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Consequences of paternal exposure to the anti-cancer drug, cyclophosphamide, on rat pre-implantation development

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirement for the degree of Doctor of Philosophy

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I dedicate this work to my parents who instilled in me the wonderful gift of perseverance under any and all circumstances I have encountered towards achieving my goals.

We choose our joys and sorrows long before we experience them.

~ Khalil Gibran ~

(1883-1931, Lebanese Poet, Novelist)

Abstract

Administration of cyclophosphamide to males targets the germ cells and causes DNA damage including single strand breaks and DNA-DNA cross links. When males are treated with a chronic low dose of cyclophosphamide and then mated to normal females, progeny loss is manifested at the pre- and post-implantation stages of development . The earliest events that lead to embryonic loss were traced to day 2 of gestation when embryos had a decreased DNA synthesis profile and lower cell numbers than control litters. I investigated the hypothesis that chronic exposure of male rats to cyclophosphamide alters zygotic gene expression thus leading to embryonic loss. To assess DNA damage in the embryo, the Comet Assay was performed on 1-cell stage embryos. A significant number of embryos sired by cyclophosphamide-treated males showed the appearance of the Comet indicative of the effect of damaged sperm on the embryos starting from the 1-cell stage. Using a candidate gene approach, the antisense RNA (aRNA), I described the presence of several DNA repair gene families in normal embryos. Progeny sired by cyclophosphamide-treated males manifested a differential expression profile for several of these genes when compared to controls, suggestive of the ability of the embryo to respond to damaged sperm through the major DNA repair systems. To study the functional capacity of progeny sired by cyclophosphamide-treated males, I assessed total RNA synthesis in both groups; while control litters showed a peak of RNA synthesis at the 4-cell stage, the treatment group showed constant low expression throughout the stages examined. I mapped the profile of a number of gene families whose roles are essential for early development in both control and

cyclophosphamide-treated groups. While control embryos showed a peak of expression for the majority of genes at the 8-cell stage, that of the cyclophosphamide-group showed an early induction at the 2-cell stage, indicative of loss of the tightly regulated transcriptional program of the embryo. The first differentiation processes in the embryo take place at the 8-cell stage when embryos undergo compaction, acquire cell polarity and form tight junctions. A comparative analysis of blastomere cell interaction revealed a markedly decreased cell number as well as a lower number of cell contacts in the treatment litters. Coupled to the morphological changes, an altered gene expression pattern of cell adhesion and scaffolding proteins at the mRNA and protein levels was observed in embryos sired by cyclophosphamide-treated males. In conclusion, I have established that paternal drug exposure has selective effects on the program of zygotic gene expression resulting in abnormal progeny outcome.

Résumé

Le traitement de mâles avec le cyclophosphamide entraîne une atteinte des cellules germinales, en causant des dommages au niveau de l'ADN, en particulier des cassures d'ADN simple brin et des liaisons de l'ADN. Quand les mâles sont traités de façon chronique avec une faible dose de cyclophosphamide puis accouplés avec des femelles non traités, on observe des pertes d'embryons à la fois aux cours des périodes pré- et post-implantatoire du développement. Le premier événement qui entraîne une perte embryonnaire fut noté dès le deuxième jour de gestation quand les embryons présentent un profil de synthèse réduit et un nombre de cellules plus faible que ceux du lot contrôle. J'ai étudié l'hypothèse selon laquelle une exposition chronique de rats mâles au cyclophosphamide perturberait l'expression des gènes du zygote entraînant une perte embryonnaire. Pour examiner l'atteinte de l'ADN au niveau des embryons, j'ai utilisé la technique des "comètes" sur des embryons au stade une cellule. Un nombre significatif d'embryons obtenus à partir de mâles traités par le cyclophosphamide présentent l'apparition d'une comète démontrant l'effet néfaste de spermatozoïdes endommagés sur les embryons; cet effet se manifeste dès le stade une cellule. A l'aide d'une approche génétique basée sur l'amplification d'ARN antisense (aRNA), j'ai pu décrire l'expression de plusieurs familles de gènes codant pour des protéines qui réparent l'ADN. Les embryons obtenus à partir de mâles traités montrent que le profil d'expression de plusieurs de ces gènes diffèrent par rapport à ceux des animaux du lot contrôle suggérant la possibilité pour les embryons d'activer ce système de correction d'ADN pour se protéger des effets néfastes de

spermatozoïdes endommagés. Pour évaluer la vitalité des embryons du groupe traité, nous avons comparé la quantité d'ARN totale synthétisé dans les deux lots. Tandis que les embryons du lot contrôle présentent une synthèse d'ARN maximale au stade quatre cellules, les animaux du groupe traité montrent une synthèse d'ARN à la fois faible et constante au cours des étapes analysées. J'ai comparé pour les groupes traité et contrôle le profil d'expression de plusieurs familles de gènes qui sont essentiels au début du développement embryonnaire. La majorité de ces gènes atteignent une expression maximale au stade huit cellules chez les embryons du lot contrôle, par contre pour les animaux du lot traités on observe une induction plus précoce dès le stade deux cellules, indiquant que la régulation précise du programme de transcription à été perturbé chez ces embryons. Les premiers phénomènes de différenciation s'opèrent au stade huit cellules quand l'embryon se compacte, que les cellules acquièrent une polarité et que des jonctions cellulaires serrées se forment. Une analyse comparative des interactions cellulaires des blastomères a révélé à la fois une diminution importante du nombre de cellules et des contacts cellulaires parmi les animaux du lot traité. Nous avons donc observé que les embryons obtenus à partir de mâles traités au cyclophosphamide présentent, en plus de certains changements morphologiques, une modification d'expression des gènes d'adhésion et de construction cellulaire à la fois au niveau des ARN et des protéines. En conclusion, j'ai pu montré que l'exposition de mâles au cyclophosphamide affecte le programme d'expression génétique du zygote, entraînant des anomalies pour la descendance.

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Acknowledgments

I would like to express my appreciation to Dr. Barbara Hales and Dr. Bernard Robaire for their assistance, constructive criticisms and their utmost attention to details throughout the development and execution of this dissertation. I am also indebted to Dr. Claudio Cuello, my advisor, for his wisdom and guidance.

I am grateful to the Medical Research Council of Canada (MRC) for the financial support of this project throughout my studies. The generous support (moral and financial) of our Department of Pharmacology & Therapeutics makes it a wonderful place to grow and pursue higher learning.

In the past five years, I have met a wonderful group of people many of whom became close friends. From the wonderful ground floor crew, I especially appreciate all the moral support and advise of: Dr. Chunwei Huang (whom I should have listened to from the very beginning), Jennifer Manley and Anna Liachenko for reminding me of the big picture and for taking all my phone calls, Patrick Syntin for instant moral support and all his help especially for translating my abstract to French, Robert Vinson for keeping the computers in a good shape, Kate Jervis for taking all the harassment (you're a superstar, kiddo!), Lu Cai (my big brother), Natali Hendersen (is it the weekend yet!). Of course the ground floor wouldn't be without the Coffee Club, a substance to which I owe many (O.K. all!) of the last many accomplishments. I especially have enjoyed interacting with Marie Ballak (our coffee club presidente), our electron microscopy specialist, and Alan Forster, our photography expert and of course Dr. Daya Varma who always made sure I was in line. The Reproduction members (past and present) who had to sit through many of my "unprepared presentation" I owe many thanks. Namely, Terence Ozolins, Seyed Moallem, Dr. Jacquetta Trasler, Carmen Merteinet (for all your energy!) and Tonia Doerkson. To the new members of the ground floor, I wish all of you luck in your endeavors as you enter the golden gates of graduate work (all the best!).

The office staff have been amazingly helpful; namely Pam Moore, Naomi Belinski, Helene Duplessis, Mona Azra, Jennifer Marleau, and Sid Parkinson.

To really be a part of the Pharmacology department, the 13th floor crew is to be acknowledged. I appreciate the help of Dr. Brian Collier and particularly recognize the input of the early days in the journal club of Dr. Moshe Szyf, especially the insights of Marc Pinard, Robert McLeod & Shyam Ramchandani into science among other things that were discussed over lunch.

Preface

Format of the Thesis

This thesis comprises three data chapters in a manuscript format. All three chapters are included in the form in which they were submitted for publication. Connecting texts are provided in accordance with section B.2 of the "Guidelines Concerning Thesis Preparation" of the faculty of Graduate Studies and Research of McGill University. These guidelines state:

"Manuscripts and Authorship:

Candidates have the option, subject to the approval of their departments, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory. The thesis must still conform to all other requirements of the "Guidelines Concerning Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. Their thesis must include, as separate chapters or section: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final

overall conclusion and/or summary. Additional material (procedural and design data, as well as description of equipment used) must be provided where appropriate and in sufficient detail (e.g. in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis. In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

The Introduction, Chapter 1, highlights briefly the salients elements involved with preimplantation development and of paternally-mediated developmental toxicities. A rationale for the studies presented in this dissertation is also provided in the Introduction.

Aspects of Chapter 2, 3, and 4 dealing with the consequences of paternal exposure to cyclophosphamide on the preimplantation progeny were carried out under the co-supervision of Dr. Robaire of the Department of Pharmacology & Therapeutics of McGill University; Thus Dr. Robaire's name appears as a co-author for these papers. In Chapter 2, experiments conducted for the Comet Assay to reveal DNA damage were performed by Alexis Codrington; thus, the name of Alexis appears as a co-author for this Chapter. This Chapter is in the final preparation stage to be submitted to *Mutation Research*. In Chapter 3, experiments for the

male pronucleus formation, BrUTP incorporation and Sp1 immunostaining were performed by Sepideh Khatabaksh; Thus, Sepideh's name appears as a co-author for this Chapter. This Chapter has been accepted by *Molecular Reproduction & Development*, in press, permission pending. In Chapter 4, embryo sections were prepared by Marie Ballak and Dr. Chunwei Huang. This Chapter is in press in *Biology of Reproduction*, Volume **63**, 74-81 (2000), permission pending. The dissertation concludes with a general discussion of the results presented in Chapter 5. This Chapter also includes a List of Original Contributions which summarizes the major findings of the results presented in chapters 2, 3 & 4.

Chapter 1.

Introduction

1. Embryogenesis

1.1 Historical perspectives

Investigating the very beginning of life is not a 20th century quest. Aristotle, in his *Generation of Animals*, was the first scientist on record to be fascinated by the mastery of details when he described the coming-into-being of an organic form and of the very nature of life. A metaphysical understanding of life was explained by some empirical and anecdotal support for the presence of four primary substances. It was not until the first quarter of the 20th century that scientists started to look into the anatomy of the coming-into-being. The advent of improved microscopy and preparatory techniques allowed researchers to record reliable observations at the cellular level thus resulting in systematic engraved illustrations and encouraging comparisons of microscopic and gross morphology. In particular, von Baer's pioneering work has led to the discovery of the mammalian egg using the dog model which allowed the delineation of four laws of development which still hold up to date. These could be summarized as: 1) The more general characters of animals appear earlier than the special ones. 2) The less general forms develop from the most general ones. 3) Every embryo separates from other forms. 4) Every embryo only resembles its own form. Von Baer went on to coin the term "spermatozoa", the male germ cells that were considered earlier by Leeuwenhoek as parasites. Pander discussed the "unique metamorphosis" that occurs in the three germ layers, namely the "serous", "mucous" and "vascular" layers within the embryo as follows:

and each hurries toward its goal; although each is not yet independent enough to indicate what it truly is; it still needs the help of its sister travelers, and therefore, although already designated to different ends, all three influence each other collectively until each has reached an appropriate level.

The next breakthrough came in the 1880's with the coming of a new generation of embryologists, including Roux, who reoriented embryology away from the comparative descriptions toward an experimental enterprise. Certainly, it is the tremendous amount of effort invested by many investigators that has founded the basis of what is known today as developmental biology (Churchill, 1985) .

1.2 Normal pre-implantation development

Due to the fact that the bulk of studies on pre-implantation mammalian development has been done in the mouse, most of the description in the introduction to the thesis will be based on results obtained using the mouse model system. I will, however, describe information relating to other species with a special mention of data on rat pre-implantation development, wherever information is available.

Starting with a single totipotent cell, the fertilized egg, an embryo undergoes the very first events of cellular differentiation which result in a highly specialized group of cells with distinct functional properties. The period of pre-implantation development refers to the period of development that encompasses

all the stages that take place between the 1-cell stage and the formation of the blastocyst. Due to intricate changes needed at these early stages, initial steps of embryogenesis in mammals are relatively long and characterized by a fairly well synchronized doubling of cell numbers until the 8-cell stage. Following compaction, asynchronous cell divisions become more frequent. At the 8- to 16-cell stage the embryo enters the uterine environment, forms a morula, a cluster of 14-22 cells (Dvorak, 1978). It is at the morula stage that cells first become committed to be outer or inner cells; at the blastocyst stage these cells become differentiated into two cell lineages, the inner cell mass cells (ICM) and the trophectoderm cells (Pedersen, 1986). The ICM cells are a clump of cells which give rise to the embryo proper and its membranes, whereas the trophectoderm cells consist of a sheet of cells which give rise to the extraembryonic placental tissues. The blastocyst undergoes expansion where the blastocoele becomes the most prominent portion of the embryo, pushing the ICM cells to one pole of the embryo. By the fifth gestational day, the embryo hatches form the surrounding *zona pellucida* and subsequently implants in the uterus. For successful pre-implantation development to occur, it is understood that all steps leading up to implantation must be well controlled and synchronized. First, gametogenesis of both the egg and the sperm have to be flawless. This is accomplished by a tightly regulated mechanism of germ-specific gene transcription and translational repression of messenger ribonucleic acid (mRNAs) accumulated in oogonia and spermatogonia. Such a regulation ensures the proper delivery of essential proteins at specific stages of

development (Sommerville & Ladomery, 1996). The second step involves the process of fertilization, where contributions of paternal and maternal genomes to the zygote are required for embryonic survival. Lastly, gene expression and repression of key molecules during pre-implantation development ensure that the embryo will implant in the mother's uterus and eventuate in a healthy live pup.

1.2.1 Oogenesis

In mammalian species, the female primordial germ cells begin their development around day 7.5-8.5 *post coitum (p.c.)*, where they divide mitotically until around day 13.5 *p.c.* before they start differentiating in a synchronous manner into meiotic cells, the primary oogonia. DNA synthesis allows for the replication of chromatin in primary oogonia which enter prophase and undergo changes leading up to the diplotene stage of meiotic prophase where they arrest until the start of ovarian cycling. At the time of birth, the ovary contains all the oocytes that the female will ever produce throughout her reproductive life. During its active phase of growth, the oocyte accumulates all the necessary components, including proteins, ribosomes, and mRNAs that will be transcribed selectively and translated as needed throughout its growth and maturation and well into the pre-implantation stages of embryonic development. Subsequently, the oocyte is arrested at metaphase II of meiosis, a quiescent state for both DNA synthesis and RNA transcription (Wassarman, 1988; Wassarman & Kinloch, 1992).

1.2.2 Spermatogenesis

In contrast to the female, where oogenesis is a single milestone in the female's life span, spermatogenesis is a periodic process which is continuous throughout the reproductive life of a fertile male; diploid spermatogonia continue to divide mitotically and differentiate into haploid spermatozoa in a normal adult male. While a group of daughter cells among dividing spermatogonia remains as stem cells, the rest of the dividing cells leave the pool of undifferentiated progenitor cells and commit to further differentiation by undergoing several mitotic divisions (5 in the rat) to form diploid spermatocytes (de Rooij, 1998). Following a number of maturational steps, spermatocytes undergo meiosis where each primary spermatocyte undergoes two meiotic divisions to give rise to four haploid round spermatids.

The process of maturation of spermatids to spermatozoa, or spermiogenesis, follows where spermatids undergo several biochemical and morphological events which result in remodeling of male germ cells before they leave the testis. Hallmarks of spermiogenesis include synthesis of the acrosome and the flagellum, replacement of histones by protamines leading to remarkable chromatin condensation, and the release by condensed late spermatids of the bulk of their cytoplasm (Reviewed by Clermont, 1972; Balhorn, 1982). Control of the intricate steps of spermiogenesis is ensured partly by regulating the translation of various key components acquired during spermiogenesis and monitored by a transcriptional system (Kleene, 1996). Premature translation of protamine 1 mRNA during spermatid differentiation results in a precocious

condensation of spermatid nuclear DNA, abnormal head morphogenesis, and incomplete processing of the protamine 2 protein (Lee et al., 1995). The time required for the completion of a full cycle of spermatogenesis differs among species; spermatogenesis spans 35 days in the mouse as compared to 49 days in the Sprague-Dawley rat (Clermont et al., 1959).

Spermatozoa traverse from the testis through the efferent ducts into the epididymis where they acquire their motility and fertilizing potential. It takes spermatozoa approximately 4-10 days for their journey through the epididymis (Robaire & Hermo, 1988). At the time of fertilization, the sperm chromatin is uniquely compacted into a highly dense, genetically inert entity (Ward & Coffey, 1991). While the egg provides the zygote with all the essential cytoplasmic and nuclear components, the only component required from the sperm for normal embryonic development to take place is an intact nucleus (Ward et al., 1999).

1.2.3 Fertilization

The process of fertilization involves the accurate execution of a number of precisely timed tasks. Spermatozoa bind to the *zona pellucida* of metaphase II arrested oocytes; this allows the sperm to undergo the acrosomal reaction and subsequently to enter the oocyte. Upon entry of a spermatozoon into a mature oocyte, a series of biophysical and biochemical processes are triggered to ensure the genetic integrity of the zygote and the success of subsequent embryonic development. These include the block of the oocyte to polyspermy, activation of the egg, completion of meiosis II, and the extrusion of the second

polar body (Yanagimachi, 1994).

The next event entails the remodeling of maternal and paternal chromosomes into functional pronuclei. The paternal pronucleus undergoes a series of events starting with the reduction of the disulfide bonds of protamines (Perreault et al., 1984). This is followed by the progressive replacement of protamines by the maternally inherited somatic histones (Rodman et al., 1981). A series of chromatin decondensation-recondensation events follows where a nuclear envelope derived from the egg cytoplasm is formed (Mouse: Adenot et al., 1991; rat: Szollosi, 1965). Oocyte factors are responsible for the successful formation of the male pronucleus as an egg is capable of remodeling a limited amount of sperm chromatin material (Harrouk & Clarke, 1993). Each pronucleus undergoes a round of DNA replication prior to combining its chromosomal complements on the first zygotic metaphase plate, thus undergoing syngamy (Perreault, 1992; Yanagimachi, 1994). The diploid one-cell embryo then undergoes mitosis to produce a 2-cell embryo with two diploid zygotic nuclei, each containing a set of maternal and a set of paternal chromosomes.

1.2.4 The one-cell embryo

Major events of the 1-cell embryo include the first round of DNA synthesis, acquisition of transcriptional permissiveness, and the first round of zygotic gene activation. Overall, the developmental program of the one-cell embryo seems to be exclusively controlled by maternal factors (Johnson, 1981). DNA replication is thought to allow maternally-derived transcription factors to

gain access to their cis-binding DNA sequences in order to regulate the genetic program of the early embryo (Wolffe, 1994). Difficulties in deciphering transcriptional events in the early embryos, attributed to a paucity of proper techniques and materials, had led scientists to conclude that the embryonic genome is transcriptionally inert until some time after the first cell cleavage (Moore, 1975). However, this early dogma has been overturned by more recent evidence demonstrating that a one-cell embryo is not transcriptionally inert. In fact, a relief of the transcriptionally repressive state has been proposed to result form DNA replication, permitting the embryo to accomplish two goals: the arrest of maternal gene expression, and the prevention of precocious and premature differentiation (Forlani et al., 1998). Microinjection studies demonstrated that reporter genes injected during the cell division (S phase) of the one-cell embryo were able to be transcribed (Ram & Schultz, 1993).

Curiously, several reports have documented that the paternal pronucleus precedes its maternal counterpart in transcription. Microinjection of reporter genes into the male pronucleus of the mouse embryo revealed that the male pronucleus was capable of expressing exogenous genes before the female pronucleus (Bouniol et al., 1995; Matsumoto et al., 1994); this phenomenon was reported as well for endogenous genes (Aoki et al., 1997). Furthermore, transcription of endogenous genes in the male pronucleus occurs at a rate of four to five times greater than that of the female pronucleus in the late one-cell embryo (Bouniol-Baly et al., 1995; Aoki et al., 1997). An explanation for this differential transcriptional ability was provided by the finding that the male

pronucleus possesses a greater nuclear concentration of transcription binding proteins (TBPs) and Sp1 transcription factors than is found in the female pronucleus (Worrad et al., 1994). Thus, the embryonic genome seems to be activated at the 1-cell stage at least in *in vitro* cultured mouse embryos (Bouniol-Baly et al., 1997).

Concomitant with the acquisition of transcriptional ability, the embryo undergoes the first burst of gene activation, a limited activity occurring between 18 and 21h *p. c.* (i.e., late one-cell) in the mouse (Flach et al., 1982). The net product of this activation is the synthesis of a small set of polypeptides restricted to heat shock proteins (Bensaude et al., 1983) and the transcription-requiring complex (TRC) (Conover et al., 1991). The coupling of the first round of DNA replication with the onset of zygotic transcription has implicated the necessity of replication for the reprogramming of gene expression (Davies & Schultz, 1997).

1.2.5 Embryonic gene activation

The lengthened cell cycle of the early mammalian embryo can be linked to one of the major events of pre-implantation development, embryonic genome activation. The first cell cycle, which takes 16-20 hours in the mouse, is ended following DNA synthesis and cell division, thus resulting in the formation of a 2-cell embryo containing two diploid zygotic nuclei. A relatively long time is consumed (18-22 hours) before the next cell division. One of the hallmarks of the 2-cell stage is the second phase of zygotic gene activation, a major activity occurring between 26 and 29 hours *p.c.* (i.e., early 2-cell) in the mouse (Flach et

al., 1982). This activity requires novel translational products (Nothias et al.,1996). In the rat, data obtained using one-dimensional gel electrophoresis have suggested that initiation of embryonic genome activation takes place at the late 2-cell stage and is followed by a burst of protein synthesis activity between the 2- and 4-cell stages of embryonic development (Zernicka-Goetz, 1994).

1.2.5.1 De *novo* synthesis of embryonic transcripts

Until the past decade, no resources existed to investigate whether embryonic activation leads to a global gene transcription or to a synthesis of independent stage-specific transcripts. Several investigators have applied the polymerase chain reaction (PCR) technique, which allows for the amplification of known gene candidates starting with a limited amount of materials, in the early embryo (Rappolee et al., 1988). However, the PCR approach does not reveal any information on novel genes. Using the subtractive hybridization of cDNA libraries constructed from poly$^+$ (A) mRNA of pre-implantation embryos, a family of cytokines was found to be differentially expressed in the early embryo, thus establishing, for the first time, that embryonic gene activation involves the differential expression of a selective set of genes rather than a global process (Rothstein et al., 1992). Some of the important regulators of the translational machinery represented by ribosomal and poly(A)$^+$ RNAs were also reported to be turned on as a result of zygotic gene activation. The expression of both factors declines at the time of germinal vesicle breakdown of the egg and resumes starting with a low level at the 2-cell stage (Bachvarova & Moy, 1985).

By the late 2-cell stage, all classes of RNAs are transcribed from the mouse embryonic genome (Clegg & Piko, 1983a). Supportive evidence for a *de novo* transcriptional activity in the early zygote was obtained from genetic profiling of enzyme variants or antigens. Data were obtained by assessing the timing of the onset of synthesis of paternally coded proteins. Since paternal genes are transcriptionally inert prior to the embryonic gene activation, any activity of a paternal pronucleus origin would imply a novel transcriptional activity (Sawicki et al., 1981).

1.2.5.2 Maternal to zygotic genome shift

The embryo relies on maternal transcripts for its survival and proper progression through development provided that such transcripts have not been made by the embryo. Embryonic transcripts become gradually expressed and progressively replace their maternal counterparts. Much investigation has been described detailing what is referred to as "maternal to zygotic genome shift". It is now well established that the shift takes place in all mammalian species examined (Telford et al., 1990).

1.2.5.2.1 *De novo* synthesis of maternally stored transcripts

The transcriptional arrest which took place in the metaphase II oocyte resumes upon meiotic maturation and after fertilization (Fox & Wickens, 1990). Dormant transcripts stored as adenylated messages in the cytoplasm of oocytes undergo translational activation upon egg activation. This renders them prone to

degradation depending on the degree of stability of individual transcripts. Among the newly translated products are a number of transitory proteins which are required for the passage from maternal to the zygotic transcriptional control. Recent evidence has revealed the presence of a major maternal transcript, *spindlin* (*spin*), which is present in the mouse during the transition from oocyte to embryo. *Spin* is an abundant maternal transcript in the unfertilized egg and the 2-cell embryo which disappears by the 8-cell stage of embryogenesis. This transitory expression has provided a potential role for *spin* in cell-cycle regulation during the transition from gamete to embryo (Oh et al., 1997). Another "maternal factor" was discovered in a strain of mutant mice, *DDK*. *DDK* is present and active in oocytes and early pre-implantation embryos and seems to be essential for normal development since embryos lacking it cannot form a blastocyst. *DDK* acts via an interaction with the paternal genome and clearly represents a maternal control over the early stages of embryonic development (Renard et al., 1994).

1.2.5.2.2 Translation of pre-existing maternal transcripts

Cytoplasmic polyadenylation of maternal mRNAs results in their translation (Richter et al., 1990). These transcripts are generally long lived; some of them persist up to the morula stage (Brinster, 1976; Richoux et al., 1991). However, not all maternally-inherited transcripts are long lived. Short lived proteins, including a 35 and a 45 Kd protein complex, have been described; they are initially expressed following fertilization and are degraded by the 2-cell stage

(Cascio & Wassarman, 1982; Howlett & Bolton, 1985).

1.2.5.2.3 Loss or decay of maternally-derived transcripts

Translation of pre-existing maternal mRNAs is responsible for the first embryonic cleavage since embryos can still cleave in the presence of α-amanitin, an RNA polymerase inhibitor (Howlett & Bolton,1985). Nonetheless, maternal transcripts are necessary but not sufficient for further embryonic development. When mouse eggs are incubated in the presence of the transcriptional inhibitor α-amanitin, development beyond the 2-cell stage is arrested (Flach et al., 1982). This indicates that new (zygotic) RNA synthesis is required for proper development to occur. At the mRNA level, a process of adenylation, deadenylation and degradation of maternal transcripts is initiated such that by mid 2-cell stage most of the maternal mRNA is destroyed or inactivated (Piko & Clegg, 1982).

The bulk of maternal mRNA turnover is processed by cytoplasmic polyadenylation of mRNAs, a post-transcriptional activity known to control the stability of transcripts (Salles et al., 1992). Concomitant with the decline in maternal mRNA is an increase in zygotically transcribed messages starting at the 2-cell stage in the mouse embryo, an indication of a shift from the use of maternal- to embryonic-derived RNA components (Clegg & Piko, 1983 b). Maternal transcripts continue to decline in abundance following the embryonic gene activation and decline further throughout the pre-implantation stages of development as they are replaced by their zygotic equivalents. A number of

genes have been shown to undergo this shift; the mRNA pools of two housekeeping genes, actin and histone 3, are shown to be reduced 10-fold at the 2-cell stage (representing the decline of maternal transcripts) followed by an increase from the 4-cell stage onwards when transcription from the embryonic genome is established (Giebelhaus et al., 1983).

1.2.6 Protein synthesis

Data using the two-dimensional gel electrophoresis system have delineated marked differences in the patterns of [^{35}S] methionine-labeled proteins synthesized by early cleavage embryos (Reviewed by Schultz & Heyner, 1992). Analysis of these data indicates that 60-85% of all proteins studied change in expression at the 1- to 2-cell transition (Latham et al., 1992). When embryos are treated with cycloheximide, a protein synthesis inhibitor, the transcriptional activity of six housekeeping genes is dramatically reduced (Wang & Latham, 1997).

Subsequent to the second burst of transcriptional activity, an activation at the level of protein synthesis is seen in embryos where some transitory proteins replace maternal products prior to their replacement by their zygotic counterparts (Davis et al., 1996,1997). Among these are the transcripts of the family of heat shock protein 70 (HSP70) (Bensaude et al., 1983). In addition to changes in protein synthesis profiles, post-translational modifications of gene products have been implicated in the reprogramming of the embryonic genome. These include an increased turnover rate of some proteins made on stable maternal mRNA

(Howlett & Bolton, 1985). Alternatively, post-transcriptional modifications such as phosphorylation, glycosylation, or proteolytic cleavage of proteins have all been shown to be implicated in the processing of maternal and embryonic gene products (Van Blerkom, 1981; Cascio & Wassarman, 1982; Pratt et al., 1983).

1.2.7 Chromatin structure

In addition to its role in establishing a transcriptionally permissive state in the zygote, the chromatin structure is involved in providing the zygotic genome with an ability to repress the activities of promoters and replication origins, thus allowing the timely execution of events (Wiekowski et al., 1997). The core histones, linker histones, and the high-mobility group (HMG) proteins package all the DNA in the nucleus within nucleosomal arrays, thus providing the necessary compaction needed for very long strands of DNA molecules (centimeters in length) into the nucleus (10^{-5} m in diameter) (Vermaak & Wolffe, 1998).

Differences in nuclear and chromatin composition exist between oocytes and sperm; the oocyte chromatin contains core histones similar to those of somatic cells, as well as some protamine-related epitopes (Clarke, 1992). Elements of the chromatin makeup, including the three major lamin products present in oocytes, are similar to those present in somatic cells. Sperm, on the other hand, do not possess a typical nuclear lamina. Up to fertilization, both the egg and the sperm have transcriptionally-inert chromatin with low levels of hyperacetylated chromatin (Thompson et al., 1998). Following fertilization, chromatin rearrangements of both genomes take place. Nucleosome

displacements, replacement of sperm-derived protamines with maternal histones in the male pronucleus, and post-translational modifications in the histones themselves involving acetylation and deacetylation of zygotic chromatin have all been reported to be involved in the programming of zygotic transcription (Stein et al., 1997). Curiously, the paternal chromatin seems to compete with the maternal chromatin for the pool of hyperacetylated histone 4 (H4) present in the egg (Thompson et al., 1998). As a result, different acetylation levels exist between the male and the female pronuclei (Thompson et al., 1998). This differential expression could lead to the preferential recruitment of transcription factors into active chromatin configurations in the male pronucleus, leading to a higher transcriptional activity in the paternal pronucleus during the S/G_2 phase of the mouse zygote. Following the first cell division, the chromatin structure of the diploid 2-cell embryo undergoes further maturational steps; these seem to be closely coordinated with the gene activation program as witnessed by their ability to regulate gene expression profiles, as has been illustrated for the heat-shock protein HSP70.1 (Thompson et al., 1995).

1.3 Major events during pre-implantation development

1.3.1 Cell division in the early embryo

A key mechanism for cell heterogeneity in the embryo is a proper cell division program (Johnson, 1985) featuring an asynchrony of division among blastomeres within a single embryo (Kelly et al., 1978). Starting with the first cell division, the first of the 2-cell blastomeres to divide maintains such a property

later on during development. This blastomere later establishes more intercellular contacts and contributes to the ICM cells of the embryo which form the embryo proper (Kelly et al., 1978; Graham & Lehtonen, 1979). Thus, the division order of cells affects the developmental fate of their progeny.

1.3.2 Cell-cell interaction

A second mechanism governing the behaviour of cells during the early cleavage stages of mouse development is continuous cell interactions (Graham & Lehtonen, 1979). Although cell interactions in the mammalian embryo are partly warranted by the presence of the *zona pellucida*, it is the number of cell contacts which determines successful embryonic development. The ability of zona-free mouse embryos to develop to term and give progeny depended on the number of total points of contacts between blastomeres; more contacts resulted in a higher number of inner cell mass cells and subsequently a higher number of live offsprings (Suzuki et al., 1995).

Contact-mediated interactions between blastomeres involve changes in the cell surface, a property influenced in part by extracellular calcium (Pey et al., 1998). This, in turn, requires an intact cytoskeletal organization (Lehtonen & Reima, 1986) since treatment of compacting embryos with a cytoskeletal inhibitor prevents compaction (Ducibella & Anderson, 1975).

The cell division movement from the 2-cell stage to the 4-cell stage depends on a brief contact between the two blastomeres. Inside the *zona pellucida*, all cells of the 4-cell embryo usually touch all other cells and they all

have the same number of cell contacts (Lehtonen, 1980). At the 8-cell stage, cells have different numbers of cell contacts and their relative contribution to the future ICM cells is a function of their number of cell contacts (Surani & Barton, 1984). A change in the cell surface of 8-cell embryos referred to as compaction represents the first differentiation event of the mammalian embryo. Compaction consists of **polarization, cell flattening and junctional communication**, all of which require cell interactions (Johnson, 1986). Although the signal to initiate compaction does not occur until the 8-cell stage, 2-cell embryos undergo morphological changes preceding the events of compaction. Ultrastructural analysis of mouse embryos demonstrates blastomere surface modifications as early as the 2-cell stage, as manifested by the loss of microvilli and endocytic activity as well as the formation of cell junctions induced at cell-cell contact points. The capacity of the plasma membrane to undergo these changes precedes any detectable activity of the embryonic genome (Mouse: Pratt, 1985; Human: Tesarik, 1989).

1.3.2.1 Polarization

Up to the early 8-cell stage, all cells of the mouse embryos are equivalent with apices exposed to the outside; any cell can contribute to the formation of either the ICM or the trophectoderm cells, the two cell lineages of the implanting embryo (Kelly et al., 1978). It is at the 8-cell stage that individual blastomeres acquire regionalization of the membrane domains as evidenced by the restricted appearance of microvilli to the free surface of the embryo (Adler & Ziomek,

1986). Polarity of early mouse blastomeres is abolished when members of the Rho family of p21 GTPases are inhibited, suggesting their involvement in the process (Clayton et al., 1999).

1.3.2.2 Cell flattening

The process regulating the spatial organization of cells within the compacting embryo is referred to as cell flattening. No *de novo* appearance of recognition molecules is seen during cell flattening. Rather, other mechanisms seem to be involved such as reorganization of pre-existing cell components in some post-translational modifications (McLachlin et al., 1983; Kidder & McLachlin, 1985). Permanent inhibition of cytokinesis inhibits intercellular flattening (Johnson, 1985).

1.3.2.3 Junctional communication

Cytoplasmic bridges between blastomeres are present starting from the 2-cell to the pre-compacted 8-cell embryo (Lo & Gilula, 1979). Tight junction adhesion between blastomeres is first seen at the 8-cell stage and gradually gains complexity as more tight junction proteins are assembled (Collins & Fleming, 1995).

Blastomeres do not initiate adhesion until they are properly signaled to do so (Collins & Fleming, 1995). Such signals are believed to depend on the zygotic gene activation program, such as the presence of a chromatin-mediated

repression at the late 1-cell stage in mammalian embryos where the zygote regulates its own transcription clock (Nothias et al., 1995).

The ubiquitous tripeptide glutathione (GSH) has been implicated in a number of cellular processes including regulation of the cell cycle, reduction of cell injury, intercellular communication, and induction of cell adhesion (Kromidas et al., 1990; Gardiner & Reed, 1995; Zeulke et al., 1997; Voehringer et al., 1998). Using a thiol-specific probe, Barhoumi and colleagues have shown a direct correlation between glutathione levels and intercellular communication in anchored cells of various origins (Barhoumi et al., 1993). The mechanism(s) mediating cell-cell contacts has been studied in the *Xenopus* embryo where disruption of cell-mediated contacts resulted in a differential response. The expression of a mesodermal gene, α-actin, was disrupted. No disruption, however, was noted for an endodermal gene, DG42, or an ectodermal gene, DG81 (Sargent et al., 1986). These results demonstrate the interplay of two processes, inductive interactions and cell-autonomous inheritance of maternal products, in a lineage-specific manner. Such an interdependence was also observed in *C. elegans* (Schnabel et al., 1997).

1.3.3 Cell adhesion

One common denominator for all three aspects of compaction (i.e., polarization, cell flattening and junctional communication) is the mutual expression of the Ca^{2+}-dependent cadherin, E-cadherin, on adjacent cells (formerly referred to as uvomorulin/cadherin). E-cadherin expression is seen as

early as the 1-cell stage and is required for the initial cell contact at the 2- and 4-cell stages (Fleming et al., 1994). Adhesion of 8-cell blastomeres during compaction is also mediated by E-cadherin. E-cadherin deletion in transgenic animals results in embryos which undergo an initial compaction. Later, cells decompact, lose polarity and fail to form a blastocyst (Larue et al., 1994; Riethmacher et al., 1995). The initial compaction is explained by the presence of maternal stores which may be sufficient for the initiation but not maintenance of compaction. Maternal proteins need to be phosphorylated. This post-translational modification of E-cadherin is not seen prior to the 8-cell stage. In fact, premature induction of E-cadherin phosphorylation at the 4-cell stage results in a premature adhesion among blastomeres; this adhesion does not require the presence of E-cadherin at later embryonic stages to occur (Collins & Fleming, 1995).

1.3.4 Cytoskeletal elements

E-cadherin interacts with a number of architectural proteins to ensure proper progression of events during early embryogenesis. Among these is the intermediate filament protein, vimentin, whose interaction with E-cadherin seems to be required to form desmosomes and the epithelia of mesenchymal type cells (Valiron et al., 1996; Vanderburg & Hay, 1996). This is accomplished by the anchoring of intermediate filaments to desmosomes through desmoplakin, an intracellular component of desmosomes. Desmoplakin co-distributes with E-cadherin along the lateral cell membranes, thus promoting the anchorage of

vimentin to the cell membrane; all three molecules thus form a "complexus adherentes". A number of other genes have been localized to cell-cell contacts, including the myosin light-chain kinase (Sobel, 1983), the tight-junction peripheral membrane protein, cingulin (Javed et al., 1993), the filamentous actin (F-actin), (Albertini et al., 1987), a microfilament bundle-membrane linking cytoskeletal protein, vinculin (Lehtonen & Reima, 1986), and spectrin (Sobel & Goldstein, 1988).

Other architectural elements include the connexin family, each of which is the product of a distinct gene; these are a family of at least twelve proteins that make up the intercellular membrane channels of gap junctions (Becker & Davis, 1995). Direct cell-cell communication via gap junctions allows for the exchange of metabolites between connecting blastomeres (Reuss et al., 1997). Among the connexins identified to date, connexin 43 seems to be the most prominent gap junction protein expressed during pre-implantation development. In the mouse, transcripts for connexin 43 are detected starting from the 4-cell stage and the protein is detected from the 8-cell stage onwards (Davies et al., 1996). In the rat, transcripts for connexin 43 are abundant in the zygote and are degraded at the 2- and 4-cell stages to reach undetectable levels in the uncompacted 8-cell embryo. Connexin 43 transcripts reappear in the compacted morula and the blastocyst (Reuss et al., 1997). However, connexin 43 is not the sole connexin responsible for proper gap junctional communication since its deletion in a mouse model did not affect embryogenesis; null mutant mice lacking connexin 43, in fact, developed normally until birth (Reaume et al., 1995). Given the

difference in the expression profile of connexin 34 in mouse and rat, it would be interesting to compare the consequences of deletion of this gene in the two animal models. Unfortunately, no rat knockout model for any connexin has been described to date.

1.3.5 Imprinting

A number of genes in the mammalian genome have been shown to be differentially expressed, depending on the origin of the parental allele, a phenomenon referred to as genomic imprinting. It is believed that the embryo regulates its growth by expressing either the paternal or maternal allele exclusively. An increasingly large family of imprinted genes has been described to date (Reviewed in Bartolomei & Tilghman, 1997). Two lines of experimental approaches have given rise to the plethora of information on the process referred to as genomic imprinting. In the first, manipulation of uniparental diploid embryos, generated by pronuclear transfer, resulted in the production of androgenotes and gynogenotes (McGrath & Solter, 1984). In the second, activation of an egg in the absence of a sperm resulted in parthenogenotes, which, when manipulated, generated diploid parthenogenotes (Surani et al., 1986). In both cases, embryos failed to develop to term, suggesting that diploidy was not a sufficient prerequisite to establish a normal pattern of gene expression guaranteeing successful embryonic development. Rather, the contribution of one copy of each of the parental genomes is necessary for normal development (McGrath & Solter, 1984). Androgenotes die around the 4-6 somite stage, with poor embryonic

formation coupled with a minor abnormality in the extraembryonic tissues. In contrast, gynogenotes survive until mid-gestation with poorly developed placentae (Surani et al., 1986). This differential effect on embryonic vs. extraembryonic tissues has led to the suggestion that a differential gene expression exists in the conceptus to balance out maternal and paternal contributions.

A more detailed dissection of the imprinting process has revealed a family of at least thirty autosomal genes contributed exclusively by either the mother or father. Insulin growth factor 2 (IGF2) was the first gene to be described as endogenously imprinted; knockout mice were observed to be reduced in size only when the gene was inherited from their fathers, but not from their mothers (De Chiara et al., 1990). As a result, IGF2 is referred to as a maternally-imprinted gene due to the exclusive silencing of the maternal allele. The same imprinting pattern has been reported for IGF2 in humans (Leighton et al., 1997) and rats (Pedone et al., 1994; Overall et al., 1997). Insulin growth factor 2 receptor (IGF2R), on the other hand, was shown to exert the opposite effect. Gene knock-out studies of IGF2R in mice resulted in progeny that were larger in size than their wild type littermates. IGF2R is expressed and inherited from the maternal allele and thus referred to as a paternally-imprinted gene (Barlow et al., 1991).

Recent data have indicated that imprinted genes seem to exist in clusters on the genome topography. For instance, the location of IGF2 on chromosome 7 in the mouse seems to be in proximity to three newly described paternally-imprinted genes. The first gene, Mash-2, a mammalian member of the *achaete-*

scute family which encodes basic-helix-loop-helix transcription factors, is a paternally-imprinted gene. Mash2 gene deletion resulted in an embryo-lethal knockout mouse; embryos died from placental failure on day 10 *p.c.* Moreover, Mash2 was found to be widely expressed throughout the pre-implantation stages of development (Rossant et al., 1998) and in the trophectoderm of mouse blastocysts (Guillemot et al., 1994), indicative of an important role during normal embryogenesis. Another paternally-imprinted gene, referred to as $p57^{kip2}$, is an inhibitor of several G_1 cyclin/cyclin dependent kinase complexes and a negative regulator of cell proliferation (Russo et al., 1996). Analysis of allelic differences between two mouse strains has shown that $p57^{kip2}$ is a maternally expressed gene (Hatada & Mukai, 1995). Apolipoprotein E (Apo E), the third imprinted gene located within the imprinting cluster of chromosome 7 (Searle & Beechey, 1990), has a reduced expression in gynogenotes, thus suggesting that ApoE is more influenced by a paternal contribution (Mann et al., 1995). No data have been published regarding expression in the pre-implantation embryo of either $p57^{kip2}$ or ApoE.

1.3.6 DNA repair systems

Genomic maintenance and propagation between generations require the presence of a surveillance system; mistakes could occur not only during mitotic cell divisions but also during events specific to meiosis such as recombination and crossing over of chromosomes. While very little has been described with regards to repair systems in germ cells, the past decade has witnessed a

plethora of information in the DNA repair field in somatic cells ranging from bacteria to humans. The major repair systems include at least five DNA repair pathways, each requiring multiple proteins: nucleotide excision repair, base excision repair, mismatch repair, double-strand break repair, and recombinational repair. A mutation in any protein associated with a particular pathway could compromise the whole process. In addition to their role as guardians of the genome, an increasingly large number of these genes are reported to be essential for normal development, as shown by gene deletion studies (Reviewed by Tebbs et al., 1998). The function of many DNA repair systems must , therefore, be essential as early as the pre-implantation stages of development.

The nucleotide excision repair system (NER) is responsible for the repair of damage by most anticancer agents, including cyclophosphamide (Andersson et al., 1996; Damia et al., 1996). NER is the most complex of the repair systems identified to date, involving up to twenty enzymes. The mechanism of repair involves two major steps, the recognition and removal of the damaged oligonucleotide, and the repair synthesis of DNA using the complementary strand as a template (Lehmann, 1995; Wood, 1997). A number of NER members are present during normal spermatogenesis, including the Excision Repair Cross-Complementing 1 gene (ERCC1), a nuclease required for the removal of damaged DNA (Walter et al., 1996). Other members of the NER pathway are important for normal embryonic development; phenotypes for the Xeroderma Pigmentosum B and D (XPB, XPD) knockouts are embryolethal for both genes starting from early stages of development (Tebbs et al., 1998).

Base excision repair (BER) recognizes and repairs endogenous DNA damage. Specific glycosylases remove the damaged base, an apurinic/apyrimidic endonuclease (APE) cleaves damaged DNA sites around the abasic nucleotide. Polymerase β and DNA ligase III then fill in the gap and religate the DNA (Lehmann et al., 1996). While this pathway involves a large number of proteins, the APE accounts solely for almost 99% of incisions at abasic sites. In a gene deletion mouse model, the absence of an APE, the APE/redox-factor 1(APE/ref-1), resulted in early embryonic death at around day 6.5 of gestation, thus demonstrating a role for this gene not only in response to DNA damage but also in normal developmental events (Xanthoudakis et al., 1996).

The role of the mismatch repair (MMR) system is to correct mismatches in base-pairing and to prevent recombination between divergent DNA homologues. In humans, a defect in the mismatch repair pathway is an indication for a predisposition to a hereditary nonpolyposis colon cancer. Whenever a mismatch is identified, a number of genes are involved, including the human paired mismatch 2 (hPMS2) protein (Kolodner, 1995). Interestingly, the MMR system has been found to be essential during normal spermatogenesis. Male PMS2 null mutant mice are infertile, exhibiting abnormal chromosome synapsis in meiosis. Surviving epididymal spermatozoa have misshapen heads and truncated flagella (Baker et al., 1995).

Double-strand breaks (DSB) are the major lethal DNA lesions incurred by toxicants. The presence of a lesion triggers chromosomal deletions and

rearrangements which, if left unrepaired, are lethal to the cell. The major participant in this repair pathway is the DNA-dependent protein kinase (DNA-PK) (Lehmann, 1995).

Recombinational repair (RCR) is the surveillance system for all recombination steps taking place during homologous recombination of meiosis. While double strand breaks are repaired by the DSB pathway, they seem to use the homologous recombination to rejoin. Thus, the RCR pathway would ensure the proper rejoining of DNA strands. In a knockout mouse model, Rad51, a yeast homologue of the bacterial recA, was shown to be essential for normal embryogenesis. Interestingly, less than 2% of total embryos assessed at the pre-implantation stages (days 2-4 *p.c.*) were Rad 51 -/- (Tsuzuki et al., 1996). Another example of the importance of this pathways to normal development is one of the genes responsible for breast cancer (Brca 1). Animal knockout studies illustrate the need for these genes during pre-implantation stages of early development (Hakem et al., 1996).

1.4 Aberrant embryonic development resulting from insults

In the human, it is estimated that one in five couples are involuntarily sterile. Over one third of early embryos die and about 15% of pregnancies abort spontaneously. Among the survivors, an estimated 3% of all new born infants are inflicted with some form of birth defects or congenital abnormality (Ferguson & Ford, 1997). A mere 20-30% of such abnormalities are estimated to be due to gene mutations in the germ cells (Hodgson & Levi, 1988). Somatic mutations

must be contributing to some of the remaining structural and functional defects of unknown etiology. Human studies have not, regrettably, concentrated on events occurring around the time of conception. The extent of the problem among human populations is illustrated by the fact that most women are not aware of the existence of pregnancy during the early stages. This would provide a very narrow window of opportunity to take appropriate measures to protect the mother and embryo.

The ability of xenobiotics to harm the developing fetus became widely recognized after the thalidomide incident in the1960's (Randall, 1990). Shortly afterwards, approximately 4-6% of birth defects were considered to be attributed to chemicals in our environment (Wilson, 1977). As the number of chemicals available on the market is consistently increasing at an average of 1000 new chemicals each year, we are faced with the consequences of an estimated 80,000 agents by the end of this year alone (Schardein, 1993).

1.4.1 Zygote exposure to insults

It has been assumed that when embryos are exposed to toxicants during the pre-implantation period, they are refractory to the toxic insult. They either die or recover and develop normally. This all-or-none response was believed to be due to the unique regulatory ability of embryos to recover and to the totipotency of the early embryonic cells. However, as detailed studies become focused on exposing early embryos to various agents, this dogma is being overturned. In fact, toxic insults to the early embryo can have a deleterious effect on the embryo

leading to fetal and neonatal death, dysmorphogenesis, and abnormalities (Dwivedi & Iannaccone, 1998). Experiments with cyclophosphamide (Spielmann & Jacob-Miller, 1981; Spielmann et al., 1981), trypan blue (Lin & Monie, 1973), actinomycin D (Epstein & Smith, 1978), and heavy metals such as methyl mercury (Matsumoto & Spindle, 1982) have all provided evidence of the detrimental effects to development when pre-implantation stage embryos are exposed to these drugs. Furthermore, within the pre-implantation embryo itself, there exists a cell lineage-specific difference in sensitivity towards various toxicants. Exposure of a blastocyst stage embryo to a X-rays has revealed that the ICM cells are more sensitive than trophectoderm cells (Goldstein et al., 1975).

The susceptibilities of germ cells and early embryos to various insults differ; while exposure of gametes produces excessive pre- and peri-implantational losses and low rates of fetal anomalies, largely due to growth retardation, that of embryos induces peri-implantational death, pan-gestational death, and fetal anomalies highlighted by hydrops, abdominal wall defects, and eye aberrations (Reviewed by Rutledge, 1997).

1.4.2 Differential effect of gamete of origin

Several lines of evidence have indicated the presence of germ line-specific mutagens. This specificity is compounded by a male-female difference in mutagenic response and in quantitative/qualitative differences in mutations when a gamete is exposed. Mutagen sensitivity at different maturational stages in male

and female germ line development varies greatly depending on the stage of differentiation that the germ cell is undergoing during the insult. This is usually manifested as a distinct mutational response experienced by the gamete. Some of the major factors which determine the differential germ cell response to toxicants include gene expression, imprinting, DNA methylation, and hyperacetylation.

Gene expression affects the repair of mutational lesions (Hanawalt, 1990 a, b). This is supported by sex differences in gene expression of transcripts expressed solely by either gamete; meg1 , a meiosis-associated gene, is differentially expressed in male and female gametes (Don et al., 1994).

Perhaps the most conclusive evidence of sex-specific mutagenesis comes from human studies where it was observed that certain chromosomal abnormalities occur preferentially in the paternal genome (Magenis, 1988), as is displayed in deletions of the tumour suppressor gene in Wilms' tumor (Huff et al., 1990). Genomic imprinting offers another explanation for sex-specific differences in response to insults. The consequence of inducing damage to imprinted genes is that such loci are potentially more likely targets for mutagenesis since one allele is normally inactive. As a result, this creates a subgroup of target genes for damaging agents. Moreover, a higher risk of mutations which affect the epigenotype of key tumor-suppressor or tumor-promoting genes could take place. Such mutations may in fact be classified as "epimutations" where it is not the DNA sequence that is affected. Rather, the post-transcriptional mechanism of epigenotype maintenance is altered in a stable fashion resulting in genomic

instability and the heritability of tumor predisposition from parent to offspring. The effect of paternal exposure to radiation on the offspring was reviewed recently (Schofield, 1998).

Through a series of experimental studies including those with knockout mouse models of DNA methyltransferase -1 (DNMT-1), the use of methylation modifying drugs, and the analysis of patterns of sequence-specific methylation in imprinted genes, it has been found that CpG methylation plays a major role in the vulnerability of the genome of the gamete (Li et al., 1993; Neumann et al., 1995; Jackson-Grusby & Jaenisch, 1996). In male germ cells, changes in methylation during spermatogenesis have been reported (Cedar, 1988; Eddy et al., 1988). As spermatogenesis progresses, specific genes change their methylation status to differentially control the DNA-protein interactions needed for transcription (Trasler et al., 1990).

Another candidate for epigenetic control is the modification of histones. In particular, hyperacetylation of histone H4 may be involved with functional alterations in the transcription of chromatin (Grunstein, 1997). Deacetylase inhibitors, such as trichostatin or butyrate, cause gross hyperacetylation of chromatin resulting in activation of normally silent genes (Xiao et al., 1997; McCaffrey et al., 1997).

1.4.3 Maternally-mediated effects on embryogenesis

Embryotoxicity that is maternally-mediated may range from aberrations in

the genetic makeup of the maternal genome to exposure of the mother to various insults during the period preceding pregnancy or throughout gestation.

Female germ cells spend most of their lifetime arrested in the dictyate stage of meiotic prophase. As this stage precedes the meiotic divisions, such a prolonged dormancy is subject to perturbations which deregulate normal chromosome disjunction and predispose to chromosomal segregation errors. The most detailed maternal effect on progeny outcome is that of the trisomy 21; more than 90% of inflicted individuals have inherited the extra chromosome from their mothers (Sherman et al., 1991). This has been attributed to meiotic chromosome structure and recombination since some trisomic patients have lower than expected recombination frequencies characterizing the extra chromosome (Hassold et al., 1991).

Some chemical mutagens seem to be female-specific for inducing damage; bleomycin, an anticancer agent, produced dominant lethal effects in maturing oocytes but not in spermatogenic cells (Sudman et al., 1992). This was explained by the fact that the diffuse state of oocyte chromatin may facilitate the intercalation of bleomycin molecules better than the highly compact spermatogenic cells. Mutagen-induced damage to female germ cells is stage-dependent (Russell & Russell, 1992). Ionizing radiation studies have indicated that resting immature cells are very resistant to the induction of mutations (Russell, 1977). However, drugs differ in their ability to affect resting oocytes, since procarbazine did induce mutations in immature oocytes (Ehling & Neuhauser-Klaus, 1988).

Upon fertilization a number of events are susceptible to damage which, if not repaired, compromises the normal development of the fetus. Prior to implantation of the embryo in the mother's uterus, the embryo is provided with some protection from the oviductal microenvironment by the presence of the *zona pellucida*. Damaging this protective layer prior to the formation of intercellular junctions at the 8-cell stage results in embryo lysis, arrest, or extruded blastomeres when transplanted into pseudopregnant mothers (Nichols & Gardner, 1989). Another example of the deleterious effects of the contents of the oviductal and uterine environment on embryo growth comes from diabetic animal models; congenital malformations and early fetal loss are the main complications of diabetic pregnancy. Embryos collected at the morula and blastocyst stages of diabetic mothers have lower cell numbers and lower protein synthetic rates when compared to control embryos (Vercheval et al., 1990; Pampfer et al., 1990).

Prior to the thalidomide disaster, it was commonly believed that the placental "barrier" protected the fetus from drugs given to the mother. Thirty years later, it is now well established that many pharmacological and chemical agents can readily pass from the mother to the fetus. Depending on its ionic charge, size, and half life, a chemical is capable of accessing the oviductal environment and altering the normal development of the early embryo, leading to fetal or neonatal death, dysmorphogenesis, or abnormalities after birth. Maternal exposure to metals (mercury, cobalt), polychlorinated biphenyls present in industrial chemicals (arcolor, 2,3,7,8-tertrachlorodibenzo-p-dioxin,

hexachlorobenene), pesticides, toluene, methanol, chlorophenols, or radiation have all been shown to affect early embryonic development (Reviewed by Dwivedi & Iannaccone, 1998). Toxic effects of alkylnitrosoureas on pregnant rodents result in implantation failure, embryonic death, and decreased birth rates (Napalkov, 1968). Organotin compounds present in agricultural products, such as tributyltin chloride, result in early embryonic loss when they are administered to rats during early pregnancy (Harazono et al., 1996).

1.4.4 Paternally-mediated effects on embryogenesis

1.4.4.1 Epidemiological Evidence

Epidemiological studies have indicated that specific paternal occupations and the exposure of the father to certain chemicals (environmental, therapeutic, or recreational) may be linked to adverse reproductive outcomes. Research on paternal exposures was initiated in the 1970s when the wives of dentists (Cohen et al., 1974) and workers in the plastics industry (exposed to vinyl chloride) (Infante et al., 1976) were reported to be at a high risk of miscarriages due to their partners' occupational exposures. Further correlatory studies have shown that men employed as firemen, janitors, forestry and logging workers, printers, or plywood mill workers are at a greater risk of siring children with birth defects (Robaire & Hales, 1993). Exposure of the father to chemicals (plastics, rubbers, solvents, anaesthetic gases, and metals, such as lead and mercury) has also been associated with an increased risk of spontaneous abortion (Savitz et al.,

1994). Finally, clinical studies have demonstrated that men treated with anticancer drugs may exhibit infertility (Miller, 1971; Watson et al., 1985).

1.4.4.2 Mechanisms of action

Male exposures to drugs can affect the outcome of progeny adversely by three general mechanisms: 1) by exposure of the conceptus to the drug via the seminal fluid, 2) by affecting germ cell development via the pituitary-testicular axis, and 3) by directly targeting germ cells during exposure.

1.4.4.2.1 Presence of chemicals in the seminal fluid

Exposure of the fetus to a drug via the seminal fluid can occur during intercourse which could coincide with fertilization or a later step of embryogenesis. This is particularly relevant to human populations since our species continues to have intercourse following the initiation of pregnancy. Thus, the potential risk of deleterious progeny outcome due to drugs in the seminal fluid might be underestimated when extrapolating animal data to human risk assessment. Several drugs have proved to affect the early embryo via this route of exposure; these include morphine, methadone, thalidomide and cyclophosphamide (Lutwak-Mann, 1964; Hales et al., 1986; Soyka et al., 1986; Robaire & Hales, 1994).

1.4.4.2.2 Effects via the pituitary-testicular axis

High public interest has spurred a number of studies concerning the effect of steroid hormones or other chemicals that interact with steroid hormone receptors on fertility and embryogenesis. This class of chemicals, referred to as endocrine disruptors due to their mechanism of action, controls fundamental events in both spermatogenesis and sexual differentiation during embryonic development. Environmental chemicals. such as metabolites of the fungicide vinclozolin and the pesticide DDT, disrupt reproductive development, resulting in male genital tract malformations, male infertility, and female breast cancer (Kelce et al., 1998). Endocrine disruptors and estrogen mimics, such as plastizicers, benzyl-butylphthalate and dibutylphthalate, the antioxidant, butyl-hydroxyanisole, the rubber additive, p-phenylphenol, and the disinfectant, o-phenylphenol, also affect the male reproductive system (Reviewed by Sonnenschein & Soto, 1998).

1.4.4.2.3 Effects on germ cells

In contrast to the exposure of a relatively uniform population of somatic cells, exposure of gametes represents a challenge in teasing out the details of an effect caused by the drug administered due to the number of factors involved; these include cell cycle patterns, accessibility of the cell, chromatin structure/function, and repair capacity of the gamete (Reviewed by Adler, 1990; Lewis, 1994; Allen et al., 1995). As a result, germ cell mutagenicity caused by drugs is manifested by two sets of insults: effect on the structure and content of DNA (resulting in mutation, aneuploidy, or polyploidy) and changes to DNA

function through epigenetic events (including DNA methylation, imprinting, and signal transduction mechanisms). Certain repeat DNA sequences selectively expressed in germ cells are hot spots for radiation-induced mutations (Allen et al, 1995). Chromosomal breakage preferentially occurs at fragile sites residing in unprotected chromatin structure where decondensation and transcriptional activity are taking place (Yunis et al., 1987).

The endpoint for testing the effect of a toxicant on male germ cells is to document an effect on either the spermatozoal quantity and/or quality. There are several causes for an alteration in spermatozoal quantity which range in manifestation from a partial loss (oligospermia) to a complete loss (azoospermia) of spermatogenesis. "Therapeutic" causes include chemotherapy, radiation therapy, and antibiotic treatment with nitrofurantoin, gentamicin or niridazole (El-Beheiry et al., 1982; Martin-du Pan & Campana, 1993). Vitamin and zinc deficiencies, as well as heat and infection, can also result in decreased numbers of spermatozoa (Chinoy et al.,1986; Kwiecinski et al.,1989; Martin-du Pan & Campana, 1993; Thonneau et al., 1998). The extent of spermatogenic arrest is dependent on several factors, including the case etiology, the dose, the duration, and the fertility status of the individual before exposure (Martin-du Pan & Campana, 1993). For example, scrotal heating in men to approximately 45^0 C for 30 minutes for a period of 12 days induces a reversible oligospermia 5-7 weeks after exposure (Kandeel & Swerdloff, 1988), while one high dose of radiation (600 rads) may result in permanent sterility (Ash, 1966).

Toxicants that affect spermatozoal quality are diverse in both their etiology and mechanism of action. Environmental reproductive toxicants include carbon disulphide, mercury, lead, vinyl, chloride, acrylamide, benzo[α]pyrene, and 1,3 butadiene (a chemical found in petroleum emissions and in the manufacture of synthetic rubber) (Cordier et al., 1991; Olshan & Mattison, 1994; Anderson et al., 1997; Marchetti et al., 1997). Therapeutics agents, including a number of anticancer drugs, may be germ cell mutagens.

Well-controlled animal studies have provided evidence for multiple mechanisms of action for a number of toxicants on male germ cells. The mechanism of action of any given drug depends on the type(s) of germ cell targeted by this drug. For instance, X- rays affect primarily spermatogonia, while mitomycin C, an antitumour antibiotic, exerts its effects primarily on spermatocytes (Adler et al., 1996). On the other hand, spermatids represent the germ cell type that is most sensitive to two anticancer agents, chlorambucil and triphosphamide, and finally spermatozoa are especially affected by acrylamide and ethylnitrosourea (Nomura, 1994). Depending on the stage at which male germ cells are exposed to a drug and the extent of damage incurred, different rates of pre-implantation loss, post-implantation loss, and congenital and behavioural malformations are found in their progeny (Russell, 1994).

Several studies have shown early embryonic loss subsequent to paternal exposure to various chemical agents. Exposure of male mice to a subchronic regimen of urethane resulted in embryonic loss at the peri-implantation stage of development (Edwards et al., 1999). Acrylamide was shown to cause a dose-

dependent increase of abnormalities as early as the pre-implantation stage among progeny of treated males. Abnormalities were manifested by growth retardation and blastomere lysis in murine blastocysts (Holland et al., 1999). Exposure of male rats to a low level of lead acetate caused a decrease in the protein synthetic capacity of progeny as early as the 2-cell stage of gestation (Gandley et al., 1999).

1.4.4.3 Repair of paternally-mediated insults by the zygote

An important hallmark of mammalian reproduction is the adaptive response to injury. This is illustrated by the robust ability to compensate for cell loss in the pre-implantation embryo; only three to four ICM cells are required to give rise to a healthy fetus (El-Shershaby & Hinchliffe, 1974). Mutagen-induced DNA damage appears to be a nonrandom process. Certain repeat DNA sequences selectively expressed in germ cells may be hotspots for toxicant-induced mutations due to the presence of hypersensitive chromatin segments associated with decondensation and transcriptional activity or the nature of their protein composition (Yunis et al., 1987; Elia & Bradley, 1992). If male germ cells are not repaired prior to fertilization, any damage incurred in their genomes should be repaired by the egg to ensure proper embryonic development (Generoso et al., 1979). Male germ cells targeted after the spermatids have recondensed their chromatin to a compacted configuration cannot repair damage until after fertilization (Sega et al., 1976).

In contrast to male germ cells, the repair capacity in females is present at all stages of germ cell maturation and well after fertilization (Pedersen & Brandriff, 1979). In the mouse, unscheduled DNA synthesis (UDS), an indication of repair, was observed starting at the pronuclear stage following irradiation with UV light (Pedersen & Brandiff, 1979). The presence of UDS after the exposure to damaging agents has been used to assess the ability of zygotes to repair DNA damage by the NER system.

X-ray-induced chromosomal damage in *Drosophila* spermatozoa was shown to be repaired when they enter the egg's cytoplasm (Pedersen & Brandriff, 1979). This observation was documented also in sea urchin eggs (Henshaw, 1932; Failla, 1962). Similarly, fertilized mouse eggs were capable of repairing damage induced in sperm and mature oocytes by X-rays (Matsuda et al., 1989a) as well as a combination of X-rays and mitomycin,(Matsuda et al., 1989 b). In a *Drosophila* study, the number of alkylation-induced mutations in male germ cells was used to assess the repair capacity in eggs. When treated males were mated to normal females, damaged sperm were successfully repaired following fertilization. However, a dramatic increase in mutations was reported in a dose-dependent manner when treated males were mated to females with a defect in their NER sytem (Vogel & Nivard, 1997). It is likely that all pathways detailed in section 1.3.6 have some role(s) in recognizing and repairing genomic aberrations in the pre-implantation embryo.

1.5 Cyclophosphamide as a male-mediated developmental toxicant

Cyclophosphamide was introduced for tumour therapy in 1958. Currently, cyclophosphamide is used as an anticancer and immunosuppressive drug. This alkylating agent is a commonly indicated drug therapy for a wide variety of human conditions (Brock, 1989); these include acute cancer treatment (non-Hodgkin's lymphoma, malignant lymphoma, and leukaemia), long term therapy for cancer, immune disorders (lupus, multiple sclerosis and Wegner's granulomatis), and organ transplants (bone marrow transplants) (Cavo et al., 1998a, b; DeRidder et al., 1998; Koldingsnes et al., 1998; Meistrich, 1998).

There is a particular concern for men who receive anticancer chemotherapeutics (Mulvihill, 1994). Cyclophosphamide is a mutagen (Anderson et al., 1995), carcinogen, and teratogen (Zemlickis et al., 1993). When administered to males, cyclophosphamide causes sister chromatid exchange (Abraham & Franz, 1983), translocations (Sotomayor & Cumming, 1989), micronucleus formation (Moreland et al., 1981; Pachierotti et al., 1983), DNA single strand breaks, and DNA-DNA cross-links in the germ cells (Skare & Schrotel, 1984; Qiu et al., 1995a). Previous studies have shown that chronic low dose administration of cyclophosphamide to male rats results in increased pre- and postimplantation loss and in growth-retarded and malformed fetuses, even though there were no apparent adverse effects on male reproduction (Trasler et al., 1986, 1987). Malformations (Hales et al., 1992) and behavioural abnormalities (Hsu et al., 1987) were seen also in the second generation.

1.5.1 Cyclophosphamide pharmacology

Cyclophosphamide is a prodrug which belongs to the nitrogen mustard family; it requires biotransformation to become a bifunctional alkylating agent which acts by covalently binding to DNA. Cyclophosphamide undergoes metabolic transformation in the liver, where it is oxidized by cytochrome P450 to 4-hydroxycyclophosphamide (Pacchieroti et al., 1983). Aldophosphamide can be oxidized to the inactive carboxycyclophosphamide by an aldehyde dehydrogenase, or be reduced to aldophosphamide by aldehyde reductase (Moore, 1991; Anderson et al., 1995).

In the cell, aldophosphamide spontaneously undergoes a β-elimination reaction to generate the toxic metabolites: acrolein and phosphoramide mustard (Moore, 1991; Anderson et al., 1995). While the biological function of cyclophosphamide is thought to be mediated by both active metabolites, their modes of action are distinct. Acrolein has been reported to bind to proteins, glutathione, and DNA (Alcaron & Meienhofer, 1971). Phosphoramide mustard, on the other hand, seems to be the primary cytotoxic metabolite with a short half-life (40 -50 minutes), acting by alkylating nucleophilic sites of the DNA, thus resulting in the formation of DNA adducts. This step is considered to be the critical chemotherapeutic action of cyclophosphamide as these effects can interfere with both transcription and replication in neoplastic cells (Fleming,1997). Cyclophosphamide is well absorbed orally and maximal concentrations in plasma are achieved 1 hour after ingestion. The half-life in plasma in humans is ~6.5 hours (Pacchieroti et al., 1983), and ~2 hours in animals (Lu & Meistrich, 1979).

Elimination of cyclophospamide, unlike its bioactivation, is a slow process. Cyclophosphamide is eliminated mostly as metabolites; very little unchanged cyclophosphamide is obtained in excrements.

1.5.2 Effects on male germ cells

One of the many consequences of cyclophosphamide treatment in men is altered male fertility (Fairley et al., 1972; Qureshi et al., 1972). Due to the nature of spermatogenesis, it is possible to extrapolate which cell type is affected by treating for specific intervals and following the sperm development through to maturity. Through a series of studies, the laboratories of Drs. Robaire and Hales' have assessed the effects of cyclophosphamide treatment on the different germ cell types (Trasler et al., 1985,1986, 1987). Chronic treatment of male rats with cyclophosphamide was found to cause germ cell stage-dependent effects on reproductive outcome. Studies have been performed to clarify how cyclophosphamide can affect the male germ cell; a chronic low-dose cyclophosphamide treatment did not affect the pituitary-testicular axis (Trasler et al., 1985), indicating that cyclophosphamide affects the male reproductive system at a local level, either via paracrine mechanisms or by affecting the testis or epididymis directly. The histology of the male reproductive system was evaluated after chronic administration of cyclophosphamide. While there were only transient effects on the male seminiferous epithelium, abnormal spermatozoa were seen in the lumen (Trasler et al, 1988). The effects of chronic cyclophosphamide treatment on the nucleus of spermatozoa have been examined (Qiu et al.,

1995b). Alkaline elution assays were performed to determine the extent of single strand breaks and DNA-DNA crosslinks in spermatozoa from the cauda epididymidis. One week of low dose cyclophosphamide exposure was found to increase single strand breaks but not DNA-DNA crosslinks, while chronic exposure (six weeks) to a low dose treatment of cyclophosphamide (6 mg/kg/day) increased both single strand breaks and DNA-DNA crosslinks (Qui et al., 1995 a). To test the ability of the spermatozoal DNA to be used as a template, tritiated thymidine incorporation was measured. Low dose drug exposure for six weeks, but not for one week, was found to impair the template function of sperm nuclei (Qiu et al., 1995b).

1.5.3 Male-mediated effect of cyclophosphamide on progeny outcome

The effects of chronic treatment of cyclophosphamide on fertility, pregnancy outcome, and progeny were assessed in male rats (Trasler et al., 1985). Paternal cyclophosphamide treatment was found to cause dose and time dependant fetal loss without affecting male fertility. Chronic treatment of rats with cyclophosphamide resulted in offspring with an increased incidence of external malformations such as dwarfism, hydrocephaly, edema, micrognathia, open eyelids and tail anomalies when progeny were sired by germ cells first exposed as spermatogonia. Pre-implantation loss, which may indicate a failure of fertilization or gross chromosomal abnormalities, was observed among progeny sired by germ cells first exposed to cyclophosphamide as spermatocytes or early spermatids. Zygotes fertilized by germ cells first exposed to cyclophosphamide as

spermatids resulted in greater than 80% post-implantation loss (Trasler et al., 1985); fertilization with spermatozoa first exposed in the epididymis resulted in 20% post-implantation loss. Effects were seen in the developing embryo as early as day 2 of gestation (equivalent to the 8-cell stage); these included a decrease in cell number and in DNA synthesis (Austin et al., 1994). Thus, cyclophosphamide is a male-mediated developmental toxicant which has dose-dependent, time-dependent, and stage-specific effects on male germ cells. Furthermore, the effect of male treatment is heritable, as there was a significant increase in malformed offspring in the F_2 generation (Hales et al., 1992).

1.6 Formulation of the project

1.6.1 Hypothesis

Previous results have shown that chronic treatment (4-6 weeks) of male rats with a low dose of the anti cancer agent, cyclophosphamide (6 mg/kg/day), produced a time-dependent and a dose-related early embryonic loss with a minimum effect on the endocrine status and the general health of the male. The earliest effect was seen among progeny of treated males starting at the 8-cell stage. Based on these results, I hypothesized that **in progeny sired by male rats treated with cyclophosphamide, the zygotic gene activation program is altered, thus leading to embryonic loss**.

1.6.2 Rationale

The chronic low dose treatment protocol used in the studies reported in this thesis was based on comparable human doses used for maintenance chemotherapy (1-2 mg/kg/day) (Livingston & Carter, 1970). Previous results have shown that treatment of male Sprague-Dawley rats with a regimen of 6 mg/kg/day of cyclophosphamide did not affect the survival or fertility of the males, but provided significant effects on progeny outcome (Trasler et al., 1987). When males subjected to this treatment regimen for 4-5 weeks were mated to normal females, a maximal post-implantation loss was seen among the progeny following implantation of the embryos to the mother's uterus, around day 6.5 of gestation (Kelly et al., 1992). Thus, developmental events occurring around, and possibly prior to, implantation must have undergone some major alterations, resulting in a high number of absorptions. Indeed, this was the case since embryos become laggard in their proliferation as manifested by a reduced cell number and DNA synthesis capacity (Austin et al., 1994). Therefore, this chronic low dose treatment regimen was chosen in order to isolate the earliest effects triggering peri-implantation death.

In the first objective of this thesis we sought to determine the extent of damage incurred upon the DNA of 1-cell embryos as a result of paternal treatment with cyclophosphamide. Using the Comet assay, or the single cell gel electrophoresis, which allows for the detection of DNA damage in single embryos, we assessed the extent of damage each sperm has imparted to the fertilized oocyte. The exposure of male germ cells to cyclophosphamide for 4-5 weeks

affects these cells starting at the spermatid stage. The chromatin structure of germ cells at this stage is highly condensed with a unique composition due to the presence of protamines, rendering the nuclei highly compacted and incapable of repairing any insult incurred on them. It is only following fertilization that sperm DNA can undergo decompaction and may be repaired inside the egg's cytoplasm, if damage is detected. Thus, we aimed to study the presence of major DNA repair pathway components in early embryos sired by control- and cyclophosphamide-treated males. To address this objective, I have adapted an situ transcription/ antisense RNA (IST/aRNA) candidate gene approach to profile the expression pattern of a number of genes whose primary role inside the cell is to repair damage, as demonstrated from studies conducted with somatic cells.

Previous data from our laboratories have indicated that the *in vitro* decondensation pattern of treated sperm is altered after cyclophosphamide treatment (Qiu et al., 1995a). We determined the time course of sperm decondensation in males treated with cyclophosphamide compared to controls. Previous reports have indicated that normal mouse sperm pronuclei become transcriptionally active prior to female pronuclei (Aoki et al., 1997). Thus, we measured the capacity of early embryos sired by control and treated sperm to synthesize mRNA transcripts by incorporating bromouridine triphosphate (BrUTP). Acquisition of a transcriptionally permissive state in the male pronuclei has been attributed to the presence of transcription factors such as Sp1. Therefore, we assessed the presence of Sp1 in embryos sired by cyclophosphamide treated males by immunofluorescence. To determine the

effect of paternal treatment on the cell division program of the zygote, we collected embryos at the 1-cell stage and cultured them in the absence or presence of an inhibitor of transcription, α-amanitin. Following an 18 hour incubation period, the percentage of embryos that underwent cell division to become 2-cell was recorded. Pre-implantation mouse embryos become capable of synthesizing all types of RNA starting at the 2-cell stage (Clegg & Piko, 1983 b). No data existed for the total RNA synthesis profile in rat embryos. Therefore, we measured the ability of pre-implantation rat embryos sired by control and drug-treated males to synthesize total RNA. To do so, we isolated pools of embryos at the 1-, 2-, 4- and 8-cell stages sired by control and drug-treated males and incubated them in the presence of ^{32}P-UTP. Total RNA was isolated and the radioactivity was measured by liquid scintillation counting. The 2-cell embryo contains two diploid zygotic nuclei, each with a set of paternal and a set of maternal chromosomes. However, parental genomes are not equivalent in their contributions to the zygote. A number of genes are expressed exclusively from one parental allele and are referred to as imprinted (Bartolomei & Tilghman, 1998). In our model, only the paternal genome is damaged by cyclophosphamide treatment. Thus, we sought to determine the effect of this treatment on the expression profile of a number of imprinted genes in the pre-implantation rat embryo (1-, 2-, 4- & 8-cell stages) using the IST/aRNA approach.

In the third objective, we analyzed the effect of paternal treatment on blastomere cell interactions of the early embryo. To address this objective, Epon-embedded embryo sections were analyzed for cell number and blastomere

interactions on day 2 of gestation (equivalent to the 8-cell stage) using light microscopy. In addition, the gene expression profiles of key cell interaction elements were assessed at the mRNA level using the aRNA approach and at the protein level using immunofluorescence of embryos at the 2-, 4-,and 8-cell stages of development.

Taken together, the purpose of this dissertation is to provide answers on the normal progression of developmental events at both the molecular and structural levels in the rat embryo. Furthermore, altered pre-implantation processes due to paternal exposure to cyclophosphamide will be explored.

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1.8 Appendix: Validation of aRNA data

A number of methodological approaches have been described to analyze the relative abundance of mRNA species in total cellular RNA. In particular, the task of identification of mRNAs from a small amount of tissue, such as the pre-implantation embryo, was daunting due to the paucity of starting material and the limit of detection for low abundance mRNAs. The powerful approach of in situ transcription/antisense RNA (aRNA) coupled with reverse Northern blotting was adopted in this thesis to profile the expression of mRNA populations in the rat pre-implantation embryo. The purpose of this appendix is to provide a comprehensive review of methods used to validate data obtained using this approach.

In their pioneer work, Eberwine and colleagues (Tecott et al., 1988) outlined the details of the validation process. The first issue to be established was the specificity of the amplification process. To this end, an oligonucleotide (46 nucleotides in length) complementary to the encoding amino acid sequence for the gene of interest was used as a primer in the initial reverse transcription process. Following amplification, the denatured transcripts were hybridized to single stranded cDNAs of the same gene of interest which had been immobilized onto nitrocellulose filters. Following rigorous washing, the hybridized transcripts were eluted from the filter and separated by electrophoresis on a polyacrylamide-urea gel. The banding patterns before and after hybridizations were clearly similar in their migration profile. Non-hybridizing transcripts failed to produce

bands following hybridization. This experiment established the bands as sequences complementary to the candidate gene cDNA (Tecott et al., 1988).

Other characteristics of the aRNA protocol including the length of the oligo-d(T)primer, the enzyme concentration, and the incubation time were established by the original investigators (Van Gelder et al., 1990). Optimal conditions were obtained when the primer was an oligo d(T)-T7 primer with a length of 57 nucleotides, containing 24 thymidine residues that hybridized to polyadenylated mRNA molecules. The concentration of the enzyme T7 RNA polymerase which was used originally was 100 units/μl but this was optimized later to 1000 units/μl. The amplification process is optimal when carried out at 37°C for 4 hours.

The ability of T7 RNA polymerase to produce transcripts of large size is assured by the fact that the 5' promoter sequence is specific for T7 RNA polymerase; this RNA polymerase is capable of amplifying transcripts up to 7 kb in length. Thus, the aRNA produced using this enzyme is expected to accurately represent the size complexity of the synthesized cDNA. To prove this, a comparison was done of the size distribution of cDNA synthesized from total RNA amplified using the 57 nucleotide primer, thus producing the aRNA sample, and that of a non-labeled portion of the same cDNA amplified with T7 polymerase. That the size distribution of the cDNAs and the aRNAs is very similar was shown by size migration on gel electrophoresis using Northern and Southern blot analyses (Van Gelder et al., 1990).

The aRNA bound to a wide range of sequences, demonstrating the sequence heterogeneity in the amplified sample (Van Gelder et al., 1990). A similar pattern of gel migration was also obtained when total RNA was compared to the aRNA products, suggesting that aRNA abundance is representative of the parent cDNA (Van Gelder et al., 1990). Together, these experiments demonstrated that the array of amplified aRNA qualitatively reflects the population of parent cDNAs. Assuming that the relative amounts of individual sequences present in cDNA approximate their relative abundance in the transcribed RNA population, the amount of specific RNA within an aRNA sample should, therefore, reflect its abundance in the original RNA population of the sample being assayed (Eberwine et al., 1992). A simple approach to determine the size distribution of aRNA products is to run a portion of the amplified aRNA sample on a denaturing gel. Typical size distribution of aRNA samples range from 1 Kb to 3.5 Kb (Vacha & Finnell, 1996).

Once amplified, aRNA samples are used to document, simultaneously, the levels of multiple mRNA populations within the same sample. This can be accomplished by combining the aRNA with reverse Northern blot analysis, thus allowing the investigator to measure the relative levels of multiple co-existing mRNAs by using the aRNA as a probe to identify specific cDNAs loaded on a slot blot. To validate this combination, a number of issues were addressed: 1) the amplification efficiency and the sensitivity of detection, 2) the specificity of the 3' amplified products, and 3) the cross-hybridizations of homologous mRNAs with the gene of interest. Using a poly(A) RNA internal standard, detection of 10

copies of the poly(A)RNA was obtained, thus establishing the validity of the amplification process (Madison & Robinson, 1998).

A final important issue in validating the aRNA approach is the linearity of the aRNA amplification. aRNA amplification is linear due to the fact that each original mRNA molecule is converted to one cDNA using the enzyme T7 polymerase, thus permitting quantitative assessment on endogenous mRNA levels (Vach & Finnell, 1996).

Adopting the aRNA method for the rat pre-implantation embryo

When the protocol of aRNA amplification was adopted for the assessment of gene expression profiles in pre-implantation stage embryos, pilot experiments were conducted to assess the success of the amplification procedure, as follows. The first objective was to compare results obtained when embryos had been stripped of their *zonae pellucidae* to those in which the *zonae pellucidae* was intact. Our data showed no difference in the amplification between the two groups (data not shown). Therefore, embryos collected for aRNA amplification were systemically processed without removal of the zonae pellucidae.

In order to ensure the integrity of mRNA within a sample, we compared groups of embryos in which an RNase inhibitor was added to each sample following collection to embryos that were stored in the absence of RNase inhibitor. Both groups of embryos were frozen on liquid N_2 or dry ice and kept at -80°C. Amplification of both groups gave similar results.

The next objective was to establish that the aRNA amplification is successful using small numbers of embryos. Pilot studies were done in which 40-70 embryos were pooled for each experiment on a per male basis for each stage and treatment (data not shown). In the next set of experiments embryos from each litter were amplified separately on a per male basis for each stage and treatment. Data collected for Chapter 2 and 3 were obtained using this method. The final group of results (as shown in Chapter 1) were obtained from single embryos. Together, we have shown that aRNA amplification is sensitive to very few cells of the pre-implantation stage and can be used in single embryos at the 1-cell stage. Our data corroborate with the earlier work of Eberwine and colleagues when they demonstrated the power of using the aRNA protocol to detect mRNA populations from single neurons (Eberwine et al., 1992).

Amplification success was monitored by trichloroacetic acid (TCA) incorporation; By comparing the radioactive label immediately after addition of radiolabeled ^{32}P and at the end of the amplification step (4 hours later), one is able to determine the radioactivity incorporated into the amplified products. This step was done on a regular basis for each experiment. Only samples that showed a 4-fold minimal increase of incorporation of radioactivity were used as probes to screen slot blot containing known quantities of cDNAs for the genes of interest.

Following the amplification and hybridization steps, the relative intensities of the hybridization of the riboprobe to the cDNAs on the slot blot were determined by scanning the radioactive signals on phosphorimager plates. The phosphorescence intensity on the plate (the raw data) were transferred from the

phosphorimager data analysis package into an excel sheet. The background was subtracted from all numbers to be analyzed by measuring a square on the blot of the size used for the candidate gene of interest and a region on the blot where no genes were immobilized; this was considered the hybridization noise and was used to eliminate experiment-to-experiment variability due to the half life of the radioactive label or the hybridization stringency. Fresh radiolabeled ^{32}P (3,000 Ci/mmol) was used within the first half-life of the products (t1/2 =14.3 days, NEN Life Science products) to ensure a consistent specific activity usage of the radioactive material. Hybridization consistency was ensured by using freshly prepared solutions and by keeping the wash times constant throughout the studies.

Choice of the internal standard

Pilot studies were conducted to monitor the gene expression profile of a wide array of candidate genes based either on an established or a possible role in pre-implantation development. Data generated from these studies were analyzed for the most consistent gene expression profile of a number of genes. These genes were considered consistent in expression if their expression profiles stayed constant upon comparison of embryonic stages and paternal treatments. Consistency was further established by running multiple statistical comparisons among constant and variable profiles. As a result, all subsequent blots were designed with three consistently expressed genes, referred to in the thesis as internal standards, blotted along with other genes under investigation.

1.8.1 References

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Chapter 2

Paternal exposure to cyclophosphamide induces DNA damage and alters the expression of DNA repair genes in the rat preimplantation embryo.

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Abbreviations

APE/Ref-1, apurinic endonuclease/redox factor-1; BER, base excision repair; CPA, cyclophosphamide; DNMT-1, DNA methyltransferse-1; MMR, mismatch repair; NER, nucleotide excision repair; IST/aRNA, in situ transcription/antisense RNA; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PVP, polyvinylpyrrolidone; RAD50, radiation sensitivity 50; RCR, recombination repair; UNG1, uracil DNA glycosylase 1; XPC, xeroderma pigmentosum complementation group C; XRCC1, X-ray repair cross-complementing gene 1.

Abstract

Chronic low dose treatment of male rats with cyclophosphamide (CPA), an anticancer alkylating agent, damages male germ cells, resulting in greater than 80% peri-implantation progeny loss. Little transcription or repair takes place in the DNA of post-meiotic male germ cells. The spermatozoal genome regains its transcriptional capacity in the fertilized oocyte. We hypothesized that: 1) DNA damage of the male genome as a consequence of exposure of male rats to CPA is transmitted to the newly fertilized egg, and 2) this damage leads to an altered DNA repair gene expression profile in the preimplantation embryo. Male rats were treated with either saline or CPA (6 mg/kg/day, 4-5 weeks) and mated to control females; embryos were collected at the 1-8 cell stages. The alkaline comet assay was used to assess DNA damage in 1-cell embryos. A significantly higher percentage (68%) of the embryos fertilized by CPA-exposed spermatozoa displayed a comet indicative of DNA damage compared to embryos sired by control males (18%). The IST/aRNA approach was used to determine if DNA damage alters the expression of DNA repair genes in the early embryo. Dramatic increases in the transcripts for selected members of the NER family (XPC, XPE, and PCNA), MMR family (PMS1) , and RCR family (RAD50) were witnessed at the 1-cell stage in embryos sired by CPA-treated males compared to control embryos, while decreases in the expression of BER family members (UNG1 and UNG2) and other RCR transcripts (XRCC1 and RAD54) were observed. In contrast, at the 2-cell stage there was 15-fold decrease in XPC and a dramatic increase in a MMR family transcript, PMS2, among the embryos sired by drug-treated males. Relatively smaller changes in the concentrations of transcripts

from all four families of DNA repair pathways were observed at the 4-cell stage. At the 8-cell stage, XPC (NER family) and MSH2 and PMS2 (MMR family) transcript levels in control embryos were elevated greatly above those in embryos sired by drug-treated males; in contrast, XPE and PCNA (NER family) as well as UNG1 (BER family) transcripts were elevated in embryos sired by drug-treated males. Therefore DNA damage incurred in spermatozoal DNA following CPA exposure is associated with alterations in the expression profiles of DNA repair genes in preimplantation embryos as early as the 1-cell stage of development. Genotoxic stress may disturb the nuclear remodeling and reprogramming events that follow fertilization and precede zygotic genome activation. The ability of the early embryo to respond to genotoxic stress may be pivotal in determining embryo fate.

Introduction

The integrity of cellular DNA is challenged constantly by endogenous and exogenous agents, resulting in an estimated 1-3 x 10^4 spontaneous lesions per cell per day [1]. DNA damage can be summarized into three broad categories: a) covalent alterations to bases, covalent joining of adjacent bases to form dimers, and base loss, b) DNA crosslinking, and c) DNA strand breaks. These various forms of damage can alter normal cellular pathways and critically affect the viability of a cell [2]. Cellular processes that can occur, singly or in combination, in an attempt to deal with the genotoxic insult include direct DNA repair of the lesion, induction of gene transcription, cell cycle arrest, or cell death [2]. To cope with DNA damage, multiple DNA repair response pathways have evolved [3].

Four major DNA repair pathways exist in mammalian cells [4]: the nucleotide excision repair (NER) pathway, which repairs the majority of DNA damage in cells [5]; the mismatch repair (MMR) pathway, which repairs mispairing errors that occur during DNA replication [6]; the base excision repair (BER) pathway, which repairs alkylation and oxidative damage [4]; and the recombination repair (RCR) pathway, which repairs strand breaks [7]. Data available to date on the mammalian repair systems have been obtained mainly in mutant rodent cell lines defective in DNA repair [8], and in patients with repair deficiency syndromes such as Xeroderma Pigmentosum and Cockayne's syndrome [9]. The deletion of several specific DNA repair genes in null mutation transgenic mice may result in embryonic lethality, which may be manifested very early during development [10], or in reduced life span

[8]. Other abnormalities obtained as a result of the deletion of specific DNA repair genes include increased susceptibility to tumors, infertility, and growth defects. Together, these data demonstrate the importance of DNA repair genes during normal development as well as in response to exposure to genotoxic agents.

The range of deleterious effects obtained when specific DNA repair genes are eliminated can be interpreted in light of their involvement in other cellular pathways besides their role in protecting cellular DNA from genotoxic agents. For example, APE/Ref-1, a BER enzyme, is involved in responding to changes in cellular redox status, and can activate several transcription factors such as Fos and Jun [11]. The NER enzymes, XPB and XPD, play additional roles in overall DNA transcription [12]. Thus, it may not be surprising that deletion of any of these three genes results in early embryonic loss [10]. Lastly, several MMR enzymes are involved in meiotic recombination [13]; deletion of PMS2, a MMR gene, results in abnormal chromosome synapsis during meiosis in male mice, rendering them infertile and prone to sarcomas and lymphomas [14].

Genotoxic insult to the early preimplantation embryo could be the result of targeting the maternal or paternal genomes, or the embryo itself. If insults are not repaired, the normal development of the conceptus is compromised. While there is evidence that post-meiotic male germ cells lose the ability to repair their DNA [15], the oocyte is equipped with DNA repair mechanisms throughout germ cell maturation and well after fertilization [16]. The capacity of the zygote to repair damaged paternal DNA has been examined previously [17] by assessing dominant-

lethal mutations [18], unscheduled DNA synthesis (UDS) [19], and the formation of chromosomal aberrations [20].

Alkylating agents affect the mammalian genome by forming DNA lesions and thus causing base substitution mutations or preventing DNA replication [21]. CPA is an anticancer alkylating prodrug which requires bioactivation to initiate its damaging effects. Active metabolites of CPA, phosphoramide mustard and acrolein, induce DNA adducts, single strand breaks, crosslinks, sister chromatid exchange, and chromosomal aberrations [22]. Activated CPA is teratogenic to postimplantation embryos [23] and to limb buds in culture [24]. Exposure of male germ cells to CPA leads to single strand breaks, crosslinks and an altered decondensation pattern of sperm and template activity in vitro [25, 26]. Although no effects on the reproductive system or sexual behavior were detected, increases in embryo deaths and in offspring with malformations have been documented among the progeny of treated males [27-30]. In particular, when male rats were treated with a chronic low dose regimen of CPA for 4-6 weeks, exposing post-meiotic male germ cells to the drug, significant increases in peri-implantation embryonic loss were observed [31]. Preimplantation embryos sired by drug-treated males showed a number of abnormalities, including a decrease in cell proliferation, a laggard cell division pattern, and a reduction in blastomere number and cell-cell contacts when compared to their control cohorts [31,32]. Furthermore, paternal drug exposure temporally and spatially dysregulated rat zygotic gene activation, altering the developmental clock as early as the 2-cell stage [33].

We hypothesize that there is an increase in DNA damage in the zygotes sired by males treated with a chronic low dose of CPA, and that paternal genomic DNA damage disrupts the programming of gene activation in the zygote. To test this hypothesis, we have used the single cell Comet assay to assess whether DNA damage is present in the zygote following paternal exposure to CPA. Using aRNA amplification we have studied the effect of such a genotoxic insult on the expression of DNA repair genes in the early embryo.

Materials and Methods

Animals

Adult male (300-315 g) and virgin female (225-250 g) Sprague-Dawley rats were obtained from Charles River Canada (St. Constant, Quebec) and housed in the McIntyre Animal Center. Food and water were provided ad libitum and animals were exposed to a 14h-light:10h-dark cycle.

Treatment, mating, and embryo collection

Male rats were randomly divided into control and CPA groups consisting of 10 rats each. Males were gavaged daily with saline or 6 mg/kg/day CPA (all chemicals were purchased from Sigma-Aldrich, St Louis, MO unless otherwise noted) for 4-5 weeks. Starting on the fifth week of treatment, each male was mated overnight with two females in proestrus. To rule out the effects of the presence of CPA in the seminal fluid, males were not gavaged on the night of mating [34]. The following morning (considered as day 0 of gestation), the presence of sperm in

vaginal smears confirmed pregnancy in mated females. To collect 1-cell embryos, pregnant females were killed on day 0 and the *ampullae* of oviducts were punctured using small forceps. Cumulus cells were removed in the presence of a medium containing 1% hyaluronidase. Embryos were either prepared for the comet assay, as described below, or snap frozen for analysis by aRNA gene expression profiling. Litters at the 2-, 4- and 8-cell stages were obtained by flushing oviducts of pregnant dams on days 1, 1.5, and 2 of gestation, snap frozen and stored at -80°C until used.

Detection of damage in individual cells using the comet assay

DNA damage was assessed using the Comet assay in embryos sired by three control and three CPA-treated males (from 24-29 embryos were analyzed pre male). The comet assay was performed as previously described with minor modifications [35]; all products for the Comet Assay were obtained from Trevigen, Inc. (Gaithersburg, MD). Following collection, embryos were washed in a warm solution of 1X phosphate-buffered saline (PBS), pH 7.4 (Mg^{2+}- and Ca^{2+}-free) containing 4 µg/ml polyvinylpyrrolidone (PVP). *Zonae pellucidae* were removed in a warm PBS/PVP solution (pH 2.5) in order to displace the polar bodies and allow DNA migration [36]. Once *zonae pellucidae* dissolved, the embryos were immediately transferred to fresh PBS-PVP medium. *Zonae*-free embryos were suspended in a 5µl drop of PBS-PVP, placed directly onto the slides, and covered with 55 µl of 0.25% molten (42^0C) low melting point agarose (dissolved in 1X PBS). A pipette tip was used to spread the agarose. The gel was allowed to solidify at 4^0C

in the dark for 10 minutes until a 0.5 mm clear ring appeared at the edge of the sample area. All subsequent steps were carried out under yellow light to prevent any additional DNA damage. Embryos were lysed to isolate the nucleus by immersing slides for 2 hours at 4°C in fresh, ice cold lysis solution (2.5% SDS, 0.025M EDTA, 1% N-lauroylsarcosine, 1% DMSO, pH 9.5) (Gibco BRL, Burlington, ON) [36]. Slides were removed from the lysis solution, drained and placed in 50 ml of freshly prepared alkaline solution (0.6g NaOH, 250µl EDTA , 200 mM , pH10.0) for 40 minutes at room temperature to unwind and denature the DNA for analysis of all breaks. They were then drained and washed twice in 1X Tris-buffered EDTA (TBE) solution for 5 minutes, placed flat side by side and equidistant from the electrodes on a gel tray submerged inTBE in a horizontal gel electrophoresis tank, allowing migration of DNA out of the nucleus. The level of 1X TBE was 2-4 mm above the slides. Electrophoresis was carried out at 23 V for 5 minutes. The slides were then drained and fixed in an ice cold methanol followed by an ice cold ethanol fixation step, each lasting for 5 minutes, and left to air dry at room temperature. Slides were stored in a desiccator at room temperature. Alternatively, slides were incubated with SYBR Green solution (4 µl 10,000X SYBR Green in 10ml TE, pH7.5) to stain the DNA and analyzed immediately using a Leica Wild MSP48 camera attached to a Wild-Leitz Laborlux D fluorescence microscope. Photographs of the comets were scanned to create digital computer images which were subsequently analyzed using the MCID-M4 image analysis program (Version 4.0, revision 1.5, Image Research Inc., St. Catharines, ON). Cells with undamaged DNA appear as intact comet heads without tails. A damaged cell has the appearance of a comet with a head (nucleus)

and tail of DNA fragments. Comet head and tail fluorescence intensity values representative of DNA content were scored. The tail/head ratio of DNA content and tail length were calculated as measures of DNA damage.

Gene expression profiles using the in situ transcription /amplified antisense amplification (IST/aRNA) procedure

Gene expression profiles were analyzed using the antisense RNA amplification approach (IST/ aRNA). The protocol for aRNA amplification in preimplantation embryos was as described previously with minor changes [37]. Individual embryos were sonicated in the presence of a lysis buffer containing 1 mg/ml digitonin, 5 mM dithiothreitol, and 1X In Situ Transcription buffer (50 mM Tris (pH 8.3), 6 mM $MgCl_2$, 0.12 mM KCl). Embryonic homogenates were reverse-transcribed at 37°C for two hours in the presence of an oligo dT-T7 primer which recognizes the poly(A) tail of mRNA populations. This in situ transcription of single-stranded cDNAs was allowed to self-prime (by hairpin loop formation) to form complementary double stranded DNA. Following excision of the self-primed hairpin loop by S1 nuclease, the double stranded cDNA was blunt ended and filled-in by T4 DNA polymerase. Double stranded cDNAs were amplified into antisense RNA in the presence of T7 RNA polymerase and ^{32}P-radiolabeled-CTP for 4 hours at 37°C.

Radiolabeled amplified RNA populations obtained from individual embryos at the 1-, 2-, 4- and 8-cell stages were utilized as probes to screen slot blots containing equimolar concentrations of cloned cDNAs for the following genes; among the NER genes we used XPC, XPD, XPE, and PCNA. From the BER family,

APE/ref-1, UNG1, and UNG2 were examined. Among the RCR genes XRCC1, RAD50, RAD54, and RAD57 were used. The MMR genes that were used were MLH1, MSH2, PMS1, and PMS2. Individual blots were pre-hybridized for 30 minutes in buffer (50% formamide, 0.12 M Na_2HPO_4, pH 7.2, 0.25 M NaCl, 7% w/v SDS) at 42°C. The heat denatured aRNA probes from each embryo sample were applied to the blots and allowed to hybridize overnight at 42°C. The following day, blots were washed at decreasing stringency from 2X sodium chloride citrate (SSC) solution down to 0.1% SSC and 0.1% SDS at 42°C for 20 minutes, each in a water bath shaker. Blots were exposed to phosphorimager plates overnight. Individual band intensities were determined after exposing the plates to a phosphorimager scanner (Molecular Dynamics, Sunnyvale, CA). Individual signals were corrected for nonspecific binding by subtracting the background values on each blot, followed by normalization of all data points to an internal standard. DNA methyltransferse-1 (DNMT-1) was chosen as the internal standard based on its consistent expression between groups (control and drug-treated) and across blots, thus facilitating experiment to experiment and blot to blot quantification. A total of 6-8 expression profiles representing individual embryos sired by different males were obtained for each stage and treatment group.

Statistical analysis

To assess the damage incurred upon the embryo by paternal exposure to CPA, we compared the presence of Comets among populations of 1-cell embryos sired by saline- or CPA-treated males using Chi-Square analysis with or without

Yate's correction. To determine the effect of paternal treatment on gene expression in the progeny, a pair-wise comparison for each developmental stage was conducted between embryos fathered by control and CPA-treated males consisting of a t -test followed by a Tukey's post-hoc test- ($p<0.05$). All statistical analyses were performed with the Sigmastat 2.3 software package (SPSS Inc., Chicago, IL).

RESULTS

DNA damage in the zygote following paternal exposure to CPA

Using the comet assay we assessed the extent of damage imparted to individual embryos at the 1-cell stage of development after fertilization with CPA-exposed spermatozoa. An embryo fertilized by a control spermatozoon with a "head" and no "tail" or comet is depicted in Fig.1A. An increase in DNA strand breaks leads to greater DNA migration out of the nucleus and into the tail of the comet. There was considerable variation among the embryos sired by drug-treated males with respect to the intensity of the head and the length and intensity of the comet tail (Fig. 1B-1F).

A significantly higher percentage of the embryos fertilized by treated spermatozoa (68%) displayed a comet indicative of DNA damage (defined as a tail/head ratio of DNA content greater than 0.02) compared to embryos sired by control males (18%). Tail/head ratios among damaged embryos sired by drug-treated males ranged from 0.06 to 1.9. The percentage of embryos ranked in designated tail/head ratio categories is shown in Fig. 2A. No DNA damage (defined as a tail/head ratio of 0-0.02) was observed among >80% of embryos sired by

control males but only a third of embryos from the CPA-sired group. Tail/head ratio values between 0.02 and 1.0, indicative of DNA damage, were found among 37% of the embryos sired by CPA-treated males, but only among 13% of control embryos. While a significant percentage of 1-cell embryos sired by CPA-treated males had tail/ head ratios in the range of 0.02 to 0.5, tail/ head ratios in the range of 0.5 to 1.0 were found in 16% of embryos sired by drug-treated males. Almost 15% of embryos from the CPA-sired group had tail/head ratios greater than 1.0, suggesting extensive DNA fragmentation (Fig. 2A); less than 5% of the embryos from the saline-sired group had tail/head ratios in this range.

DNA fragments migrated a distance 1 to 3 times their nuclear diameter (~27 μm), resulting in tail lengths varying from 62 μm to 173 μm. Significant differences in comet tail lengths were observed between embryos sired by saline-treated males and those sired by CPA-treated males (Fig. 2B).

Expression of DNA repair genes in early embryos sired by control and CPA-treated males

NER family gene expression

In 1-cell control embryos, the expression of XPC, XPE, XPF, and PCNA was barely detectable. In contrast, XPD was highly expressed (Fig. 3 A). By the 2-cell stage, steady state concentrations of XPC, XPE, and PCNA were increased dramatically; only XPF remained low. Embryos at the 4-cell stage expressed low

levels of XPC and XPF; all NER genes with the exception of XPF showed abundant transcript levels by the 8-cell stage in control embryos (Fig. 3 A-D, grey bars).

Embryos sired by CPA-treated males exhibited a different profile of expression of NER family genes compared to controls. Transcripts found only in low levels in the control 1-cell stage embryo, XPC, XPE, and PCNA, were present at high concentrations in embryos sired by CPA-treated males. In contrast, concentrations of the transcript for another NER family gene, XPF, remained low. At the 2- cell stage a dramatic decrease in the expression of XPC was observed among the progeny of CPA-exposed males; XPC transcripts were at the limit of detection in 4-cell and 8-cell stage embryos sired by drug-treated males, but expresssed abundantly in control embryos at the 8-cell stage. Interestingly, transcripts for XPD, XPE and PCNA remained abundant in embryos from both treatment groups from the 2-cell stage on (Fig. 3 A-D, black bars).

BER gene expression

The expression profiles of three BER family genes, APE/Ref-1 and uracil-DNA glycosylases, UNG1 and 2, were assessed (Fig. 4 A-D, grey bars). Transcript levels for APE/Ref-1 were low at all stages in embryos sired by control and CPA-treated males. In contrast, UNG1 and 2 were consistently expressed at abundant levels up to the 4-cell stage. The expression of UNG1 was dramatically decreased at the 8-cell stage.

A significant decrease in the steady state concentrations of the transcripts for both UNG1 and UNG2 was observed at the 1-cell stage in embryos sired by

CPA-treated males compared to their control counterpoints; no difference was observed at the 2-cell stage and a small increase was found in 4-cell stage embryos. Interestingly, UNG1 expression was significantly higher among the progeny of drug-treated males at the 8-cell stage, while UNG2 expression was not altered among the embryos sired by CPA-treated males at this stage (Fig. 4 A-D, black bars).

MMR gene expression

Transcripts for MLH1 were below the limit of detection in both embryonic groups at all stages examined (Fig. 5 A-D); in contrast, expression of MSH2, were low at the 1-cell stage and increased to peak at the 8-cell stage. PMS1 expression was low at the 1-cell stage in control embryos, increased at the 2-cell stage, and persisted at subsequent stages. Like MSH2, PMS2 expression was not detected prior to the 8-cell stage in control litters.

One MMR family transcript, PMS1, displayed a dramatic increase in expression at the 1-cell stage in embryos sired by CPA-treated males compared to controls (Fig. 5A). PMS1 continued to be expressed at all subsequent stages in embryos from both treatment groups. PMS2 showed an exclusive peak of expression among litters of treated males at the 2-cell stage (Fig. 5 B). At the 8-cell stage, when MSH2, PMS1 and PMS2 transcript were abundant in control embryos, MSH2 and PMS2 transcripts at the limit of detection in embryos sired by CPA-treated males (Fig. 5D).

RCR gene expression

In control embryos, XRCC1 was expressed at the 1-cell stage and decreased in subsequent stages (Fig. 6 A-D, grey bars). Among the RAD genes, only RAD54 was highly expressed at the 1-cell stage among control litters; RAD54 remained abundant in expression at subsequent stages. Steady state concentrations of the transcripts for RAD50 and RAD57 were low embryos sired by control males at all stages examined.

Among the embryos sired by CPA-treated males, transcripts for XRCC1 and RAD54 were significantly lower than in their control 1-cell counterparts, while RAD50 expression was significantly elevated. Only relatively smaller differences in the expression profile of RCR genes were observed between embryos sired by control and CPA-treated males subsequently, at the 2-cell, 4-cell or 8-cell stages (Fig. 6 A-D, black bars).

Discussion

The comet assay is a highly sensitive method for detecting DNA damage in single cells [38]. Data obtained with this assay revealed that the extent of damage was significantly greater in 1-cell embryos sired by CPA-treated males compared to those sired by control males. At low damage levels, migration of individual pieces of DNA is unlikely to occur. Instead, supercoiled DNA is relaxed and stretching of attached strands occurs to form a small tail [38]. Increasing the number of breaks would allow DNA pieces to migrate freely, thereby creating a

long tail of greater DNA content compared to the head. Therefore, the size of migrating DNA is reflected in the tail length, whereas the number of relaxed or broken fragments of DNA is represented by the intensity of DNA in the tail. Our data suggest that the degree of damage varied among litters sired by CPA-treated males, as indicated by the range of tail/head ratios of DNA content and tail length values. The percentage of DNA found in the majority of comet tails was not high; the possible explanations for this finding are that: 1) few DNA breaks were present, therefore low levels of DNA damage resulted in stretching of relaxed DNA and not the migration of DNA fragments; 2) CPA caused an increase in DNA-DNA cross links in spermatozoal DNA, preventing DNA migration; and/or 3) an increase in DNA breaks and DNA-DNA cross links occurred, therefore the intensity of the comet tails was lower than the intensity of the heads. Alkaline elution studies demonstrated that chronic CPA exposure induced a significant increase in single strand breaks and DNA-DNA cross-links in rat spermatozoal nuclei [26].

The DNA damage induced in male germ cells is transmitted to the fertilized egg. It may be that the DNA repair capacity of the fertilized egg is what determines the fate of the conceptus. Our results indicate that in normal 1-cell embryos, the expression of many important DNA repair genes is limited. There were members of each of the four DNA repair pathways which were expressed only at or below the level of detection in 1-cell stage embryos. This observation suggests that the 1-cell embryo is completely dependent on maternal proteins for DNA repair; the zygote may be able to regulate repair efficiency only after the

first cell division. Interestingly, the expression of members of the four families of DNA repair genes was regulated in an embryo-stage specific manner. Transcription of NER family members was decreased with activation of the embryonic genome, whereas transcription of MMR DNA repair family genes was abundant only in 8-cell stage embryos. To our knowledge, this is the first report profiling the expression of DNA repair pathways during early embryo development.

The deletion of a number of DNA repair genes in null mutation transgenic mice has greatly assisted in the elucidation of their roles in normal development [8]. Among the BER genes, APE/ref-1 was shown to play a role in normal development as null mutation mice die shortly after implantation [39]. We found that APE/ref-1 transcripts were present at the limit of detection in the stages of early embryo development examined. We cannot exclude that APE/Ref-1 becomes more abundant in mRNA expression during later stages of peri-implantation development, the time at which its presence becomes critical for normal embryonic development. The homologous-recombination machinery includes the DNA-dependent ATPase RAD54 which is involved in a number of aspects of DNA metabolism such as transcription and repair. RAD54-knockout yeast, chicken, and mouse cells are irradiation-sensitive and display a reduced level of homologous recombination [40]. However, a role during development has not been documented. The high level of expression for RAD54 in rat embryos sired by control- and CPA-treated males could be explained by the role this gene plays in transcription and repair processes. Deletion of PMS2 results in male

mice with abnormal chromosome synapsis in meiosis, rendering them infertile and prone to sarcomas and lymphomas [14, 45]. No data exist to date on the possible role of this family during normal embryonic development, making it difficult to propose an explanation for the dramatically high transcript levels for specific MMR genes at specific embryonic stages.

We have demonstrated that the presence of CPA-induced DNA damage in the paternal genome alters the regulation of various DNA repair genes in the embryo as early as in the 1-cell stage. Embryonic genome activation is thought to occur in the late 1-cell or 2-cell embryo [41]. CPA exposure may alter chromatin structure of the male pronucleus, allowing transcription factors earlier/easier access to promoter and enhancer regions of these genes. Alternatively, CPA treatment may alter transcription factor activity, leading to changes in the coordination of gene expression. Previous studies from our laboratories have shown that the male pronucleus was formed earlier in embryos sired by cyclophosphamide-treated male rats than in those sired by controls [33]. Furthermore, early male pronucleus formation was followed by alterations in the gene activation program. BrUTP incorporation into RNA and Sp1 transcription factor immunostaining were increased and spread over both cytoplasmic and nuclear compartments in 2-cell embryos sired by cyclophosphamide-treated males compared to controls [33]. The increase in expression of PCNA in 1-cell embryos sired by CPA-treated males may be due to enhanced DNA transcription, since PCNA is involved in RNA polymerase II-mediated DNA transcription [5].

Previous studies have shown the importance of DNA repair genes in the response to CPA exposure; gene mutations for two NER enzymes, ERCC1 and XPF, resulted in greater cellular sensitivity to 4-hydroperoxycyclophosphamide, an activated form of CPA, in a cell culture system [42]. Paternally-mediated effects of CPA on progeny outcome may be the result of DNA damage at specific target genes. CPA damage to a limited number of loci in the paternal genome would result in low tail/head ratios of DNA content; strand breaks in close proximity at these locations would generate DNA fragments small enough to create the different tail lengths observed. Specific-locus mutation tests have shown the presence of loci in the genome that are more susceptible to mutations [43]. DNA damage in the paternal genome may be converted into chromosome aberrations at the first metaphase in the fertilized egg [44].

In conclusion, this study reveals that early preimplantation embryos possess some, but not all, of the DNA repair machinery found in other mammalian cells, and that exposure to a genotoxic agent can alter the expression dynamics of this machinery. In embryos fertilized by damaged spermatozoa, the DNA may act as a trigger to alter chromatin conformation and the regulation of transcription. These changes in gene expression may be translated into an altered DNA repair capacity which is critical to cope with DNA lesions and an eventual decrease in embryonic loss. The extent to which the DNA repair capacity of early embryos contributes to preventing early pregnancy loss and thus infertility and adverse pregnancy outcome in humans is not known.

Acknowledgments

These studies were supported by the Medical Research Council of Canada. We should like to thank the following investigators for providing us with the following cDNA probes:JH Hoeijmakers for XPD, CK Chao for XPE, J Trasler for DNMT-1, YC Liu for PCNA, S Xantoudakis for Ref-1, H Krokan for UNG1 & 2, K Brookman for XRCC1, J Petrini for RAD 50, R Kanaar for RAD 54, N de Wind for PMS1, and M Liskay for PMS2. Probes for XPC and XRCC were purchased from ATCC.

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Figure legends

Figure 1

Fluorescence images of embryonic DNA after the Comet Assay. A,1-cell embryo sired by a control male. B-F, 1-cell embryos sired by CPA-treated males. B, tail/head ratio of DNA content= 0.06, tail length= 62 μm. C, tail/head ratio= 0.13, tail length= 100 μm. D, tail/head ratio= 1.5, tail length= 51 μm. E, tail/head ratio= 1.1, tail length= 161 μm. F, tail/ head ratio= 1.9, tail length= 173 μm. Bars represent 20 μm.

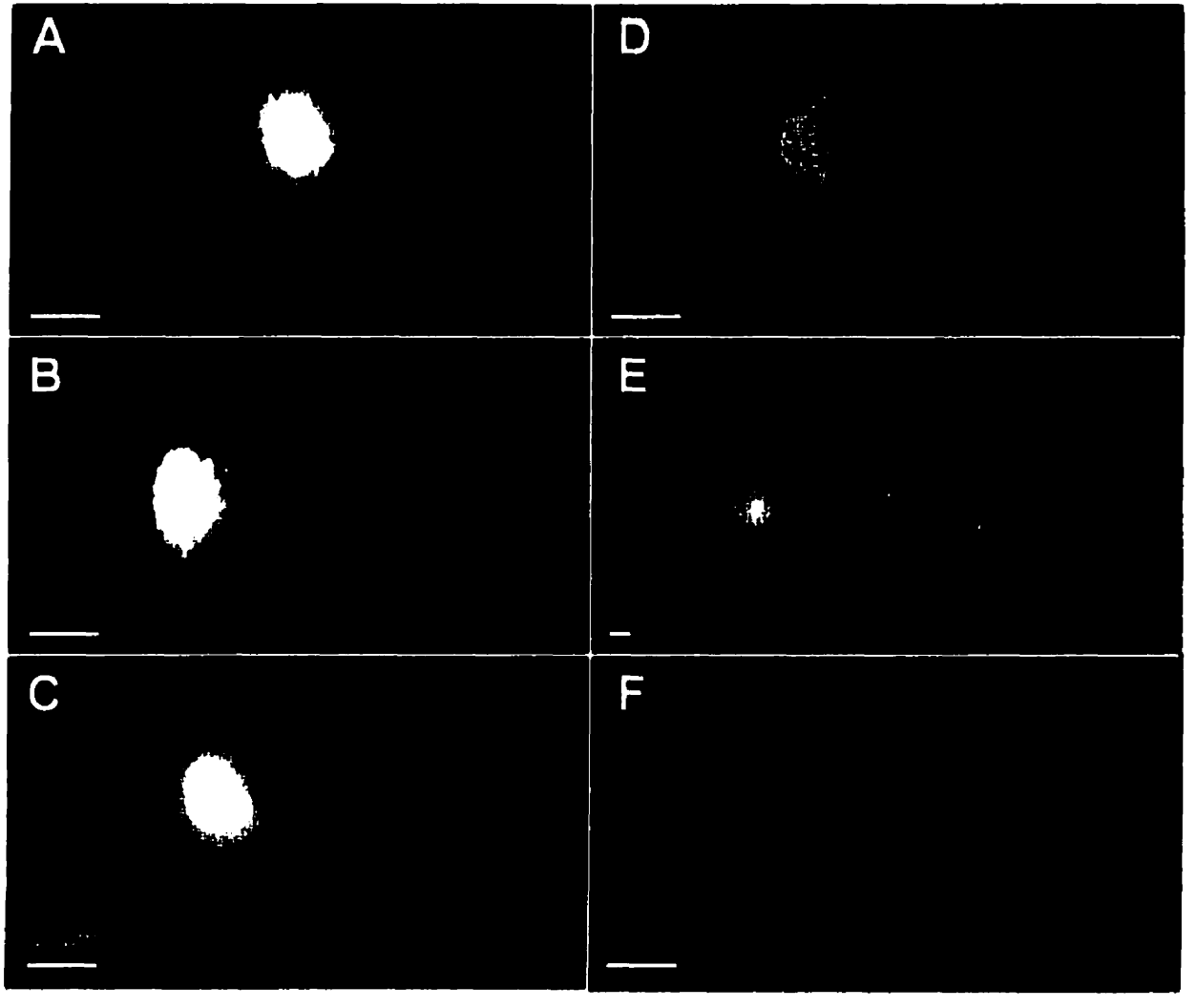
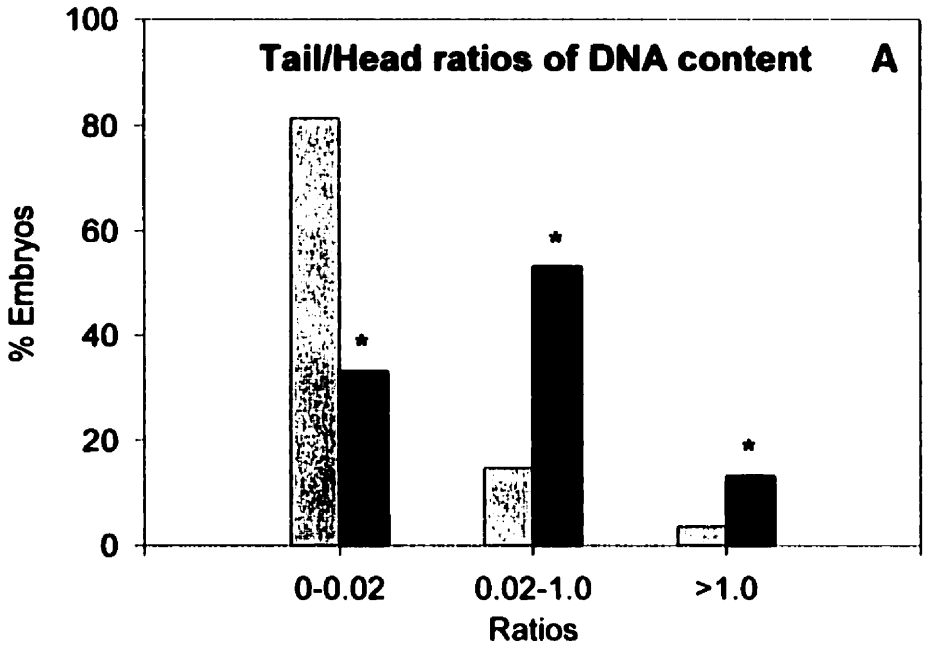

A
D
B
E
C
F

Figure 2

Effect of chronic cyclophosphamide treatment of male rats on DNA of 1-cell embryos. A, A comparison of the percentage of control embryos (grey bars) to embryos sired by CPA-treated males (black bars) which depicted various tail/head ratios of DNA content is shown. B, A comparison of control (grey bars) and experimental (black bars) embryos which migrated during electrophoresis to produce Comet tails is shown. Using these parameters, CPA treatment caused a significantly higher embryonic DNA damage ($p<0.001$).


Tail/Head ratios of DNA content
A
% Embryos
100
80
60
40
20
0
0-0.02
0.02-1.0
>1.0
Ratios
*
*
*

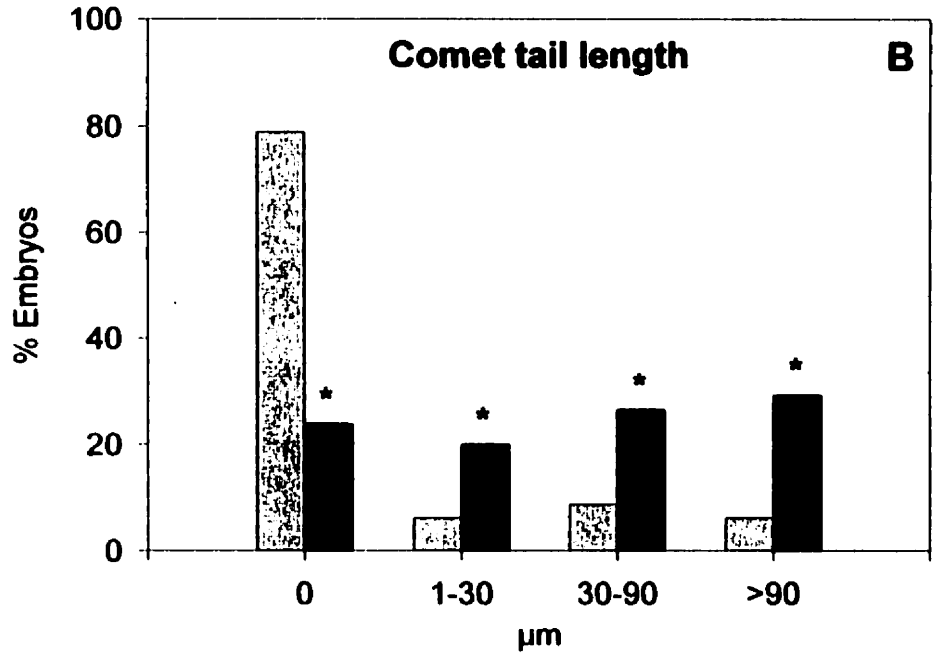

Comet tail length
B
% Embryos
100
80
60
40
20
0
0
1-30
30-90
>90
µm
*
*
*
*

Figure 3

aRNA analysis of the expression profiles of nucleotide excision repair (NER) genes in 1- cell (A), 2-cell (B), 4-cell (C) and 8- cell (D) rat embryos sired by control (grey bars) and CPA-treated male rats (hatched bars). Results are expressed as the means ± the standard errors of the mean (SEM) (n = 8) of the intensity of expression of each probe as a percentage of the internal standard, DNA methyltransferase (DNMT-1). A, at the 1-cell stage, control embryos displayed low levels for all genes except for XPD. In contrast, embryos sired by CPA-treated males displayed an increase of expression of: XPC, XPE and PCNA. B, by the 2-cell stage, transcripts levels increased in control embryos such that most genes were expressed in both groups with no dramatic differences. This pattern continued into the 4- (C) and the 8-cell (D) stages. Asterisks indicate a significant difference from control by Student's t-test ($P \leq 0.05$) at that stage. Numbers above each bar denotes a significant difference from that stage, one-way ANOVA and Tukey's test ($P<0.05$).

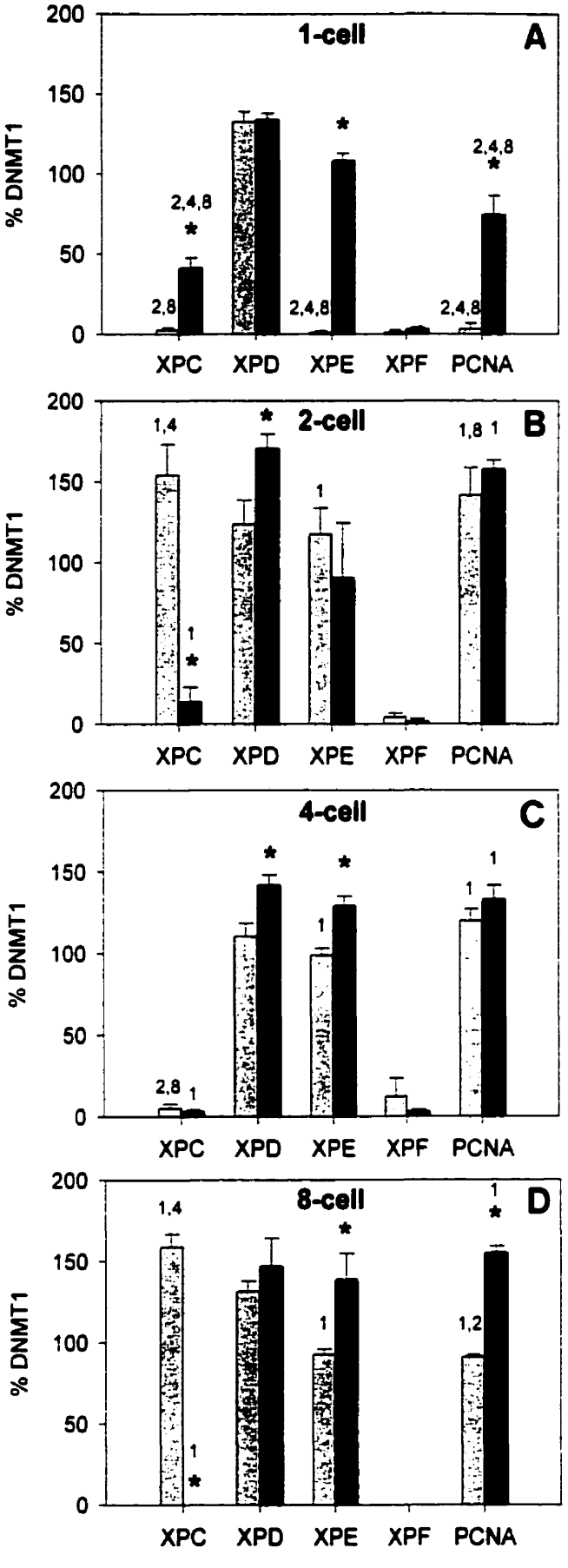

1-cell
A
2-cell
B
4-cell
C
8-cell
D
% DNMT1
200
150
100
50
0
XPC
XPD
XPE
XPF
PCNA
2,8
2,4,8
1,4
1
1,8
1,2
*

Figure 4

aRNA analysis of the expression profiles of base excision repair (BER) genes in 1-cell (A), 2-cell (B), 4-cell (C) and 8-(D) cell rat embryos sired by control (grey bars) and CPA-treated male rats (black bars). Results are expressed as the means ± SEM (n = 8) of the intensity of expression of each probe as a percentage of the internal standard, DNMT-1. A, at the 1-cell stage, Ref-1, UNG1 and UNG2 showed an increased expression among control embryos which was absent in sires of treated males. At the 2-cell stage (B) and 4-cell stage (C), no differences were seen among the 2 groups. D, UNG1 expression was dramatically higher in embryos sired by CPA-treated males compared to control embryos. Asterisks indicate a significant difference from control by Student's t-test ($P \leq 0.05$) at that stage. Numbers above each bar denotes a significant difference from that stage, one-way ANOVA and Tukey's test ($P<0.05$).

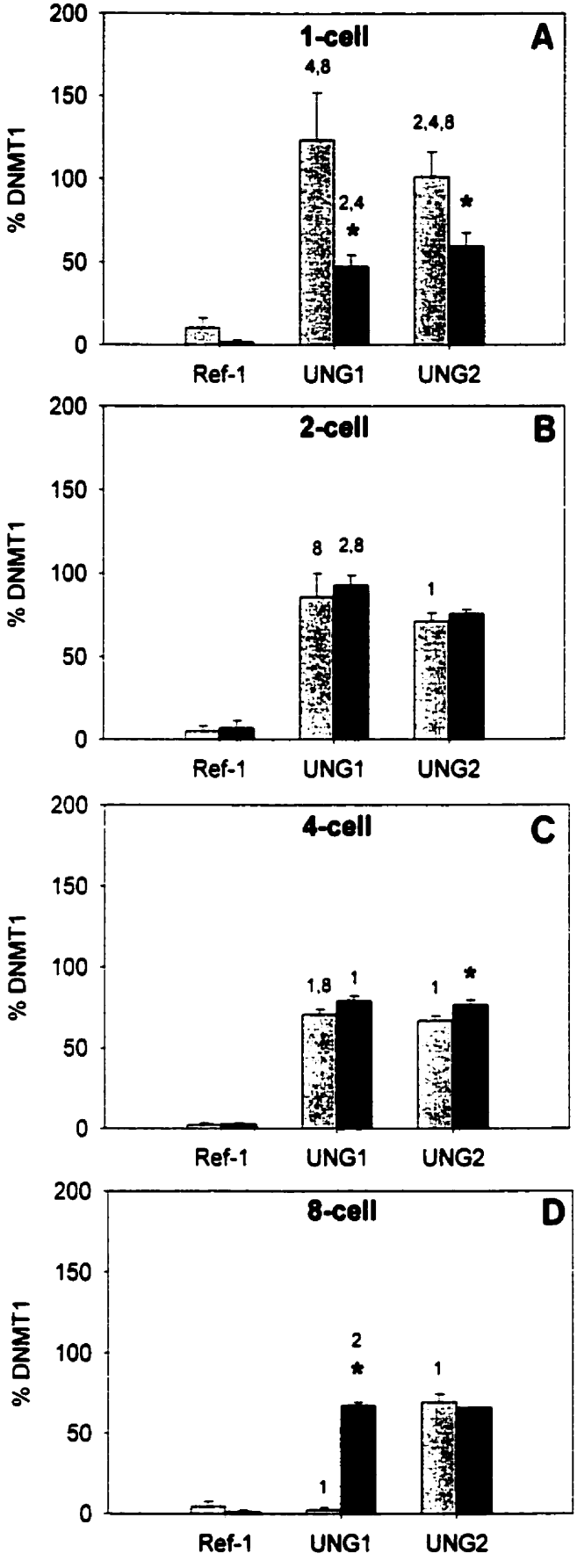

1-cell
A
% DNMT1
200
150
100
50
0
4,8
2,4
2,4,8
*
*
Ref-1
UNG1
UNG2
2-cell
B
% DNMT1
8
2,8
1
Ref-1
UNG1
UNG2
4-cell
C
% DNMT1
1,8
1
1
*
Ref-1
UNG1
UNG2
8-cell
D
% DNMT1
2
*
1
1
Ref-1
UNG1
UNG2

Figure 5

aRNA analysis of the expression profiles of mismatch repair (MMR) genes in 1- cell (A), 2-cell (B), 4-cell (C) and 8-(D) cell rat embryos sired by control (grey bars) and CPA-treated male rats (black bars). Results are expressed as the means ± SEM (n = 8) of the intensity of expression of each probe as a percentage of the internal standard, DNMT-1. A) At the 1-cell stage, no expression of MMR genes was seen among control embryos. In contrast, only PMS1 transcripts were highly abundant among embryos sired by CPA- treated males. B, While control embryos expressed PMS1 at comparable levels to the treated group, a significant increase for PMS2 were seen among embryos sired by CPA-treated males compared to control embryos. C, PMS1 was the only gene which continued to be expressed in both groups. D, by the 8-cell stage, Levels for MSH2, PMS1 and PMS2 were all higher in control embryos compared to their treated counterparts. Asterisks indicate a significant difference from control by Student's t-test ($P \leq 0.05$) at that stage. Numbers above each bar denotes a significant difference from that stage, one-way ANOVA and Tukey's test ($P<0.05$).

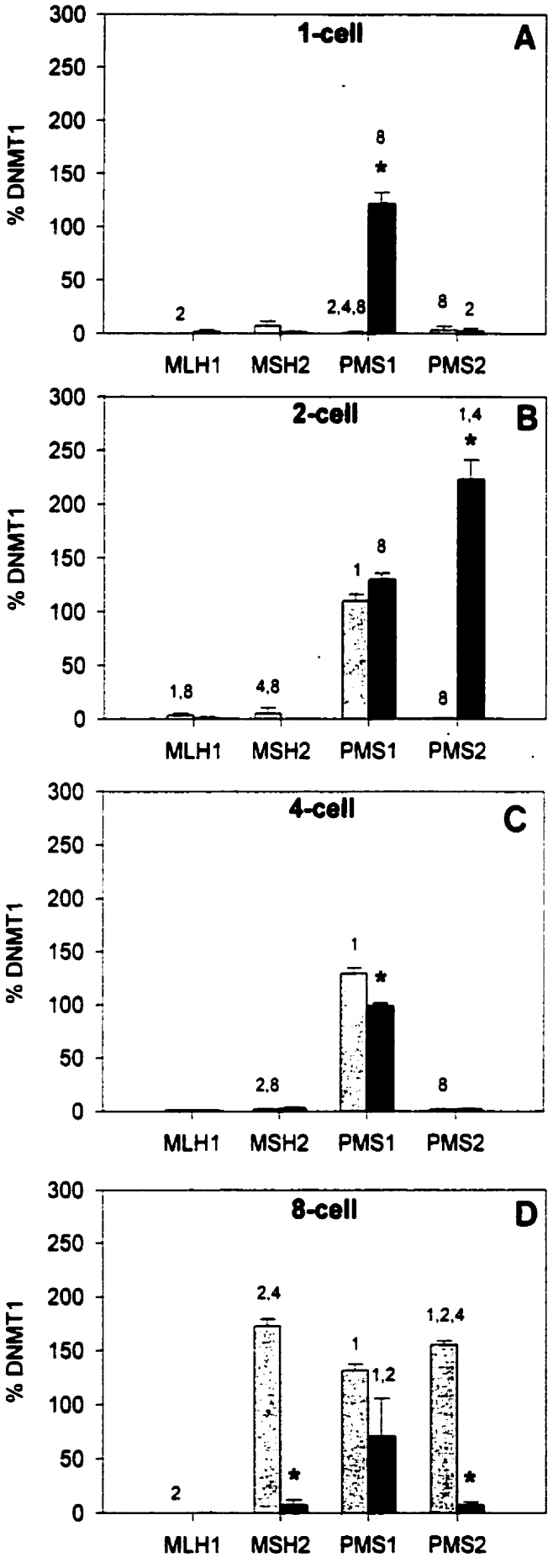
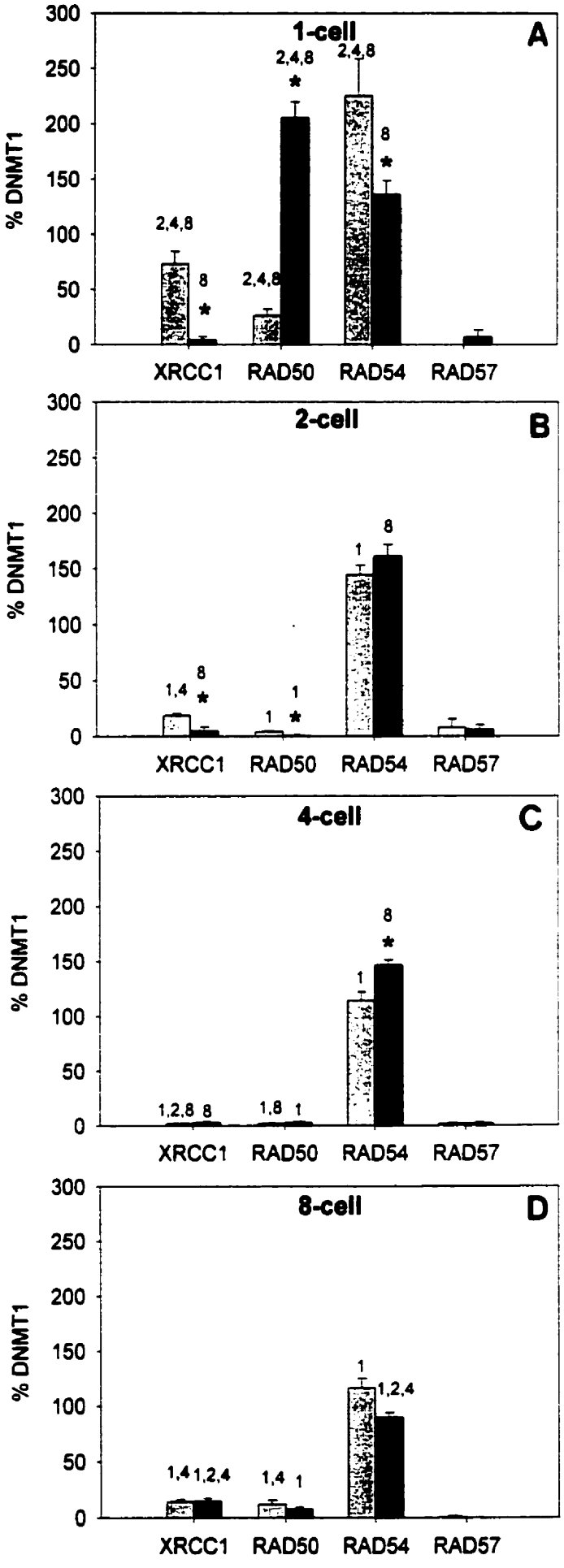

1-cell
A
% DNMT1
300
250
200
150
100
50
0
2
8
*
2,4,8
8
2
MLH1
MSH2
PMS1
PMS2
2-cell
B
1,4
*
1
8
1,8
4,8
8
MLH1
MSH2
PMS1
PMS2
4-cell
C
1
*
2,8
8
MLH1
MSH2
PMS1
PMS2
8-cell
D
2,4
1
1,2
1,2,4
*
*
2
MLH1
MSH2
PMS1
PMS2

Figure 6

aRNA analysis of the expression profiles of the recombination repair (RCR) enzymes in 1- cell (A), 2-cell (B), 4-cell (C) and 8-(D) cell in rat embryos sired by control (grey bars) and CPA-treated male rats (black bars). A, at the 1-cell XRCC1 and RAD 54 were strongly expressed among control embryos. In contrast, among progeny of CPA-treated males, RAD 50 and RAD 54 were strongly expressed. Only RAD54 was significantly expressed in both groups, compared to barely detectable levels for all RCR genes at the 2-cell (B), 4-cell (C) and 8-cell stages. Asterisks indicate a significant difference from control by Student's t-test ($P \leq 0.05$) at that stage. Numbers above each bar denotes a significant difference from that stage, one-way ANOVA and Tukey's test ($P<0.05$).


1-cell
A
% DNMT1
300
250
200
150
100
50
0
2,4,8
8
2,4,8
2,4,8
2,4,8
8
XRCC1
RAD50
RAD54
RAD57
2-cell
B
1,4
8
1
1
8
4-cell
C
1,2,8
8
1,8
1
8
1
8-cell
D
1,4
1,2,4
1,4
1
1
1,2,4

Connecting Text

In the previous chapter (Chapter 2) the effect of treating males with cyclophosphamide was shown to have deleterious effects on the DNA of embryos sired by treated males. This was manifested at two levels; at the DNA level, a significant increase in DNA damage was seen among 1-cell embryos sired by treated males when compared to controls. Second, the expression profiles of major DNA repair pathways were demonstrated to be differentially altered in these embryos compared to controls; thus demonstrating that the pre-implantation embryo employs specific DNA repair genes in response to damage brought in during fertilization by the damaged spermatozoon. Therefore, in the next chapter we sought to determine the effects of DNA damage and induction of DNA repair in 1-cell embryos on subsequent events of the pre-implantation development of the rat embryo.

Chapter 3

Paternal exposure to cyclophosphamide dysregulates the gene activation program in rat preimplantation embryos

Wafa Harrouk, Sepideh Khatabaksh, Bernard Robaire and Barbara F. Hales

Abstract

Although there has been progress in determining the mechanisms by which maternal toxicant exposure affects progeny, there is little information on the actions of drugs administered to the father. We investigated the effects of pre-conceptional paternal exposure to cyclophosphamide, an anti-cancer agent, on embryonic gene activation in the rat. The male pronucleus was formed earlier in embryos sired by cyclophosphamide-treated male rats than in those sired by controls; early male pronucleus formation was followed by alterations in the gene activation program. BrUTP incorporation into RNA and Sp1 transcription factor immunostaining were increased and spread over both cytoplasmic and nuclear compartments in 2-cell embryos sired by cyclophosphamide-treated males compared to controls. Total RNA synthesis was constant in 1-8 cell embryos sired by drug-treated fathers, while in control embryos RNA synthesis increased four-fold to peak at the 4-cell stage. In 2-cell embryos sired by drug-treated males, the relative abundance of candidate imprinted genes was elevated significantly above control; a peak in the expression of these genes was not observed until the 8-cell stage in control embryos. Thus, paternal drug exposure temporally and spatially dysregulated rat zygotic gene activation, altering the developmental clock.

Key words: IST/aRNA, imprinted genes, male pronuclear formation, BrUTP, Sp1.

Introduction

The exposure of males to various drugs and environmental agents may result in adverse progeny outcome, even when spermatogenesis appears to function normally (Olshan and Mattison, 1994). There is particular concern for men who receive anticancer chemotherapeutics (Mulvihill, 1994). The alkylating anti-neoplastic drug, cyclophosphamide, is a widely prescribed agent for cancer therapy and is a mutagen, carcinogen, and teratogen (Andersen et al., 1995; Zemlickis et al., 1993). When administered to males, cyclophosphamide causes sister chromatid exchange (Abraham and Franz, 1983), translocations (Sotomayer and Cumming, 1989), micronucleus formation (Moreland et al., 1981; Pachierotti et al., 1983), DNA single strand breaks, and DNA-DNA cross-links in the germ cells (Skare and Schrotel, 1984; Qiu et al., 1995a). Previous studies have shown that a chronic low dose administration of cyclophosphamide to male rats results in increased pre- and post-implantation loss and in growth-retarded and malformed fetuses, even though there are no apparent adverse effects on male reproduction (Trasler et al., 1986, 1987). Malformations (Hales et al., 1982) and behavioural abnormalities (Hsu et al., 1987) are seen also in the second generation. As an early sign of cyclophosphamide damage, spermatozoa from treated males undergo an altered in vitro decondensation pattern; this pattern is characterised by a normal initial nuclear swelling followed by an aberrant nuclear elongation pattern, possibly due to alkylation of nuclear proteins or DNA by cyclophosphamide or its metabolites (Qiu et al., 1995b). Embryos fertilised with treated spermatozoa manifest their earliest defects on

day 2 of gestation (representing the 8-cell stage); these include a delay in cell proliferation, a longer and asynchronous cell doubling time, and a decreased incorporation of [^{3}H] thymidine, reflecting a decrease in the rate of DNA replication (Kelly et al., 1994).

Upon entry into the egg, sperm chromatin undergoes extensive remodelling which results in reorganisation of the haploid paternal genome into a pronucleus. Although the female pronucleus is typically formed ahead of the male pronucleus, the latter precedes its maternal counterpart in acquiring transcriptional capacity for both endogenous (Bouniol et al., 1995) and exogenous genes (Aoki et al., 1997). This observation may be explained by the presence of higher concentrations of transcription factors (such as Sp1) and of transcription binding proteins (TBPs) in the paternal pronucleus (Worrad et al., 1994).

The maternal and paternal pronuclei undergo a round of DNA synthesis before entering the first mitosis to produce a 2-cell embryo containing two diploid zygotic nuclei, each with a set of paternal and maternal chromosomes. In the mouse, formation of the 2-cell embryo marks the transition from maternal to zygotic control, a process referred to as zygotic gene activation. From this point onwards, it is the zygotic clock that regulates all aspects of development, including transcription and translation (Nothias et al., 1995). An initial round of transcription occurs at the late G_1/S phase of the 1-cell embryo followed by a more extensive one after 2-cell formation (Nothias et al., 1996). Although maternal transcripts continue to be present until later stages of development, the

zygote initiates its own transcription and translation machinery following zygotic gene activation. The extent to which maternal and paternal genomes contribute to the early events of embryogenesis is not equivalent. Indeed, the paternal genome does not need to be associated with the maternal genome prior to the 8-cell stage for normal embryonic development to take place (Renard et al., 1991). Profiling of a family of genes referred to as imprinted genes (Bartolomei and Tilghman, 1997), where allelic expression is inherited from either parent, has provided evidence for a differential parent-of-origin genomic contribution to the zygote. A number of imprinted genes are expressed starting from the pre-implantation stages of development.

Fetal wastage in humans has been estimated to be from one-half to two-thirds of all fertilised ova. The extent to which paternal exposures contribute to fetal loss (developmental toxicity) is unknown. To address this problem, it is imperative to elucidate the underlying mechanisms by which paternal drug exposures adversely affect embryo fate. To accomplish this goal, we have assessed the effects of paternal exposure to a commonly used anticancer agent, cyclophosphamide, on early embryonic development.

Materials and Methods

Animals

Adult male (300-325g) and virgin female (225-250) Sprague-Dawley rats were obtained from Charles River Canada (St. Constant, Quebec) and housed in the McIntyre Animal Centre. Food and water were provided ad libitum and animals were exposed to a 14h-light:10h-dark cycle. Female Syrian hamsters (6-9 weeks old) were obtained from bio-breeders (Fitchburg, MA) and maintained on a 14h-light:10h-dark cycle with food and water ad libitum.

Treatment, mating, and embryo collection

All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise noted. Male rats were randomly divided into a control and a cyclophosphamide group consisting of 10 rats each. Males were gavaged daily with saline or 6 mg/kg/day cyclophosphamide for 4-5 weeks. This treatment regimen results in more than 85% post-implantation loss (Trasler et al., 1987). Post-implantation loss was seen as early as day 6.5 of gestation when the inner cell mass population was significantly reduced in embryos sired by cyclophosphamide-treated males (Kelly et al., 1992). Starting on the fifth week of treatment, each male was mated overnight with two females in proestrus. To rule out effects of the presence of cyclophosphamide in the semen, males were not gavaged on the night of mating (Hales et al., 1986). The following morning, considered as day 0 of gestation, the presence of sperm in vaginal smears was used to confirm pregnancy in mated females. Pregnant females were killed on

days 0, 1, 1.5 and 2 of gestation in order to collect preimplantation embryos at the 1- ,2-, 4- and 8 cell stages, respectively, by flushing oviducts.

In vitro fertilization (IVF) and pronuclear formation

Males were treated with either saline or 6 mg/kg/day cyclophosphamide for 4-5 weeks. The cauda epididymides were removed and punctured with a very fine needle to release spermatozoa into TALP-PVA medium (114.0 mM NaCl, 3.16 mM KCl, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10.0 mM Na Lactate (60%), 0.35 mM NaH_2PO_4, 5.0 mM glucose, 25 mM $NaHCO_3$, 20,000 U Penicillin G/200 ml, and 0.1% polyvinyl alcohol) supplemented with PHE (100X) (20 M D-Penicillamine, 100 μM hypotaurine, and 1 μM epinephrine) and 30 μg/ml BSA (Farrell and Bavister, 1984). For the *in vitro* study, spermatozoa were incubated in this medium for 1 hr at 37°C in 5% CO_2 in air to undergo the acrosome reaction. Female hamsters were superovulated by intraperitoneal injection (IP) with 25 IU/ml of pregnant mare serum gonadotropin followed, 48 hours later, with another 25 IU/ml IP injection of human chorionic gonadotropin; oocytes were collected the following morning. *Zonae pellucidae* were removed using a pre-warmed solution of acid Tyrode's buffer (pH 2.5). Following several washes in TALP-PVP medium, eggs were incubated with capacitated spermatozoa (the final concentration of spermatozoa was 10^6/ml), covered with mineral oil, and incubated at 37°C with 5% CO_2 in air in TALP-PVP/PHE medium. At half-hour intervals, hamster eggs were examined and the ones which had sperm attached were removed and placed into a drop of fresh medium without spermatozoa to

decrease the incidence of polyspermic eggs. Six control and six drug treated males were used; a total of 1008 hamster oocytes were examined, 476 for the control and 532 for the drug treated males. For the *in vivo* studies, females rats were mated to control or cyclophosphamide treated males and sacrificed four (n=3), four and a half (n=7) or twenty four hours (n=23) afer mating in order to collect 1-cell oocytes/zygotes from the oviducts; a total of 318 rat oocytes were examined. Cells were fixed at the specified time points in 4% paraformaldehyde for 1h at 37°C and stained with Hoechst 335258 dye to visualise the pronuclei. Using phase contrast microscopy, sperm tails were identified in order to confirm that the eggs were fertilised. Male pronuclei were distinguished from their female counterparts due to their larger size and the presence of sperm tails still attached to them. Embryos were mounted in Immunomount (Shandon, Pittsburgh, PA) and observed immediately under an epifluorescence microscope equipped with a UV filter.

Bromouridine triphosphate (BrUTP) incorporation and immunostaining

Two-cell embryos were collected by puncturing the oviduct with fine forceps in a HEPES-buffered KSOM medium (Ho et al., 1995) (a simplex-optimised medium containing high sodium and potassium concentrations) and were labelled with BrUTP as described previously (Aoki et al., 1997). Control embryos (n=21) were obtained from three females; the embryos sired by cyclophosphamide treated males (n=18) were also obtained from three females. Embryos were washed in phosphate-buffered saline (PBS) and plasma

membranes permeabilised by treating the embryos for 1-2 min with 0.05% Triton X-100 in transcription buffer (TB) containing 100 mM potassium acetate, 30 mM KCl, 1 mM $MgCl_2$, 10 mM Na_2HPO_4, 1 mM $MnCl_2$, 50 mM ammonium sulfate and the following nucleotides: 2 mM ATP, 0.4 mM each of GTP, CTP, and BrUTP. Embryos were then washed with a physiological buffer (PB) containing 10 mM potassium acetate, 30 mM KCl, 1 mM $MgCl_2$, 10 mM Na_2HPO_4, 1 mM ATP supplemented with 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride, and 50 units/ml of RNasin (Gibco BRL, Burlington, ON), transferred to a drop of TB, incubated for 10 min at 33°C, and then washed three times in PB. Embryonic nuclear membranes were permeabilised in Triton X-100 (0.2% in PB) for 3 min., washed three times in PB, and fixed overnight with 3.7% paraformaldehyde in PB.

The incorporation of BrUTP was detected by immunostaining with an anti-BrdU antibody (Sigma Chemical Company). Embryos were washed five times in 15μl drops of PBS containing 3 mg/ml BSA (PBS/BSA) over a period of 15 min and then incubated for 60 min with 2 μg/ml of a monoclonal anti-mouse BrdU antibody in blocking solution. Embryos were then washed three times with PBS/BSA for 15 min and incubated in the dark for 60 min in a fluorescein-conjugated anti-mouse IgG antibody (0.5 μg/ml) in PBS/BSA. Fluorescence was detected using a laser-scanning confocal microscope (LSM-410 invert, Carl Zeiss, Thornwood, NY).

Sp1 immunofluorescence

All steps were performed at room temperature unless otherwise noted. Embryos (control, n=10; cyclophosphamide-sired group, n=16) were fixed in 4% paraformaldehyde overnight at 4°C. Fixation was followed by rigorous washing in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2 $HPO_4.7H_20$, 1.4 mM KH_2PO_4) containing 3 mg/ml polyvinylpyrrolidone (PBS/PVP). Embryos were permeabilised in a solution of 0.1% Triton X-100, 1% BSA in PBS for 15 min and blocked for 1 hr in 3% BSA, 0.1% Triton X-100 in PBS. Embryos were incubated for 1hr in a humidified chamber with an anti-mouse Sp1 antibody (1µg/ml in blocking solution; Molecular Probes; Eugene, OR), washed for 3x20 min in blocking solution, incubated in a mouse FITC-conjugated secondary antibody for 1 hr in the dark, and washed 3x10 min in PBS/PVP. Negative controls consisted of replacing the primary antibody with an affinity purified normal rabbit IgG or by omitting the primary antibody incubation step (data not shown). Slides were allowed to dry overnight in the dark before examination under a Zeiss laser-scanning confocal microscope.

Quantitative analysis of RNA synthesis: radioisotope labelling with ^{32}P-UTP

The assessment of total RNA synthesis was done as described previously (Clegg and Piko, 1993). Following collection, a minimum of 20-30 embryos per group were pretreated with 1% isolecithin to permeabilise the *zonae pellucidae*, ensuring access of the radioisotope to the embryo, and incubated in KSOM containing 5 μM ^{32}P-UTP (3000 µCi specific activity, Amersham) under oil (4 hrs

at 37°C, in 5% CO_2, 5% O_2, 90% N_2). At the end of the incubation period, embryos were washed in Tris-buffered saline (TBS, 100mM Tris.Cl, pH 8.0; 0.9% NaCl) containing BSA (5 mg/ml) and transferred to microcentrifuge tubes containing 20 µg glycogen as a carrier and 40 μl potassium perchlorate (PCA, 0.5 N) in a final volume of 50 μl. Labelled nucleotides were extracted by repeated cycles of freeze/thawing in a dry ice-ethanol bath. PCA-precipitable material was collected by centrifugation at 13,000x rpm for 10 min at 4°C. Supernatants were removed and represented the overall non-specific incorporation of label. The pellet containing the PCA-precipitable material (incorporation into RNA) was hydrolysed (100 μl KOH for 1h at 37°C). Following centrifugation, supernatants were neutralised with 20 μl KOH, precipitated on ice for 10 minutes, and centrifuged. Pellets were dissolved in 100 μl NaOH (0.5N). Radioisotope incorporation was determined by scintillation spectrometry. Supernatants were pooled and represented the non-specific radioisotope incorporation. RNA synthesis was calculated as the specific uptake/non-specific incorporation per embryo after subtracting background levels; these were obtained using culture medium containing 5 μM ^{32}P-UTP without embryos. Negative controls were obtained using dead (heat killed) embryos.

Gene expression profiles using the in situ transcription /antisense RNA (IST/aRNA) amplification procedure

mRNA profiles were analysed using the antisense RNA amplification approach (IST/ aRNA). The protocol used for IST/aRNA amplification in

preimplantation embryos was as described previously with minor change (Taylor et al., 1995). Embryos from individual litters were sonicated in the presence of lysis buffer containing 1 mg/ml digitonin, 5 mM DTT, and 1X in situ transcription (IST) buffer (50mM Tris, 6mM $MgCl_2$, 0.12mM KCl, pH 8.3). Embryonic homogenates were reverse-transcribed at 37°C for 2 hrs in the presence of an unlabeled oligo dT_{24}- extended at the 5' end with the T7 RNA polymerase promoter (50 ng) which recognises the poly(A) tail of mRNA populations resulting in complementary cDNA (cDNA) synthesis. Reverse transcription was done using the avian myeloblastosis virus reverse transcriptase (50 U; NEB, Montreal, Canada) which binds to the primer and copies single stranded mRNAs to cDNAs in the presence of deoxyribonucleotides (Boehringer Mannheim, Montreal, Canada). Following a phenol/chloroform extraction, first strand cDNAs were precipitated with ethanol and glycogen (4 mg). The pellet was resuspended in 10μl water, heated to 95°C to denature the DNA:RNA hybrid, and chilled on ice. Single-stranded cDNAs were allowed to self-prime (by hairpin loop formation) to form double cDNA strands (ds-cDNAs). Following excision of the self-primed hairpin loop by S1 nuclease (2 U) for 3 min at 37°C, ds-cDNAs were blunt-ended and filled-in with the addition of T4 DNA polymerase (5 U) and Klenow fragment of *Escherichia coli* (5 U) in the presence of dATP, dCTP, dGTP and dTTP nucleotides. The resulting ds-cDNAs which contained the promoter region for the bacteriophage T7 RNA polymerase (NEB) were extracted with phenol/chloroform and amplified into RNA (aRNA) in the following mixture: 40 mM Tris pH 7.5, 6 mM $MgCl_2$, 10 mM dithiotheitol (DTT), 400 mM of ATP, GTP,

UTP, and 4 mM CTP, 20 U RNasin, 100 U T7 RNA polymerase, and ^{32}P-radiolabeled-CTP (10 mci/ml; 3000 Ci/mmol specific activity) in a final volume of 25 μl. This reaction was conducted for 4hrs at 37°C.

Reverse Northern blots and genetic expression profiles

Equimolar concentrations of cDNA clones were calculated based on the approximate size of the insert relative to the vector size, which served to normalise the total amount of each of the cDNA clones immobilised on slot blots and hybridised with the amplified aRNA samples. Radiolabelled amplified RNA populations obtained form embryos at the 1-, 2-, 4- and 8-cell stages were utilised as probes to screen slot blots containing: the non-imprinted growth hormone, insulin growth factor-I (IGF I, Shimatsu and Rotwin, 1987) gene and the following imprinted genes: insulin-like growth factor II (IGFII, DeChiara et al., 1990), insulin like growth factor II receptor (IGFIIR, Lau et al., 1994), Mash-2, a mammalian member of the achaete-scute family encoding basic-helix-loop-helix transcription factors (Johnson et al., 1990), the apolipoprotein gene, ApoE (Mann et al., 1995), and p57^{kip2} (Hatada et al, 1995), an inhibitor of several G1 cyclin/CDK complexes and a negative regulator of cell proliferation. Individual blots were pre-hybridised for 30 min in buffer (50% formamide, 0.12 M Na_2HPO_4, pH 7.2, 0.25M NaCl, 7% w/v SDS) at 42°C. Heat-denatured aRNA probes obtained from each embryonic sample were applied to the blots and allowed to hybridise overnight at 42°C. The following day, blots were washed at decreasing stringency from 2X sodium chloride citrate (SSC) solution down to 0.1% SSC and

0.1% SDS at 42°C for 20 minutes in a shaking water bath for 20 min each. Blots were exposed to phosphorimager plates overnight. Individual band intensities were determined after exposure of the plates to a phosphorimager scanner (Molecular Dynamics, Sunnyvale, CA). Individual signals were corrected for nonspecific binding by subtracting the background values on each blot and normalised to our internal standard, retinoic acid binding protein -1 (RABP-1, Douglass et al., 1991). The use of such an internal standard allows comparisons between different blots, as the individual hybridisation intensities of each cDNA on a blot can be expressed as the ratio of its expression to that of the internal standard. RABP-1 was chosen as an internal standard based on its consistent expression between groups (control and drug-treated) and across blots, thus facilitating experiment to experiment and blot to blot quantification and comparison. As the cDNA population should reflect accurately the abundance of the original mRNA population, the intensity of the aRNA hybridisation signal reflects the abundance of the original mRNA for any given gene. Six to eight expression profiles, each representing individual litters, were obtained for each stage and treatment group. The litters were obtained from 6 different males for each treatment group.

Statistical analysis

Differences in the rate of pronuclear formation between embryos fertilized by germ cells from control and cyclophosphamide-treated males were analysed using Chi-Square analysis followed by Yates' post-hoc test ($p<0.05$). A similar

test was used to assess differences in the percentage of embryos cleaving to the 2-cell stage. To reveal effects of paternal treatment on gene expression in progeny, a pair-wise comparison for each developmental stage was conducted between litters fathered by control and cyclophosphamide-treated males by a t-test followed by Tukey's post-hoc test ($p<0.05$). Statistical analysis was done using SigmaStat 2.3 (SPSS Inc., Chicago, IL).

Results

Chronic treatment of male rats with cyclophosphamide results in earlier male pronucleus formation

The time course required for the formation of male pronuclei following fertilisation of normal hamster oocytes with spermatozoa from control or cyclophosphamide-treated males is shown in Figure 1. Spermatozoa from control and drug-treated males were collected from the cauda epididymidis, capacitated in vitro, and incubated with superovulated eggs from normal females for 3-7 hrs. After a 3 hr incubation period, no pronuclei had formed in eggs fertilised with control spermatozoa; in contrast, 15% of the eggs fertilised by spermatozoa from cyclophosphamide-treated rats had formed male pronuclei (Figure 1). Eggs fertilised by spermatozoa from drug-treated males continued to have a significantly higher proportion of embryos which formed male pronuclei compared to their control counterparts until 6 hrs of incubation. At 6.5 and 7 hrs, comparable results were obtained for both groups. To confirm that the timing of pronuclear formation after paternal drug treatment was also advanced after in vivo fertilization, the presence of male pronulcei was examined in rat oocytes at specific time-points. After four hours, none of the oocytes from females mated to control males had male pronuclei, whereas 18% had male pronuclei if the father had been treated with cyclophosphamide. By four and a half hours, 24% of controls had male pronuclei ($p<0.05$, Chi Square Analysis), while there was a sharp increase to 80% for zygotes from cyclophosphamide-treated males. By 24 hours, pronuclear formation among the controls was increased to 60% while the

treated group remained essentially unchanged (87%). Together, these data clearly demonstrate that chronic cyclophosphamide treatment advances the timing of male pronuclear formation.

BrUTP incorporation is not confined to the nucleus in 2-cell embryos sired by cyclophosphamide-treated males

To determine whether earlier pronuclear formation in embryos sired by drug-exposed males led to alterations in the gene activation program, the incorporation of 5-bromouridine 5'-triphosphate (BrUTP) was assessed in 2-cell embryos. The presence and localisation of newly synthesised RNA was compared in embryos sired by control and cyclophosphamide-treated males by immunostaining embryos cultured in BrUTP for a short period (10 min). An increase in BrUTP incorporation was seen in >50% of the 2-cell embryos sired by cyclophosphamide-treated males, compared to their control counterparts. Moreover, while BrUTP incorporation was exclusively nuclear in control embryos (Figure 2A), it spread to the cytoplasm in 2-cell embryos sired by drug-treated males (Figure 2B). BrUTP incorporation represented RNA synthesis since the signal was essentially eliminated when RNase was added; the addition of RNase-free DNase did not eliminate the signal (data not shown).

Sp1 expression is enhanced in embryos sired by cyclophosphamide-treated males

Sp1, a multifunctional housekeeping transcription factor, has been well characterised during early stages of embryonic development (Worrad and Schultz, 1997). Using immunofluorescence and confocal laser microscopy, we determined the localisation of Sp1 in 2-cell embryos. Sp1 immunostaining was principally nuclear in control embryos (Figure 3A). Most embryos (>70% of embryos examined) sired by cyclophosphamide-treated males displayed a dramatic increase in Sp1 immunostaining in the cytoplasm as well as in the nucleus (Figure 3B). Thus, there is a defect in embryos sired by cyclophosphamide-treated males at the level of the transcriptional machinery.

Effects of paternal exposure to cyclophosphamide on embryonic development: from the pronucleus to the 2-cell stage

It is likely that early male pronucleus formation and dysregulation of the transcriptional machinery affect the subsequent development of preimplantation embryos. To determine if this was true and the role of zygotic gene activation, embryos were collected on day 0 of gestation (corresponding to the 1-cell stage) and were incubated for 18 hrs in the presence or absence of α-amanitin, an RNA polymerase II inhibitor. *In vitro*, in the absence of the inhibitor, 80% of control embryos and 67% of embryos sired by cyclophosphamide-treated males developed to the 2-cell stage ($p<0.05$, Chi-Square Analysis). In the presence of α-amanitin, the proportion of control embryos dividing to the 2-cell stage was

reduced to 67%; surprisingly, a higher proportion, 88%, of the embryos sired by cyclophosphamide-treated males reached the 2-cell stage ($p<0.05$, Chi-Square Analysis). Thus, inhibition of RNA synthesis with α-amanitin increased the percentage of embryos sired by drug-treated males capable of undergoing the first cell division. One explanation for this finding may be that the male germ cells exposed to cyclophosphamide alter the zygotic gene activation program by inactivating a repressor whose role is to inhibit precocious division to ensure the normal contribution of each parental genome

Total RNA synthesis does not peak in embryos sired by cyclophosphamide-treated males

To assess the overall ability of embryos sired by control and drug-treated males to synthesise RNA, zygotes were collected at the 1-, 2-, 4- and 8-cell stages of development and incubated in the presence of ^{32}P-UTP for 4 hrs. The capacity of control embryos to synthesise total RNA was low in 1- and 2-cell embryos and dramatically increased to peak at the 4-cell stage; incorporation at the 8-cell stage fell to levels similar to those at the 1- and 2-cell stages (Figure 4). Surprisingly, constant levels of RNA synthesis were maintained in embryos sired by treated males throughout the stages examined (Figure 4). While RNA synthesis in embryos sired by cyclophosphamide-treated males was significantly higher compared to controls at the 1- and 8- cell stages, the 4-cell stage peak in RNA synthesis was completely absent. These data provide evidence of an

altered gene activation program in embryos sired by cyclophosphamide-treated males.

Temporal alterations in the expression of imprinted genes in progeny sired by cyclophosphamide-treated fathers

mRNA expression was analysed using in situ transcription/anti-sense RNA coupled with reverse Northern blotting (IST/aRNA). This approach allows the analysis of mRNA populations with low abundance; the resultant amplified aRNA population proportionally represents the size and complexity of the original sample. The amplification is directed by the addition of T7 RNA polymerase which, unlike the Taq polymerase enzyme used in RT-PCR techniques, allows for a linear amplification, thus permitting direct quantification of the relative abundances of individual mRNA species (Kacharmina et al., 1999). Radiolabelled aRNA can then be used to probe Northern blots which contain candidate genes of interest, resulting in a "gene expression profile" where expression of multiple genes can be coordinately monitored from the same sample. Using this approach, we have mapped the gene expression profile of a group of growth factors and imprinted genes with well established roles during pre-implantation development. In control embryos, transcripts for IGFI, a non-imprinted gene, and for IGFII and IGFIIR, two imprinted genes, showed low levels of expression in embryos spanning the 1- to 4-cell stages (Figure 5); at the 8-cell stage, a peak of expression was seen ($p<0.001$). In contrast, analysis of these gene expression profiles among progeny sired by cyclophosphamide-

treated males revealed a dramatic peak of expression for all three transcripts at the 2-cell stage (Figure 5). Steady state concentrations of the transcripts for IGFI, IGFII, and IGFIIR declined subsequently, at the 4- and 8-cell stages. This could be explained partly by the fact that some of the embryos sired by cyclophosphamide-treated males collected on day 2 of gestation do not reach the 8-cell stage; thus, gene expression profiles could reflect their arrested developmental profiles.

Expression profiles of another group of imprinted genes, ApoE, Mash-2, and $p57^{Kip2}$, are shown in Figure 6. ApoE and Mash-2 were expressed at high levels during preimplantation rat embryo development; steady state concentrations of the transcripts for both genes were maximal at the 8-cell stage. In contrast, $p57^{Kip2}$ transcripts were near the level of detection at all of the stages examined. A significant elevation in the level of Mash-2 transcripts was observed at the 2-cell stage among the embryos sired by drug-treated males; by the 8-cell stage transcript levels for Mash-2 in embryos sired by cyclophosphamide-treated males fell below those of controls. For ApoE, significant elevations in the levels of transcripts were observed at the 1-cell, 2-cell, and 4-cell stages among embryos sired by drug-treated males; like Mash-2, transcript levels for ApoE dropped below those of controls in embryos sired by cyclophosphamide-treated males by the 8-cell stage (Figure 6). A decrease in the expression of $p57^{Kip2}$ was observed at the 1- and 4-cell stages in embryos sired by cyclophosphamide-treated males (Figure 6). Together, the aRNA data demonstrate that treatment of males with cyclophosphamide alters the gene

expression profile of a number of growth factors and imprinted genes in embryos sired by treated males.

Discussion

Chronic, low dose, cyclophosphamide treatment exposes all post-meiotic germ cells to the drug during spermiogenesis and sperm maturation. We have shown that the gene activation program is altered in the newly fertilised rat conceptus sired by drug-exposed spermatozoa. We report here, for the first time, that the formation of male pronuclei can be advanced in embryos sired by males treated with a xenobiotic. Damage of sperm DNA, and/or its nuclear proteins, by alkylation with active metabolites of cyclophosphamide may shorten the time required for pronuclear formation by decreasing the time needed to replace protamines with somatic histones in the nuclei of spermatozoa of treated males. A previous study showed that the decondensation pattern was altered in spermatozoa of males treated with this drug (Qiu et al., 1995b). The sulfhydryl groups in protamines are reduced early after entry into the egg's cytoplasm, prior to pronuclear formation; these sites may be alkylated, thus altering the rate of sperm decondensation and pronuclear formation (Perreault et al., 1987). In the hamster, the timing of nuclear sperm decondensation and male pronucleus formation are related to sperm nuclear disulfide bond content (Perreault et al., 1987).

Embryos sired by cyclophosphamide-treated males began to display overt morphological abnormalities by day 2 of gestation (corresponding to the 8-cell stage) (Kelly et al., 1994). In contrast to control embryos, which displayed a peak of RNA synthesis at the 4-cell stage, embryos sired by cyclophosphamide-

treated males had constant levels of RNA synthesis at all stages examined. The 4-cell stage peak of RNA synthesis in control embryos is likely to represent zygotic gene activation (Zernicka-Goetz, 1994). A peak of RNA synthesis has been documented in the mouse embryo at the 2-cell stage (Piko and Clegg, 1982); most of the RNA synthesised was ribosomal, indicative of new synthesis originating from the genome of the zygote. Important factor(s) necessary for the normal 4-cell stage peak in RNA synthesis may be deficient in embryos sired by cyclophosphamide-treated males. In addition, there were abnormalities in the regulation of transcription, as immunoreactive Sp1 in 2-cell embryos sired by treated males was increased in the nucleus and spilled into the cytoplasm. Interestingly, this latter observation was manifested also in the BrUTP data, where incorporation was not confined to the nucleus, but spread to the cytoplasm of the 2-cell embryos sired by drug-treated males. These data suggest that there may be a targeting mechanism which is absent (or defective) in 2-cell embryos fathered by drug-treated males.

In the mouse, a number of enhancers and repressors, inherited by maternal factors, are turned on at the 2-cell stage (Nothias et al., 1995, 1996). Repression is believed to regulate the zygotic clock whereby transcription is delayed until the chromosomes of both parental genomes are remodelled from a post-meiotic state to a transcriptionally-permissive state (Henery et al., 1995); this ensures the proper execution of temporal and spatial events taking place in the zygote. One consequence of paternal treatment with cyclophosphamide may be the alleviation of a maternal repression that is required for normal

development; the absence of this repression in embryos sired by cyclophosphamide-treated males leads to a dysregulated and precocious transcription.

Expression profiling has led to the development of robotic methods for arraying thousands of cDNAs on microarrays. When screened with labelled aRNA or cDNA, these arrays generate a molecular fingerprint of a specific cell type. Using this powerful candidate gene approach, we studied the coordinate expression profile of imprinted genes among progeny sired by control and cyclophosphamide-treated males. A defect in the expression profile of specific genes in embryos sired by treated males was observed. A peak of expression was seen at the 8-cell stage in control embryos, possibly indicating *de novo* synthesis of zygotic transcripts. If zygotic gene activation in the rat is initiated at the 4-cell stage, an accumulation of zygotic transcripts may take place between the 4- and 8-cell stages, culminating in a peak in expression at the 8-cell stage. In the mouse, a three-fold increase in RNA content has been documented in 8-cell embryos compared to 2-cell embryos (Piko and Clegg, 1982). Three possibilities could explain the high accumulation of message levels found in the 1- and 2- cell embryo after paternal cyclophosphamide treatment; there could be an increase of transcription (induction), reduced turnover rates reflective of either slow degradation of maternal message or an absence of new synthesis from the embryonic genome, or an increased polyadenylation of existing maternal RNAs.

Here, we report disturbances in the gene expression profile of embryos fathered by cyclophosphamide-treated males starting at the 2-cell stage, prior to

the appearance of any morphological abnormalities. This dramatic increase in transcript levels at the 2-cell stage in embryos sired by cyclophosphamide-treated males is especially surprising because damage to the paternal genome leads to delays in DNA replication and cell division (Kelly et al., 1994). Our data show that paternal drug exposure is an important determinant of early embryo loss, and emphasise the need for an array of complementary tools in the analysis of male-mediated development toxicity.

Acknowledgments

We should like to thank the following investigators for providing us with the following cDNA probes: P. Rotwein for IGFI, C. Polychronakis for IGFII, R. McDonald for IGFIIR, M. H. Lee for $p57^{kip2}$, R. Quirion for ApoE, D.J. Anderson for Mash-2, and J. Douglass for RABP-1. We also thank H. Clarke and O. Mohamed for their advice with the ^{32}P-UTP experiment, and R. Finnell for introducing us to the aRNA technique.

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Figure 1

Male pronucleus formation in hamster eggs. Spermatozoa from the cauda epididymidis of control (white bars) and cyclophosphamide- (hatched bars) treated male rats were collected, capacitated *in vitro*, and incubated with normal eggs for 3-7 hrs. Eggs were fixed and stained to visualise the presence of pronuclei. The data are expressed as the percentage of the total number of embryos analysed for each time point which contained a male pronucleus. Differences in male pronucleus formation between eggs fertilised by drug-exposed and control spermatozoa were analysed by chi-square, with a threshold of significance of $P \leq 0.05$(*). Sperm collected from six control and six cyclophosphamide treated males (n=6) were used to fertilize a mean of 84 eggs/male (the number of eggs varied from 60 to 107/male).

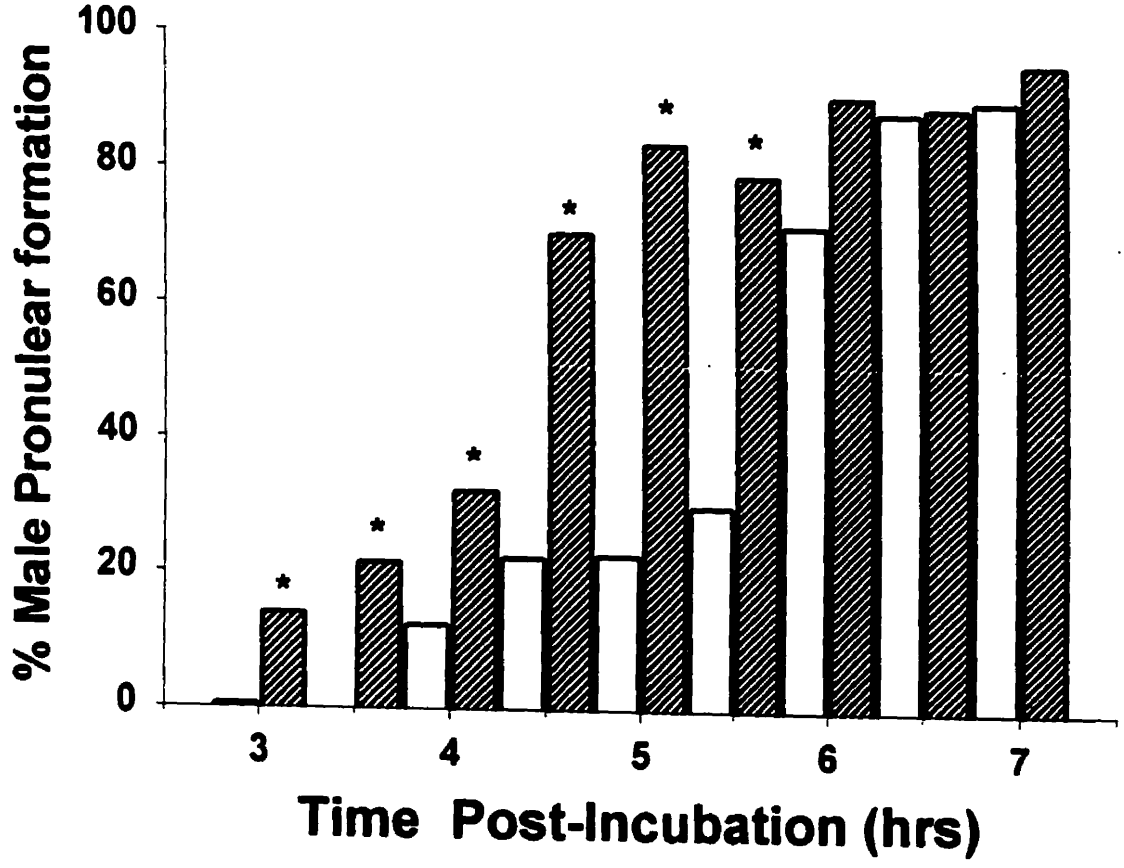

100
80
60
40
20
0
% Male Pronulear formation
*
*
*
*
*
*
3
4
5
6
7
Time Post-Incubation (hrs)

Figure 2

BrUTP incorporation into newly synthesised RNA in 2-cell embryos. Embryos were collected on day 1 post coitum (p.c.), permeabilised and incubated in the presence of BrUTP. Immunostaining with an anti-BrdU antibody and confocal microscopy were done to determine the presence and localisation of BrUTP incorporation in embryos sired by control (A) (n=21) and drug-treated (B) (n=18) males. Optical sections consisting of 1μm each was recorded for each embryo. Settings for the brightness and contrast were kept constant for all samples evaluated in both control and treatment groups. Magnification (400X).

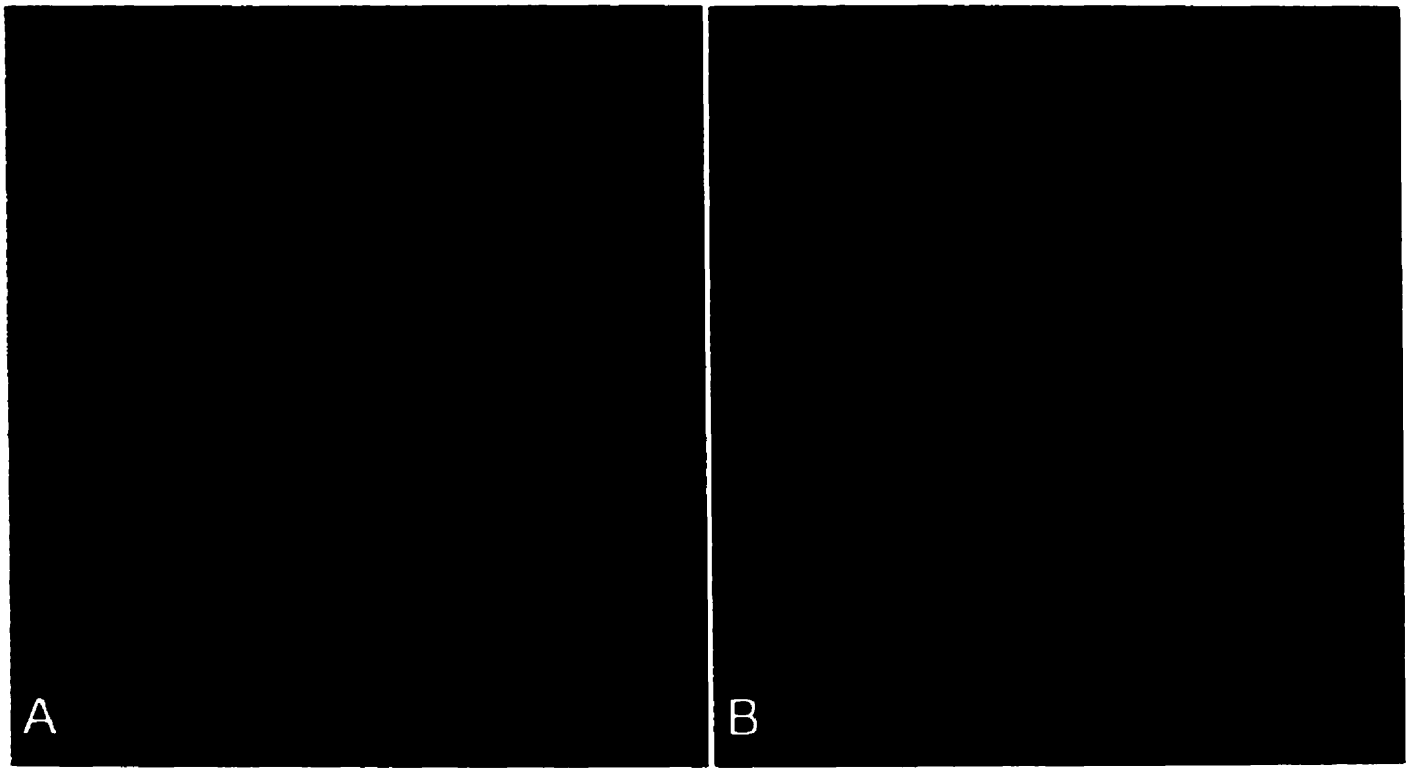

A
B

Figure 3

Sp1 transcription factor localisation in 2-cell embryos. Embryos were collected on day 1 p.c., fixed and immunostained with an Sp1 antibody. A clear difference in localisation pattern was obtained when comparing embryos sired by control (A) (n=10) and cyclophosphamide (B) (n=16) treated males. Similar settings for confocal examination, as described for the BrUTP experiment above, were used for the Sp1 immunostaining. Magnification (400X).

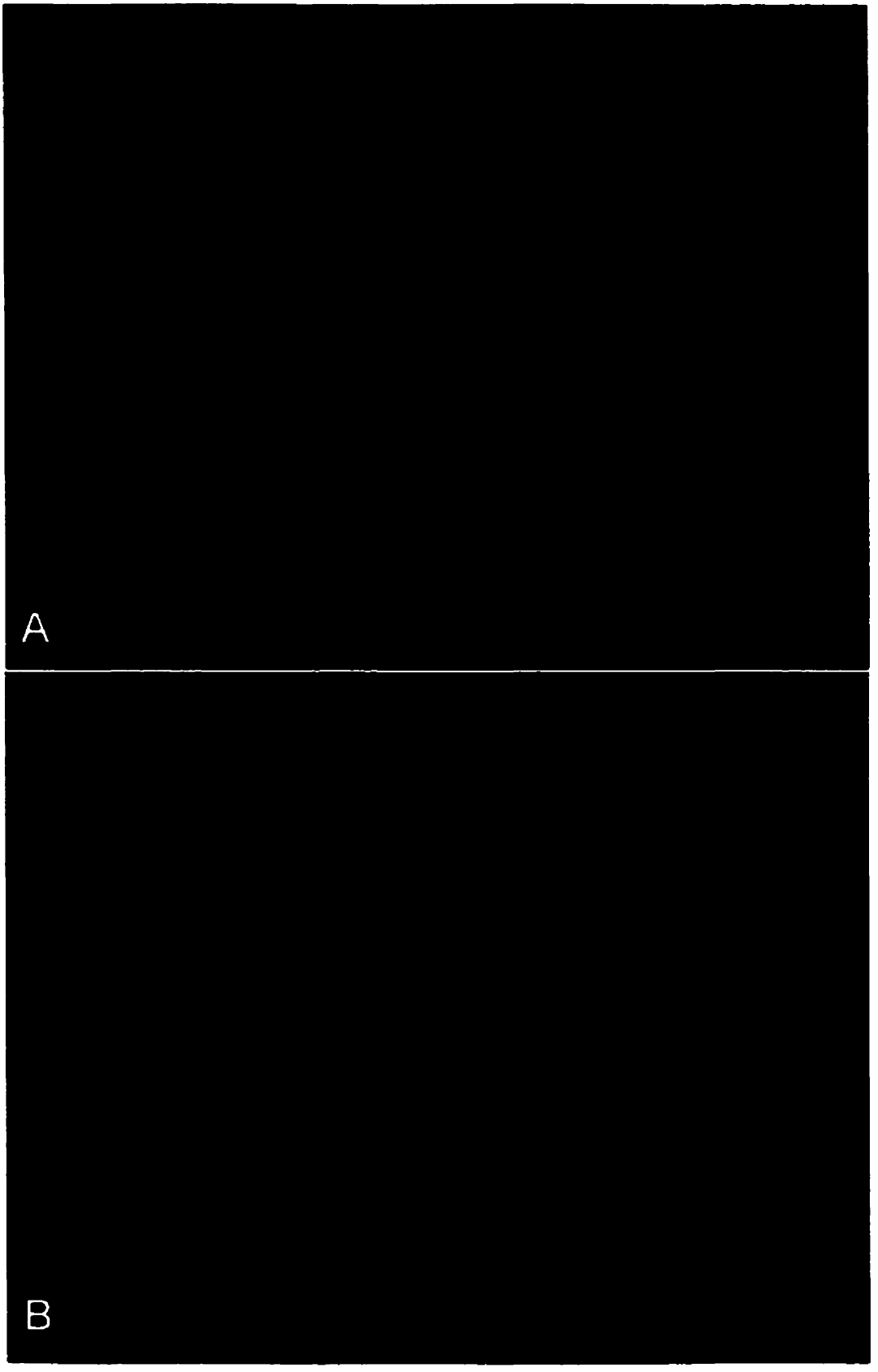

A
B

Figure 4

Total RNA synthesis in pre-implantation embryos. Embryos (n= 30-50 per stage per treatment) were collected on days 0-2 p.c., permeabilised, and incubated in the presence of ^{32}P-UTP for 4 hours. Total RNA synthesis per embryo is represented as the total count per minute (cpm) obtained. To assess the difference in the capacity of embryos sired by control (white bars) and cyclophosphamide (hatched bars) exposed males to synthesise RNA, a t-test analysis was conducted followed by Tukey's post-hoc test with a threshold of significance value of $P \leq 0.05$ (*). Each data point was repeated 3-5 times from 6 different males for each treatment group. Error bars represent the standard error of the mean.

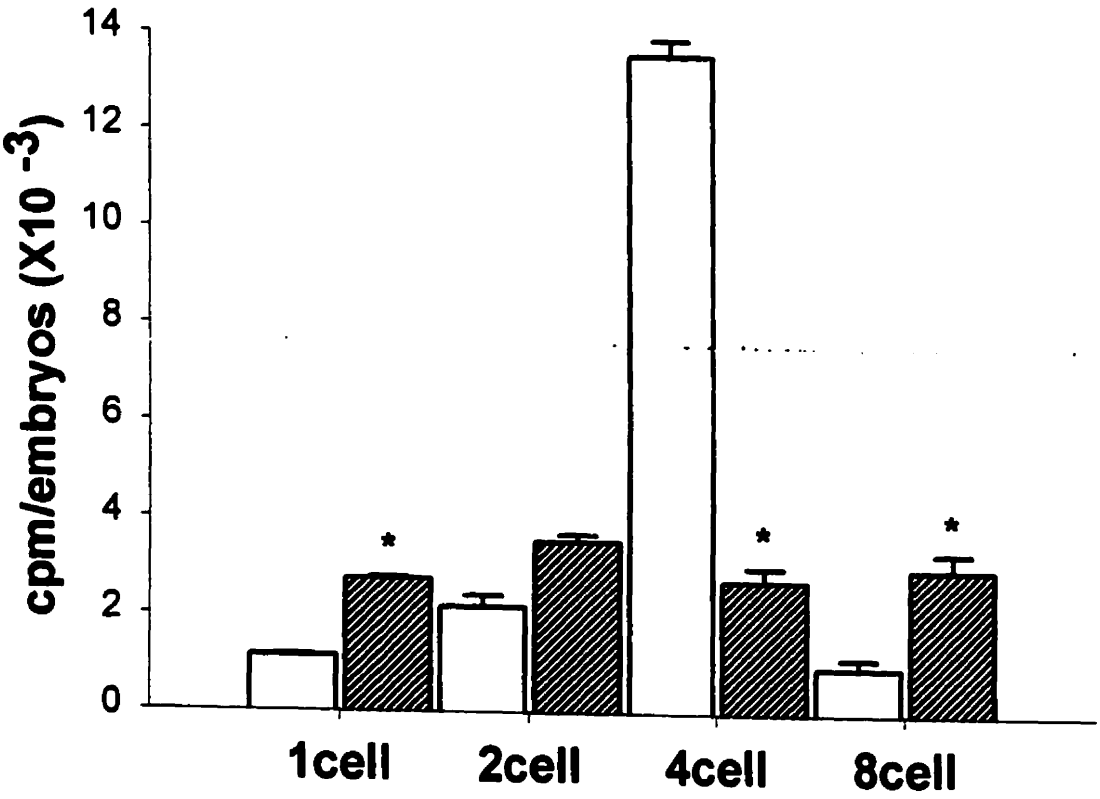
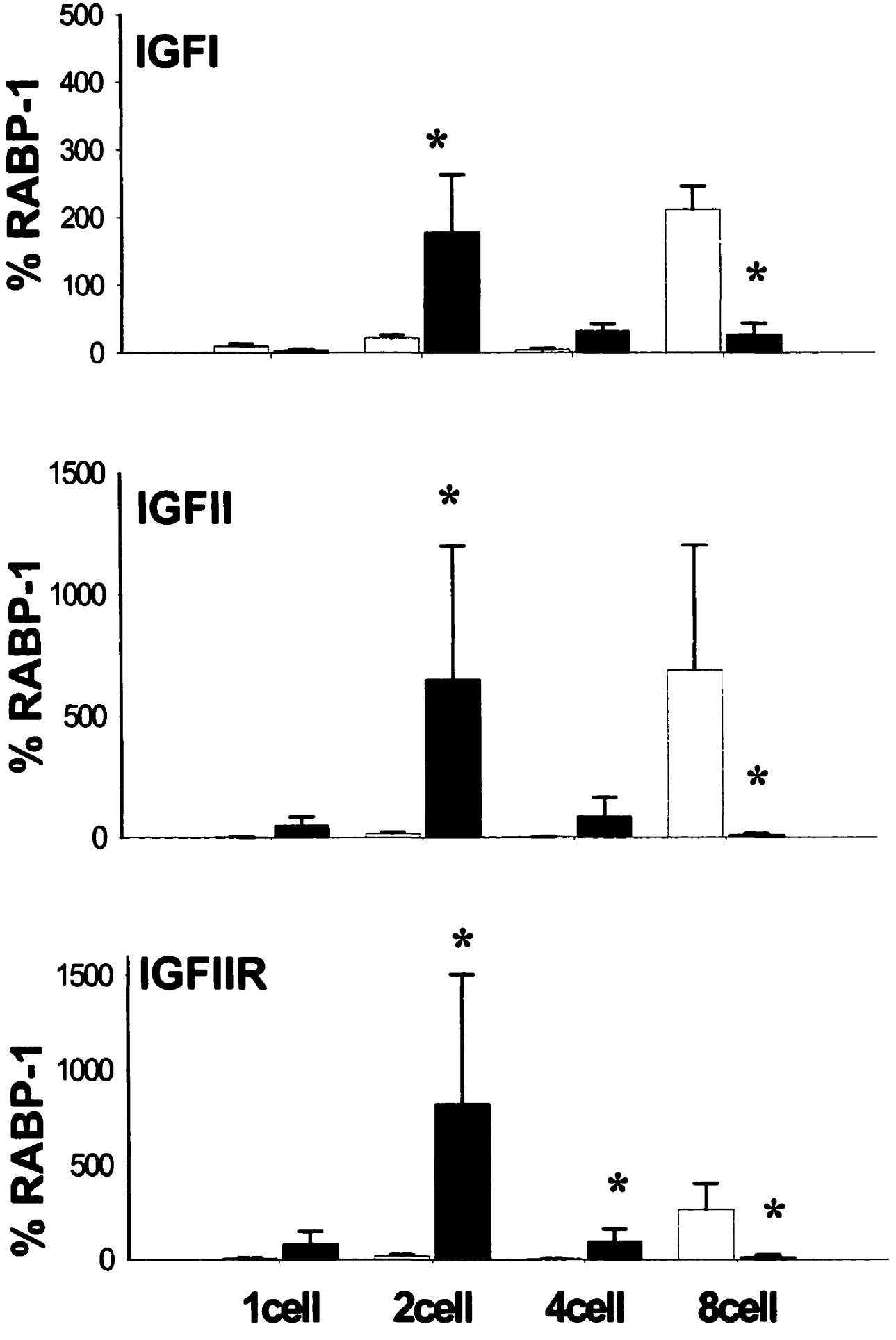

14
12
10
8
6
4
2
0
cpm/embryos (X10 -3)
*
*
*
1cell
2cell
4cell
8cell

Figure 5

IST/aRNA analysis of growth factor gene expression profiles in pre-implantation embryos. Control embryos, grey bars; embryos sired by cyclophosphamide-treated males, black bars. At the 2-cell stage, the relative abundance of transcripts was low for all genes in control embryos; embryos sired by cyclophosphamide-treated males revealed a significant increase in expression of most transcripts ($p<0.001$-0.02). At the 4-cell stage, expression remained low in control embryos; some expression persisted in embryos sired by cyclophosphamide treated males. By the 8-cell stage, control embryos showed an increase in expression for all genes examined. Embryos sired by cyclophosphamide-treated males showed a consistently low expression at this stage. Results are expressed as the means ± the standard errors of the mean ($n=8$ samples representing different litters from 4 different males for each treatment group) of the intensity of expression of each probe as a percentage of the internal standard, RABP-1. Asterisks indicate a significant difference of the treated group compared to controls ($P \leq 0.05$) at that stage.


IGFI
% RABP-1
500
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100
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*
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IGFII
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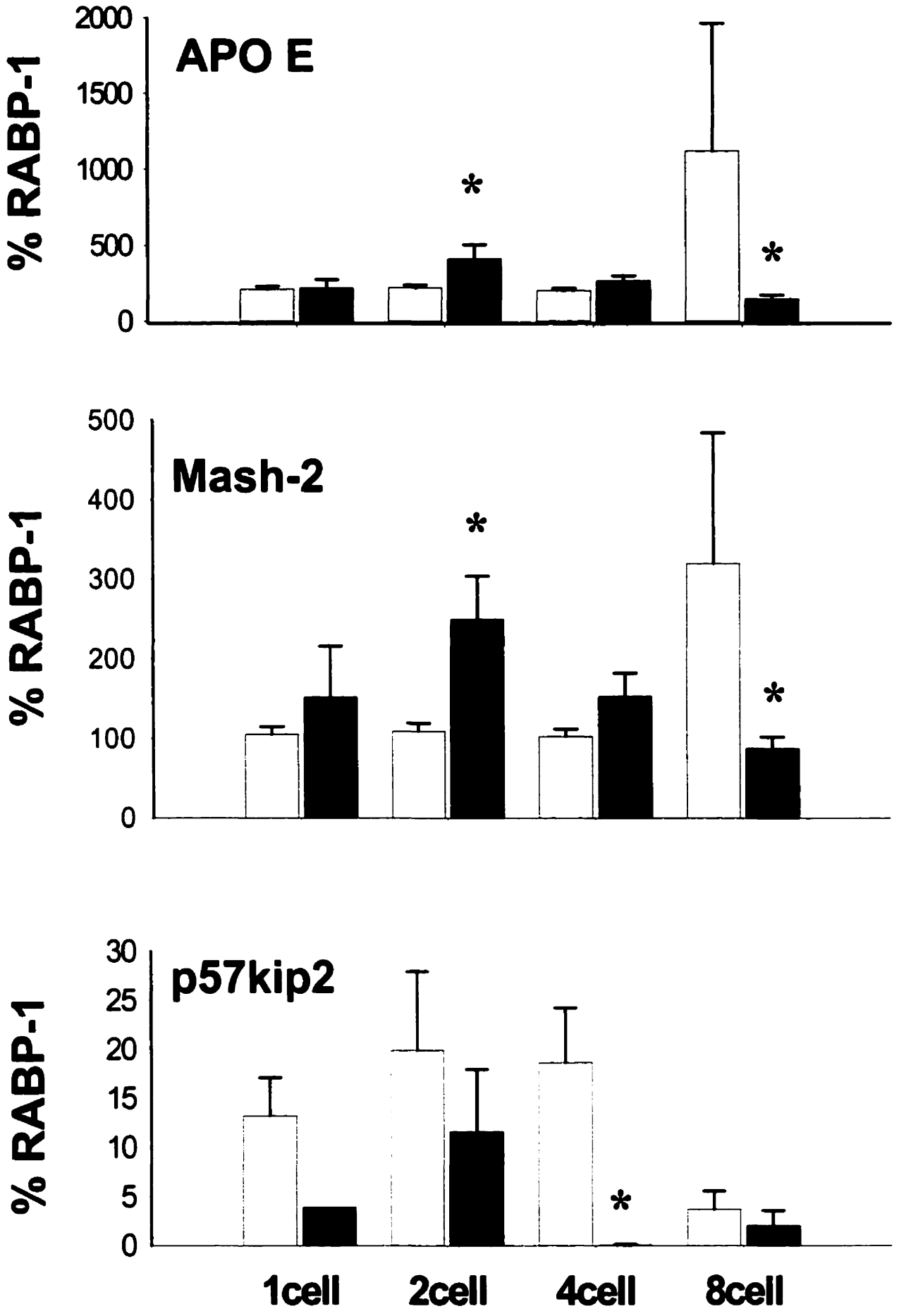
IGFIIR
% RABP-1
1500
1000
500
0
*
*
*
1cell
2cell
4cell
8cell

Figure 6

IST/aRNA analysis of the expression profiles for the imprinted genes: ApoE, Mash-2 and $p57^{kip2}$ in preimplantation embryos. Control embryos, grey bars; embryos sired by cyclophosphamide-treated males, black bars. All data were analysed as described in Figure 5. In contrast to the growth factors expression patterns, ApoE and Mash2 were expressed at moderate levels for all stages analysed for control embryos; $p57^{kip2}$ expression was close to the limit of detection. Results are expressed as the means ± the standard errors of the mean (n=8 samples representing different litters from 4 different males for each treatment group) of the intensity of expression of each probe as a percentage of the internal standard, RABP-1. Asterisks indicate a significant difference from control ($P \leq 0.05$) at that stage.


APO E
% RABP-1
2000
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*
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Mash-2
% RABP-1
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200
100
0
*
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p57kip2
% RABP-1
30
25
20
15
10
5
0
*
1cell
2cell
4cell
8cell

Connecting Text

Data presented in the previous chapter employing complementary approaches demonstrated that the zygotic gene program of the newly fertilized embryo was dysregulated. This was manifested by an earlier formation of the male pronucleus, misincorporation of BrUTP, scattered immunolocalization of Sp1, and an absent peak of total RNA synthesis. Coupled with these events, we observed a precocious peak of mRNA expression for growth factors and imprinted genes among embryos sired by cyclophosphamide treated males when compared to controls. Therefore, we decided to analyze the consequences of the dysregulated gene expression of the newly fertilized embryo on subsequent events occurring in the precompacting embryo using three complementary approaches; First, day 2 embryo sections were assessed for cell numbers and cell contacts. Second, the expression at the mRNA level of cytoskeletal and cell adhesion molecules for 2-, 4- and 8-cell stages was assessed. Third, the expression at the protein level of E-cadherin was determined in embryos sired by control and cyclophosphamide treated males.

Chapter 4

Paternal exposure to cyclophosphamide alters cell-cell contacts and activation of embryonic transcription in the pre-implantation rat embryo

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Biology of Reproduction **63**, 74-81 (2000)

Running title: Paternal drug exposure affects embryonic cell contacts

ABSTRACT

Paternal exposure to chronic low doses of cyclophosphamide, an anti-cancer agent, results in aberrant embryonic development of the progeny. We hypothesized that paternal exposure to cyclophosphamide disturbs zygotic gene activity regulating proper progression through pre-implantation development and that this disturbance results in improper cell-cell interactions. To test this hypothesis, we analyzed cell-cell interactions and the expression of cytoskeletal elements in pre-implantation embryos sired by male rats gavaged with saline or 6 mg/kg/day cyclophosphamide for 5 weeks. Embryos from control litters had 4-12 cells on day 2 of gestation; cell-cell contacts were observed consistently. Embryos from litters sired by cyclophosphamide-treated males were frequently abnormal, had lower cell numbers, and decreased cell-cell contacts. Steady state concentrations of the mRNAs for cell adhesion molecules (cadherins and connexin 43) and structural proteins (ß-actin, collagen, and vimentin) were low in 2- and 4-cell control embryos; expression increased dramatically by the 8-cell stage. In contrast, embryos sired by cyclophosphamide-treated males displayed the highest expression of most trancripts at the 2-cell stage. In parallel with the mRNA profiles, E-cadherin immmunoreactivity was nearly absent in 2-cell control embryos and strong by the 8-cell stage; immunoreactivity in embryos sired by drug treated fathers was strong at the 2-cell stage, but absent at later stages. Thus, drug exposure of the paternal genome led to dysregulated expression of structural elements and decreased cell interactions during pre-implantation embryonic development.

INTRODUCTION

A key mechanism governing the behavior of cells during the early cleavage stages of mammalian development is a continuous series of cell-cell interactions. The number of cell-cell contacts determines successful embryonic development [1]. Although cell interactions in mammalian embryos are partly assured by the presence of the *zona pellucida* [2], the ability of zona-free mouse embryos to develop to term depends on the number of total points of contact between blastomeres; more contacts result in a higher number of inner cell mass cells and, subsequently, a higher number of live offspring [3].

A change in the cell surface of eight-cell embryos, associated with the process of compaction, represents the first differentiation event in the mammalian embryo [4]. Compaction consists of polarization, cell flattening, and junctional communication, all of which require cell-cell interactions [5]. Contact-mediated interactions in the compacting embryo require an intact cytoskeletal organization; treatment of compacting embryos with a cytoskeletal inhibitor prevents compaction [6]. Although the signal to initiate compaction does not occur until the eight-cell stage, two-cell embryos undergo morphological changes preceding the events of compaction. Ultrastructural analysis of human embryos reveals that blastomere surface modifications occur as early as the two-cell stage, as manifested by the loss of microvilli, the acquisition of endocytic activity, and the formation of cell junctions at cell-cell contact points [7]. In the mouse, the membrane proteins required for cell-cell interactions and cytoskeletal- membrane interactions are already synthesized and assembled at the two to four-cell stage [8]. The capacity of the plasma membrane to undergo these changes precedes any detectable activity of the embryonic genome.

One common denominator for all three aspects of compaction (i.e., polarization, cell flattening, and junctional communication) is the requirement for expression of the Ca^{2+}-dependent cadherin, E-cadherin, on adjacent cells. E-cadherin expression spans all stages of pre-implantation development, starting at the one-cell stage [9] and is required for the initial cell-cell contacts at the two- and four-cell stages [10]. Deletion of the E-cadherin gene in mice results in embryos which undergo an initial compaction but subsequently decompact; blastomeres lose their polarity and fail to form a blastocyst [11,12]. Among the genes which interact with E-cadherin is the intermediate filament protein, vimentin; together, they form desmosomes in the mesenchymal epithelium [13, 14]. In the early embryo, a number of other proteins have been localized to cell-cell contacts, including filamentous actin [15] and members of the connexin family; the latter are involved in making up the intercellular membrane channels of gap junctions. Of the members of the connexin family, connexin 43 has the highest expression during pre-implantation development [16].

Chronic treatment of adult male rats with a low dose of the alkylating agent cyclophosphamide affects male germ cells without an effect on the male reproductive system [17], or on the ability of a spermatozoon to fertilize an oocyte [18]. A five-week treatment with cyclophosphamide affects spermatozoa which are first exposed to the drug as late spermatids, thus encompassing spermiogenesis (maturation from spermatids to mature spermatozoa). Starting from the late spermatid stage, the chromatin of male germ cells is tightly packaged due to the replacement of somatic histones with male germ-cell-specific proteins, the protamines [19]. Little, if any, DNA repair or transcription takes place during this period of maturation [20]. It is not until after fertilization that the genome of the spermatozoon is capable of transcriptional activity [21].

As a result, any damage to the drug-treated sperm will only be repaired following fertilization. When mated to normal females, the progeny of treated males start dying at the pre-implantation (15%) and early post-implantation stages (80%); some of the live pups (5%) have malformations [22]. Eight-cell embryos sired by cyclophosphamide-treated fathers have decreased cell numbers and a longer cell doubling time, coupled with a decrease in DNA synthesis capacity, when compared to litters sired by control males [23]. The presence of these abnormalities in eight-cell embryos, at the stage when the first events in differentiation requiring cell interactions take place, suggests that there is a disturbance in the gene expression program regulating major events. Thus, we hypothesize that the zygotic gene activity regulating cell division and the proper progression through pre-implantation development is disturbed as a result of paternal exposure to cyclophosphamide, and that this disturbance results in improper cell-cell interactions. The objectives of the current study were to elucidate the gene expression profiles of key regulators of the cytoskeletal architecture in pre-implantation embryos sired by drug-treated males and to investigate the establishment of cell-cell contacts in these embryos.

MATERIALS AND METHODS

Animals

Adult male (300-325g) and virgin female (225-250) Sprague-Dawley rats were obtained from Charles River Canada (St. Constant, Québec) and housed in the McIntyre Animal Centre, McGill University. Food and water were provided *ad libitum* and animals were exposed to a 14hours-light:10hours-dark cycle.

Treatment, mating, and embryo collection

Male rats were randomly assigned to the control or cyclophosphamide groups, each consisting of six rats. Males were gavaged daily with saline or 6 mg/kg/day cyclophosphamide (Sigma, St Louis, MO) for 5 weeks. Starting on the fifth week of treatment, each male was mated overnight with two females in proestrus. On the night of mating, males were not treated in order to avoid the presence of the drug in the semen [24]. The following morning (considered as day 0 of gestation), females were checked for pregnancy as judged by the presence of spermatozoa in vaginal smears. Sperm positive females were euthanized on days 1, 1.5, or 2 of gestation. To ensure that embryos were collected at consistently similar stages of development, vaginal smears and embryo collection were performed always at the same time of day. Embryos were flushed from oviducts and collected; they were either snap frozen in liquid nitrogen and stored at -80° C until used for the antisense RNA (aRNA) profile analysis or fixed for immunofluorescence or embryo sectioning and light microscopy. For the aRNA analysis, six-eight litters were collected at the two-, four-, and eight-cell stages from both the control and cyclophosphamide-treated groups and each was assayed separately with the protocols outlined in this paper. Each stage was carefully assessed under light microscopy prior to further processing.

Embryo sectioning and light microscopy

Following collection, day 2 embryos were transferred to a depression slide containing 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) (GA) and fixed for 45 minutes at room temperature, followed by two washes in phosphate buffer and one wash in Dulbecco's phosphate buffer supplemented with 10%

bovine serum albumin (BSA), (BSAD) (Gibco BRL, Burlington, ON). Embryos were then transferred to plastic capsules (MECA Lab Ltd., Montreal, QC) containing 10 µl BSAD and allowed to settle to the bottom of the capsule for 30-40 minutes. Capsules were centrifuged horizontally for 15 minutes at 1800 x g at room temperature. Three drops of GA were placed on top of the BSAD and capsules were centrifuged again for 60 minutes at 1800 x g; the capsules were then filled with GA and kept at 4°C overnight. The following day, the molds were removed and the bottoms of the capsules were cut away using a razor blade. Molds were then subjected to a post fixation step using osmium tetroxide (1% in phosphate buffer), dehydrated in serial alcohol washes, infiltrated by propylene oxide alone followed by a mixture (50:50) of propylene oxide and Epon, and finally Epon alone. Epon-embedded molds were left in an oven at 60°C overnight [25]. Thin sections (4μm) were cut on a Reichert Ultramicrotome, stained with toluidine blue O (0.5%) (Fisher Scientific Ltd., Nepean, ON) for 30 seconds, dried, and stored until examination under a Leitz MPS60 phase microscope.

Amplified antisense (aRNA) procedure

mRNA profiles were analysed using the antisense RNA amplification approach (IST/ aRNA). The protocol used for aRNA amplification in pre-implantation embryos was as described previously with minor changes [26]. Embryos from individual litters (an average number of 9 embryos per litter was collected and considered as one sample) were sonicated in the presence of lysis buffer containing 1 mg/ml digitonin, 5 mM dithiothreitol (DTT), and 1X in situ transcription (IST) buffer (50mM Tris, 6mM $MgCl_2$, 0.12mM KCl, pH 8.3). Embryo homogenates were reverse-transcribed at 37°C for two hours in the presence of an unlabeled oligo dT_{24}- extended at the 5' with the T7 RNA polymerase

promoter (50 ng); recognition of the poly(A) tail of mRNA populations results in complementary cDNA synthesis. Reverse transcription was performed using the avian myeloblastosis virus reverse transcriptase (50 U; NEB, Mississauga, ON) which binds to the primer and copies single stranded mRNAs to cDNAs in the presence of deoxyribonucleotides (Boehringer Mannheim, Laval, QC). Following a phenol/chloroform extraction, the first strand cDNAs were precipitated with ethanol and glycogen (4 mg). The pellet was resuspended in 10 μl water, heated to 95°C to denature the DNA:RNA hybrid, and chilled on ice. Single-stranded cDNAs were allowed to self-prime (by hairpin loop formation) to form double cDNA strands (ds-cDNAs). Following excision of the self-primed hairpin loop by S1 nuclease (2 U) for 3 min at 37°C, ds-cDNAs were blunt ended and filled-in with the addition of T4 DNA polymerase (5 U) and Klenow fragment of *Escherichia coli* (5 U) in the presence of dATP, dCTP, dGTP and dTTP nucleotides. The resulting ds-cDNAs, which contained the promoter region for the bacteriophage T7 RNA polymerase (NEB), were extracted with phenol/chloroform and amplified into aRNA in the following mixture: 40 mM Tris pH 7.5, 6 mM $MgCl_2$, 10 mM DTT, 400 mM of ATP, GTP, UTP, and 4 mM CTP, 20 U RNasin, 100 U T7 RNA polymerase, and ^{32}P-radiolabeled-CTP (30 μl) in a final volume of 25 μl. This reaction was conducted for 4h at 37°C.

Reverse Northern blots and genetic expression profiles

Equimolar concentrations of cDNA clones were calculated based on the approximate size of the insert relative to the vector size, which served to normalize the total amount of each of the cDNA clones immobilized on slot blots and hybridized with the aRNA probes. Radiolabelled amplified RNA populations obtained form embryos at the one-, two-, four-, and eight-cell stages were

utilized as probes to screen slot blots containing the following cloned cDNAs: E-cadherin [27], N-cadherin [28], P-cadherin [29], vimentin [30], collagen [31], connexin 43 [32], ß-actin [33] and retinoic acid binding protein-1 (RABP-1) [34]. Individual blots were pre-hybridized for 30 min in buffer (50% formamide, 0.12 M Na_2HPO_4, pH 7.2, 0.25 M NaCl, 7% w/v SDS) at 42°C. The heat denatured aRNA probe from each embryo sample was applied to the blots and allowed to hybridize overnight at 42°C. The following day, blots were washed at decreasing stringency from 2X standard saline sodium chloride (SSC) solution down to 0.1% SSC and 0.1% SDS at 42°C for 20 minutes in a shaking water bath. Blots were exposed to phosphorimager plates overnight. Individual band intensities were determined after exposure of the plates to a phosphorimager scanner (Molecular Dynamics, Sunnyvale, CA). The individual signals were corrected for nonspecific binding by subtracting the background values on each blot and normalized to the internal standard. The use of an internal standard allows comparisons between different blots, as the individual hybridization intensities of each cDNA on a blot are expressed as a ratio of expression to that of the internal standard. The three internal standards on each blot were retinoic acid binding protein-1 (RABP-1), DNA methyltransferase-1, and γ-glutamylcysteinyl synthase 50; all three cDNAs showed similarly consistent results between stages and groups (data not shown). Subsequent data are expressed as a percent of RABP-1 transcript concentrations; steady state concentrations of RABP-1 mRNA were consistent between groups (control and drug-treated) and across blots, thus facilitating experiment to experiment and blot to blot quantification and comparison.

As the cDNA population should reflect accurately the abundance of the original mRNA population, the intensity of the aRNA hybridization signal reflects the abundance of the original mRNA for any given gene. Six to eight expression

profiles, each representing individual litters, were obtained for each stage and treatment group. A representative blot displaying the hybridization signal resulting from a litter at the two-cell stage is shown in Figure 1; bands representing the genes discussed in this paper have been identified.

Immunofluorescence and laser-scanning confocal microscopy

Embryos were fixed in a solution of 95% ethanol and glacial acetic acid (9:1 v/v) for 15 minutes; this step eliminated the need for removal of the *zona pellucida* which made the embryos very sticky and caused disaggregation of blastomeres. Fixation was followed by rigorous washing in 1x phosphate buffered saline (PBS) / polyvinylpyrolidone (1%). Embryos were permeabilized (0.05% Triton X-100, 1% BSA in PBS for 15 minutes, blocked for 1 hour in 3% BSA, 0.1% Triton X-100 in PBS), and then incubated overnight at 4°C in a humidified chamber with the primary antibody for E-cadherin (a polyclonal anti-rabbit antibody, Signal Transduction Laboratories, Mississauga, ON) at a 1:50 dilution in blocking solution. For the negative controls, the primary antibody was omitted from the protocol. Embryos were washed for 3 x 20 minutes in blocking solution, incubated in a fluorescein-conjugated mouse anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) for 1 hour, washed 3 x 10 minutes, and incubated in propidium iodide (1μg/ml) (Molecular Probes) in blocking solution to stain nuclei, followed by rigorous washing. To mount embryos, a drop of immunomount (Shandon, Pittsburgh, PA) was placed on a Superfrost microscope glass slide and embryos were pipetted into the drop. Embryos were allowed to settle to the bottom of the drop to prevent them from floating and covered with a glass cover slip. Slides were allowed to dry overnight in the dark before examination under a Zeiss LSM410 laser scanning microscope.

Statistical analysis

Analysis of the developmental regulation of gene expression for each embryonic stage was conducted separately on litters sired by control- and cyclophosphamide- treated males using a pairwise multi-comparison procedure (ANOVA) followed by a Tukey's post hoc analysis ($p<0.05$). To reveal any effect of paternal treatment on gene expression in the progeny, a pair-wise comparison consisting of a t-test followed by a Tukey's post-hoc test ($p<0.05$) for each developmental stage was conducted between litters fathered by control and cyclophosphamide-treated males. Statistical analysis was done using a Sigmastat 2.3 (SPSS Inc., Chicago, IL) software package.

RESULTS

Morphological assessment of cell numbers and cell-cell interactions

Analysis of pre-implantation embryos at the light microscope level revealed that control embryos on day 1.5 of gestation were usually at the four-cell stage with the presence of cell-cell contacts (Fig. 2A). A significant delay in cell division starting from day 1.5 postcoitum was seen in litters sired by cyclophosphamide-treated male rats, when compared to controls (data not shown). By day 2 of gestation, control litters had divided to reach up to the 16-cell stage (Fig. 2B). Even though progeny of cyclophosphamide-treated males had developed to the four-cell stage and beyond, a significant number of embryos contained dead blastomeres (Fig. 2C,E), some individual blastomeres looked slightly shrunken (Fig. 2D); thus contributing to a reduction in cell contacts in these embryos. Other abnormalities observed in litters sired by treated males included a higher percentage of abnormal/degenerated embryos (Fig. 2F).

A comparison of the cell numbers and cell contacts in approximately 100 embryos per group on day 2 of gestation is shown in figure 3. Analysis of cell numbers among control embryos revealed a range of cell numbers from 4-6 (39%), to 8 (42%), or 12-16 (15%) (Fig. 3A, grey bars). Analysis of cell numbers in progeny of cyclophosphamide-treated males revealed that these embryos were capable of progressing to the four-six (41%) and the eight-cell stage (21%). However, embryos in this group manifested a number of abnormalities compared to those from control litters; these included an increase in the percentage of embryos classified as abnormal or degenerating (26%) and an arrest of some embryos at the two-cell stage (9%); these findings explain the

lower number of embryos that advanced to the eight-cell (21%) and the 12-16-cell stages (3%) when compared to control embryos (Fig. 3A, black bars).

Analysis of the percentage of embryos having cell-cell contacts on day 2 of gestation revealed that most control embryos at all stages examined exhibited the presence of cell contacts (approximately 95%) (Fig. 3B, grey bars). In contrast, less than half of the embryos sired by cyclophosphamide-treated fathers displayed such cell-cell contacts at all of the stages examined with the exception of the 12-16 cell stage (Fig. 3B, black bars).

Temporal expression of cell adhesion molecules and cytoskeletal elements during rat pre-implantation development

Control embryos expressed low levels of transcripts at the two- and four-cell stages for all of the genes examined; only vimentin was expressed with a relatively moderate abundance. At the eight-cell stage, an increase in expression was seen for most of the genes examined; this was most marked for N-cadherin, E-cadherin and vimentin ($p< 0.01$, 0.003, 0.04, respectively, Fig. 4). Analysis of the gene expression profiles among the embryos sired by cyclophosphamide-treated males revealed a remarkable shift of maximal gene expression, for many of the genes examined, to the two-cell stage ($p<0.02$-0.001). A decrease ensued by the 4-cell stage, and a further dramatic decrease was observed at the eight-cell stage (Fig. 4). A comparison of the embryos in each treatment group was conducted separately for each developmental stage. An eight-fold increase in expression for all genes except for β-actin was seen at the two-cell stage in embryos sired by treated fathers ($p<0.001$). By the four-cell stage, embryos in the paternally drug-treated group still showed a dramatic increase in transcripts over controls, especially for N-cadherin, P-cadherin, and vimentin ($p<0.004$,

0.006, 0.025, respectively). At the eight-cell stage, except for connexin 43, the expression for all genes examined was remarkably reduced ($p<0.003$-0.01) among the progeny of cyclophosphamide-treated fathers. These data suggest that a precocious increase in the steady state concentrations of cell structural gene transcripts among embryos sired by cyclophosphamide-treated fathers may be incompatible with normal embryonic development.

Immunofluorescence of E-cadherin

Immunostaining of E-cadherin in two-, four-, and eight-cell embryos, sired by control and cyclophosphamide-treated fathers, revealed a time-shift in maximal immunoreactivity (Fig. 5). In control embryos, E-cadherin immunoreactivity was low at the two- and four-cell stages and localized between blastomeres in the regions of cell-cell contacts. At the eight-cell stage, this expression increased to encompass a cytoplasmic staining which was not seen in earlier stages (Fig. 5A, B, C). The strong staining in the cytoplasm overwhelmed the staining at cell-cell contacts in the embryo shown in this figure. Embryos sired by cyclophosphamide-treated males showed a strong E-cadherin immunoreactivity at the two-cell stage, in a pattern that was not restricted to cell-cell contacts; staining was evident throughout the cytoplasm and perinuclear areas. At the four- and eight-cell stages, embryos from the treated group showed cytoplasmic staining that was reduced substantially from that seen at the two-cell stage and in stage-matched control embryos (Fig. 5D, E, F).

DISCUSSION

Chronic administration of the anti-cancer agent, cyclophosphamide, to male rats results in a dose- and time-dependent embryonic loss starting from the

pre-implantation stages of development. In this study, we examined the spatial and temporal events that precede this embryonic loss. Using light microscopy, we traced the early morphological abnormalities to day 2 of gestation, a critical time in embryonic development that corresponds to embryonic compaction and to the first signs of the differentiation program of the embryo. A diagrammatic representation of some key abnormalities found in progeny after paternal treatment with cyclophosphamide is shown in figure 6; abnormalities include a delay in cell division, a higher proportion of embryos lacking cell-cell contacts, and a high number of degenerated and abnormal embryos.

The mechanisms underlying the loss of cell interactions have been studied in several other model systems. Disrupting cell-cell interactions in *Xenopus* embryos results in a differential response in mesodermal and ectodermal lineages; whereas expression of α-actin, a mesodermal gene, is inhibited when cell interactions are disrupted, that of DG81, an ectodermal gene, is not affected [35]. In *C. elegans* embryos, cell-cell interactions are pivotal in the production of regions during embryogenesis [36]. In our model, a dysregulation in the gene expression program of cytoskeletal elements in the embryo, starting during pre-implantation stages, leads to distinct abnormalities at various levels of cell lineage allocation in the developing embryo. This dysregulation is evident as early as the two-cell stage when steady state concentrations of all transcripts, except collagen, were high; a subsequent decrease was observed in all transcripts, except for connexin 43, at the eight-cell stage. The accumulation of transcripts in these embryos may be due to either an increase in transcription (induction) of the zygotic genome or a decrease in the rate of maternal mRNA degradation (reduced turnover). Our current experimental design does not distinguish between these two possibilities.

In accord with the aRNA data, embryos sired by cyclophosphamide-treated males showed a similar precocious increase of E-cadherin immunoreactivity at the two-cell stage that was not sustained in later stages. Interestingly, the immunoreactive E-cadherin was not targeted to the expected cellular compartment. In control embryos at the eight-cell stage E-cadherin undergoes phosphorylation, which causes displacement to the cytoplasm [37]. This might explain the presence of a strong cytoplasmic signal in control embryos.

Starting at the 16-32 cell transition during normal development, the extent to which outer blastomeres divide to contribute to either the inner cell mass (the non-polar cells) or trophectoderm cells (the outer polar cells) is related to the extent of flattening of the outer blastomeres. When the flattening of the latter cells is less extensive, their contribution to non-polar cells is higher, a phenomenon referred to as differentiative division. The inside cells, by forcing outwards on polar cells, might delay or even inhibit division itself [38]. In our model, there is a general decrease in cell number (both inside and outside), thus the pressure exerted by the inner cells on the outer cells is not established. Of particular note, analysis of embryonic morphology at the eight-cell stage reveals a high number of embryos missing inner cells (Fig. 6). As a result, it is tempting to speculate that the lower cell numbers may result in an initial delay of the differentiative division of eight-cell blastomeres and an eventual inhibition of division itself, thus potentiating the retardation and resulting in eventual failure of the embryos to proceed through implantation.

Normal cell associations are maintained in pre-implantation embryos by the presence of a number of cytoskeletal and cell adhesion proteins (*Xenopus laevis*: [35]; Human: [39]). In turn, cell interactions regulate the expression profile

of a number of genes, including cingulin [40], syndecan [41], spectrin [42], and myosin [43]. In the present study, control embryos exhibited a dramatic increase in expression of cytoskeletal and cell adhesion molecules at the eight-cell stage. During normal development, a number of the transcripts important for cell proliferation, cavitation, and blastocyst formation accumulate in abundance in a second wave of gene activation, concomitant with compaction [44]. In contrast, embryos sired by cyclophosphamide-treated rats had a premature peak of expression at the two-cell stage. This was unexpected since the morphological data suggested a retardation of cell division and development. On the basis of differences in protein synthetic profiles from the one-cell to the four-cell stage [45], it is likely that the activation of embryonic transcription in rats occurs at the two-cell stage, as it does in mice and other rodents [46]. In the mouse, two-cell embryos acquire a repression of gene activation prior to any cell division, a property inherited from maternal factors [47]. Such an activity is lacking in male pronuclei and the cytoplasm of early one-cell embryos [48]. This suppression has been proposed to coordinate the zygotic clock of the embryo and to ensure a proper sequence of transcriptional and translational events in the embryo [49]. If this suppression is also an important feature controlling the timing of zygotic gene activation in the rat embryo, a disturbance in its regulation may be one of the consequences of exposure of the paternal genome to drugs.

We have shown previously that the nuclei of spermatozoa from male rats treated with cyclophosphamide decondense in vitro at a faster rate than do the nuclei of spermatozoa from control rats [50]. It is possible that the initial decondensation of cyclophosphamide-exposed spermatozoa is also faster in the oocyte cytoplasm. This might hasten the initial events of gene activation and transcription in these embryos, as observed here. However, in this study we

have shown that this initial gene activity was not sustained until later stages, as transcript levels fell by the eight-cell stage, a period when transcripts are required in control litters for differentiation events. The embryo loss among litters sired by drug-treated males could be a result of this abnormal, precocious, gene activity.

It is likely that an intact sperm nucleus is an essential requirement for normal embryonic development. A recent report illustrating the need for a normal sperm nucleus lends support to this hypothesis [51]. Exposure of males to cyclophosphamide resulted in an increased incidence of DNA breaks in their germ cells [52]. In addition, cross-links in spermatozoal chromatin have been reported following exposure to other alkylating agents [53]. The genome of spermatozoa obtained from cyclophosphamide-treated males had a more open conformation, as assessed by initial in vitro DNA template function, than did the genome of spermatozoa from control males [52]. Exposure of the paternal genome to cyclophosphamide may affect DNA synthesis in the first round of zygotic DNA replication. Early DNA replication in embryos sired by cyclophosphamide-treated males might relieve the transcriptionally repressive state that normally arrests maternal gene expression, thus leading to premature differentiation [54].

When two- and four-cell mouse embryos were incubated in the presence of aphidicolin, a reversible inhibitor of DNA polymerase a, during the G1 phase, only embryos incubated at the two-cell stage were affected adversely [55]. These effects were manifested as delayed DNA replication and the absence of cell division; there was no flattening or polarization, events that occur only later in eight-cell embryos. These data suggest that it is early inhibition of DNA replication that has a maximal impact on later events in the differentiation of the pre-implantation embryo. Dysregulation of the pattern of gene expression in two-

cell stage embryos sired by cyclophosphamide-treated males may result from inhibition of DNA synthesis which is manifested later as a failure of these differentiative events.

The DDK syndrome is a genetic mouse model that shows an early embryonic lethal phenotype caused by an incompatibility between a maternal and a paternal factor of DDK origin [56]. Transmission of the gene through the maternal genome contributes to the defect [57]. One of the characteristics of the DDK syndrome is that embryos have a defect in cell interactions starting at the 8-cell stage [58].

It is tempting to speculate, based on the results obtained from the DDK model and from paternal exposure to cyclophosphamide, that there is a differential parent-of-origin effect that influences major events of the embryonic program such as the establishment of cell-cell interactions. Such events must be sensitive turning points which are susceptible to various insults and require components contributed by both the maternal and paternal genomes.

ACKNOWLEDGMENTS

We should like to thank the following investigators for providing cDNA probes: G. Batist for connexin 43; M. J. G. Bussemakers for vimentin; A. C. Cuello for β-actin; J. Douglass for retinoic-acid binding protein-1(RABP-1, BC-1); N. Morris for the α1(XI) chain of collagen; and M. Takeichi for E-, N-, and P-cadherins. We should also like to thank R. H. Finnell for introducing us to the aRNA technique and M. Ballak and C. Huang for excellent technical assistance with embryo embedding and sectioning.

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Figure 1

A representative blot illustrating the banding pattens resulting from hybridization of aRNA from a 2-cell stage litter sired by a cyclophosphamide-treated male to a slot blot membrane. Bands representing hybridization to the cDNAs discussed in this paper are identified.

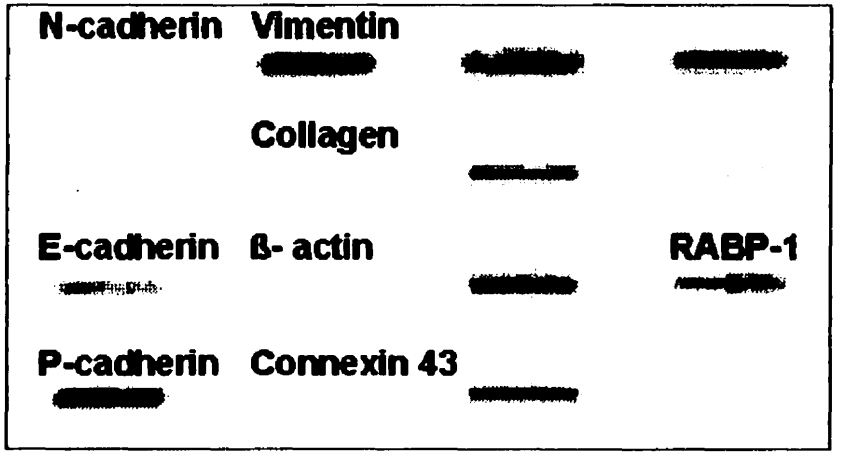

N-cadherin
Vimentin
Collagen
E-cadherin
ß- actin
RABP-1
P-cadherin
Connexin 43

Figure 2

Representative embryonic morphology examined under light microscopy on days 1.5 (A) and 2 of gestation (B-F). Embryos were scored for cell numbers, the presence of cell-cell contacts and any abnormal structures within the *zonae pellucidae*. On day 1.5, control embryos were at the 4-cell stage (A). Day 2 control embryos had advanced to the 8-12-cell stages (B). Embryos sired by cyclophosphamide-treated fathers (C-F) displayed a number of abnormalities: loss of individual blastomeres (C, D), degenerate or abnormal morphology (F) as well as absence of cell-cell contacts (E).

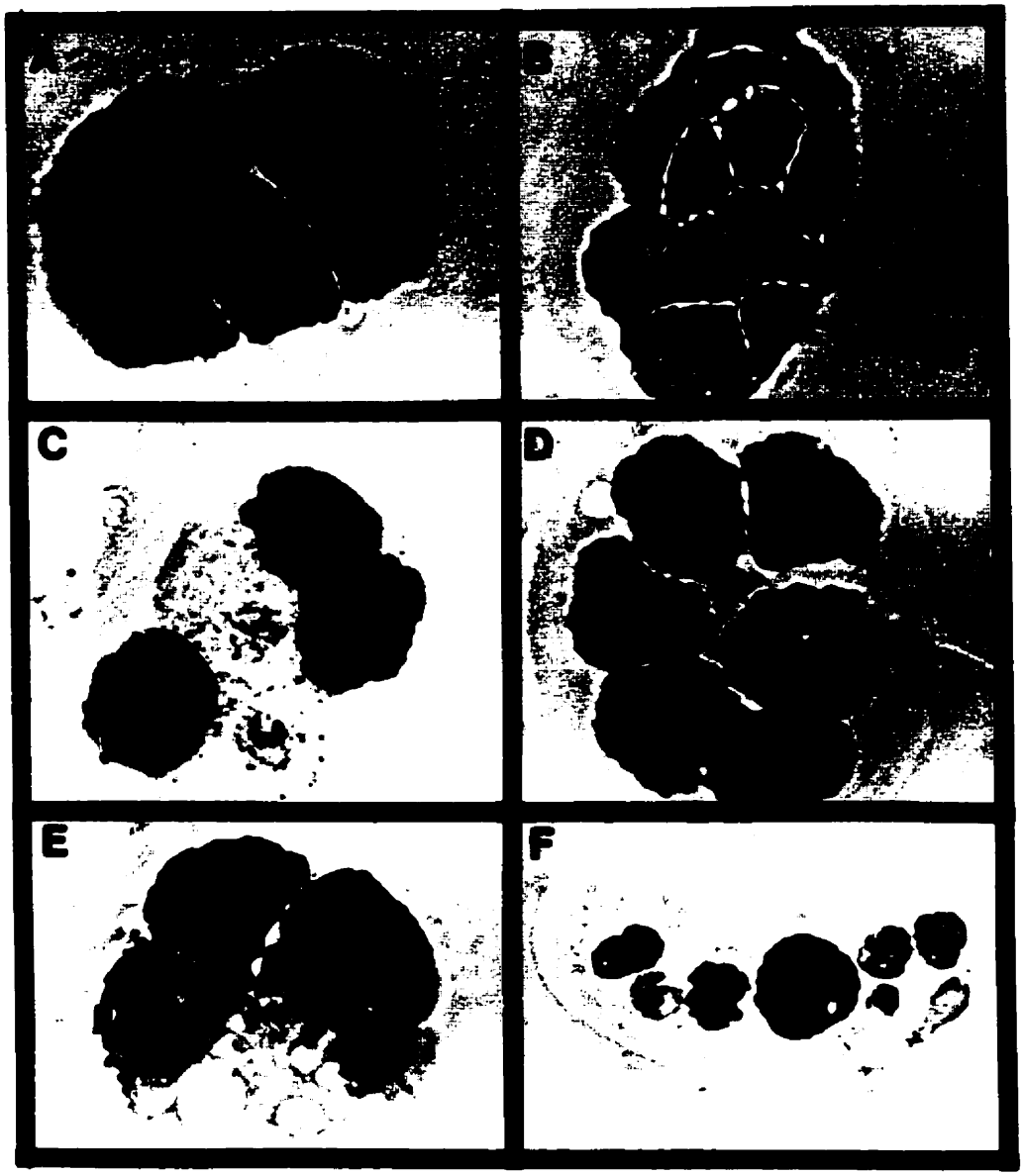
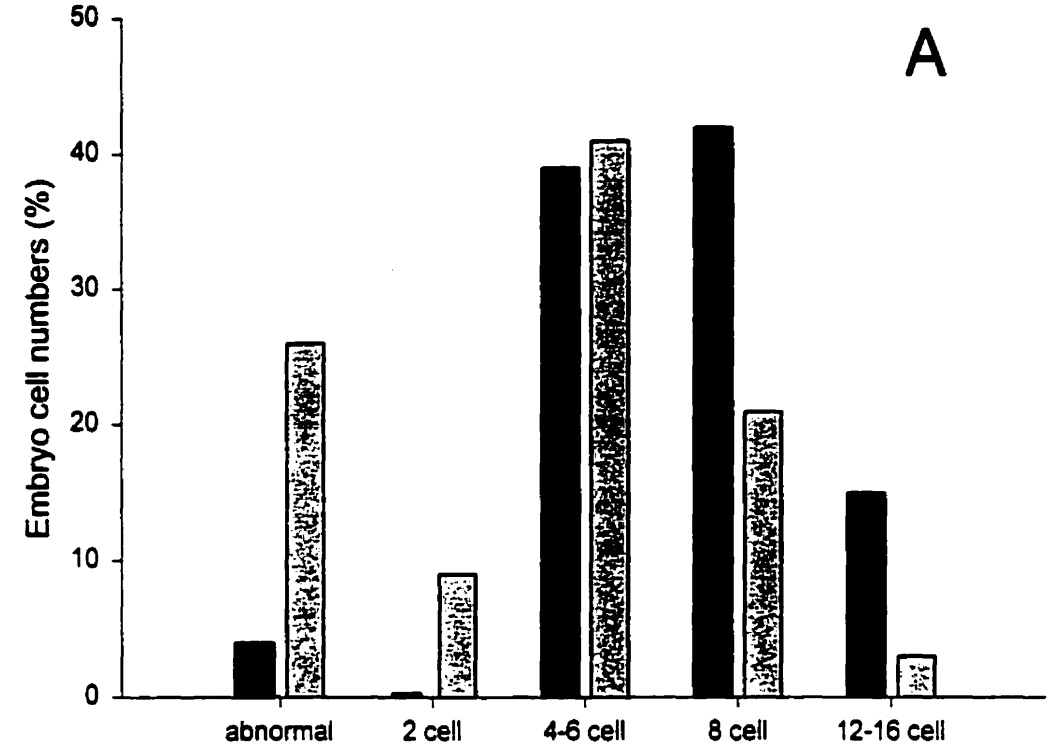

C
D
E
F

Figure 3

Cell numbers (A) and cell contacts (B) in progeny sired by control and cyclophosphamide-treated fathers on gestational day 2. Embryos sired by control males (n= 95) (grey bars, A) had cell numbers that ranged from the 4-8-cell stages with some reaching the 12-cell stage. Embryos sired by cyclophosphamide-treated males (n=100) (black bars, A) had lower cell numbers and a higher percentage of abnormal, fragmented, or retarded embryos. The majority of embryos in control litters displayed cell contacts (grey bars, B), whereas a markedly reduced number of embryos with cell contacts were seen among the progeny of drug-exposed males (black bars, B).


A
Embryo cell numbers (%)
50
40
30
20
10
0
abnormal
2 cell
4-6 cell
8 cell
12-16 cell

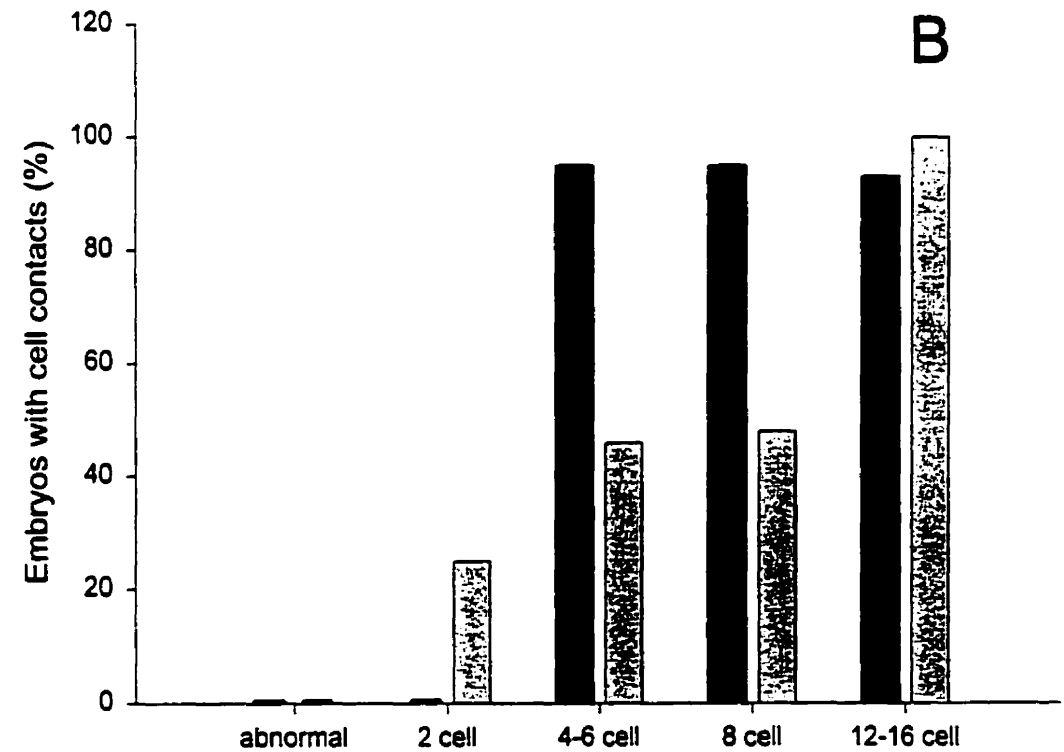

B
Embryos with cell contacts (%)
120
100
80
60
40
20
0
abnormal
2 cell
4-6 cell
8 cell
12-16 cell

Figure 4

aRNA analysis of the expression profiles of cell adhesion and cytoskeletal genes in preimplantation rat embryos sired by control and cyclophosphamide-treated male rats. Results are expressed as the means ± standard errors of the mean (n = 8) of the intensity of expression of each probe as a percentage of the internal standard, RABP-1. Control embryos, light grey bars; embryos sired by cyclophosphamide-treated males, black bars. At the 2-cell stage, transcripts were low for all genes in control embryos; embryos sired by cyclophosphamide-treated males revealed a marked and significant increase in expression of most transcripts ($p<0.001$-0.02). At the 4-cell stage, expression remained low in control embryos; some expression persisted for E-, & P-cadherin as well as vimentin in embryos sired by cyclophosphamide-treated males, all of which were significantly higher than controls ($p< 0.004$-0.026). By the 8-cell stage, control embryos showed an increase in expression of most genes examined with a peak seen in transcripts for N- ($p<0.01$) and E-cadherin ($p<0.003$), vimentin ($p<0.04$) and collagen ($p<0.042$); embryos sired by cyclophosphamide-treated males showed a consistently low expression at this stage. Asterisks indicate a significant difference from control ($P \leq 0.05$) at that stage.

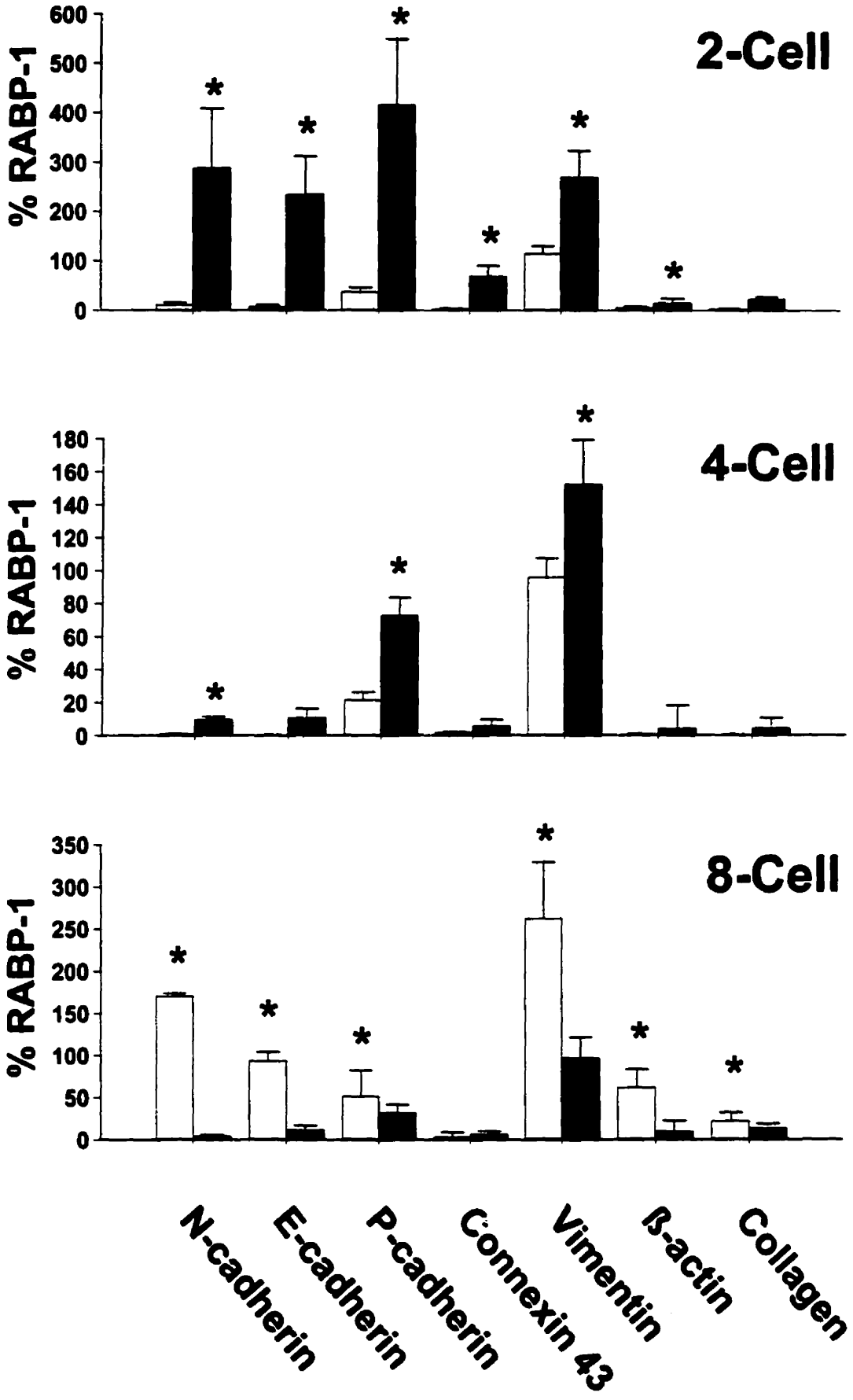

2-Cell
% RABP-1
600
500
400
300
200
100
0
4-Cell
% RABP-1
180
160
140
120
100
80
60
40
20
0
8-Cell
% RABP-1
350
300
250
200
150
100
50
0
N-cadherin
E-cadherin
P-cadherin
Connexin 43
Vimentin
ß-actin
Collagen

Figure 5

Immunolocalization of E-cadherin in 2-, 4-, and 8-cell rat embryos. Immunostaining of E-cadherin epitopes is depicted in green whereas nuclear staining is seen in red. Embryos sired by saline-treated males (left panels) showed staining at cell-cell contacts at the 2- and 4-cell stages (A, B). This staining became more cytoplasmic by the 8-cell stage (C). Immunostaining of E-cadherin in embryos sired by cyclophosphamide-treated males (right panels) at the 2-cell stage was not confined to cell contacts; it spread to the cytoplasm and the perinuclear area (D). Four- and 8-cell stage embryos did not have a detectable signal for E-cadherin (E and F).

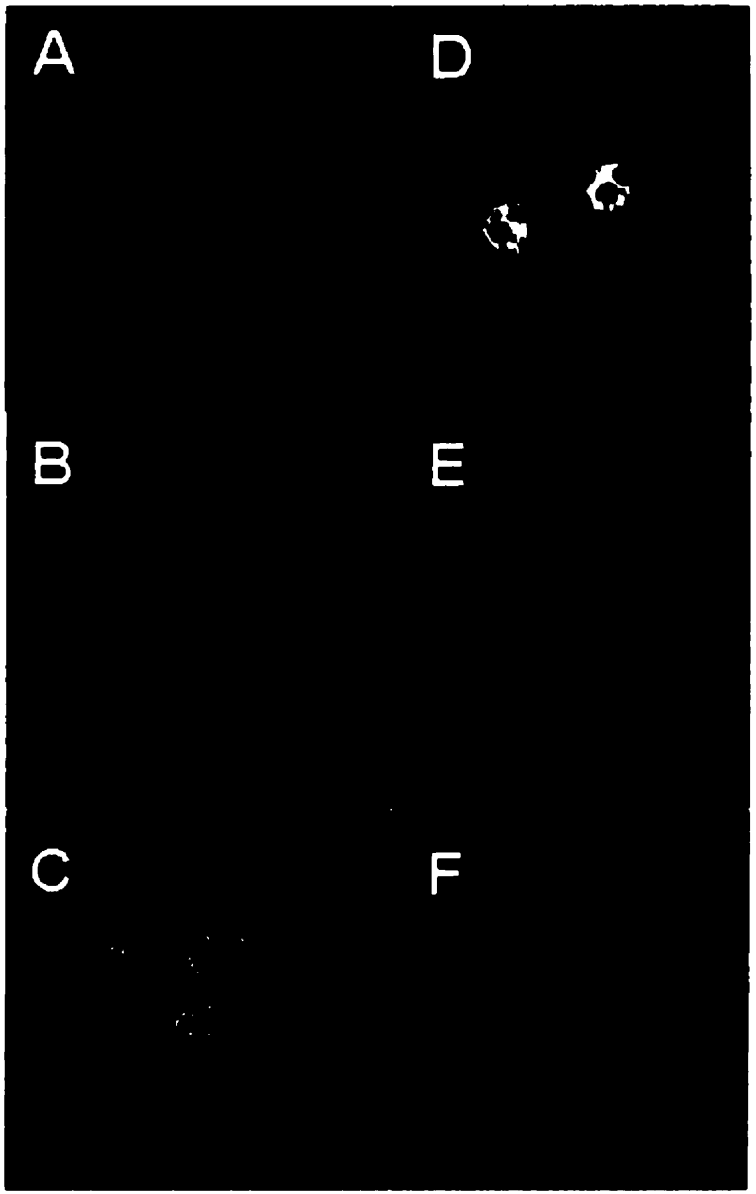
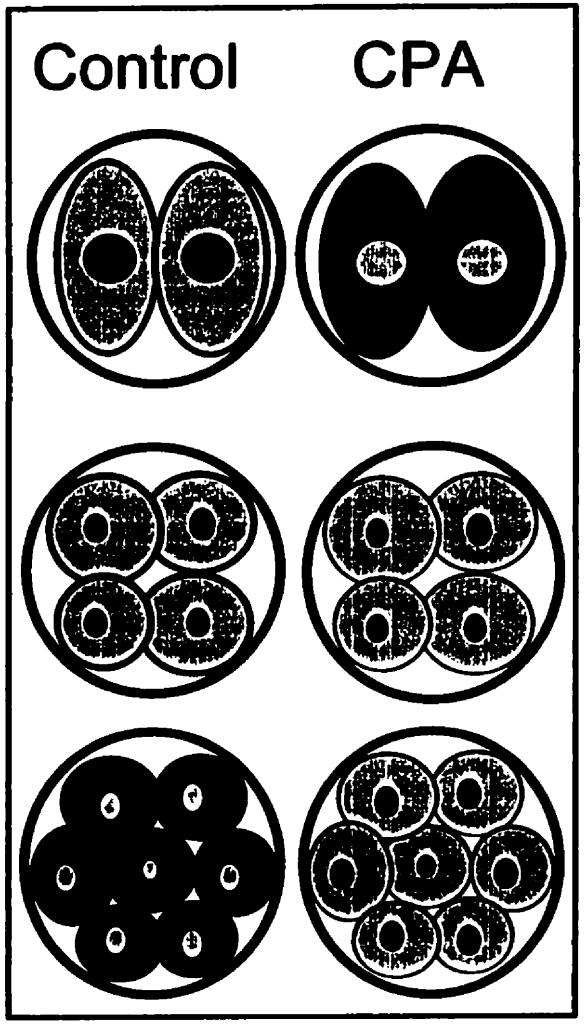

A
D
B
E
C
F

Figure 6

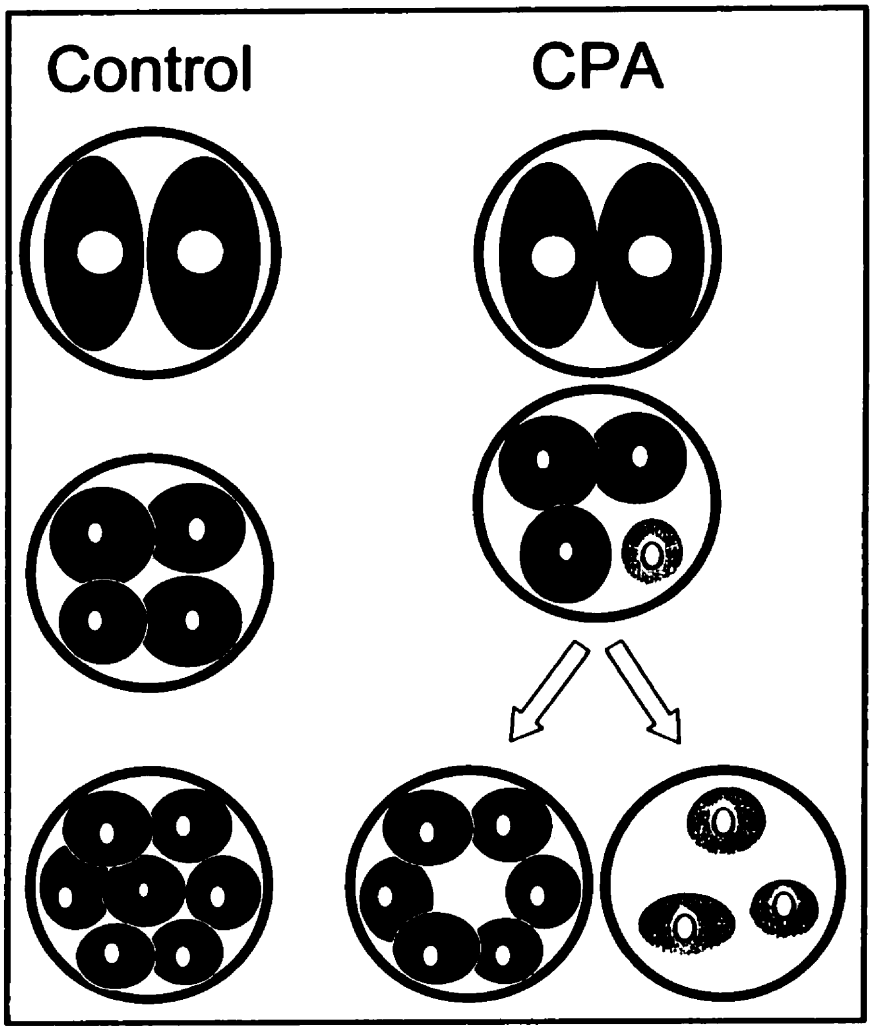
Diagrammatic representation of gene expression, cell numbers, and cell-cell contacts in rat pre-implantation embryos. Data presented in the paper examined the expression patterns for cytoskeletal elements at the 2-, 4-, and 8-cell stages. While the expression of most genes peaked at the 8-cell stage in control embryos, embryos sired by cyclophosphamide-treated fathers displayed a precocious expression at the 2-cell stage. Immunolocalization data for E-cadherin showed that this increase was not confined to cell interaction regions but expanded throughout the embryo. Analysis of cell numbers indicated that cell numbers in the treated group lagged at the 4-cell stage, when loss of individual blastomeres was often seen. By day 2 of gestation, cell loss continued to be seen and abnormal and degenerate embryos became more frequent. Lastly, blastomere interaction was compromised in the treatment group, as manifested on both day 1.5 and 2 of gestation.

Cell-Cell contacts

Gene expression



Blastomere interaction



Chapter 5

Discussion

5.1 Relevance of pre-implantation stage embryos to studies of male-mediated developmental toxicity

During the past two decades investigations of the normal development of pre-implantation embryos have opened many avenues into some of the most amazing technologies used today in the fields of transgenics and assisted reproduction. As far as the field of reproductive toxicology is concerned, however, post-implantation development has been considered as the phase of development that is most relevant to study effects of toxicants during development (Rutledge, 1997). Nonetheless, since abnormal pre-implantation stage embryos may not simply die, but eventuate in the birth of abnormal progeny (Dwivedi & Iannaccone, 1998), it is imperative to assess the health of the conceptus at early stages to predict and hopefully prevent any aberrations in the new born.

Chemotherapy protocols in which drugs are used in combination to combat various ailments still are being developed. However, the effects of such drugs, alone or in combination, on the health and fertility of surviving patients of reproductive age has, for the most part, been elusive. To elucidate the mechanisms of action of toxicants (including chemotherapy drugs) on the normal cascade of events occurring during normal embryogenesis, the use of animal models, a well controlled medium where all factors could be analyzed, has been instrumental.

The aim of this thesis was to delineate the extent to which paternal exposure to the anti-cancer agent, cyclophosphamide, affects the zygotic program of the conceptus starting from the early stages following fertilization by drug exposed sperm. Previous results from the laboratories of Drs. Robaire and Hales have established a paternally-mediated effect of cyclophosphamide on the progeny. This effect was traced to the 8-cell stage, at a point when the embryonic genome is active and the first differentiation events associated with cell lineage allocation, namely the inner mass and

trophectoderm cells, are being established. However, no data existed relating to the earliest events occurring after fertilization with regards to the extent of damage incurred on the fertilized egg and the capacity of the egg to respond to the damaged sperm. Data presented in this dissertation represent a combination of approaches in an effort to delineate the underlying cause(s) for early embryo loss following paternal exposure to cyclophosphamide. My findings have wide applications encompassing various exposure protocols which are relevant not only to the animal model but extend to risk assessment studies of human exposure.

In this chapter, I will review the effects of cyclophosphamide treatment on the male genome. Then I will summarize the consequences of these effects on the pre-implantation embryo by highlighting the most salient results obtained in the course of this work, and attempt to place them in the larger context of the program of pre-implantation gene regulation. Wherever possible, I will offer some proposals to future plans where this project could be further pursued in an effort to resolve some of the vexing elements relating to embryonic loss.

5.2 Effects on the male genome of exposing male rats to cyclophosphamide

Most chemotherapeutic agents present a "risk-benefit" dilemma, a reality stemming from the fact that drugs disrupt basic cellular processes in healthy cells via similar mechanism(s) by which they target cancerous cells. Prescribing cyclophosphamide in chemotherapy protocols falls in this line of "risk-benefit" dilemma. Cyclophosphamide bioactivation results in the production of two active agents, phosphoramide mustard and acrolein, both of which are capable of binding to DNA, RNA, and protein (Murthy et al., 1973; Hemminiki, 1985). Cyclophosphamide acts to alkylate DNA, resulting in DNA single and double strand breaks, DNA-DNA, and DNA-protein cross-linkages.

When cyclophosphamide is administered to male rats for 4-6 weeks, post-meiotic germ cells in the testis are exposed to the drug throughout spermiogenesis and the remainder of spermatogenesis. Alterations to the germ cells seen at the end of the treatment period represent an accumulation of attacks spanning the presence of germ cells in the testis and the epididymis. While still in the testis, spermatid cells undergo DNA condensation where somatic histones in the chromatin are replaced with sperm-specific nuclear proteins, the protamines (Balhorn, 1982). This male germ cell specific packaging ensures the compaction of the male genome into one tenth of its size, a step required for its ultimate goal, fertilization. As a result of this compaction, segments of the DNA, the DNase hypersensitive sites, as well as the exposed sites of chromatin, are more vulnerable to the effects of alkylating agents (McLeod, 1995). The continuous exposure of male germ cells to cyclophosphamide for 4-6 weeks results in an accumulation of alkylated sites in both DNA and protamines. As spermatozoa progress through the epididymis, the sulfhydryl groups present in the protamines become gradually oxidized resulting in disulfide bond formation (Shalgi et al., 1989). This event represents another potential site for cyclophosphamide action on treated spermatozoa. Previous results using a chronic low dose exposure protocol (similar to the protocol used in this study) demonstrated that cyclophosphamide reduced the number of free sulfhydryl and disulfide linked groups in epididymal spermatozoa. Furthermore, an altered decondensation pattern of treated spermatozoa was reported, possibly due to the inability of chromatin to condense tightly (Qiu et al., 1995a). Following fertilization, sperm nuclei undergo a number of remodeling steps which are initiated by a reduction in disulfide bonds of protamines and their replacement with histones present in the egg. Experiments using commercially available antibodies to recognize histone epitopes present in the egg cytoplasm following fertilization would provide answers to the type of remodeling that treated sperm undergo in the cytoplasm of a normal egg compared to

controls. Another undertaking would be to assess the influence of the chromatin structure, which is altered due to the cyclophosphamide exposure, on the induction of double strand breaks in the male genome. Using pulsed field gel electrophoresis, Elia and Bradley (1992) assessed the importance of individual histone components in the induction of DNA double strand breaks in isolated intact nuclei which were treated with ionizing radiation. Using a similar protocol on drug-treated spermatozoal nuclei would resolve the issue of whether protein components of the chromosomes are affected as a result of cyclophosphamide treatment. In the likely event that proteins are involved, the extent of involvement of individual chromosomal protein components, such as protamines and histones, can be examined.

Alternatively, cyclophosphamide may act via a "direct hit" on spermatozoal DNA. The post-meiotic germ cells are genetically inert (Zirkin et al., 1976); thus, any damage incurred at this stage via alkylation or cross-linkage of germ cell DNA is not repaired until after fertilization. Following fertilization, the transcriptional restriction imposed on germ cells starting from the late spermatid stage is lifted, and DNA synthesis can take place (Naish et al., 1987). As sperm decondensation progresses, the availability of the DNA template increases gradually. Assessing the DNA template availability in spermatozoa from chronically treated rats revealed that the template availability in treated spermatozoa occurs more rapidly faster than in controls (Qiu et al., 1995b). This is confirmed further by our new findings (Chapter 3) that sperm pronuclear formation occurred earlier in zygotes fertilized by drug-treated spermatozoa when compared to controls. Using the gene expression array technology, it would be feasible to quantitatively compare gene expression profiles between treated and control germ cells. A detailed comparison of gene expression profiling between control and treated germ cells would allow to pinpoint the most sensitive targets of the treatment on specific

stages of spermatogenesis. Such data would provide leads into the mechanism of action of other alkylating agents on the single gene level.

5.3 Evidence of damage in 1-cell embryos sired by cyclophosphamide-treated males

The first objective of this thesis was to determine whether we could detect the damage incurred upon the 1-cell embryo as a result of paternal treatment with cyclophosphamide in a chronic low dose regimen (6 mg/kg/day for 4-5 weeks) of drug exposure. Using the Comet assay, or the single cell gel electrophoresis, which allows for the detection of DNA damage in single embryos, we detected a significant increase in DNA damage (Chapter 2) among progeny of treated males relative to controls. We observed variability in the extent of damage seen among fertilized eggs as assessed by the tail/head ratio of the comet. We can speculate that the various fates (pre- and post-implantation losses as well as malformations) of progeny sired by males exposed to this drug treatment regimen (Trasler et al., 1985, 1986, 1987) may reflect varying degrees of DNA damage. Measurement of the tail length is an indicator of the size of the migrating DNA fragments, such that smaller fragments migrate greater distances. Although embryos sired by cyclophosphamide-treated spermatozoa traveled significantly longer distances than controls, this migration was rather conservative; this implies that cyclophosphamide treatment results in large DNA fragments that travel short distances. Nevertheless, a small yet significant percentage of comets had tail lengths up to six times their nuclear diameter, suggesting the presence of highly fragmented DNA among these embryos. It would be interesting to follow up on the fate of each subset of embryos to determine whether the extent of damage correlates with specific abnormalities when embryonic loss takes place (day 6.5-7 of gestation). Obviously, a more direct approach would be to conduct the same assay on treated spermatozoa to

determine the extent of damage among treated sperm populations. Preliminary results obtained from ongoing studies point to a comparable distribution of DNA damage among treated spermatozoal populations as described for the 1-cell embryos.

5.4 Presence of DNA repair pathways in the rat pre-implantation embryo during normal development

The second objective of this project was to determine the presence of transcripts for key DNA repair enzymes during normal development in the rat (Chapter 2). The major repair systems include five major DNA repair pathways, each requiring multiple proteins: nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), double-strand break repair (DSB) and recombinational repair (RCR). These pathways play a role of a surveillance system where genomic maintenance and normal propagation between generations is maintained. The presence of DNA repair pathways in somatic cell lines from yeast, *Drosophila* and mammalian systems is now well established. However, less data are available concerning their roles during normal development, especially during the pre-implantation stages (See Chapter 1). Thus, we sought to determine the expression profile of some of these genes during normal pre-implantation stages of rat development. To address this objective, we used the candidate gene approach, in situ transcription/antisense RNA (aRNA), to profile the expression pattern of gene families whose reported primary roles is to repair damage.

Profiling the NER genes in normal embryos revealed a developmentally differential profile for XPC, XPD, XPE, XPF and PCNA. XPC peaked in expression at the 2-cell and 8-cell stages, possibly reflecting mRNA processing of maternal and zygotic transcripts, respectively. This temporally coordinated burst of mRNA production might not be essential for normal embryogenesis since the absence of XPC in a knockout mouse model resulted in normal fertile adults (Sands et al., 1995).

Conversely, XPD deficiency in knockout mice was found to be embryonic lethal (Tebbs et al., 1998). Our data show that XPD is a predominant transcript in the rat embryo (Chapter 2). Besides its role in DNA repair, XPD is involved in basal transcription; such an essential role may not be compensated for by other transcriptional subunits and could partly account for the embryolethality seen in transgenic mice. Transcripts for XPE, a gene involved in the recognition of damaged DNA, and PCNA, a gene required for cell replication, were low at the 1-cell stage but increased by the 2-cell stage in control rat embryos. Although their specific roles in early development have not been mapped out, it is conceivable that both play essential roles. Lastly, XPF was consistently expressed below the threshold of detection in all stages examined. This is intriguing since disruption of XPF produced runted neonates that died within the first month of life from liver failure (McWhir et al., 1993). XPF may not play an essential role during the pre-implantation stages of embryonic development.

The BER repair pathway recognizes and repairs endogenous DNA damage. This pathway relies mostly on the normal functioning of the APE/Ref-1, which accounts for almost 99% of incisions at abasic sites. A gene deletion mouse model of ref-1 resulted in early embryonic death at around day 6.5 of gestation (Xanthoudakis et al., 1996). As with results described for XPF above, control embryos showed low levels of detectable Ref-1 transcripts. Due to its role during development as well as in metabolism, a detailed profiling of Ref-1 in the embryo should clarify the temporal regulation of mRNA transcripts during subsequent stages of development. Pre-implantation rat embryos expressed differential levels for other members of the BER repair pathway, the uracil DNA glycosylases. Examination of the two isoforms of the uracil DNA glycosylase, which are generated by alternative splicing of the UNG gene (Nilsen et al., 1997), revealed consistently abundant transcripts throughout the stages examined with the only exception of a dramatic decrease of UNG1 at the 8-cell stage. The finding that rat pre-

implantation embryos expressed high levels of both isoforms begs the question of whether there is an essential role for UNG1 and UNG2 during normal development. To date, no null mutation models for this enzyme have been described. However, these two variants possess a special feature with regards to their cellular distribution; while UNG1 is mitochondrial, UNG2 is nuclear (Nilsen et al., 1997). Future studies could be performed taking advantage of their localization patterns pending the availability of antibodies specifically directed towards these two epitopes.

The role of the MMR repair system has been underlined by findings from human studies where defects in this pathway predispose inflicted individuals to a hereditary nonpolyposis colon cancer (Kolodner, 1995). No transcripts for MLH1 were detected between the 1-cell and 8-cell stages. MSH2 and PMS2 were only detected at the 8-cell stage in control embryos. Only PMS1 was expressed consistently from the 2-cell stage onwards. While a detailed characterization of the role of specific members of the MMR family still needs to be established, it is possible that the early embryo does not require all members of MMR family in order to survive. Rather, it is interesting to speculate that these genes share overlapping functions during early embryogenesis, where each assumes a similar role in guarding against mismatches that could occur. Supportive evidence for such a mechanism of action for the MMR genes comes from knockout mouse models; Prolla and colleagues (Prolla et al., 1998) have generated mice deficient in MLH1 and PMS1 genes. They found that while both animal models showed tumor susceptibilities, the type of tumors differed depending on the knockout, suggesting that these genes share overlapping but non-identical roles (Prolla et al., 1998). This hypothesis will have to await further investigation.

The RCR pathway is the surveillance system for all recombination steps taking place during homologous recombination of meiosis. The X-ray cross-complementing -1 (XRCC1) gene is involved in DNA strand-break repair, homologous recombination, and

sister chromatid exchange (Walter et al., 1996). When deleted in mouse models, it is embryo-lethal (Tebbs et al., 1996). The timing of embryonic death is reminiscent of knockouts for Ref-1 and in embryos sired by cyclophosphamide-treated males. Interestingly, control rat embryos express XRCC1 at higher levels exclusively at the 1-cell stage. Only RAD 54 was consistently expressed at high levels in all stages examined. Although its role in repair of double-strand DNA breaks is well established (Essers et al., 1997), no data concerning its role during normal embryogenesis has been described. In the rapidly expanding field of DNA repair, a number of molecular tools such as antibodies and enzymatic assay protocols are becoming increasingly available. Using some of these tools would permit a detailed characterization at the level of expression as well as function of each of the pathways profiled in this thesis.

5.5 Differential expression of DNA repair genes in the embryo following paternal drug exposure

Exposure of male germ cells to a drug for 4-5 weeks affects these cells starting at the spermatid stage. The chromatin structure of late spermatids is highly condensed with a unique composition due to the presence of protamines; this renders them highly compacted and incapable of repairing any insult incurred on them. It is only following fertilization that spermatozoal DNA can undergo decompaction and repair inside the egg cytoplasm, if damage is detected. Thus, in my third objective I assessed the ability of embryos sired by cyclophosphamide-treated males to respond to the damaged paternal genome. Using the aRNA approach, I compared the expression profiles of the same genes discussed in section 5.2 between control and treatment groups.

The capacity of eggs to repair damage is not a new concept; in the '70s Pedersen's laboratory showed that mammalian eggs and early embryos are capable of repairing both radiation- and drug-induced damage (Pedersen & Brandriff, 1979); others

used damaging agents such as X-rays and mitomycin (Matsuda et al.,1989), methanesulfonates (ethyl, isopropyl), benzo[a]pyrene (Generoso al., 1979). Data from this thesis confirm and further extend these earlier studies. The element of novelty in these new findings is the mapping of specific gene expression profiles that are differentially altered in response to treatment by a drug that is not traditionally thought of as a selective agent. This is illustrated by the following example showing the difference in patterns of genes belonging to the NER and the BER families. The NER repair system is responsible for the repair of damage by most anticancer agents, including cyclophosphamide (Damia et al., 1996; Andersson et al., 1996). Progeny of cyclophosphamide-treated male rats expressed especially high levels of XPD, XPE and PCNA starting from the 1-cell stage. The presence of transcripts for these genes at significantly high levels at the 1-cell stage strongly suggests that maternal transcripts already available in the egg are turned on in response to the paternally-damaged genome. In contrast, the expression of both UNG1 and UNG2, two BER enzymes, was relatively constant at all stages examined in embryos sired by cyclophosphamide-treated males.

Despite the presence of an altered expression profile of major DNA repair pathways in embryos sired by cyclophosphamide treated rats, these embryos continue to develop and about 10% of them make it to term. It is likely that individual embryos are capable of repairing damaged DNA to a different extent, depending on the degree of damage present. To test the capacity of individual embryos to repair paternal DNA damage, new commercially available products would help elucidate some of the key elements involved; for instance, a new assay, referred to as the" fragment length analysis using repair enzymes" (FLARE) allows for the uncovering of all damaged bases in the DNA of a single cell, not only those that are alkali labile (Trevigen Inc.). Using a similar protocol to the Comet Assay, FLARE uses glycosylases, the class of repair

enzymes which remove damaged bases from the phospho-ribose backbone of the DNA leaving behind intact apurinic/apyridinimic sites. Using this assay on embryos sired by cyclophosphamide-treated males would provide clear answers as to whether damaged DNA could be repaired by this pathway. Cyclophosphamide-damaged sperm could be repaired in vitro using this assay and then used for in vitro fertilization with normal ovulated eggs. The fate of the resulting embryo could be then compared to that of control embryos. Another possible avenue to address is to take advantage of the knockout models generated to date. A combination of these models with a protocol similar to the one used in this study would shed some light into the precise role of various DNA repair pathways in the embryo.

5.6 Dysregulation of the embryonic gene activation program in progeny of treated males

Previous results have established a paternally-mediated effect of cyclophosphamide on the progeny starting at the 8-cell stage. By this stage, the zygotic genome has become increasingly independent of maternal factors as these latter gradually degrade to be replaced by zygotic transcripts. Since a set of haploid chromosomes is contributed from the maternal and paternal genomes to form the diploid zygotic genome, it is likely that basic events taking place starting at the 1-cell stage are affected in embryos sired by cyclophosphamide-treated males as a result of paternal exposure to the drug. Thus, my goal was to characterize some of functional aspects of these early events prior to any overt abnormalities.

As a first objective, I determined the overall ability of embryos sired by control and drug-treated males to synthesize RNA by measuring the incorporation of radioactively labeled uridine triphosphate (UTP), thus assessing the capacity of embryos to synthesize total RNA. Data from mouse embryos demonstrated that all major classes

of RNA are synthesized by the 2-cell stage (Clegg & Piko, 1982). Results presented here indicate that control embryos start RNA synthesis at the 1-cell stage. However, the peak of synthesis is not seen until the 4-cell stage, likely indicative of zygotic gene activation. Embryos sired by cyclophosphamide-treated males, on the other hand, failed to undergo this peak of total RNA synthesis, suggesting a deficiency or alterations in major transcriptional pathway(s) important for the regulation of early development. Instead, this group of embryos showed a dramatically higher transcriptional activity at the 1-cell stage. In the mouse, transcription promoters and origins of DNA replication are strongly repressed starting at the pronucleus stage (Henery et al., 1995). This repression is believed to regulate the zygotic clock such that transcription is delayed until the chromosomes of both parental genomes are remodeled from a postmeiotic state to one in which transcription can be regulated. Therefore, it is plausible that the cyclophosphamide-treated male genome has inactivated the activity of such a repressor(s). To address this possibility, it would be important to characterize regulatory elements acting as repressors during the early rat development. Microinjection experiments of plasmid DNA have been used in the mouse embryo to establish the extent of promoter repression, and the ability of enhancers to stimulate promoter activity starting from the 2-cell stage (Nothias et al., 1996). A similar approach could be utilized for the rat embryo.

The second objective of this chapter was to determine the effect of a precocious RNA synthesis profile among embryos sired by cyclophosphamide-treated males on cellular events. First, we found that the male pronuclear formation was initiated earlier and completed faster in embryos sired by cyclophosphamide-treated males when compared to controls (Chapter 3). Furthermore, culture of embryos at the 1-cell stage resulted in a higher percentage of embryos sired by cyclophosphamide-treated males that reached the 2-cell stage as compared to controls (Chapter 3). When embryos were

cultured in the presence of α-amanitin, a transcriptional inhibitor, a significant number of embryos sired by cyclophosphamide-treated males were arrested at the 1-cell stage (Chapter 3), supporting the hypothesis that paternal treatment with cyclophosphamide has caused the premature initiation of transcription among these embryos. Together, these data suggest that paternal treatment with cyclophosphamide leads to a dysregulated programming of the zygotic clock starting from the pronucleus stage.

The repression of gene activity is differentially regulated in the early embryo; microinjection experiments have proved that transcription of endogenous genes occurs in the male pronucleus at a rate of four to five times greater than that of the female pronucleus in the late 1-cell embryo (Bouniol-Baly et al., 1997; Aoki et al., 1997). This differential transcriptional ability may be explained by the finding that the male pronucleus possesses a greater nuclear concentration of transcription binding proteins (TBPs) and Sp1 transcription factors (Worrad et al., 1994). Both factors continue to be expressed at the 2-cell stage, indicating their importance for embryonic development. To assess whether dysregulation of the zygotic program among embryos sired by treated males is associated with altered expression of such transcription factors, we used an artificial UTP analogue, BrUTP, to assess the localization of new RNA synthesis in 2-cell embryos from both groups. In accordance with previous data, embryos sired by cyclophosphamide-treated males showed a significantly higher incorporation of BrUTP. Furthermore, the localization of BrUTP incorporation was irregular. Control embryos showed an exclusively nuclear staining pattern whereas those sired by cyclophosphamide-treated males displayed cytoplasmic and nuclear BrUTP incorporation. A similar pattern was obtained for Sp1 immunostaining in 2-cell embryos (Chapter 3). Further studies are required to establish downstream genes regulated by these transcription factors. One possible approach would be to perform gel shift assay where binding of these transcription factors to candidate gene products,

such as those that displayed an altered expression using the aRNA protocol, would be evaluated.

5.7 Temporal alterations in the gene expression profile of imprinted genes among embryos sired by treated males

The phenomenon of genomic imprinting is a process whereby the embryo regulates its growth by expressing exclusively either the paternal or the maternal allele. Two separate sets of experimental manipulations, the generation of uniparental diploid embryos by pronuclear transfer and parthenogentic activation of eggs in the absence of a sperm, have established the necessity of an equal contribution of one copy of each parental genome for normal embryonic development (Bartolomei & Tilghman, 1997). Knockout animal models have characterized the roles of specific imprinted genes in establishing specific traits within the animal. For instance, deletion of the Insulin growth factor 2 (IGF2) gene resulted in litters that were reduced in size only when the gene was inherited from fathers, but not from mothers (De Chiara et al., 1990). Insulin growth factor II receptor (IGF2R), on the other hand, was shown to exert the opposite effect. Gene knock-out studies of IGF2R in mice produced animals that were larger in size than their wild type littermates (Lau et al., 1994).

In our model, only the paternal genome is affected following exposure of the male germ cells to cyclophosphamide. Therefore, analysis of the expression profiles of imprinted genes in progeny of treated males would predict the importance of these genes during normal development and as targets of cyclophosphamide action. In control embryos, the expression of a number of imprinted genes did not peak until the 8-cell stage. Embryos sired by cyclophosphamide-treated males, on the other hand, showed a significant increase in steady state transcript concentrations for most of the genes studied at the 2-cell stage (Chapter 3). These data could be explained in light of the

repression of gene activity, a mechanism which might be absent in the treatment group, thus leading to a dysregulated and precocious transcription. Such a dysregulation was sustained in later stages; transcript levels fell dramatically after the 2-cell stage and the peak seen among control embryos at the 8-cell stage was absent in embryos sired by the treated males.

Based on the results obtained in this thesis, two powerful approaches can be adopted to investigate the exact contribution of paternally imprinted genes to embryonic development using the model described in this project. First, new fluorescent probes for specific chromosomal regions (FISH) have allowed the evaluation of stable chromosomal rearrangement and aneuploidy in single cells. This approach allows for whole chromosome painting in metaphase chromosomes as well as chromosomal breakage and aneuploidy for cells in interphase (reviewed by McGregor et al., 1995). Embryos sired by cyclophosphamide-treated males could then be genotyped for specific chromosomal aberrations starting from the 1-cell stage. This would allow the establishment of a link between the untimely peak of expression at the transcript level and the specific chromosomal genotype. Second, the availability of strain-specific polymorphisms in genomic DNA of imprinted genes could be used. Specific polymorphisms were detected for two imprinted genes, IGF2 and H19, between two inbred rat strains, the Sprague-Dawley and the Brown Norway (Overall et al., 1997). Treating Sprague-Dawley male rats with a similar regimen as used in this study and mating them to Brown Norway females would deliver a definitive answer to the origin of the transcripts found in the early embryos. It is tempting to speculate that various degrees of embryonic abnormalities would be correlated with a specific parental origin.

The adverse effects of paternal exposure to cyclophosphamide on progeny outcome have been shown to be heritable (Hales et al., 1992). This indicates that certain permanent abnormalities (such as mutations), which manifest themselves in

later generations, have occurred on the paternal genome as a result of the drug exposure. Data presented in this thesis point to a differential gene expression of imprinted genes among progeny of treated males. Paternal exposure to radiation has been proposed to predispose the progeny to leukemia (Gardner et al., 1990) and results in genomic instability and mutations (Schofield, 1998). Evidence for potential involvement of imprinting has also been suggested for unstable DNA diseases such as Huntington's disease and myotonic dystrophy (Petronis, 1996). Such examples of gamete-of-origin specific effects on inheritance of diseases or malformations provide us with the possibility that imprinted genes are targets for instability. This hypothesis deserves further analysis, especially in light of the interpretation of epidemiological data of exposed individuals. The use of allele specific polymorphisms should help in providing some answers.

5.8 Cell-Cell contacts: A link between activation of gene expression profiles and morphology

Mammalian embryos undergo their initial differentiation events at the 8-cell stage, when the embryo gradually loses its totipotent state to develop specialized cell lineages. This is manifested by the establishment of two cell lineages, the inner mass and the trophectoderm. For these events to occur properly, the embryo depends on cell interactions among blastomeres starting from the 2-cell stage (Graham & Lehtonen, 1979). When male rats were treated with a chronic low dose of cyclophosphamide, the earliest morphological events were seen at the 8-cell stage when cell proliferation (Austin et al., 1994) and cell contacts (Chapter 4) faltered. Among embryos scored with a low cell count, a significant number failed to establish cell contacts. However, cell contacts were not only affected in embryos with low numbers of blastomeres; of the embryos sired by cyclophosphamide treated males, a number that did divide to form 4-

and 8-cell stages included shrunken blastomeres which did not establish proper cell contacts (Chapter 4). Of particular note, a number of embryos scored at the 8-cell stage consistently lacked the inner cell. This observation is important in light of the fact that it is the inner cell which controls the cell division program of the outer cells at the 8-cell stage. Later events, including polarization, compaction, cell flattening, and junctional communication, all depend on the proper signaling between inner and outer cells in order to differentiate into a healthy embryo.

To analyze the molecular aspects underlying these morphological changes, I assessed the capacity of embryos sired by cyclophosphamide-treated males to express, at the mRNA and protein level, some of the key regulators of cell-cell interactions. At the mRNA level, expression of cell adhesion and cytoskeletal elements in control embryos revealed a peak in expression at the 8-cell stage. On the other hand, in the treatment group this increase was manifested at the 2-cell stage (a pattern reminiscent of that of imprinted genes; Chapter 3). Furthermore, immunofluorescence results correlated with mRNA results; E-cadherin, a key molecule involved in cell adhesion, was overexpressed only at the 2-cell stage among embryos sired by cyclophosphamide-treated males (a pattern similar to that seen for BrUTP and Sp1; Chapter 3). Together, results from this chapter provide evidence for a coordinate expression pattern at the mRNA and protein levels among cell adhesion molecules among the progeny of both control and drug-treated males.

To follow up on this study, a rescue of embryos which are lacking in cell contacts could be envisaged. Wiley and her colleagues (Straume et al., 1993) developed a chimera assay in which control or treated blastomeres could be tagged with a fluorescent label permitting later identification. Applying this approach to our model, embryos with a low number of cell contacts (on day 2 of gestation) could be tagged and aggregated with embryos with a normal cell contact count. Following in vitro culture, it

would be possible to assess whether blastomeres from normal embryos are capable of establishing cell contacts with those from abnormal embryos. Another experiment using the same approach could be conducted where chimeric embryos are constructed from the 2-cell stage prior to the appearance of any morphological abnormality. At this stage the gene expression profile of major cell adhesion and cytoskeletal elements was already disturbed in embryos sired by cyclophosphamide-treated males (Chapter 4). Results from this experiment would provide answers as to whether the alteration(s) present in the embryo is (are) reversible and, if so, at which stage of development.

5.9 Integration of present findings and future directions

The shortage of ideal anticancer drugs is due in part to the fundamental difficulties associated with the development of any safe effective drug. An effective chemotherapeutic must selectively kill tumor cells. However, since most anticancer drugs have been discovered by serendipity, the molecular alterations that provide selective tumor cell killing are unknown. Even understanding the molecular mechanism by which a drug acts provides little insight into why the treated tumor cell dies. As an example, cisplatin, a DNA crosslinking agent, is an effective chemotherapeutic for most germ line testicular tumors; however, its specific mechanism of action is poorly understood (Koberle et al., 1997).

Nonetheless, approaches using a combination of traditional methods in genetics and the new wealth of genomic information for both human and model organisms promise to open up strategies by which drugs can be profiled for their ability to selectively kill cells in a molecular context. On the one hand, genetic models, where a single gene is disabled in a cell, allow us to understand the function of specific genes in isolation from the whole organism. Certainly, the use of single gene knockouts in murine embryonic stem cells has offered opportunities without which many of the results

obtained in this dissertation would have been impossible to achieve. Big strides have been accomplished in mapping the genome of several organisms, including the human. Thus, the combination of the two fields will bring about a better understanding, not only of normal processes taking place in an organism, but also of new targets for chemotherapeutics. Until then, several tools have become available which allow the dissection of the molecular mechanisms of action of drugs such as cyclophosphamide. DNA chip technology, where thousands of genes can be profiled, has become available. Designing such a chip for the pre-implantation embryo would allow us to map all the genes involved in this stage of development. A comparison between embryos sired by control and cyclophosphamide-treated males would reveal the exact targets of the drug on the male genome which are contributing to embryonic loss. Large scale cDNA analysis of gene expression during pre-implantation mouse development has revealed profound changes in gene expression, including the transient induction of transcripts at each stage (Ko et al., 2000). It has been suggested that development is driven by the action of a series of stage-specific expressed genes, with a new and unusual gene expression profile during each cell cycle. Unveiling specific targets of drugs on known genes would allow an assessment of gene profiling of those more prone to damage than others. This information could then be used in evaluating the risk of an adverse reproductive outcome for men on chemotherapy who are in the reproductive age, and in the subsequent development of treatment.

Another aspect which must be addressed is the correlation between the expression and the function of specific genes. This thesis was mostly concerned with mapping changes at the RNA level. However, steps into understanding downstream events must be pursued. One approach would be to conduct a similar project on the protein level. An effort has been made to map the mouse protein profile by the 2-dimensional gel electrophoresis (2D-PAGE) (Latham et al., 1992). Exploration of the

possibility that the expression of some protein is altered or simply absent would add invaluable information to integrate into the data that we observed at the RNA level. Such information could also be coupled to the gene array technology where transcript and protein maps can be produced.

5.10 References

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List of Original Contributions

1) I adopted the "In Situ Transcription/antisense RNA approach (aRNA)" to profile gene expression of candidate genes in preimplantation embryos. This has allowed me to amplify mRNA populations from even a single embryo at the 1-cell stage.

2) I demonstrated, for the first time, that specific transcripts for genes involved in the nucleotide excision repair, the base excision repair, the recombination repair, and the mismatch repair pathways are present in single normal rat pre-implantation embryos, thus establishing the availability of specific markers for DNA repair pathways in the newly fertilized embryo.

3) I established that genes in the DNA repair pathways are differentially expressed in embryos sired by cyclophosphamide treated males when compared to controls; thus the pre-implantation embryo is capable of altering the transcript profile of specific DNA repair genes in response to damage brought in during fertilization by a damaged spermatozoon.

3) I found that the total RNA synthesis profile of rat preimplantation embryos showed a peak of expression at the 4-cell stage, compared to a constant profile of synthesis among embryos sired by cyclophosphamide treated males, demonstrating a dysregulation of the zygotic gene program in embryos sired by treated males.

4) I demonstrated that the gene expression profile of several growth factors and imprinted genes displayed a peak of expression at the 8-cell stage in normal rat embryos, likely reflective of transcript production following activation of the zygotic gene program. The expression of growth factors and imprinted genes peaked early at the 2-

cell stage in embryos sired by cyclophosphamide treated males; this precocious peak of expression may be due to an absence of transcriptional inhibitors (repressors) normally activated in pre-implantation embryos.

5) I*n vitro* culture experiments showed that a higher proportion of embryos sired by cyclophosphamide treated males underwent the first cell division compared to control embryos. This precocious cell division was partially delayed when the treatment group was cultured in the presence of a transcriptional inhibitor, confirming the proposed hypothesis stated in point 4.

6) I found that there was an association between cell numbers and cell contacts in the pre-compacting rat embryo. A higher proportion of day 2 embryos sired by cyclophosphamide treated males underwent blastomere loss and failed to establish proper cell contacts among their blastomeres compared to controls.

7) Gene profiling of cell adhesion and architectural elements necessary for the cellular cytoskeleton revealed a peak in expression at the 8-cell stage in control embryos. In contrast, embryos sired by cyclophosphamide treated males showed this peak at the 2-cell stage. The dysregulation of cytoskeletal elements may have a role in perturbing signals necessary for cell division and cell contacts in the early embryo.

8) Using immunofluorescence and laser confocal microscopy, the expression of E-cadherin, a major cell adhesion molecule, was delineated in 2-, 4- and 8-cell embryos. In control embryos. Immunolocalization was restricted to cell contacts at the 2- and 4-cell stages. In contrast, 2-cell embryos sired by cyclophosphamide treated males displayed an increased and scattered staining throughout the embryo, followed by a

loss of signal in later stages. These data demonstrate that the coordinate expression at the mRNA and the protein level of cytoskeletal elements is associated with a decrease in cell numbers and cell contacts.