APE/Ref-1 is involved in regulating the response of the conceptus to oxidative stress (Ozoliņš and Hales, 1999). The highly proliferative status of the embryo would argue for a role for APE/Ref-1 during development. In fact, APE/Ref-1 is essential for embryonic development, as null mutant mice embryos die around the time of organogenesis (Xanthoudakis et al., 1996; Ludwig et al., 1998); yeast strains lacking the homologous APN1 gene are hypersensitive to both oxidative stress as well as alkylating agents (Ramotar et al., 1991). High levels of APE/Ref-1 mRNA were found throughout the rat embryo on GD14 and 17 using in situ hybridization (Wilson et al., 1996). APE/Ref-1 expression has been linked also to the proliferative status of cells in the developing mouse brain (Ono et al., 1995), while oxidative stress can increase APE/Ref-1 transcript levels (reviewed in Evans et al., 2000). Therefore, increased APE/Ref-1 expression in the embryo on GD12 may be due to its role in protecting proliferative cells from oxidative, and potentially genotoxic, damage.
The BER components required for replacing the damaged base and ligating the DNA, *LigIII*, *PolB*, and *XRCC1* (Memisoglu and Samson, 2000), were expressed at low levels in the yolk sac, as well as in the embryo until GD12. In fact, *LigIII* was the only gene that showed a decrease in expression, between GD10 and 12 in the yolk sac; this may indicate a diminished capability to complete BER during this period of development.

**Mismatch repair during organogenesis**

The MMR pathway is responsible for removing mismatches that occur during replication and recombination, as well as mismatches arising from the spontaneous deamination of 5-methylcytosine (Modrich, 1991). Both types of mismatches can occur with greater frequency in rapidly proliferating cells, arguing for the necessity of MMR during embryonic development. Of the MMR genes studied, only *PMS1* was expressed at high levels in the conceptus throughout organogenesis. The role for PMS1 in MMR is still not fully understood, although its necessity for maintaining genome stability is evidenced by the higher rate of mutations in null mutant animals lacking *PMS1* (Prolla et al., 1998). As well, the increase in expression of other MMR genes examined in the embryo on GD12 suggests increased repair ability at this time point.

The MMR pathway interacts with other DNA repair pathways. The interaction of MMR proteins with components of the NER pathway (Mellon et al., 1996) argues for a role in protecting cells from other forms of genotoxic stress. The NER pathway is a major DNA repair pathway in mammalian organisms (reviewed in Sancar, 1996), and is involved in repairing lesions caused by...
cyclophosphamide. In addition, the MMR pathway has been implicated in repair of specific forms of alkylation damage. A complex of MSH2 and MSH6 can bind to lesions caused by the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in vitro (Ceccotti et al., 1996; Duckett et al., 1996); these lesions are normally repaired by the BER enzyme $\text{O}^6$-methylguanine DNA methyltransferase (MGMT) (Kawate et al., 1995). MNNG is a known teratogen (Inouye and Murakami, 1978). MSH2 and MSH6 are implicated also in the induction of apoptosis by alkylating agents (Hickman and Samson, 1999); increased apoptosis may result in abnormal tissue growth and patterning during development. Transcript levels for both MGMT and MSH2 were low in the conceptus between GD10 and 12; this argues for enhanced susceptibility of the conceptus to alkylating agents during mid-organogenesis.

Recombination repair during organogenesis

The mammalian RCR pathway is still largely unresolved. Mammalian RCR genes are homologues of yeast genes, which are themselves known by their functional similarity to bacterial proteins such as RecA (reviewed in Morrison and Takeda, 2000). The involvement of RCR genes with proper chromosome metabolism has been demonstrated by the range of effects produced by deficiencies in RAD genes (Morrison and Takeda, 2000). The predominant role of the RAD gene family is in homologous recombination, repairing double strand breaks. The role of RAD54, the RCR gene with the highest expression in this study, is suggested by the increased sensitivity to ionizing radiation, mitomycin C, and MMS in null mutant animals lacking this gene (Essers et al., 1997). The high
levels of transcripts of *RAD54* observed on GDs 10-12 may indicate a protective role in the conceptus.

**Effect of alkylation damage during development**

Cyclophosphamide is a well-studied chemotherapeutic agent and genotoxic teratogen (Mirkes, 1985); DNA damaged induced by cyclophosphamide is repaired by members of multiple DNA repair pathways. DNA crosslinks (ICLs) are believed to be the most cytotoxic lesions created by bifunctional alkylating agents (Murnane and Byfield, 1981); therefore, the ability to repair these lesions may be an indicator of susceptibility to 4-OOHCPA. Recent evidence suggests that repair of ICLs occur through an intermediate involving DSBs (McHugh *et al.*, 2000), which are repaired by the RCR pathway in mammalian cells (De Silva *et al.*, 2000). As well, the role for recombination repair appears to coincide with cell division in *S. cerevisiae* (McHugh *et al.*, 2000); if this is found in mammalian cells, then the RCR pathway is critical for repair of these lesions during development.

The results from this study show that there is no induction of DNA repair genes following exposure to 4-OOHCPA. Conversely, expression of the majority of DNA repair genes examined decreased to levels below the limit of detection following exposure to 4-OOHCPA; the decreased expression of repair genes may lead to the exacerbation of the teratogenicity of 4-OOHCPA. The inability of the organogenesis-stage conceptus to increase DNA repair gene expression following genotoxic teratogen exposure may indicate a lack of capacity to respond to the insult. The differential effect of 4-OOHCPA treatment in yolk sac
versus embryo proper may have an effect on tissue sensitivity. While the time
course of the depression of gene transcript levels may suggest a non-specific
toxic effect of 4-OOHCPA, this decrease is not universal, as genes from other
DNA repair families did not decrease in expression following 4-OOHCPA
exposure (Vinson and Hales, 2001a). Decreased DNA repair gene expression
following exposure to nitrogen mustard has been described previously (Yuan R.
et al., 1999a). Nitrogen mustard decreases also DNA binding of various
transcription factors (Fabbri et al., 1993; Chen X.M. et al., 1999a,b). Together,
these varied mechanisms of action of nitrogen mustards may account for the
specific decreases in DNA repair gene expression observed in this study.

Genes involved in downstream responses to DNA damage, such as p53,
are a determinant in the teratogenicity of genotoxicants (Nicol et al., 1995;
Moallem and Hales, 1998). As these genes are required for successful cellular
proliferation, it can be assumed that genes encoding DNA repair enzymes are
required also when faced with genotoxic stress. We hypothesize that, as with
tumour sensitivity to genotoxic agents (Dabholkar et al., 1994; Boland, 1996; Li
et al., 1998; Khanna and Jackson, 2001), there is a link between the expression of
DNA repair genes and developmental outcome after exposure to a genotoxic
agent. The expression of DNA repair enzymes may make the difference between
embryonic survival and death following genotoxic insult during development.
3.6 **Acknowledgements**

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The previous work elucidated the expression profiles of three specific DNA repair pathways during organogenesis in the rat conceptus. We wished to further examine the possibility of alterations in gene transcript levels following teratogen exposure using another agent, MTX, whose genotoxic lesions are repaired by the BER enzyme UNG.
CHAPTER FOUR

Expression and Activity of the DNA Repair Enzyme Uracil DNA Glycosylase During Organogenesis in the Rat Conceptus and Following Methotrexate Exposure In Vitro

Robert K. Vinson and Barbara F. Hales
4.1 Abstract

The RNA base uracil is incorporated into DNA following conditions that limit thymidine biosynthesis, and is removed from DNA by two isoforms of uracil DNA glycosylase (UNG), UNG1 (mitochondrial) and UNG2 (nuclear). We hypothesize that UNG is important in protecting the mid-organogenesis stage (gestational day (GD) 10-12) conceptus against conditions that limit nucleotide pools during organogenesis. Transcripts for both UNG isoforms were highly expressed in rat yolk sac and embryo; a greater than 400% increase in the transcripts for both isoforms was found exclusively in the embryo between GD11 and 12. GD10 and 11 yolk sacs showed the highest levels of putatively active UNG2 protein, with little UNG1; the yolk sac had diminished active UNG2 on GD12. The embryo expressed moderate levels of both active UNG2 and UNG1 on GDs 10, 11 and 12. Unlike the transcript profiles, there was no significant increase in either UNG protein isoform in the embryo on GD12. UNG activity was higher in yolk sacs than embryos on GDs 10 and 11, and mirrored protein levels in both tissues. Exposure to methotrexate (MTX), a potent dihydrofolate reductase inhibitor, results in the depletion of cellular thymidine pools, causing uracil incorporation into DNA and cell death due to genotoxic stress. Dose-dependent decreases in developmental growth parameters were observed in embryos exposed to MTX (0.5, 2.5, or 5μM) for 24 or 44hr. Decreased yolk sac vasculature and increased malformations, such as kinked tail, retarded limb development, blebs, and eye defects, were observed after exposure to MTX. UNG transcripts were elevated 30-40% in both yolk sac and embryo samples.
after culture for 6hr with 0.5μM MTX; UNG protein concentrations and activity were not affected. Thus, UNG expression was developmental stage- and tissue-specific; interestingly, transcript and protein levels did not match, indicating post-translational regulation of UNG during development. Importantly, nucleotide depletion in the conceptus due MTX exposure caused malformations but did not modify UNG protein expression or activity, indicating an inability to activate this final defence against MTX-induced genotoxic stress.
4.2 Introduction

The ability to remove misincorporated nucleotides is crucial for ensuring genomic integrity, particularly during development due to the specific program and timing of gene expression required for proper embryonic growth. Genotoxic stress has the ability to drastically alter cellular function and viability. Both dietary folic acid deficiency and exposure to methotrexate (MTX) induce genotoxic damage and malformations. Folic acid deficiency during pregnancy results in neural tube defects (NTDs), congenital heart defects, renal and urinary tract defects, limb malformations, and facial clefts (palate and lip) in the newborn (Hall and Solehdin, 1998). NTDs, such as spina bifida and anencephaly, occur with a frequency of one per 1000 births in the United States, and are among the most common forms of birth defects (Campbell et al., 1986) and causes of perinatal mortality observed (Hall and Solehdin, 1998); in the United Kingdom, 15% of perinatal deaths are attributed to NTDs (Copp et al., 1990). MTX, a structural analog of folic acid, causes malformations that are similar to those induced by folic acid deficiency. The teratogenic effects of MTX have been reported in rats (Berry, 1971), mice (Darab et al., 1987), rabbits (Jordan et al., 1977), cats (Khera, 1976), and humans (Milunsky et al., 1968; Warkany, 1978).

Both folic acid deficiency and MTX alter the biosynthesis of thymidine, a necessary component of DNA. Folic acid is an essential cofactor for the enzyme dihydrofolate reductase (Gready, 1980). Reducing folic acid intake in vivo decreases thymidine levels and increases uracil incorporation into DNA (Pogribny et al., 1997; Duthie et al., 2000). Inhibition of dihydrofolate reductase is the main mechanism of MTX-induced teratogenesis (Sutton et al., 1998); MTX lowers
cellular thymidine levels and causes uracil misincorporation into DNA (Goulian et al., 1980a; Blount and Ames, 1994). Incorporation of uracil into DNA leads to inhibition of DNA synthesis, DNA breaks and DNA fragmentation (Lorico et al., 1988), loss of DNA repair capacity (Borchers et al., 1990), and cell death (James et al., 1997; Duthie and Hawdon, 1998).

The DNA base excision repair enzyme uracil DNA glycosylase (UNG) is the principal mammalian enzyme that removes misincorporated uracil from DNA. The mammalian UNG gene encodes two enzyme isoforms via separate promoters: UNG1, which localizes to mitochondria, and UNG2, which is nuclear in humans (Haug et al., 1996; Nilsen et al., 1997) and both mitochondrial and nuclear in mice (Nilsen et al., 2000a). These UNG products make up the majority of uracil excision activity in cells (Slupphaug et al., 1995). Tissue from adult Ung null-mutant mice exhibit levels of uracil in DNA that are elevated over four-times those found in normal cells (Nilsen et al., 2000a). Human UNG1 and UNG2 are both expressed highly in proliferative tissues, such as testis, colon, and thymus (Haug et al., 1998); the expression of rodent isoforms is also correlated with the proliferative status of the tissue (Weng and Sirover, 1993; Nilsen et al., 2000a). UNG activity, but not transcript or protein levels, has been examined late during rat development (Weng and Sirover, 1993), but not during mid-organogenesis (gestational days (GD) 10-12), a critical period during which time a variety of tissues first begin to differentiate into organ systems.

We hypothesize that UNG is important in protecting the embryo against the consequences of limited thymidine levels during organogenesis. The first goal of this study was to characterize the expression and activity of UNG during
mid-organogenesis. Furthermore, we hypothesize that MTX-induced thymidine
deprivation may affect UNG expression. To test these hypotheses we have
characterized *UNG* gene and protein expression, as well as activity, in the rat
conceptus *in vivo* and examined the consequences of MTX exposure *in vitro* on
UNG expression and activity.
4.3 Materials and Methods

Tissue preparation. Timed-pregnant female Sprague-Dawley rats (200-225g) were obtained from Charles River Canada (St. Constant, Québec) and housed in the McIntyre Animal Resource Centre. Food and water were provided ad libitum, and animals were exposed to a 14hr light:10hr dark cycle. All treatments were in accordance with a protocol approved by the Animal Care Committee of McGill University. Gestational day (GD) zero was defined as the morning following mating. On GD10, 11, and 12, uteri were removed and embryo and yolk sac tissues were dissected immediately and either frozen individually in liquid nitrogen for gene expression analysis or pooled for protein analysis, and stored at -80°C.

Embryo culture. The whole embryo culture model removes any confounding maternal effects that may occur following drug exposure. GD10 conceptuses with an intact yolk sac and ectoplacental cone were cultured for 6, 24 or 44hr in the presence of vehicle (sterile water) or in the presence of 0.5, 2.5, or 5μM MTX (Sigma-Aldrich, St. Louis, MO), as previously described (New, 1978). Following culture, embryos were removed and dissected as described above.

Antisense RNA (aRNA) technique. The antisense RNA (aRNA) technique was used to examine UNG gene expression in individual embryos (van Gelder et al., 1990; Taylor et al., 1995), and was performed as previously described (Vinson and Hales, 2001a,b). Total RNA from individual samples of embryo and yolk sac
was extracted, and mRNA from this pool was converted into cDNA by a combination of an oligo(dT) primer attached to the T7 RNA polymerase promoter, reverse transcriptase (Gibco BRL, Burlington, Ontario), S1 nuclease (Gibco BRL), T4 DNA polymerase (Gibco BRL), and Klenow fragment (New England Biolabs, Mississauga, Ontario). Antisense RNA was amplified from the cDNA templates using T7 RNA polymerase (New England Biolabs) and labelled with α\(^{32}\)P-CTP (10mCi/mol; Amersham Pharmacia Biotech, Baie d’Urfé, Québec). Labelled aRNA was hybridized overnight to nylon membranes (Zeta-Probe GT, Bio-Rad, Mississauga, Ontario) previously slot-blotted with equimolar amounts of cDNAs encoding the UNG DNA repair gene isoforms. The membranes were washed in solutions of decreasing stringency, and were exposed overnight to phosphorimager plates. The linearity and reproducibility of this amplification reaction was determined by trichloroacetic acid precipitation of α\(^{32}\)P-CTP incorporated into the acid-insoluble fraction (data not shown).

Quantification and analysis of aRNA data. Images of the blots were obtained using a STORM phosphorimager (Molecular Dynamics, Sunnyvale, CA). Quantification of gene expression was done using ImageQuant 5.0 software (Molecular Dynamics). Data are expressed as the mean % of UNG expression relative to vimentin from the same membrane ± SEM of tissues from five to seven separate GD10-12 samples for in vivo results, or four to five separate samples for culture alone or for MTX treatment, each obtained from separate litters. The data are represented relative to the structural protein gene vimentin, as its expression
did not change more than 2-4% of the total blot intensity between the different timepoints and tissues examined (data not shown).

**Protein extraction.** Embryonic tissue was sonicated on ice (Vibracell, Sonics & Materials, Danbury, CT) in RIPA buffer with a protease inhibitor cocktail, and spun at 10,000rpm for 10min at 4°C; the supernatant was removed for protein analysis. Protein concentrations were determined by the Bio-Rad Protein Assay. Samples were frozen at -80°C until used.

**Western blot analysis.** Protein samples (40μg) were mixed with loading buffer, boiled for 5min, centrifuged briefly, and electrophoresed on a 10% polyacrylamide gel for 2.25hr at 100V. Samples were transferred to Hybond-C Super nitrocellulose membranes (Amersham Pharmacia Biotech) at 130V for 2hr at 4°C. Membranes were dried briefly, washed with TBS plus 0.1% Tween-20 (TBS-T) for 2x5min, then incubated with TBS-T supplemented with 5% non-fat dry milk (5%M-TBS-T) for 1hr to block non-specific antibody binding. Membranes were washed, then incubated with the primary anti-UNG antibody (1:5000 final dilution; a gift from G. Slupphaug) in 5%M-TBS-T overnight at 4°C. Donkey anti-rabbit (1:2500; Amersham Pharmacia Biotech) secondary antibody conjugated to horseradish peroxidase was used to detect specific antibody interactions. Specific antibody binding was visualized with the ECL-Plus Kit (Amersham Pharmacia Biotech) and Hyperfilm ECL (Amersham Pharmacia Biotech). Protein band molecular weight determination was done by visualization of Precision
prestained protein standards and biotinylated SDS-PAGE broad range standards (Bio-Rad) with Streptavidin-HRP conjugate (Amersham Pharmacia Biotech) at 1:5000 incubation for 5min in TBS-T. Adult rat testis and recombinant UNG (New England Biolabs) were used as positive controls. Actin expression was used as a loading control by incubating membranes with anti-actin antibody (1:500; Santa Cruz, Santa Cruz, CA) for 3hr at RT in 5%M-TBS-T.

DNA substrates for UNG assay. Oligonucleotide substrates were synthesized by the Sheldon Biotechnology Centre (McGill University, Montréal, Québec). The sequences of the oligonucleotides were as follows:

30U: 5'-GGATGGCATGCATGATCC(U)GAGGCCGCGCG-biotin-3'
30T: 5'-GGATGGCATGCATGATCC(T)GAGGCCGCGCG-biotin-3'
30compl: 5'-CGCGCGGCCTCAGGATCATGCATGCCATCC-3'.

Double-stranded oligonucleotide substrates (ds30U, ds30T) were prepared by annealing equimolar amounts of 30U and 30compl for ds30U, or 30T and 30compl for ds30T, for 5min at 85°C, and allowing them to cool slowly to RT. Annealing efficiency was determined by electrophoresis on a non-denaturing 20% polyacrylamide gel.

In vitro UNG activity assay. The same tissue extracts used for Western blot analysis (20μg) were incubated with 10ng oligonucleotide substrate (either ds30U or ds30T) in 20mM Tris-HCl pH8.0, 1mM EDTA, 1mM DTT, 0.1mg/ml BSA, and 10mM MgCl₂ in 50μl final volume. The repair reaction was carried out for 30min
at 37°C. Recombinant human APE (10U; used along with recombinant UNG (10U) as a positive control) was from Trevigen (Gaithersburg, MD).

**Denaturing gel electrophoresis.** UNG activity assay products (5µl) were mixed with an equal amount of denaturing loading buffer (8M urea, 20mM EDTA, 5mM Tris-Cl ph7.5), heated for 5min at 90°C, and separated by electrophoresis on a 20% polyacrylamide mini-gel containing 3.9M urea, 22% formamide and 1X TBE buffer. Gels were pre-run at 240V for 1hr, samples added and run at 240V for 50min in 1X TBE buffer. A 10bp ladder (Gibco BRL) was used as a sizing standard for visualization of samples under short-wave UV light following ethidium bromide staining of the gel. The samples were blotted onto Zeta- Probe GT nylon membrane (Bio-Rad) using a Trans-Blot SD semi-dry transfer apparatus (Bio-Rad) running at 210mA (constant) and 7V for 10min in 0.5X TBE buffer. The membrane was baked 30min at 80°C, then samples visualized by Phototope-Star Detection Kit (New England Biolabs), and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech). Quantification of UNG activity was done using a ChemilImager 4000 imaging system (Alpha Innotech, San Leandro, CA) with AlphaEase 3.3b software.

**Statistical analysis.** Statistical analyses were done with SigmaStat version 2.03 Windows PC software (SPSS, Chicago, IL).
4.4 Results

**UNG gene expression in vivo.** Transcripts for both UNG1 (Figure 4.1A) and UNG2 isoforms (Figure 4.1B) were expressed at high levels relative to vimentin in GD10 to 12 embryo and yolk sac samples. Quantification of this data showed a dramatic 400% increase in both transcript levels on GD12, specifically in the embryo.

**UNG protein expression in vivo.** To determine whether changes in the steady state concentrations of the UNG transcripts corresponded to differences in protein levels, the expression of both UNG isoforms was examined by Western blot analysis (Figure 4.2). High levels of the nuclear isoform, UNG2, were observed in the yolk sac. In particular, two specific bands were detected. A higher-MW, phosphorylated species has been observed following Western blot analysis, and is believed to be the active form of the isozyme (G. Slupphaug, personal communication). This band was apparent on GDs 10 and 11 in the yolk sac, but not on GD12. In contrast, the embryo from GD10 to 12 displayed only the higher-MW UNG2 band, at consistently lower levels than yolk sac. Levels of the mitochondrial isoform, UNG1, were moderate in the embryo at all three timepoints; however, little or no UNG1 was observed in yolk sac samples. An additional, uncharacterized band appeared slightly above that for UNG1, which may be a modified form of the enzyme. Adult rat testis, used as a positive control, showed both UNG1 and UNG2 bands, as well as a yet uncharacterized lower MW band; recombinant *E. coli* UNG ran as the expected single band.
Figure 4.1. Quantitative analysis of (A) UNG1 and (B) UNG2 transcript expression profiles in the rat conceptus during organogenesis. UNG gene expression was analyzed from arrays probed with aRNA samples from yolk sac (light grey bars) or embryo (dark grey bars) tissues from GD10 to GD12. Data are expressed as the mean % of vimentin expression +/- SEM of tissue from five to seven separate GD10-12 yolk sac and embryo samples obtained from different litters. *, significantly different from GD10 and 11, p<0.05 ANOVA and Tukey’s test.
Figure 4.2. Expression of UNG1 and UNG2 proteins during organogenesis in vivo by Western blot analysis. UNG2 is seen as two bands (phosphorylated and non-phosphorylated). Rat testis extract and recombinant UNG protein were used as positive controls. Actin was used as a loading control. This experiment is representative of results from three different sets of tissue samples from separate litters. ys, yolk sac; emb, embryo.
**UNG enzyme activity in vivo.** To determine the capacity of the conceptus to remove uracil from DNA, tissue samples were incubated with a double-stranded 30-mer oligonucleotide containing a single uracil residue at position 19 (ds30U); UNG activity causes the substrate DNA band at 30bp to disappear, forming an 11bp fragment due to the removal of uracil by UNG and the subsequent cleavage at the AP site by AP endonuclease. UNG activity assay results are shown in Figure 4.3. At all three developmental timepoints examined (GD10-12), both yolk sac and embryo samples had the ability to excise uracil from DNA. GD10 and 11 yolk sacs had dramatically reduced amounts of the substrate 30-mer oligonucleotide (Figure 4.3A, lanes 3,5, upper band), along with increased intensity of the 11bp fragment (Figure 4.3A, lanes 3,5, lower band), indicating high uracil removal ability. GD12 yolk sac had a much-reduced incision of the substrate (Figure 4.3A, lane 7), and was similar to the GD12 embryo (Figure 4.3A, lane 8). Quantification of the intensity of the 11bp fragment is shown in Figure 4.3B. The high activity found in GD10 and 11 yolk sac samples mirrored the high protein levels found in those tissues by Western blot analysis (Figure 4.2).

Additional bands, between the 30bp full-length substrate and the 11bp cleavage product, were visible in the GD10 and 11 yolk sac samples. Addition of 100μM aurintricarboxylic acid (ATA), an inhibitor of nucleases (Hallick et al., 1977; Gonzalez et al., 1980), to the reaction mixture caused the additional bands to disappear (data not shown), with a concomitant decrease in the 11bp fragment intensity and an increase in the intensity of the 30bp band, indicating that ATA
Figure 4.3. In vitro UNG activity assay. (A) Repair reaction products were separated on a 20% denaturing polyacrylamide gel: lane 1, 2ng ds30U oligonucleotide only; lanes 2-8, aliquots of reaction samples containing 10ng ds30U oligonucleotide and: 10U each recombinant UNG and APE (lane 2); GD10 yolk sac (lane 3) and embryo (lane 4); GD11 yolk sac (lane 5) and embryo (lane 6); and GD12 yolk sac (lane 7) and embryo (lane 8) tissue extracts. (B) Semi-quantitative analysis of assay shown above. Data are represented as the intensity of the 11bp oligonucleotide band. This experiment is representative of results from three different sets of tissue samples from separate litters. ys, yolk sac; emb, embryo.
also inhibits UNG activity. Addition of a specific bacteriophage uracil glycosylase inhibitor peptide (UGI; New England Biolabs) (Wang et al., 1991) did not remove the additional bands, indicating that they are not due to UNG activity (data not shown). The additional bands remained when an oligonucleotide containing a thymidine, instead of a uracil, at position 19 (ds30T) was used in the reaction assay (data not shown). Therefore, the additional bands are not due to UNG activity and are not specific for uracil-containing DNA. Inhibition by ATA suggests that they may be due to other DNA nucleases present in the yolk sac on GDs 10 and 11.

**MTX teratogenicity in vitro.** To assess the *in vitro* teratogenicity of MTX during mid-organogenesis, GD10 embryos were cultured for 24 or 44 hr with 0.5, 2.5, or 5 μM MTX, then examined for normal development by using the Brown-Fabro scoring system (Brown and Fabro, 1981), and by counting somite numbers. Following 24 hr culture, a concentration-dependent decrease in embryonic growth was observed (*Figure 4.4*; 2.5 μM MTX not shown). Decreased yolk sac vasculature and malformations such as kinked tails, retarded limb development, blebs, eye defects, and a general decrease in overall embryo size were observed even at the lowest concentration used (0.5 μM MTX; *Figure 4.4B*) compared to control embryos (*Figure 4.4A*); embryo defects were even more severe at the highest concentration (5 μM MTX; *Figure 4.4C*). The developmental retardation was quantitated both as a decrease in the Brown-Fabro scores (*Figure 4.5A*) and somite numbers (*Figure 4.5B*) with all three doses of MTX. Both
Figure 4.4. Dark-field photographs of embryos with intact yolk sacs (left) or with yolk sacs removed (right) following 24hr in vitro embryo culture with (A) vehicle alone, (B) 0.5μM, or (C) 5μM MTX. All pictures are to the same scale.
Figure 4.5. Quantification of developmental parameters following in vitro culture with vehicle alone, 0.5μM, 2.5μM, or 5μM MTX for (A) Brown-Fabro score and (B) somite number following 24hr culture. Data are expressed as the mean +/- SEM from 11-13 embryos. *, significantly different from control, p<0.05 by ANOVA on Ranks followed by Dunn’s post-hoc test (Brown-Fabro Score) or one-way ANOVA followed by Tukey’s test (somite number).
developmental parameters were further decreased following 44hr culture with MTX (data not shown).

**UNG gene expression following MTX exposure.** The effect of MTX on UNG gene expression was examined following short-term (6hr) culture *in vitro* (Figure 4.6). Low-dose (0.5μM) MTX induced a 30-40% increase in transcripts for both UNG isoforms in both yolk sac and embryo for UNG1, and in the yolk sac for UNG2. Exposure to 5μM MTX had no significant effect on UNG transcript levels.

**UNG protein expression following MTX exposure.** To determine if UNG protein levels increased in parallel with gene expression, conceptuses were cultured with MTX for 6hr and examined for UNG1 and UNG2 protein expression (Figure 4.7). Unlike the *in vivo* results, UNG1 was present in equal amounts in the yolk sac and embryo of cultured embryos. As for the *in vivo* results, both non-phosphorylated and phosphorylated forms of UNG2 were found, at equivalent levels in the yolk sac in control samples, while the embryo expressed only the active, phosphorylated UNG2 form; UNG2 protein concentration did not change following MTX exposure. After MTX exposure for 24hr (data not shown) similar levels for both UNG isozymes were found, compared to 6hr exposure in either yolk sac or embryo.

**UNG enzyme activity following short-term culture with MTX.** UNG activity following 6hr culture with MTX are shown in Figure 4.8.
Figure 4.6. Expression of *UNG* transcripts for (A) UNG1 or (B) UNG2 following 6hr culture with vehicle alone, 0.5μM, or 5μM MTX. *UNG* gene expression was analyzed from arrays probed with aRNA samples from yolk sac (light grey bars) or embryo (dark grey bars). Data are represented as the mean % of vimentin expression +/- SEM of tissue from four to five separate yolk sac and embryo samples obtained from different litters. *, significantly different from control, p<0.05 Student's t-test with Bonferroni correction.
Figure 4.7. Expression of both UNG protein isoforms by Western blot analysis following 6hr culture with either 0.5µM or 5µM MTX. Rat testis extract and recombinant UNG protein were used as positive controls. Actin was used as a loading control. This experiment is representative of results from three different sets of tissue samples from separate litters. ys, yolk sac; emb, embryo.
embryos had slightly higher activity levels than MTX-exposed tissues (Figure 4.8A; lane 3). In addition, all three yolk sac samples showed additional bands between the substrate and cleaved oligonucleotide product. Both yolk sac and embryo proper, exposed to either dose of MTX, had similar activity levels. Quantification of UNG activity is shown in Figure 4.8B.
Figure 4.8. UNG activity assay following 6hr culture of GD10 conceptuses with MTX. (A) Repair reaction products were separated on a 20% denaturing polyacrylamide gel: lane 1, 2ng ds30U oligonucleotide only; lanes 2-8, aliquots of reaction samples containing 10ng ds30U oligonucleotide and: 10U each recombinant UNG and APE (lane 2); tissue extracts from yolk sac (lane 3) and embryo (lane 4) cultured with vehicle alone; tissue extracts from yolk sac (lane 5) and embryo (lane 6) cultured with 0.5μM MTX; and tissue extracts from yolk sac (lane 7) and embryo (lane 8) tissue extracts cultured with 5μM MTX. (B) Semi-quantitative analysis of assay shown above. Data are represented as the intensity of the 11bp oligonucleotide band. This experiment is representative of results from three different sets of tissue samples from separate litters. ys, yolk sac; emb, embryo.
4.5 Discussion

This study provides evidence of differential expression and activity of the major uracil removing glycosylases during mid-organogenesis in the rat conceptus. Steady state concentrations of the transcripts for both UNG isoforms were high in the conceptus, in agreement with a previous study which showed that both UNG isoforms were expressed in murine embryos during mid-gestation (Nilsen et al., 2000a); these data suggest an increased need to remove uracil from DNA during this period of development. In fact, transcripts for UNG1 and UNG2 are among the highest-expressed DNA repair transcripts from any repair pathway in the rat conceptus during this period of development (Vinson and Hales, 2001a,b). The high levels of expression of UNG during development may indicate the importance of this pathway as a compensatory mechanism to ensure that genetic integrity is maintained during a highly proliferative state.

Interestingly, while UNG transcript levels increased on GD12, protein levels did not parallel this increase. As well, while UNG transcripts were expressed at similar levels, there were distinct differences in the protein concentrations of the two UNG isozymes. In particular, concentrations of nuclear UNG2 were higher than those of the mitochondrial isozyme. As well, there was much greater expression of UNG2 in the yolk sac; the UNG2 apparent in the embryo was exclusively of the higher-MW, putatively phosphorylated variety, which is hypothesized to be the active form of the enzyme. Conversely, UNG1 was expressed at higher levels in the embryo than the yolk sac. Why the yolk sac would require high levels of UNG2, and not UNG1, during development is
unknown; perhaps thymidine pools are lower in yolk sac cells, thereby requiring a
greater ability to remove misincorporated nucleotides in nuclear DNA.

Further differences between yolk sac and embryo proper with respect to
UNG protein levels were found. Higher-MW protein bands were observed via
Western blot analysis at all three developmental time points examined in vivo
(data not shown). These high-MW species have been hypothesized to represent
ubiquitinated UNG (G. Slupphaug, personal communication). Bands for specific
ubiquitinated protein products overlapped those of the higher-MW UNG products
(data not shown), strengthening this hypothesis. While the precise role of
ubiquitination of UNG is unknown, ubiquitination is the major method of targeting
proteins for degradation, and altering protein turnover rate (reviewed in
Wilkinson, 2000). Higher protein turnover rates may help to explain the lack of
correspondence between transcripts (high levels, particularly on GD12 in the
embryo) and protein (low levels). Nilsen et al. (2000) proposed that UNG
expression during development is modulated by transcription altering factors,
such as MZF-1, which are present during development. Both UNG (Vilpo, 1988)
and MZF-1 (Hromas et al., 1991) are highly expressed in haematopoietic cells; as
the yolk sac is the main site of haematopoiesis at this time during development
(reviewed in Palis and Yoder, 2001), the high concentrations of UNG2 in this
tissue may be in haematopoietic cells within this tissue.

Maximal UNG activity was observed previously around birth in the rat
(Weng and Sirover, 1993), although the only pre-natal time point examined in this
study was four days prior to birth. Nevertheless, several organs that were
examined demonstrated the highest levels of UNG activity before birth, with
activity correlated with the proliferative status of the organ. Another study examining uracil removal activity during neuronal development also demonstrated developmental regulation (Focher et al., 1990). The activity profile found in this study suggests that the yolk sac predominantly has the nuclear form, although the disappearance of the higher-MW protein band on GD12, coupled with the decrease in activity at the same time point, argues for decreased ability to remove uracil on GD12 in this tissue. Why UNG protein levels and activity decrease at this time point remains uncertain.

Folic acid deficiency has been implicated in the defects induced by a variety of teratogenic exposures, varying from heat shock (Shin and Shiota, 1999), to methanol exposure (Sakanashi et al., 1996), or arsenic exposure (Ruan et al., 2000). The importance of folic acid during development is due to its absolute requirement for the production of DNA nucleotide precursors in all eukaryotic cells; failure to synthesize adequate levels of dTTP results in uracil incorporation into DNA, which can lead to cell death. The limitation of thymidine biosynthesis during development is a well-known teratogenic condition. This is the first study to examine the regulation of UNG during rat embryo organogenesis in vivo and in vitro following MTX exposure. We have found that culture with MTX induces time- and dose-dependent malformations in vitro. Many of the malformations observed in vitro are similar to those found after whole-animal dosing, validating the culture system for examining MTX and the biochemical pathways leading to MTX-induced malformations. Interestingly, while the rat conceptus modulates UNG transcript levels following low-dose MTX, this alteration in transcript levels does not correspond to alterations in protein levels.
or UNG activity.

The presence of high levels of UNG during organogenesis may lead to exacerbation of the genotoxic effects of MTX. A futile cycle of excision of uracil from DNA by UNG, and reincorporation of dUTP due to lack of dTTP, leads to DNA strand breaks and cell death (Goulian et al., 1980a,b). The loss of cell viability following decreases in thymidine levels is termed "thymidineless death" (Seno et al., 1985), and may contribute to the teratogenicity of MTX. In particular, high levels of UNG in the yolk sac may lead to futile repair and further genomic instability. Of note is the fact that embryos cultured with MTX had much less vasculature and blood cells in the yolk sac; perhaps these cells were killed via thymidineless death.

Differences between mitochondrial and nuclear UNG expression following MTX exposure may play a role in the aetiology of MTX-induced malformations. Although several teratogens have been shown to interact with mitochondria via inhibition of energy and respiration pathways (Mackler et al., 1975; Ranganathan et al., 1989) and induction of oxidative stress (Liu and Wells, 1994), none have been shown to induce mitochondrial DNA damage in the conceptus, with the possible exception of diethylstilbestrol (Beyer et al., 1987; Thomas and Roy, 1995). Examining the relative amounts of uracil incorporated into mitochondrial versus nuclear DNA would help determine whether differential genotoxic effects occur following MTX exposure, and whether this effect is related to specific malformations induced by MTX or folic acid deficiency.

The results from this study indicate that the conceptus has a very limited ability to modify the UNG DNA repair pathway in response to MTX; this may
indicate susceptibility during this critical window of development to conditions that
limit thymidine pools. While there is a genetic component for a small proportion
of NTDs (Papapetrou et al., 1996; Shaw et al., 1998; Barber et al., 1999), studies
demonstrate that neither single mutations in critical genes of the folate metabolic
pathway nor decreased activity of these enzymes account for the majority of
NTDs in infants born to folate-deficient mothers. Therefore, an understanding of
the downstream pathways that utilize folate one-carbon pathway products may
shed light on the aetiology of NTDs. We suggest that the inability of the embryo
to respond to genotoxic stress during development is critical in mediating the
malformations that are induced following folic acid deficiency and MTX exposure.
4.6 **Acknowledgements**

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The ability of the conceptus to modulate the expression of genes involved in direct repair of genotoxic stress has been examined in the previous three Chapters. Another important factor that determines the effect of a genotoxic agent are the genotoxic stress response pathways that activate downstream repair pathways. Members of the PI3K family are involved in these genotoxic stress response pathways. We therefore examined whether these pathways are present in the conceptus, and whether they respond to oxidative stress and alkylation damage, their primary triggers. One method of inducing transient oxidative stress is by placing embryos in culture; the expression of specific transcription factors responsive to oxidative stress are induced following the onset of culture (Ozoliņš and Hales, 1997). Therefore, we examined whether embryo culture could alter genes involved in the genotoxic stress response following oxidative and alkylation stress via culture with 4-OOHCPA.
CHAPTER FIVE

Genotoxic Stress Response Gene Expression in the Mid-Organogenesis Rat Conceptus

Robert K. Vinson and Barbara F. Hales
5.1 Abstract

Genotoxic stress response pathways alert the cell to DNA damage, eliciting cell cycle arrest, DNA repair, and apoptosis. The ability of the conceptus to sense genotoxic stress may be critical for normal development and may influence the outcome of exposure to genotoxic teratogens. Members of the phosphatidylinositol 3-kinase (PI3K) superfamily are involved in controlling cell cycle activity and maintaining genomic stability. The goal of this study was to determine the expression profile of PI3K family members, ATM, ATR, and the catalytic subunit of DNA-PK (DNA-PKcs), and of downstream genes, p53, GADD45, and p21, in the mid-organogenesis stage rat conceptus (gestational day (GD) 10-12) in vivo and following exposure to genotoxic stress in vitro. ATM was the highest expressed PI3K family member in both yolk sac and embryo proper, with transcript levels increasing ~4-fold in the embryo from GD10 to GD12. Transcript levels for ATR, DNA-PKcs, and downstream genes were low in both tissues, with all genes having increased transcript levels exclusively in the GD12 embryo.

Culture of embryos in vitro induces transient oxidative stress. While short term culture had no effect on transcript levels in either tissue, culture for 24 or 44hr significantly decreased ATM transcript levels in both embryo and yolk sac; downstream genes were unaffected. To determine whether the conceptus can react to alkylation stress embryos were cultured with 10μM 4-hydroperoxycyclophosphamide (4-OOHCPA), an activated form of the
teratogenic nitrogen mustard cyclophosphamide (CPA). Transcript levels for all genes examined were unaltered following culture with 4-OOHCPA. Therefore, while transcripts for genotoxic stress response genes are found in the mid-organogenesis-stage rat conceptus, the expression of these genes is not regulated by transient oxidative stress or nitrogen mustard exposure. The inability of the conceptus to regulate these transcripts in response to insult may indicate increased susceptibility to stressors during organogenesis.
5.2 Introduction

The ability to sense and signal within the cell that DNA damage has occurred is one of the most important cellular defences against genotoxic agents. DNA damage sensors activate downstream cellular targets that delay the cell cycle, leading to checkpoint arrest (reviewed in: Mercer, 1998; Lowndes and Murguia, 2000), modify DNA repair activity, and activate the apoptotic cascade (reviewed in: Durocher and Jackson, 2001; Shiloh, 2001). Members of the phosphatidylinositol 3-kinase (PI3K) superfamily are serine/threonine protein kinases that act as genotoxic stress sensors. This family includes Ataxia-Telangiectasia Mutated (ATM; Savitsky et al., 1995), ATM and Rad3-related (ATR; Cimprich et al., 1996), and the DNA-dependent protein kinase (DNA-PK; Carter et al., 1990; Jackson et al., 1990; Lees-Miller et al., 1990). ATM is activated following ionizing radiation (IR; Canman and Lim, 1998; Rotman and Shiloh, 1999) and oxidative stress (reviewed in Rotman and Shiloh, 1997a), ATR following UV radiation (UVR; Tibbetts et al., 1999) and exposure to alkylating agents such as cisplatin (Damia et al., 2001), and DNA-PK in response to double strand breaks (DSBs; reviewed in Smith and Jackson, 1999). Activation of ATM and ATR leads to the activation of p53 (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999), which, in turn, transcriptionally upregulates p21 and the Growth Arrest and DNA Damage Inducible gene 45 (GADD45; Fornace et al., 1989a; Levine, 1997; Wang et al., 1999). GADD45 triggers G₂/M checkpoint arrest (Wang et al., 1999; Zhan et al., 1999), while activation of p21 leads to G₁/S checkpoint arrest (reviewed in Sherr and Roberts, 1999), and can activate also the apoptotic machinery (reviewed in Dotto, 2000). DNA-PK, composed of a
catalytic subunit, DNA-PKcs and the Ku70/80 heterodimer (reviewed in Smith and Jackson, 1999), binds to DNA DSBs and activates the apoptotic machinery (Wang et al., 2000).

Both oxidative stress (reviewed in Wells et al., 1997) and alkylating agents are teratogenic (reviewed in Glantz, 1994), inducing specific malformations during susceptible stages of development. Model systems to examine these teratogens have utilized the embryo culture system. Transient oxidative stress is induced within 30min following the initiation of culture (Ozoliņš and Hales, 1997). Exposing conceptuses to the nitrogen mustard CPA is used to model alkylating teratogen action (reviewed in Mirkes, 1985). The rat conceptus is most sensitive to the effects of genotoxic teratogens during mid-organogenesis, gestational days (GD) 10-12 (Platzek et al., 1982; Jirakulsomchok and Yielding, 1984; Little and Mirkes, 1987; Liu and Wells, 1995). Specific birth defects seen following teratogen exposure may be due to impaired responses to DNA damage. Previously, we examined the consequences of exposure of the mid-organogenesis conceptus to 4-OOHCPA, an activated analog of CPA (Slott and Hales, 1988), on the gene expression profiles of the major DNA repair pathways (Vinson and Hales, 2001a,b); however, little is known about the expression of DNA damage sensor genes in the conceptus. The present study was undertaken to elucidate the expression of these genes during mid-organogenesis in the rat conceptus, and determine whether exposure to transient oxidative stress or to 4-OOHCPA alters the expression profile of these genes.
5.3 **Materials and Methods**

**Tissue preparation.** Timed-pregnant female Sprague-Dawley rats (225-250g) were obtained from Charles River Canada (St. Constant, Québec) and housed in the McIntyre Animal Resource Centre (McGill University). Rat chow (Purina, St. Louis, MO) and water were provided *ad libitum*, and animals were exposed to a 14hr light: 10hr dark cycle. All treatments were in accordance with a protocol approved by the Animal Care Committee of McGill University. Gestational day (GD) zero was defined as the morning following mating. On GD10, 11, and 12, uteri were removed and embryo and yolk sac tissues were dissected immediately, frozen individually in liquid nitrogen, and stored at -80°C.

**Embryo culture.** Rat conceptuses were explanted from timed-pregnant dams on GD10 and cultured using established techniques (New, 1978). The whole embryo culture model removes any confounding maternal effects that may occur following drug exposure. For short-term oxidative stress studies, conceptuses with an intact yolk sac and ectoplacental cone were cultured for 0.5, 1.5, 3, and 6hr at 37°C in 90% heat-inactivated rat serum supplemented with penicillin and streptomycin. For 4-OOHCPA studies, conceptuses were cultured as above in the presence of vehicle (sterile water) or 10μM 4-OOHCPA (a gift from M. Colvin) for either 24 or 44hr. In culture, 4-OOHCPA breaks down spontaneously in solution to its active metabolites (Low *et al.*, 1982; Slott and Hales, 1988), phosphoramide mustard and acrolein. Following culture, embryos were removed and dissected as described above.
**Antisense RNA (aRNA) technique.** The aRNA technique was used to examine the DNA repair gene expression profile on a per embryo basis, as previously described (Eberwine et al., 1992; Vinson and Hales, 2001a). This technique is essentially a “reverse Northern blot”, allowing for the simultaneous examination of multiple gene transcripts from a single tissue sample (van Gelder et al., 1990; Taylor et al., 1995). Individual embryos and yolk sacs were sonicated on ice in lysis buffer (1mg/ml digitonin, 5mM DTT, 50mM Tris pH8.3, 6mM MgCl2, 0.12mM KCl), and reverse-transcribed for 2hr at 37°C in the presence of an oligo(dT) primer attached to a T7 RNA polymerase promoter, which recognises and binds to the poly(A) tail of mRNA in the tissue sample. Self-priming allowed the formation of double-stranded cDNA by a combination of T4 DNA polymerase (Gibco BRL, Burlington, Ontario) and Klenow fragment (New England Biolabs, Mississauga, Ontario). Antisense RNA was created from the cDNA pool by T7 RNA polymerase (New England Biolabs) and simultaneously radiolabelled with $\alpha^{32}$P-CTP (10 mCi/mol; Amersham Pharmacia Biotech, Baie d’Urfé, Québec) for 4hr at 37°C. The linearity and reproducibility of this amplification reaction were determined by trichloroacetic acid precipitation of $\alpha^{32}$P-CTP incorporated into the acid-insoluble fraction (data not shown).

**Gene array membranes and hybridization.** To examine the expression of multiple genes using a single tissue sample, gene expression arrays were created. In particular, the expression of six $ATM$ pathway and family member genes was examined. These were: Ataxia-Telangiectasia Mutated ($ATM$), Ataxia-
Telangiectasia Related (ATR), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), growth arrest and DNA damage inducible gene 45 (GADD45), p21, and p53. To create these arrays, nylon membranes (Zeta-Probe GT, Bio-Rad, Mississauga, Ontario) were slot-blotted (Bio-Dot SF, Bio-Rad) with equimolar amounts of cDNAs, as per the manufacturer's protocol. Wherever possible, rodent cDNA probes were used. Non-rodent probes were chosen based on their high homology to rodent cDNAs for the genes of interest. Gene expression arrays were pre-hybridized for 30 min in hybridization buffer (7% SDS, 0.12M Na$_2$HPO$_4$ pH7.2, 0.25M NaCl, 50% formamide). Heat denatured radiolabelled aRNA probes were hybridized overnight to arrays at 42°C.

Following hybridization, the arrays were washed in solutions of decreasing stringency (from 2X SSC/0.1% SDS to 0.1X SSC/0.1% SDS) at 42°C for 20 min each, and exposed to phosphorimager plates overnight. Arrays were stripped of probe by boiling in 0.1X SSC/0.5% SDS twice for 20 min each. Stripping efficiency was determined by exposing membranes to phosphorimager plates overnight. Arrays were reused a maximum of five times, at which point no appreciable degradation in signal was observed (data not shown).

Quantification and analysis of aRNA data. Images of the gene expression arrays were obtained using a STORM phosphorimager (Molecular Dynamics, Sunnyvale, CA), and gene expression quantified using ImageQuant 5.0 software for Windows NT (Molecular Dynamics). Gene expression intensity values on each membrane were normalized relative to the expression of vimentin, a structural protein; vimentin was chosen because its expression remained
constant between the different time points and tissues. The intensity value from pUC18 plasmid DNA blotted onto each array (non-specific hybridization) was also subtracted from each gene intensity value. In order to ensure the consistency and reliability of the data (Lee et al., 2000), several replicates for each tissue and treatment were performed, each from separate litters. Data are from four separate GD10-12 yolk sac and embryo samples for in vivo data, from three to six separate samples for culture alone data, and from three samples for culture with 4-OOHCPA data, each obtained from separate litters.

Statistical analysis. Statistical analysis was done on an individual gene basis with SigmaStat version 2.03 Windows PC software (SPSS, Chicago, IL).
5.4 Results

Expression of PI3K superfamily and downstream stress-response genes during organogenesis in vivo. We first determined the expression pattern of PI3K family members ATM, ATR, and DNA-PKcs in rat yolk sac and embryo proper on GDs 10, 11, and 12 (Figure 5.1A-C). ATM was the highest expressed PI3K family gene examined in both tissues at all timepoints (Figure 5.1A). ATM expression was similar in both tissues on GD10 and GD11; a dramatic ~4-fold increase in the steady state concentrations of ATM transcripts occurred between GD11 and 12, exclusively in the embryo. ATR and DNA-PKcs were expressed at low levels in the yolk sac between GDs 10-12 (Figure 5.1B,C). The embryo proper displayed transcript levels near the limit of detection for ATR and DNA-PKcs on GD10, with higher transcript levels for both genes observed on GD11 and 12 (Figure 5.1B,C).

The expression profiles for downstream genes p53, GADD45, and p21 are shown in Figure 5.1D-F. The expression of all three genes was low in both tissues. Transcript levels did not change significantly during organogenesis in the yolk sac; in the embryo, GADD45 and p21 transcript levels mirrored the increase seen for ATM, increasing on GD12 compared to GD10 (for GADD45; Figure 5.1E) or compared to GD10 and 11 (for p21; Figure 5.1F).

Effect of embryo culture on PI3K superfamily and downstream stress-response gene expression. Embryo culture induces short-term oxidative stress (Ozoliņš and Hales, 1997). To determine if this oxidative stress affects gene expression, embryos were cultured for 0.5, 1.5, 3, and 6 hr and the expression of
Figure 5.1. PI3K superfamily and downstream stress response gene expression during mid-organogenesis in rat yolk sac (light grey bars) and embryo proper (dark grey bars): ATM (A), ATR (B), DNA-PKcs (C), p53 (D), GADD45 (E), and p21 (F). Values represent the mean transcript concentrations +/- SEM relative to vimentin from four replicates. *, expression significantly different from GD10 or ** both GD10 and GD11 by one-way ANOVA and Tukey Test, p<0.05. †, expression significantly different from yolk sac by Student’s t-test, p<0.05.
members of the PI3K superfamily and downstream genes was determined; the results are shown in Figure 5.2. Short-term culture did not affect ATM (Figure 5.2A), ATR (Figure 5.2B), or DNA-PKcs transcript levels (Figure 5.2C), nor did it alter transcript levels for downstream targets, p53, GADD45, and p21 (Figure 5.2D-F).

Embryos are cultured frequently for 24 or 44hr in the presence and absence of putative teratogens to elucidate the effects of these substances on organogenesis. To compare expression profiles of the ATM family of stress response genes during organogenesis in vivo with that from embryos cultured in vitro, GD10 embryos cultured for 24hr were compared to GD11 embryos in vivo, whilst GD10 embryos cultured for 44hr were compared to GD12 embryos in vivo (Figure 5.3). While a slight decrease in ATM transcript levels was observed in yolk sac following 24hr culture compared to GD11 in vivo values (Figure 5.3A), a dramatic decrease to 15% of GD12 levels was observed following 44hr culture (Figure 5.3B). p53 and GADD45 transcript levels were unaffected following either culture period (p21 levels were not examined). In the embryo, ATM transcript levels decreased to 22% of GD11 (Figure 5.3C), and 35% of GD12 values (Figure 5.3D), after 24 and 44hr culture, respectively. Apart from a decrease in GADD45 transcript levels to undetectable levels following 44hr culture (Figure 5.3D), p53 and GADD45 transcripts were unaffected by prolonged culture.

Effect of 4-OOHCPA on PI3K superfamily and downstream stress-response gene expression. We next examined whether the alkylating agent and
Figure 5.2. PI3K superfamily and downstream stress response gene expression following short-term embryo culture in yolk sac (light grey bars) and embryo proper (dark grey bars): ATM (A), ATR (B), DNA-PKcs (C), p53 (D), GADD45 (E), and p21 (F). Values represent the mean transcript concentrations +/- SEM relative to vimentin from three to six replicates.
Figure 5.3. *ATM* family stress response gene expression in yolk sac (A, B) and embryo proper (C, D) following long-term embryo culture for 24hr (grey bars; A, C) and 44hr (grey bars; B, D) compared to GD11 (black bars; A, C) or GD12 *in vivo* (black bars; B, D). Values represent the mean transcript concentrations +/- SEM relative to vimentin from three to six replicates. *, expression significantly different from *in vivo* value by Student's t-test, *p*<0.05.
teratogen 4-OOHCPA could alter the expression of DNA damage sensor genes. GD10 embryos were exposed to vehicle or drug (10μM) in culture for 0.5, 1.5, 3, and 6hr (Figure 5.4). No significant changes in ATM, ATR or DNA-PKcs expression occurred following 4-OOHCPA exposure (Figure 5.4A-C). ATR and DNA-PKcs were expressed at or near the limit of detection in the embryo at most timepoints (Figure 5.4B,C). p53, GADD45, and p21 transcript levels were also unaffected by 4-OOHCPA exposure, in either yolk sac or embryo (Figure 5.4D-F).
Figure 5.4. PI3K superfamily and downstream stress response gene expression following short-term embryo culture with 10μM 4-00HCPA in yolk sac (light grey bars) and embryo proper (dark grey bars): ATM (A), ATR (B), DNA-PKcs (C), p53 (D), GADD45 (E), and p21 (F). Values represent the mean transcript concentrations +/- SEM relative to vimentin from three replicates.
5.5 **Discussion**

A key aspect in the response to DNA damage is co-ordination of the cell cycle to allow DNA repair to take place before DNA synthesis and cell division occur. During development, the cell cycle is short compared to adult cells (MacAuley *et al.*, 1993); tight control of the cell cycle is necessary to ensure proper cell growth and tissue formation. The importance of PI3K family members for normal growth and development is apparent in the null-mutant animals. *Atm*-null mice exhibit growth retardation, chromosomal instability, and increased cancer susceptibility (Elson *et al.*, 1996; Xu *et al.*, 1996); *Atr* null-mutant mice die in early development (Brown and Baltimore, 2000; de Klein *et al.*, 2000), while severe combined immunodeficiency (SCID) mice, harbouring mutations in DNA-PKcs (Blunt *et al.*, 1995; Kirchgessner *et al.*, 1995; Peterson *et al.*, 1995), are hypersensitive to IR (Fulop and Phillips, 1990; Biedermann *et al.*, 1991; Hendrickson *et al.*, 1991). While some checkpoint functions may overlap between the PI3K family members, loss of more than one genotoxic stress sensor is not compatible with life; deficiencies in both *Atm* and other members of recombination and/or DNA damage sensor pathways are embryolethal, e.g. *Atm* null in DNA-PK null-mutant backgrounds (Gurley and Kemp, 2001; Sekiguchi *et al.*, 2001). Null-mutant mice lacking downstream targets of PI3K family members also exhibit increased tumour susceptibility and checkpoint problems (Deng *et al.*, 1995; Levine, 1997; Hollander *et al.*, 1999; Vogelstein *et al.*, 2000; Hollander *et al.*, 2001; Martin-Calabbero *et al.*, 2001). Analysis of the expression profile of
these genes during a susceptible period of development was undertaken to help unravel the ability of the conceptus to respond to genotoxic teratogens.

**Expression of PI3K family members and downstream genes in vivo**

Studies which examined the expression of PI3K family members during development found high expression levels in areas undergoing rapid cell division, consistent with a role for ATM in genome maintenance during cell division (Chen and Lee, 1996; Soares *et al.*, 1998). The length of the cell cycle in the conceptus differs with the tissue type (Mirkes *et al.*, 1989) and proliferative status (MacAuley *et al.*, 1993). ATM was the highest-expressed PI3K family member examined in both tissues. While the overall cell cycle length of the GD12 rat conceptus has, to the best of our knowledge, not been determined, the dramatic increase in ATM transcript levels on GD12 argues for a role for ATM during cellular proliferation and differentiation and may be due to high levels of metabolic activity and DNA metabolism, both of which may increase the occurrence of internally-generated DNA damage. While ATM is classically regulated at the level of kinase activity, ATM transcript or protein levels are induced in several model systems in which cells are rapidly proliferating and/or differentiating (Clarke *et al.*, 1998; Fukao *et al.*, 1999). ATM may be regulated either by alterations in the transcription of pre-existing ATM mRNA or by post-transcriptional mechanisms (Savitsky *et al.*, 1997). ATM may therefore be regulated transcriptionally and post-transcriptionally by specific proliferation-responsive signals within the rat conceptus. Both ATR and DNA-PKcs transcript levels were at or near the limit of detection until GD12 in the embryo proper; once again, this would indicate a
requirement for checkpoint control and the genotoxic stress response during this latter period of mid-organogenesis.

Genes downstream of the ATM superfamily kinases were expressed at very low levels in the conceptus. Earlier studies found that $p53$ transcript levels are highest during organogenesis in the mouse (Rogel et al., 1985), with tissues undergoing differentiation exhibiting higher levels of $p53$ transcripts (Schmid et al., 1991). In the rat conceptus, a trend towards higher transcript levels appeared on GD12, perhaps coinciding with differentiation of $p53$-dependent tissues. In previous studies $GADD45$ expression was found in the rat embryo during organogenesis on GD12 and later (Rees et al., 1999). In this study, transcripts for both $GADD45$ and $p21$ start to appear between GD11 and 12 in the embryo, mirroring $ATM$ and $DNA-PKcs$ transcript profiles, and in parallel to the expression profile for $p53$.

**Transient oxidative stress and PI3K pathway gene expression**

Alteration of $ATM$ expression may be beneficial during organogenesis to protect the embryo against increased oxidative stress as a result of high levels of metabolism or xenobiotic exposure (Wells et al., 1997). Embryo culture elicits transient oxidative stress in the embryo, increasing oxidized glutathione and altering AP-1 transcription factor mRNA levels (Ozoliņš and Hales, 1997). Oxidative stress did not affect $ATM$ transcript levels. Nevertheless, long term culture, for 24 or 44hr culture, did result in decreased $ATM$ mRNA levels; this decrease was specific for $ATM$, as several other DNA repair pathway genes were unaffected by culture conditions (data not shown). The explanation for this
decrease is not clear; embryos cultured for 24 or 44hr have normal oxidized to reduced glutathione levels, indicative of a lack of oxidative stress at these time points (Ozoliņš and Hales, 1997). The decrease in ATM transcripts may be due to other factors acting in the culture system, such as depletion of specific growth factors and other nutrients, which may lead to dysregulation of cell functions. It is possible that alteration of ATM expression may be stressor-specific, or that the response of the embryo to genotoxic stress is unique. Limitations of factors required for proper embryonic growth following these extended periods of culture may also play a role in gene downregulation.

Effect of 4-OOHCPA on PI3K family genes and downstream targets

Several DNA repair pathways repair the damage caused by CPA (Sancar, 1996; Cai et al., 1999; De Silva et al., 2000; McHugh et al., 2000). In particular, the non-homologous end joining (NHEJ) pathway is involved in repairing DSBs formed by nitrogen mustards (McHugh et al., 2000); DNA-PK and associated Ku subunits are involved in this recombination repair pathway (reviewed in Lieber, 1999). Therefore, the expression of these genes might be crucial in repairing the genotoxic damage caused by CPA.

Previous studies have demonstrated a link between genotoxic stress and checkpoint arrest during organogenesis. Embryos cultured with 4-OOHCPA exhibit an accumulation of S-phase cells and G2/M checkpoint arrest, suggesting a relationship between cell cycle arrest and malformations (Chernoff et al., 1989; Little and Mirkes, 1992). CPA doses that are genotoxic but do not lead to malformations have been shown to perturb the cell cycle (Francis et al., 1990),
suggesting that the embryo has the ability to repair genotoxic stress and that checkpoint arrest is integral in the prevention of teratogenesis. The perturbation of the cell cycle occurs approximately 5hr after CPA exposure (Little and Mirkes, 1992, data not shown); it was hypothesized that the G2/M arrest was due to the presence of DNA cross-links that inhibited initiation of mitosis, rather than an active arrest process. In this study, no changes in gene expression occurred near this time point for any PI3K family members or their downstream targets following 4-OOHCPA exposure. If cell cycle arrest in the conceptus requires active transcription of these genes, then perhaps the conceptus is unable to activate these checkpoints, and the arrest seen is indeed due to genotoxic damage-induced structural abnormalities within the genome.

Several genes examined in this study have been examined previously following teratogen exposure. Cell death due to 4-OOHCPA may be p53-dependent or independent (Moallem and Hales, 1998). Interestingly, there are differential tissue-specific requirements for p53; as well, there is a link between p53 levels and G1 checkpoint arrest, following exposure to CPA (Torchinsky et al., 1999). p21 was upregulated following genotoxic stress in the murine conceptus (Macleod et al., 1995), while GADD45 transcript levels increased in primary rodent embryonic cell cultures exposed to the teratogen methylmercury (Ou et al., 1997). The present data suggest that if checkpoint arrest was induced following 4-OOHCPA, it is independent of GADD45 or p21 transcriptional activation and does not require transcriptional upregulation of the PI3K family members.
Apoptosis is another hallmark of the teratogenicity of CPA (Chen et al., 1994; Moallem and Hales, 1995; Mirkes and Little, 1998). While CPA can induce DSBs, the role of PI3K family members in the response to these breaks is unclear. ATM and DNA-PK have been implicated in activation of the apoptotic pathway via c-Abl (Agami et al., 1999; Gong et al., 1999; Yuan Z.M. et al., 1999b). Therefore, it is possible that PI3K family members play a role in both the normal programmed cell death that occurs during development, as well as the aberrant cell death following teratogen exposure.

DNA repair capability and downstream cellular responses to DNA damage are largely unknown during mammalian development. Only when the capacity of the conceptus to sense and respond to genotoxic teratogens is established can the importance of these responses in the teratogenicity of genotoxic agents be determined. We suggest that one of the determinants of the ability of the conceptus to respond to oxidative stress and other genotoxic agents may be the expression of PI3K family members. The results of this study demonstrate that PI3K family members, as well as their downstream targets, are expressed in the rat conceptus during mid-organogenesis, in a time- and tissue-dependent manner, and that they are not upregulated following genotoxic stress.
5.6 Acknowledgements

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6.1 The Consequences of Genotoxic Teratogen Exposure

DNA damage caused by genotoxic agents can impact on virtually any cellular process due to the ability of DNA damage to affect gene expression and subsequent gene products. The importance of repairing DNA damage is evidenced by the variety of DNA repair pathways that have evolved in all living organisms, and the human syndromes caused by a lack of this repair ability.

There exist three fundamental choices for a cell once its genome is damaged. The first is to remove the lesions from the genome, and return to as close a pre-exposure condition as possible. The second option is to continue functioning in spite of the damage; this may result in further damage and alterations in gene transcription and DNA replication. This almost certainly impacts on cellular metabolism, and can lead to carcinogenesis or cell death. Finally, the cell can choose to activate its apoptotic cascade, leading to cell death. The choice between these three pathways can greatly affect the functioning of the whole organism; a single genotoxic molecule, creating a single DNA lesion in a single cell, has the ability to trigger cellular events leading to cell death and potentially the death of the whole organism.

Studies as early as 1977 noted the similarity between DNA damage-mediated chemical carcinogenesis and teratogenesis (Nomura, 1977). The cellular decisions following genotoxic teratogen exposure of somatic cells in the
embryo are identical to those facing somatic cells in the adult following genotoxic
carcinogen exposure, and ultimately influence the developmental outcome of the
organism. As DNA repair, cell cycle checkpoints, and apoptosis are all intimately
related, it is ultimately the balance between all three that determines the ability of
a genotoxic teratogen to induce malformations (Figure 6.1).

Of over 2500 chemicals tested for teratogenicity, roughly 1200 can cause
malformations when administered to laboratory animals; however, the list of
known human teratogens stands at around 40 (Shephard, 1998), with far fewer
having been studied extensively for their genotoxic potential. This incredible
discrepancy is primarily due to the lengthy process of confirming the teratogenic
effects of a compound in humans, particularly if only a small number of women
have been exposed to a suspect chemical. The vast majority of known
teratogens have been identified as a result of the gross malformations induced in
the exposed offspring of lab animals or humans. It is becoming clear, however,
that not all teratogenic effects are visible to the naked eye. Alterations in gene
expression or enzyme activity during critical periods of development could have a
tremendous impact on the health of the newborn. Determining the effects of
potential teratogens, therefore, is becoming increasingly complex. Elucidating
the molecular pathways underlying developmental processes will clarify the
teratogenic potential of single compounds as well as whole classes of
compounds.

It has been shown that organogenesis-stage conceptuses display
increased apoptosis following exposure to genotoxic teratogens (reviewed in
Knudsen, 1997). If the conceptus cannot modify its DNA repair capability
Figure 6.1. The relationship between DNA repair and genotoxic stress response pathways and teratogenesis.
following DNA damage, then perhaps the only recourse is the activation of cell death machinery. Aberrant cell death via apoptosis has a dramatic impact on the growth and development of the conceptus; it has been demonstrated for a variety of teratogens that unscheduled cell death occurring after teratogen exposure leads to either malformations or loss of viability of the conceptus. Therefore, the conceptus must be able to remove genotoxic lesions from its genome to ensure proper growth.

6.2 Summary of Thesis

The ideas for the studies presented in this thesis were brought about by one simple question: does the conceptus have the cellular components necessary to repair the damage caused by well-known and well-studied genotoxic teratogens? The fact that this question has gone unanswered for so long is due, in part, to the difficulties in examining the expression of multiple genes in embryonic tissues, as well as the relatively recent uncovering of many components of mammalian DNA repair pathways. Gene expression, protein expression, and enzyme activity were examined in order to provide a comprehensive picture of DNA repair capability in the conceptus. Many of the genes examined in this thesis have not previously been studied in the conceptus, while some of the other data complement expression studies during other periods of development.

The field of teratology has been at the cross-roads of science for several years. Much of the early research on teratogens that are still studied today was
done at the gross anatomical and morphological level. Differences between the yolk sac and embryo proper, or between the “head” and “trunk” regions of the conceptus, were the limit of tissue-specific examination using biochemical techniques. The integration of molecular biology techniques into the field of teratology has allowed a much closer examination of small areas of the conceptus. However, in order to integrate new molecular biology-based approaches with the previous body of knowledge, a “transition” phase appears necessary to bridge this gap between knowledge at the whole embryo level and at the individual cell level. Consequently, this thesis examined gene transcript, protein levels, and repair activity in the whole yolk sac and the whole embryo proper, in order to lay the foundation for future, more detailed studies examining specific cell groups or tissues in the conceptus.

6.2.1 Expression of DNA Repair Pathways in the Organogenesis-Stage Rat Conceptus

The ability to examine the gene expression of all members of a cellular pathway is one of the strengths of array-based expression technology. In the case of DNA repair pathways, this yields a more complete view of the repair system and gives an indication of potential “bottlenecks” in the repair process. Virtually the entire set of core genes from the NER, MMR, and RCR pathways, as well as a broad spectrum of BER glycosylases and genotoxic stress checkpoint genes, were examined in this thesis using the aRNA technique. This technique has been used almost exclusively to examine transcript levels during
development, due to its ability to examine gene expression in minute amounts of tissue. This is the first time such DNA repair gene expression profiles have been determined during mammalian development. The results obtained demonstrate that the majority of DNA repair genes examined are expressed in the conceptus during mid-organogenesis, although several were at or near the limit of detection of the aRNA assay. Furthermore, the expression profiles of virtually all repair genes were similar in both the yolk sac and embryo proper on GDs 10 and 11; only on GD12 did the embryo display increased transcript levels for many of the genes examined, compared both to GD10 and 11 embryo as well as GD12 yolk sac values.

Of the DNA repair genes examined at various time points during the lifetime of an organism, many have time-specific expression patterns during development \textit{e.g.} APE/Ref-1 (Ono \textit{et al.}, 1995; Wilson \textit{et al.}, 1996), ATM (Chen and Lee, 1996), MPG (Kim \textit{et al.}, 2000), and UNG (Nilsen \textit{et al.}, 2000b). The data described in this thesis add to this knowledge of developmental gene expression. That transcript levels for genes in the same pathway differed dramatically suggests specific requirements for certain DNA repair enzymes during development. While alterations in gene expression do not necessarily imply a change in DNA repair capability, this is one of the key mechanisms by which DNA repair activity can be modified. Some of the repair enzymes examined are involved in other cellular pathways, which might explain their time- and tissue-specific expression during organogenesis (Sections 2.5 and 3.5). However, the majority of genes examined are primarily involved in DNA repair, and no links to other processes have been established. The close relationship...
between embryo and yolk sac gene expression on GDs 10 and 11 argues for a similar requirement to repair damage at these time points, with increased repair necessary in the GD12 embryo. Therefore, it is possible that the pattern, and not the levels of gene expression, ultimately determines developmental susceptibility to genotoxic teratogens.

6.2.2 DNA Repair Activity During Development

The ability to remove genotoxic lesions from the genome is the ultimate proof of the functionality of a DNA repair pathway. While the corresponding repair activities of all pathways examined in this thesis would have given a more comprehensive view of DNA repair in the mid-organogenesis stage conceptus, difficulties exist in examining repair during development. In particular, the ability to dissect DNA repair from DNA replication in rapidly dividing cells is an inherent limitation with most repair assays that examine the larger pathways such as NER, MMR, and RCR. Only four studies to date have examined the ability of the conceptus, or cells derived from embryonic tissues, to repair genotoxic lesions (see Table 1.1). An attempt to create an NER assay using the whole conceptus was performed during this thesis, which failed due to significant embryotoxicity of the assay itself. Therefore, only the activity of the BER UNG pathway was examined, due to the direct relationship between assay activity in vitro and biological activity in vivo. The UNG repair pathway is isolated, in that no other repair pathway can mimic its function of removing uracil due to thymidine limitation from DNA. The results obtained from this assay demonstrate a
capacity to repair genotoxic lesions, as well as a correlation between UNG protein levels and enzyme activity in vivo. This important pathway is a critical link between folic acid deficiency, one of the most studied teratogenic conditions, and the malformations caused by thymidine depletion.

6.2.3 Teratogens and DNA Repair Gene Expression

One of the goals of this thesis was to test the hypothesis that genotoxic stress produced by genotoxic teratogens would alter DNA repair gene expression and enzyme activity in the conceptus, and thus affect the ability of the conceptus to respond to this stress. As the rat conceptus is the major model for drug toxicology and teratogenicity testing, it is essential to understand the ability of the conceptus to respond to teratogens. For genotoxic teratogens, the ultimate response would be alteration of DNA repair capability. One of the most striking results found in these studies is the near absence of response at the gene, protein, or activity level of the rat conceptus towards genotoxic teratogens. Of the four repair pathways and the genotoxic stress checkpoints examined, transcript levels for only one gene, UNG, increased following genotoxic teratogen exposure, and only following low-dose exposure. This lack of induction of DNA repair or checkpoint genes following exposure to 4-OOHCPA, transient oxidative stress, or high-dose MTX demonstrates the rigidity of the developmental gene expression program. Curiously, for several genes there was a striking decrease in gene expression following genotoxic teratogen exposure. Obviously, the protein and activity levels of these pathways must be examined in detail before
any concrete conclusions can be drawn concerning the ability of the conceptus to modify cellular responses to DNA damage. Given that many of the genes examined in this thesis are regulated at the transcript level, rather than post-transcriptionally, the lack of increased gene expression following teratogen exposure appears to indicate that the repair capacity of the conceptus is unaltered. This does not mean that the conceptus is unable to respond to genotoxic teratogens; while DNA repair pathways are only one part of a complex system of biochemical pathways that can respond to teratogens, other cellular responses to genotoxic damage will not resolve the fundamental problem, the need to repair the genotoxic lesions. The ability to alter DNA repair gene transcript levels following genotoxic stress has been demonstrated in other models (Fornace et al., 1989b; Laval, 1991; Lefebre et al., 1993; Labudova et al., 1998; Evans, 2000; Scherer et al., 2000). The studies presented in this thesis demonstrate that the ability of the conceptus to control gene expression during development is different than that in cells that are not in a state of rapid proliferation. The observed decreases in gene expression could lead to exacerbation of the effects of genotoxic teratogens, and indicate a lack of checkpoint activation ability following oxidative stress.

6.2.4 UNG Repair Activity Following Genotoxic Teratogen Exposure

The studies presented in this thesis show a complete lack of responsiveness at the level of UNG repair activity following exposure to MTX. This is the first time the repair activity of a DNA repair enzyme has been studied
following genotoxic teratogen exposure during development. Underlying susceptibility factors to MTX teratogenicity have been difficult to determine; the results presented in this thesis show that the conceptus is vulnerable and incapable of modifying key cellular pathways to counteract MTX teratogenicity. In fact, the UNG repair pathway is the last defence against MTX-induced genotoxic stress. If this is the case for other genotoxic teratogens, it would indicate a considerable susceptibility to genotoxic stress during development. Ultimately, examination of UNG expression and activity in specific tissues of the embryo proper would help determine the exact correlation between UNG activity and tissues that are targeted for MTX-induced malformations. As well, a more detailed examination of other DNA repair activities following teratogen exposure, once new assays become available, will shed light on the "black box" process of other teratogen-induced malformations.

6.3 Conclusions

The results obtained from the experiments presented in this thesis lead to the following conclusions:

1) The gene expression of four major DNA repair pathways varies in a time- and tissue-dependent manner in the mid-organogenesis stage rat conceptus, with some DNA repair genes not expressed at all or expressed at levels below the limit of detection.
2) Noticeable differences exist between DNA repair protein/activity levels and their corresponding gene expression values.

3) Exposure of the rat conceptus to three specific genotoxic teratogens (4-OHCPA, MTX, oxidative stress) demonstrated an almost complete lack of inductive response at the gene, protein, or activity level at teratogen concentrations shown to either induce malformations or elicit other cellular stress response pathways.

These conclusions formulate a global view of DNA repair gene expression in the mid-organogenesis stage rat conceptus, and are synthesized into a schema of DNA repair/genotoxic stress response pathways during development in response to genotoxic teratogens (Figure 6.2). With the increased sensitivity of molecular biology tools, it will become easier to examine levels of genotoxic stress, as well as the responses to that stress, in order to define the genotoxic potential of teratogens. Only once these pathways are elucidated fully during development can we hope to understand the mechanism of action of genotoxic teratogens, and to potentially devise ways to reduce this risk.
Figure 6.2. DNA repair pathway and cell cycle checkpoint gene expression in the rat conceptus during organogenesis in vivo, and following teratogen exposure in vitro. Thickness of line indicates relative transcript abundance (not to scale).
6.4 **List of Original Contributions**

The overall aim of this thesis was to shed light on the expression and activity of various DNA repair pathways during development. Consequently, specific original contributions to the scientific body of knowledge were achieved. They are:

1. The gene expression of four main mammalian DNA repair pathways (NER, BER, MMR, and RCR), as well as DNA damage-responsive checkpoint members was determined for the first time in the mid-organogenesis stage rat conceptus.

2. Age- and tissue-dependent differences in DNA repair gene expression were found in all pathways examined.

3. The conceptus was found to be unable to upregulate the expression of the majority of DNA repair genes following genotoxic teratogen exposure.

4. The expression of specific DNA repair genes in the conceptus was found to decrease following genotoxic teratogen exposure.

5. The embryo culture system was found to negatively regulate the expression of genes involved in the oxidative genotoxic stress response pathway in the conceptus following long-term culture.
6. The expression of several NER proteins/enzymes was determined for the first time in the conceptus, and were found to have tissue and developmental time-specific expression profiles.

7. The expression and activity of both isoforms of the BER enzyme UNG was determined for the first time \textit{in vivo} and following MTX exposure \textit{in vitro} in the conceptus.

8. The teratogenic effects of MTX \textit{in vitro} in the conceptus was determined to be similar to \textit{in vivo}, whole-animal studies, validating this system for examining the effects of folic acid deficiency.
Epilogue

While this thesis, in my opinion, is a well-rounded study, it is nowhere near my original concept, or that of my supervisor, for my Ph.D. thesis. In fact, the work done in my first year and a half in the lab is not mentioned in this thesis, and will not be published in any paper. Many graduate students experience this same frustrating course of events during their thesis work, but rarely is it made public, except perhaps to their close friends and colleagues. I, however, believe that this demonstrates the capacity of new, inexperienced graduate students to struggle against great odds, try every possible angle, attempt every possible modification to their protocols, with the firm belief that it WILL work. When it becomes apparent that it will, in fact, NOT work, and their project is modified accordingly, it can be the most humbling, exasperating, even depressing event of their lives. However, these trials and tribulations are the best way to understand the hardships, and reality, of scientific research. I wish to offer my humble congratulations to all graduate students past, present and future, who have or will experience the frustration of working on a project that, unbeknownst to them, was doomed from the start, not by any fault in their hypothesis, but due to limitations of the current state of scientific knowledge, methods, and reagents available to them at the time.
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