INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

REGULATION AND EXPRESSION OF DNA METHYLTRANSFERASE IN THE MOUSE GERMLINE

By: Carmen Mertineit

A thesis submitted to the Faculty of Graduate Studies and Research
of McGill University in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy.

Submitted: August 1999

Department of Pharmacology and Therapeutics
McGill University
Montréal, Québec
Canada

This thesis is dedicated with love to my family:
Lieselotte and Dietmar Mertineit, my loving parents
Tanja and Dirk, my little sister and little brother
And
James Blair Thompson, my Pookie-Bear.

My deepest respect and appreciation to my parents. My education has always been their top priority.
My deepest thanks to my companion Blair who has always been by my side in these difficult years as a graduate student. His tremendous support, sacrifice and care were greatly appreciated.
I am indebted to the kindness and love of my family.

TABLE OF CONTENTS

Abstract

The epigenetic modification of DNA by methylation at cytosine in CpG dinucleotides plays an important role in X-chromosome inactivation, genomic imprinting, host defense and cancer. In this thesis, I investigated the role of the predominant mammalian DNA methyltransferase, Dnmt1, in the establishment and maintenance of DNA methylation patterns in the germline. In a comparative study of the expression of Dnmt1 during male and female germ cell development, I identified putative windows in which Dnmt1 may set down sex-specific methylation patterns for imprinted genes. I also identified and characterized sex-specific exons in the 5' region of *Dnmt1* which control the production and localization of enzyme during specific stages of gametogenesis by a mechanism that involves alternative splicing. An oocyte-specific 5' exon is associated with the production of a functional Dnmt1 protein that is truncated at the N-terminus, accumulates to high levels during oocyte growth and later persists through preimplantation development, while a spermatocyte-specific 5' exon leads to the production of a larger, non-translated message. I further examined the dynamics in the expression of the oocyte-specific Dnmt1 isoform in populations of isolated oocytes obtained at different stages of development and their association with the methylation status of the maternally imprinted *Snrpn* gene. The data suggest that the tightly regulated expression of the oocyte-specific Dnmt1 during postnatal oogenesis may play a role in the establishment of maternal methylation imprints at this time. Furthermore, since the oocyte-specific isoform persists during preimplantation development, at a time when the somatic isoform of Dnmt1 is not detected, I propose that the oocyte isoform may also be important for the propagation of maternally- and paternally-derived imprints in the early embryo.

Résumé

Les modifications épigénétiques de l'ADN par méthylation des cytosines aux dinucléotides CpG jouent un rôle important dans l'inactivation du chromosome X, l'empreinte génétique, les défenses immunitaires et le cancer. Dans cette thèse j'ai étudié le rôle de la méthyltransferease d'ADN de mammifères, Dnmt1, dans la formation et le maintien des sites de méthylations de l'ADN dans les cellules germinales. En comparant l'expression de Dnmt1 dans les cellules germinales mâles et femelles au cours du développement, j'ai identifié une fenêtre de temps potentielle durant laquelle Dnmt1 pourraît déterminer la méthylation de certains gènes possédant une empreinte génétique. J'ai aussi identifié et caractérisé des exons dans la région 5' du gène Dnmt1 qui sont spécifiques du sexe des cellules germinales et qui contrôlent la production et la localisation de l'enzyme au cours de certaines étapes de la gamétogenèse par un mécanisme qui implique un épissage alternatif. L'un de ces exons spécifiques des ovocytes est responsable de la production d'une protéine Dnmt1 tronquée au niveau N-teminal mais qui est fonctionnelle. Cette protéine s'accumule en quantité importante durant la croissance ovocytaire et persiste ensuite au cours du développement préimplantatoire. Au contraire, un autre exon en position 5' spécifique des spermatocytes entraîne la transcription d'un ARN messager plus long mais qui ne peut être traduit. J'ai aussi étudié le profil d'expression de l'isoforme Dnmt1 spécifique des ovocytes à partir d'ovocytes isolés à différents stades de leur développement ainsi que l'état de méthylation des gènes Snrpn maternelles. Ces données suggèrent que l'expression du gène Dnmt1 spécifique des ovocytes et qui est précisément régulé au cours de l'ovogénèse post-natal pourraît jouer à cette période un rôle dans l'établissement des profils de méthylation maternelles. De plus, comme l'isoforme spécifique des ovocytes persiste au cours du développement préimplantatoire, à une période où l'isoforme Dnmt1 spécifique des cellules somatiques n'est pas encore détecté il est possible

que l'isoforme spécifique des ovocytes puisse jouer un rôle très tôt dans l'embryon pour la diffusion des empreintes génétiques d'origine maternel et paternel.

List of Figures

Chapter Three

Chapter Four

Preface

Format of the Thesis

This thesis is composed of three chapters of scientific experimentation and analyses, all of which are manuscripts that have been submitted, will be submitted for publication soon, or have been published. Connecting texts are provided in accordance with section C of the "Guidelines for the Preparation of a Doctoral Thesis" of the Faculty of Graduate Studies and Research, McGill University, which states the following:

> "Manuscripts and Authorship:
>
> Candidates have the option, subject to the approval of the Department, of including, as part of the thesis, copies of the text of a paper(s) submitted or to be 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 for Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (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 research, (4) a comprehensive general review of the background literature to the subject of the thesis, (in addition to that covered in the introduction to each paper), and (5) a final overall conclusion and/or summary. Additional material (procedural and experimental design, as well as descriptions of the equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis. In the case of co-authored papers that are included in the thesis, the candidate must have made a substantial contribution to all papers in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent in a section entitled

"Contributions of Authors". The supervisor must attest to the accuracy of such claims at the Doctoral Oral Defense."

The Introduction, Chapter One, includes a general review of the background literature regarding DNA methylation and the role of DNA methyltransferase-1 in the male and female germlines, with emphasis on the establishment of gametic imprints. The rationale for the studies presented in this thesis, the primary hypothesis, and the research objectives are also presented in Chapter One. Chapter Two has been submitted for publication. Chapter Three has been published in *Development* (125: 889-897, 1998). Chapter Four will be submitted for publication soon. A general discussion of the results is presented in Chapter Five. A List of Original Contributions, that summarizes the major findings of Chapters Two through Four, is included at the end of Chapter Five. A comprehensive List of References for all chapters is provided in Chapter Six and is followed by an Appendix containing signed waivers from publishers for previously published copyright-protected material that has been included in this thesis.

Contribution of Authors

In Chapter Two, I performed all experiments, with the exception of the Northern blot that was performed by Guylaine Benoit. I also analyzed all the data, wrote the manuscript and prepared the figures. Dr. T. Taketo provided the expertise for the early gonad isolation and histological examination and for the genetic sexing of embryos. Dr. P. Moens provided the antibodies, technical protocols and advice on the preparation and immunostaining of meiotic chromosome spreads. Dr. T. Bestor provided the anti-DNA methyltransferase-1 (Dnmt1) antibody, pATH52, and Dnmt1 probes for the Northerns. Dr. S. Igdoura provided help in the staging of the mouse seminiferous epithelium.

In Chapter Three, I carried out the tissue and cell collection, all morphological analyses and participated in the preparation and writing of the manuscript. Dr. J. Yoder identified and characterized the sex-specific exons

in the 5' end of *Dnmt1* using RACE cloning, cDNA library screening and Rnase H mapping. He carried out the immunoblot and RT-PCR assays using samples that I prepared. Dr. T. Taketo provided help with the isolation of the embryonic gonads and the analyses of the data. Dr. D. Laird provided expertise and training on the confocal microscope. Dr. T. Bestor supervised the experimental work done in New York by his student J. Yoder. Dr. Trasler supervised my experimental work carried out in Montreal.

In Chapter Four, I carried out all the experiments, analyzed the data, prepared the figures and wrote the manuscript. Guylaine Benoit provided technical assistance with the bisulfite sequencing assay. C. Howell, Dr. F. Ding, and Dr. R. Chaillet generated and affinity-purified the amino terminus anti-Dnmt1-antibody, UPT82. Dr. T. Bestor provided the pATH52 antibody. Dr. H. Clarke provided expertise in the isolation, culture and preparation of mouse oocytes and embryos for RNA extraction and RT-PCR, immunocytochemistry and Western blotting assays.

Acknowledgements

First and foremost, I would like to thank my supervisor **Dr. Jacquetta Trasler** for her guidance, encouragement, and kindness throughout my studies. She has been instrumental in teaching me scientific reasoning and allowed me to leave home-base and work in other laboratories to develop my project in an independent fashion. The birthday cakes and Christmas gifts were always nice gestures.

A special thanks to my friend, **Dr. Suleiman Igdoura** for his criticisms and support and the one hundred, or so, cups of coffee.

My great thanks to **Drs. Bernard Robaire and Barbara Hales** for their helpful and constructive criticisms of my project at our weekly lab meetings.

I am grateful to my advisor, **Dr. Dusica Maysinger**, for her support of my research and her concerns of my personal well-being.

I thank the **Department of Pharmacology and Therapeutics** and the **Montreal Children's Hospital Research Institute** for the opportunity to study in an excellent teaching and research environment.

I would like to acknowledge the financial support of the following groups: **The Montreal Children's Hospital Research Institute**, the **Faculty of Medicine**, and the **Faculty of Graduate Studies and Research** at McGill University.

I am grateful to **Dr. Timothy Bestor**, **Dr. Jeffrey Yoder**, and **Dr. Richard Chaillet** and his students **Carina Howell** and **Feng Ding** for their beneficial collaborations and for providing me with the experimental tools necessary to develop this project.

My greatest thanks to **Dr. Teruko Taketo** and **Dr. Hugh Clarke** for allowing me to work in their laboratories and join in on their lab meetings and journal clubs, and for their ongoing support of this project.

I am thankful to **Dr. Peter Moens** for his antibodies to the chromosomal components of the synaptonemal complex and his helpful discussions.

I would like to thank **Dr. Dale Laird** for providing training and use of the confocal microscope in the **Department of Anatomy and Cell Biology**.

My appreciation to **Dr. Moshe Szyf** for his enthusiasm and unique approaches towards unravelling the mysteries of DNA methylation and demethylation.

My appreciation and regard to **Dr. Daya Varma** and **Dr. Brian Collier** for their wisdom and truthfulness and to **Dr. Riaz Farookhi** for his sense of humour and helpful discussions.

I am thankful to **Dr. Patrick Syntin** for translating my Abstract into French.

My greatest thanks to **Guylaine Benoit** for her outstanding technical assistance and excellent lab management.

My gratitude to my fellow lab members: **Tonia Doerksen**, **Amin Ladak** and **Tamara Kelly**.

Finally, I would like to thank all the graduate students I have met throughout the course of my doctoral studies: **Chung-Hae Lee**, **Roksana Nasseri**, **Marc Champigny**, **David McLay**, **Othman Mohamed**, **Patricia Hurtubise**,

Germaine Fu, Patrick Allard, Wafa Harrouk, Robert Vinson, Valérie Serre, Terence Ozolins, Seyed Moallem and all the new kids on the block.

CHAPTER ONE

Introduction

1. Purpose of the Investigation

One of the major thrusts of molecular biology research is to elucidate the mechanisms governing gene expression during development. It is well established that normal development of mammalian embryos requires the genetic contributions of both parents via the highly specialized germ cells, the spermatozoon and the oocyte. Following fertilization, the subsequent differentiation of cells during embryogenesis involves a complex program of changes in gene activity that dictates the progression of cells into functionally specialized tissues. Since all somatic cells in an organism contain the same genetic information, developmental switches are used to turn on tissue-specific genes and suppress those genes characteristic of other cell types. One factor that is involved in the regulation of gene expression is the epigenetic modification of the DNA (Holliday and Pugh, 1975). For more than 40 years, it has been known that mammals tag their DNA by the covalent addition of a methyl group to cytosine residues creating an additional DNA base, 5-methylcytosine. However, until recently, the functional significance of this modification has remained speculative.

A series of discoveries over the last few years has driven DNA methylation firmly into the mainstream of biology and medicine where it has been postulated to play a role in many processes including: gene regulation, cellular differentiation, X-chromosome inactivation, genomic imprinting, genome defense, and cancer. Methylation of CpG sites, especially of the promoter region of mammalian genes, has been associated with transcriptional repression and provides a mechanism to regulate gene expression (Meehan et al., 1992; Kass et al., 1997a). Aberrant promoter methylation of tumor suppressor genes has now emerged as an epigenetic inactivation pathway contributing to cancer (Laird and Jaenishch, 1994). There is increasing evidence to support the notion that ectopic methylation is a contributor to mutagenesis in mammals, whereby deamination of 5-methylcytosine can lead to C→T transition mutations (Bestor and Coxon, 1993; Jones, 1996). Much excitement has revolved around the role of DNA

methylation in genomic imprinting, a phenomenon in which parental alleles of the same gene are expressed at unequal dosages (Barlow, 1993). Abnormal expression of imprinted genes is currently implicated in several human disorders, such as the Prader-Willi, Angelman and Beckwith-Wiedemann syndromes (Davies, 1992; Ogawa et al., 1993; Rainier et al., 1993; reviewed by Lalande, 1997; Tycko et al., 1997), and more instances seem likely to be discovered. The entire field of DNA methylation, with its foundations resting largely on the strength of correlative data, took a powerful step forward with the direct demonstration that normal mammalian development was perturbed by a disruption in the gene encoding the DNA methyltransferase-1 enzyme (Li et al., 1992). These mutant embryos exhibited a significant decrease in global methylation levels (Li et al., 1992), abnormalities in X-chromosome inactivation (Beard et al., 1995), a loss of genomic imprinting (Li et al., 1993a; Li et al., 1993b), and died by mid-gestation (Li et al., 1992). However, little is known about the role of DNA methylation in male and female germ cells.

Sex- and sequence-specific patterns of DNA methylation are thought to originate during gametogenesis and are subjected to further modifications during embryo development. These allelic differences in methylation patterns are most prominent at imprinted loci (Tremblay et al., 1997) and retroviral sequence elements (Yoder et al., 1997a; Walsh et al., 1998). Methylation differences between male and female germ cells have been described for single and low copy genes (Sanford et al., 1987; Kafri et al., 1992), but with respect to global DNA methylation, the sperm genome is more methylated than that of the oocyte (Monk et al., 1987). A better understanding of the mechanisms that regulate the establishment and maintenance of DNA methylation patterns is needed to determine the role of DNA methylation in gametogenesis.

My thesis project seeks to comprehend the epigenetic phenomenon of DNA methylation in the germline by examining the expression, localization and regulation of the mammalian DNA (cytosine-5)-methyltransferase-1 enzyme (hereafter designated simply as Dnmt1) at the level of the gene,

mRNA and protein. To date, Dnmt1 is the best characterized component of the DNA methylating system and remains the only mammalian enzyme with demonstrated DNA methyltransferase activity and function *in vivo* (Carlson et al., 1992; Li et al., 1992; Yoder et al., 1997b). The studies in this thesis will identify putative windows in which Dnmt1 may set down sex-specific methylation patterns in the male and female germlines; provide evidence of gamete-specific regulation of the *Dnmt1* gene; and examine the dynamics and role of a novel form of Dnmt1 during oocyte development.

It is a difficult task to cover all aspects of DNA methylation comprehensively in this chapter. However, an overview of DNA methylation, the enzymes involved in this process, and their roles during development will be discussed, with special attention given to the germline. But first, an overview of male and female germ cell development in the mouse will be provided. Much of the literature on DNA methylation is based on the mouse or cell lines derived from this species, and as such, this dissertation will largely focus on studies using the mouse as a model.

1.1 Gametogenesis

Gametogenesis is a unique system. The products of this lineage, spermatozoa in the male and oocytes in the female, are highly differentiated cells that retain the genetic information to direct the development of all cell lineages in the new individual following their union at fertilization. It forms the fragile link between one generation and the next, and so is of central importance for the survival and evolution of living organisms. In this way, the germline carries the potential for both totipotency and immortality.

1.1.1 The Origin of the Germline

Since the beginning of the century, several studies have investigated the origin of the germline (Brambell, 1927; Eddy and Hahnel, 1983; McLaren, 1988; Gomperts et al., 1994a; McLaren 1998). It was discovered that primordial germ cells (PGCs) are the founder cells of the germline and that

their descendants will form the functional gametes of the adult animal. In many organisms, including the mouse, PGCs are characterized as a population of migratory cells that are rich in alkaline phosphatases (Chiquoine, 1954; Ginsburg et al., 1990). The presence of these enzymes and their chemodifferentiation property renders the PGCs in histochemical preparations readily distinguishable from all other cells during early embryo development (Chiquoine, 1954). At about 7 days post-coitum (dpc), a small cluster comprising 10 or so alkaline phosphatase-positive cells can first be identified at the posterior end of the primitive streak in the extraembryonic mesoderm (Ginsburg et al., 1990). Prior to this time, there are no markers to identify the mouse germline and it has yet to be determined exactly how many and at what stage germ cells are set aside in early development. Towards the end of gastrulation, these PGCs number between 50 and 80. Between 8 and 9 dpc, the population of about 100 PGCs increases to about 350 and is mainly found in the hindgut endoderm and also at the base of the allantois. From here, they actively migrate in a dorsal direction along the hindgut mesentery toward the developing genital ridges, which they begin to colonize at 10.5 dpc. PGCs proliferate mitotically at a rather uniform rate during this period of migration, and continue to divide within each genital ridge, reaching a maximum number of about 25,000 by 13.5 dpc in the sexually-differentiated gonad of the mouse (Tam and Snow, 1981; Donovan et al., 1987).

Little is known about how the PGCs find their way to the genital ridges, although contact guidance, differential adhesion and chemotaxis have all been postulated. There is evidence that many of the germ cells are linked together by cellular processes to form networks (Gomperts et al., 1994b). At least three cell surface carbohydrates appear to specifically mark PGCs during their migration and have been used to generate specific antibodies. These include the stage-specific embryonic antigens (SSEA)-1 and –3, and the Forssman antigen (for review, see Wylie et al., 1986). The best characterized of these, SSEA-1, is found on clusters of germ cells specifically during the period when they emerge from the hindgut endoderm until they

colonize the genital ridges, raising the possibility that this surface antigen may play a role in guiding germ cell migration through germ cell adhesion. The survival and proliferative capacity of migratory PGCs requires a functional c-kit/steel factor signal transduction pathway. Disruption of either the c-kit receptor on PGCs, or its steel factor ligand, which is expressed along the migration pathway and in the genital ridges, greatly reduces the number of germ cells which colonize the genital ridges and abolishes or reduces fertility (McCoshen and McCallion, 1975; Orr-Utreger et al., 1990; Motro et al., 1991). A dysfunctional c-kit receptor or the absence of steel factor are linked to mutations in the *dominant white-spotting (W)* and *steel (Sl)* loci, respectively. The *germ cell deficient (gcd) locus*, which is associated with an insertional mutation, leads to a deficiency in PGC number, suggesting that this locus may play a role in germ cell proliferation (Pellas et al., 1991). In addition to steel factor (Godin et al., 1991), other growth factors which have been found to influence germ cell proliferation or their direction of movement *in vitro* include basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), interleukin-4 (IL-4), and transforming growth factor-β_1(TGF-β_1) (Godin and Wylie, 1991; Matsui et al., 1991; Resnick et al., 1992; Cooke et al., 1996). In summary, the elements affecting the survival, proliferation, migration and chemotaxis of PGCs are complex and will require further experimentation using tools in molecular biology, such as conditional and germline specific gene targetting, to explore these avenues.

The developmental potential of mouse germ cells can be examined using four general types of cells: primordial germ cells (PGCs), embryonal carcinoma (EC), embryonic stem (ES) and embryonic germ (EG) cells. While EC cells are derived from culturing teratocarcinomas (Martin, 1980), ES cells are acquired from culturing blastocyst-stage embyros (Evans and Kaufman, 1981; Martin, 1981). The long-term culture of PGCs on feeder layers from 8.5 dpc embryos has led to the development of immortalized germ cell lines termed EG cells (Matsui et al., 1992; Resnick et al., 1992). One test of a cell's developmental potential is to determine the extent of its contribution to

the various cell lineages in chimeras after transplantation into a blastocyst. PGCs that are directly explanted from embryos appear incapable of contributing to any cell lineage in chimeras (Wylie and Heasman, 1993). EC cells contribute largely to somatic lineages but rarely to the germline (Martin, 1980). Like ES cells, EG cells derived at 8.5 dpc can contribute to most if not all the somatic tissues, as well as the germline, and to this extent they are totipotent (Matsui et al., 1992; Stewart et al., 1994). EG cell lines have also been derived from both male and female germ cells isolated at 11.5-12.5 dpc. However, these cell lines do not resemble normal ES cells as closely, since on injection into blastocysts, they cause fetal overgrowth and skeletal abnormalities (Tada et al., 1998).

1.1.2 Fetal Germ Cell Development

Throughout the migratory period, the appearance and behaviour of PGCs are indistinguishable in male and female embryos, but following their colonization of the genital ridges, their fates diverge (McLaren, 1995). The male pathway of germ cell development is characterized by a 'prespermatogenic' wave followed by a period of mitotic arrest prior to birth, and a second proliferative wave that initiates a process of continuous meiosis after birth (Oakberg, 1956a; Oakberg, 1956b; Kluin and de Rooij, 1981; McLaren, 1984; Hilscher, 1991). The female pathway, in contrast, is characterized by a single proliferative wave of oogonia followed by entry into meiosis prior to birth (McLaren, 1984; Hilscher, 1991). Both the 'prespermatogenic' and 'oogenic' waves of germ cell proliferation are similar and occur between 10.5 and 14.5 dpc in the mouse. During this time, the genital ridge begins to show signs of sexual differentiation into either a testis or an ovary. Prior to 12.5 dpc, the gonads are morphologically indifferent, but after this time, the sex of an embryo can be determined by the presence of the seminiferous cords in the male and by their absence in the female (Hogan et al., 1986). For sexually indifferent gonads, genetic sexing of the embryo for the presence of genes linked to the Y-chromosome (Nagamine et al.,

1989; Taketo-Hosotani et al., 1989) or the detection of sex chromatin bodies in amniotic cells (Palmer and Burgoyne, 1991) are used to determine if the embryo is male (XY) or female (XX), respectively.

A) The Fetal Testis

The morphology and kinetics of germ cell development in the prenatal testis are largely based on light and electron microscopic and autoradiographic studies (Sapsford, 1962; Hilscher et al., 1974; Kluin and de Rooij, 1981). In general, fetal germ cells or gonocytes can be divided into two subtypes based on their cell cycle activity. Gonocytes during the period of multiplication are termed M-prospermatogonia and during the transitional period of mitotic arrest are called T-prospermatogonia (Hilscher et al., 1974). Masculinization at 12.5 dpc involves the formation of a prominent blood vessel along the coelomic border of the testis and the gonocytes are clumped together into cords surrounded by a layer of somatic cells, the pre-Sertoli cells. The interstitial region encompassing the sex cords contains the precursors to the Leydig and the peritubular myoid cells. At 15.5 dpc, a redistribution of the sex cords into tubules occurs so that the gonocytes occupy the centre of the tubules while the pre-Sertoli cells are situated at the periphery (Sapsford, 1962; Kluin and de Rooij, 1981). Between 15.5 dpc until shortly after birth, the T-prospermatogonia, which are characterized by diffuse chromatin and prominent nucleoli, remain arrested in G1 of the cell cycle (McLaren, 1984). The next cycle of DNA replication does not take place until early after birth, at which time, the T-prospermatogonia resume their mitotic activity and descend towards the basement membrane giving rise to primitive type A spermatogonia (Sapsford, 1962; Bellvé et al., 1977). The primitive type A spermatogonia are considered to be the progenitor cells of adult type A spermatogonia. Proliferation and differentiation of mature type A spermatogonia result in the formation of Intermediate (In) and, subsequently, type B spermatogonia in an age-dependent manner in the early prepubertal testis (Nebel et al., 1961). The first meiotic cells are seen in the normal

mouse testis about 10 days after birth (See Figure 1; Nebel et al., 1961; Bellvé et al., 1977).

B) The Fetal Ovary

The developmental history of female germ cell proliferation and early meiosis is, for the most part, descriptive and based on cytological and histological studies (Brambell, 1927; Borum, 1961; Bakken and McClanahan, 1978; Speed, 1982; Dietrich and Mulder, 1983). In contrast to the male, germ cells in the ovary, the oogonia, enter meiosis prenatally at about the same time as the gonocytes enter mitotic arrest. After their final mitotic division, the oogonia undergo one further round of DNA replication, known as the preleptotene stage, before entering prophase I of meiosis (See Figure 2). The first meiotic prophase includes the leptotene, zygotene, pachytene and diplotene stages, which represent specific events during the pairing and unpairing of homologous chromosomes. At 14 dpc, the first leptotene-stage oocytes mark the onset of meiotic prophase by their characteristic shortening and thickening of duplicated chromosomes. Over the next few days, they pass through zygotene, when homologous chromosomes begin pairing to form a synaptonemal complex, and subsequently through pachytene, when complete formation of the synaptonemal complex and genetic recombination take place. At birth or shortly afterwards, most oocytes in the mouse have reached the diplotene stage, which is characterized by the segregation of homologues and results in the generation of a unique combination of genes. As the oocytes progress through meiotic prophase, connective tissue and blood vessels invade the parenchyma, dividing the ovary into two anatomical regions, the medulla and the cortex. The more advanced oocytes occupy the centre of the cortex and the less advanced oocytes are usually found in the periphery (Borum, 1961). Oocytes enter and pass through meiotic prophase with some degree of synchrony; thus a fetal ovary of a given age contains a majority of oocytes in one or two stages of meiosis (Bakken and McClanahan, 1978). During this meiotic progression, many oocytes degenerate through a

process of atresia. This loss peaks to 50% at about the time of birth, during the pachytene to diplotene transition (Borum, 1961). Unlike the male where meiosis is a continuous process, oocytes progress through meiotic prophase collectively only once and remain arrested in the diplotene or dictyate stage until they are recruited into the growth phase and hormonally stimulated to mature. Meiosis then resumes shortly before ovulation.

C) Sexual Differentiation

The acquisition of a sexually dimorphic phenotype is an important aspect of mammalian development that depends on critical steps during embryogenesis. Sexual differentiation is characterized by three sequential events: the establishment of genetic sex at fertilization, growth and differentiation of the gonads, and the development of the proper sexual phenotype. Prior to 12.5 dpc, the genital ridges and the urogenital tracts are morphologically indifferent in male and female embryos, yet their germ cells are genetically distinct, as XY and XX respectively. If the embryo is of XY karyotype, the indifferent gonads differentiate into testes and secrete specific hormones, testosterone and Müllerian inhibiting substance (MIS), which trigger the development of male accessory sex organs (Jost, 1953; Jost et al., 1973). Testosterone, a steroid hormone produced by the Leydig cells, induces the differentiation of the Wolffian ducts into the seminal vesicles, epididymides, vas deferens, and ejaculatory ducts. MIS, a glycoprotein secreted by the Sertoli cells, causes regression of the Müllerian ducts. In the absence of these two hormones, the Wolffian ducts degenerate and the Müllerian ducts develop into the oviducts, uterus, cervix and upper vagina.

Sex determination and differentiation of both the germ cells and somatic cells in the gonads are, to some extent, dependent on the activity of the sex chromosomes. Studies have indicated that the presence of the Y chromosome directs development of the indifferent gonad towards testicular differentiation through the action of the testis-determining gene, *Sry* (Gubbay et al., 1990; Koopman et al., 1991). It has been hypothesized that *Sry* is the

master controller of male sex determination; however, other transcription factors such as Dosage-sensitive sex reversal, adrenal hypoplasia congenita, X-linked-1 (Dax-1), steroidogenic factor-1 (Sf-1), Wilm's tumour-1 (Wt-1) and *Sry* related HMG box-9 (Sox9) also play critical roles in the processes of sex determination and differentiation (reviewed by Haqq and Donahoe, 1998; Parker et al., 1999). Apart from the Y chromosome, X-linked gene expression is equalized between the sexes by the transcriptional silencing of one X chromosome, termed X inactivation, in female somatic cells (Lyon, 1961; reviewed by Migeon, 1994). During preimplantation development, female embryos express genes from both X chromosomes until the blastocyst stage, at which time, X inactivation occurs in the extraembryonic lineages (Monk and Harper, 1979). It is presumed that X inactivation occurs in the germline at the same time as in the other epiblast cells, since female (and male) PGCs during their migration and colonization of the genital ridges have only one active X chromosome (McMahon et al., 1981; McLaren, 1983). In the female pathway, reactivation of the silent X chromosome occurs in the fetal ovary at about the time of onset of meiosis (Monk and McLaren, 1981). In the male pathway, inactivation of the X chromosome occurs at a later stage, in pachytene spermatocytes (Lifschytz and Lindsley, 1972).

1.1.3 Spermatogenesis

Spermatogenesis is an elaborate process of cell proliferation and differentiation starting with mitotically-dividing spermatogonial stem cells and terminating with fully differentiated motile spermatozoa (Figure 1). In mammals, spermatogenesis continues throughout life and, in man, leads to the production of about 100 million new spermatozoa each day (Amann, 1981). It is one of the few examples in the adult of a system where differentiated cells pass through a series of developmental phases: mitosis, meiosis, differentiation, and maturation.

The male reproductive system is intricately designed to produce and deliver functional spermatozoa to the female reproductive tract. The testis is

the site of production of male gametes and in addition, functions as an endocrine gland, producing a number of hormones. The development of spermatozoa takes place within narrow coiled seminiferous tubules that form the bulk of the testis. These tubules have a central fluid-filled lumen and a cellular component called the seminiferous epithelium comprised of germ cells and somatic cells, the Sertoli cells, which support and nourish the germ cells. The germ cells are arranged so that successive generations of cells form concentric layers around the central lumen of the seminiferous tubules. The least mature germ cells are found near the basement membrane, while the most mature cells lie next to the lumen. Junctions between adjacent Sertoli cells form the blood-testis barrier which protects the more advanced stages of spermatogenesis against blood-borne noxious agents and immune cells (Dym and Fawcett, 1970). The interstitial cells surrounding the tubules contain blood vessels, nerves, immune cells, and the steroidogenic Leydig cells that produce androgens. At the termination of spermatogenesis, the spermatozoa along with the fluid secreted by the Sertoli cells, are released into the lumen and pass through the rete testis and efferent ducts into the epididymis. The epididymis provides a specialized luminal microenvironment that is distinct within each segment (initial segment, caput, corpus and cauda), and during epididymal transit, the spermatozoa mature and acquire the ability to fertilize an egg (reviewed by Robaire and Hermo, 1988). Spermatozoa then pass through the vas deferens where they combine with seminal vesicle and prostatic secretions to form semen and subsequently exit through the penile urethra.

The production of spermatozoa is regulated by the hypothalamo-pituitary-testicular axis. The hypothalamus synthesizes and releases, in a pulsatile manner, gonadotropin-releasing hormone (GnRH) which stimulates the production of gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), in the anterior pituitary (Everett, 1994). FSH and LH are the primary hormonal mediators of testicular function, whereby LH stimulates the production of testosterone by the Leydig cells and FSH

maintains the function of Sertoli cells (Fritz, 1978; Griswold, 1993). FSH plays a significant role in the initiation of spermatogenesis at puberty but its role in the adult is less certain. The production of LH is regulated by feedback inhibition of circulating testosterone on the pituitary and hypothalamus, whereas suppression of FSH at these sites is mediated by feedback inhibition of inhibin and androgen-binding protein (ABP) from Sertoli cells (Sharpe, 1994).

Spermatogenesis can be divided into three distinct phases, each involving a unique class of germ cells that are coordinately arranged in the seminiferous epithelium. Figure 1 illustrates the events that occur during spermatogenesis in the mouse (Bellvé et al., 1977). The tight junctions of the Sertoli cells separate the seminiferous epithelium into a basal compartment containing the mitotic germ cells and the adluminal compartment with the meiotic and haploid germ cells (Dym and Fawcett, 1970).

The initial phase of spermatogonial proliferation and stem cell renewal occurs in the basal compartment. Although the mechanism of stem cell renewal remains controversial, it is postulated that a pool of A_I spermatogonia serving as stem cells divide by mitosis to produce new A_I stem cells and type A_{II} spermatogonia that commit to further differentiation and the exponential increase in daughter cells (Monesi, 1962). The continued mitotic proliferation of type A_{II} spermatogonia forms, in sequence, type A_{III}, type A_{IV}, intermediate and type B spermatogonia (Oakberg, 1956a; Oakberg, 1956b; Monesi, 1962). In an alternate model of spermatogonial renewal, type A_{IV} spermatogonia can also contribute to the production of A_I stem cells (De Rooij and Kramer, 1967). The type B spermatogonia subsequently divide to form preleptotene spermatocytes which duplicate their chromosomes during the final S-phase before entering meiosis.

The second phase of spermatogenesis, meiosis, is characterized by two reductive divisions of spermatocytes to produce haploid spermatids in the adluminal compartment of the seminiferous epithelium. As in the female, meiotic prophase encompasses the leptotene, zygotene, pachytene and

diplotene stages. However, unlike the diplotene stage which is long-lived in the female, the pachytene stage has a long duration in the male. In the mouse, pachytene takes about one week (Oakberg, 1956b; Russel et al., 1990) and includes an early, mid and late component during which the cell and its nucleus progressively increase in volume. Meiotic prophase terminates in the first reductive division with the formation of secondary spermatocytes. The latter cells quickly enter the second reductive division to form the haploid spermatids.

Spermiogenesis, the final phase of spermatogenesis, consists of a complex transformation of haploid germ cells that culminates with the release of spermatozoa into the lumen of the seminiferous tubule. There are 16 steps of spermiogenesis in the mouse (Oakberg, 1956b) during which spermatids undergo condensation and elongation of the nucleus; acquire an acrosomal cap; release a droplet of cytoplasm called a residual body; and develop a motile flagellum. The packaging of DNA into the sperm head is facilitated by testis-specific histones called transition proteins which coil the DNA into nucleosomes and are later replaced by protamines (Oko et al., 1996). In the mouse, the development of a spermatozoon takes 34.5 days (Oakberg, 1956a).

Morphological studies have shown that germ cells in spermatogenesis are arranged into distinct 'cellular associations' called 'stages of the cycle of the seminiferous epithelium' (Leblond and Clermont, 1952a; Leblond and Clermont, 1952b; Oakberg, 1956b). Due to the precise and regular timing of the phases of spermatogenesis, spermatids at a specific time of development are always associated with the same types of spermatocytes and spermatogonia (Clermont, 1972). Even in the prepubertal testis, the coordinated entry of spermatogonia into meiosis is time-dependent; thus, enriched populations of different types of spermatogonia, spermatocytes and spermatids can be isolated from one another on specific days to facilitate molecular and biochemical studies of individual germ cell types (Nebel et al., 1961; Bellvé et al., 1977). In the adult mouse, there are 12 stages of germ

cell associations that have been identified based largely on the shape of the acrosome and on the degree of chromatin condensation in the differentiating spermatids (Leblond and Clermont, 1952a; Oakberg, 1956b). In a given area of the seminiferous tubule, progression to the next stage involves the synchronous maturation of each germ cell type to the succeeding step of development. Since such a sequence must repeat itself indefinitely, the seminiferous epithelium and all its stages evolve in a cyclic manner representing the 'cycle of the seminiferous epithelium' (Leblond and Clermont, 1952b). The duration of one cycle of the seminiferous epithelium in the mouse is 8.6 days (Oakberg, 1956a).

There are several intriguing features of the spermatogenic process. One is that, for a given species, each phase of spermatogenesis has a constant duration; thus, germ cell differentiation unfolds as if regulated by a rigid time-scaled program (Clermont, 1972). Another feature is that the developing male germ cells fail to complete cytoplasmic division, or cytokinesis, during mitosis and meiosis, so that all the differentiating daughter cells descended from one maturing spermatogonium remain connected by cytoplasmic bridges. These bridges are thought to promote the synchronous development of germ cells in the tubules through the exchange of ions and other molecules (Fawcett et al., 1959; Dym and Fawcett, 1971). In addition, unscheduled DNA synthesis in post-mitotic germ cells is associated with repair to damaged DNA following insult (Orlando, 1984; Bently and Working, 1988). Unlike somatic cells, the global transcriptional machinery of developing male germ cells shuts down during mid-spermiogenesis due to changes in chromatin condensation and conformation (Schäfer et al., 1995; Sassone-Corsi, 1997). Thus post-meiotic cells either synthesize and store important proteins prior to terminating transcription, or store the transcripts encoding these proteins until a later time when they can be translated (Hecht, 1995; Schäfer et al., 1995; Sassone-Corsi, 1997). Since spermatogenesis is possibly one of the most complex processes of cell differentiation taking place in the adult individual, many of its facets remain to be studied and clarified at the molecular level.

1.1.4 Oocyte Growth and Maturation

The oocyte is a unique and extremely specialized cell. In mammals, it is formed in the fetal ovary and later suspended at the diplotene stage of meiosis where, depending on the species, it may wait for days to years before it starts to grow and mature. It is not until these events have been completed that the oocyte can resume meiosis and undergo fertilization. Unlike the continuous production of spermatozoa in man, the human female produces only one egg per month for 35 or so years of the reproductive lifespan. Major changes in the number, morphology and biochemistry of the oocyte occur during growth and maturation, and a molecular program for embryo development is established (Reviewed by McLaren, 1988; Wassarman and Albertini, 1994; Gosden et al., 1997).

The female reproductive system is designed to prepare oocytes for fertilization and promote the development of a new individual. The mature ovary is the site of gamete and hormone production. It consists of a medullary region containing a rich vascular bed with loose connective tissue, and a cortical region where ovarian follicles housing oocytes predominate. Although oogonia enter meiosis during fetal life, nuclear development progresses no further than diplotene until the oocytes begin to ripen by hormonal cues in the Graafian follicles of the adult (McLaren, 1988). Thus, in the mature ovary, germ cells undergo a temporally and spatially ordered development from an oocyte to an ovum that requires the nutritional and regulatory support of their neighbouring follicular cells, the granulosa cells. As a follicle develops from a primordial stage to a Graafian stage, its oocyte simultaneously increases in size and accumulates organelles and stores of mRNAs and proteins that will be used to support the early development of an embryo. Unfortunately, the fate of most oocytes is to die along the pathway of growth and maturation by a process of follicular atresia (Byskov, 1974). Following the preovulatory gonadotropin surge, an oocyte within a healthy Graafian follicle completes maturation into an ovum and is subsequently

ovulated into the ostium of the oviduct where it is carried by cilia to the ampulla region. In order for fertilization to proceed, spermatozoa that are deposited by the male into the vagina must swim to the ampulla in search of the ovum. The union of a single spermatozoon and an ovum results in the formation of a zygote. As the zygote travels through the oviduct, it undergoes several cleavage divisions and later implants in the uterine wall for further development. If the ovum is not fertilized, however, it begins to degenerate and the uterine epithelium either undergoes deterioration and leucocytosis, as in the estrous cycle of the mouse (Allen, 1922), or is shed, as in the menstrual cycle of humans and primates (Dollar et al., 1979). After ovulation, the Graafian follicle collapses and forms a temporary endocrine gland called the corpus luteum which secretes progesterone and estrogens. Overall, the entire process of oocyte growth and maturation along with changes in the accessory sex organs is tightly regulated by endocrine factors and their appropriate feedback pathways.

The transformation of a non-growing oocyte into a mature ovum is accompanied by dynamic changes in cellular composition and morphology, biochemical activities, and nuclear events (Albertini, 1992; Wassarman and Albertini, 1994). In general, oocyte development can be divided into two separate phases: growth and maturation. While the growth phase involves major changes in the morphology and biochemistry of the oocyte, the maturation phase, by and large, deals with nuclear progression.

The growth phase begins with the recruitment of a small number of oocytes from the non-growing pool into the growing pool. Despite considerable effort, the signals that trigger the entry of oocytes into the growth phase are unknown. Yet, non-growing oocytes cannot develop to term in isolation from their granulosa cells, which provide structural support, growth factors and hormones, and regulate their overall development (Wassarman and Albertini, 1994). Oocyte growth and the proliferation of their surrounding granulosa cells usually occur coordinately in a stage-specific manner. In general, there are three basic stages of follicular development starting with a

primordial follicle, which becomes a growing follicle, and then later matures into a Graafian follicle. The primordial follicle consists of a non-growing primary oocyte surrounded by a single layer of flattened follicular cells; however, upon commencing the growth phase, the follicular cells become cuboidal and then multilayered. A cavity containing follicular liquor appears, and during its expansion, the oocyte moves from a central position to one side of the follicle. During mid-growth, the stroma immediately around the follicle differentiates to form a thecal layer, comprising the theca interna and the theca externa, which provides additional support and nutrition to the follicle. In the mature Graafian follicle, the oocyte is positioned on a small hillock of cells called the cumulus oophorus and is surrounded by two or more layers of granulosa cells (cumulus cells), with the innermost layer designated the corona radiata. Most of oocyte growth in the mouse has occurred by the time the first two layers of granulosa cells have been laid down and is complete when the follicular antrum starts to form. Near the end of the growth phase, oocytes acquire the property of meiotic competence (Wickramasinghe et al., 1991), which refers to the oocyte's ability to reinitiate meiosis spontaneously when released from the follicular environment.

Major structural alterations take place during the growth period that are linked to an increase in cellular metabolism. There are changes in the subcellular distribution and ultrastructure, and increases in the number of mitochondria, ribosomes, microtubule networks, and Golgi complexes along with the formation of cortical granules (Wassarman and Josefowicz, 1978; Mattson and Albertini, 1990; Ducibella et al., 1994). Remodeling of the nucleus, or germinal vesicle, involves the progressive wrapping of heterochromatin around the nucleolus (Mattson and Albertini, 1990), which also transforms from a diffuse structure into a slightly larger, dense and uniform mass during oocyte growth (Chouinard, 1991). Among the best documented biosynthetic changes are those involving the production and accumulation of maternal mRNA, ribosomal RNA and proteins required either for oocyte structure or for fertilization, such as the zona pellucida proteins

(Schultz, 1986; Philpott et al., 1987; Roller et al., 1989). Both homologous gap junctions between granulosa cells and heterologous ones with the oocyte are maintained throughout growth to permit the passage of small molecules (Anderson and Albertini, 1976). Overall, the high rates of transcription and translation along with the impressive morphological changes reflect the activation of a developmental program that prepares growing oocytes for the final stages of meiosis, fertilization and preimplantation development.

The meiotic progression of the oocyte nucleus occurs in the adult ovary and is associated with the maturation phase. A summary of oocyte meiosis is shown in Figure 2 (Tsafriri, 1978). Shortly after delivery, the mouse ovary is populated with approximately 10,000 non-growing oocytes arrested at diplotene, or the so-called dictyate stage, of the first meiotic prophase. After puberty, only fully grown oocytes in Graafian follicles resume meiosis and complete the first meiotic reduction just prior to ovulation. Resumption of meiosis can be mediated by a hormonal stimulus *in vivo*, namely by LH (Bingel and Schwartz, 1969; Schwartz, 1974), or simply by the release of oocytes from their ovarian follicles into a suitable culture medium *in vitro* (Szybek, 1972; Sorensen and Wassarman, 1976). Meiotic maturation takes 12-14 hours in the mouse and begins with germinal vesicle breakdown (GVBD) followed by the continuation of meiosis. The steps terminating the first meiosis involve: desynapsis of homologous chromosomes in diplotene followed by diakinesis; the alignment of chromosome pairs at the equatorial plate in metaphase; the separation of homologues in anaphase; and the equal division of chromosome pairs between the oocyte and the first polar body in telophase. Immediately after expulsion of the first polar body, the nucleus of the ovum starts the second meiotic division and arrests, for a second time, in metaphase II until fertilization. Ovulation follows next and penetration of a spermatozoon restores the diploid number of chromosomes and serves as a stimulus for the ovum to complete the second meiotic division and cast off the second polar body. This task can also be accomplished by artificial activation. In the second reductive division, there is

a separation of the sister chromatids along the centromere. An unusual feature of both meiotic divisions in the female is that the oocyte receives almost all of the cytoplasm while the polar bodies receive very little. Only oocytes that have undergone successful meiotic maturation are capable of being fertilized and developing normally.

Morphological studies of the prepubertal and adult mouse ovaries have indicated distinct types of developing follicles (Peters, 1969). A classification of oocytes and follicles has been proposed which is based on the size of the oocyte and the morphology and size of the follicle in ovarian sections (Pedersen and Peters, 1968). There are three classes of oocytes: small oocytes with a diameter of less than 20 μm; growing oocytes with a diameter of 20-70 μm; and large oocytes which have reached their final size of 70 μm. The follicles are divided into types according to the numbers and layers of granulosa cells, the presence or absence of a follicular antrum, and the position of the oocyte within the follicle. According to this classification system, there are eight different types of follicles in the mouse (Pedersen and Peters, 1968). Alternate models that classify isolated oocytes into different stages of growth are based on changes in chromatin configuration (Debey et al., 1993) or a combination of chromatin and microtubule organization (Mattson and Albertini, 1990).

The mouse oocyte grows from 12 to 80 μm in diameter over a period of 2-3 weeks and undergoes roughly a 300-fold increase in volume, thus making it one of the largest cells of the body. Accordingly, the ratio of cytoplasmic to nucleoplasmic volume increases from about 8:1 in small oocytes to about 64:1 in fully grown oocytes (Wassarman and Albertini, 1994). As in the male, there is a time-dependent synchronous wave of germ cell development following birth in the female. Thus, pure populations of immature oocytes can be isolated from preantral follicles in prepubertal mice, whereas fully grown meiotically-competent oocytes can be obtained from antral follicles in 3-4 week old mice using the techniques of Eppig and Telfer (1993). Under normal laboratory housing conditions, a sexually mature mouse ovulates

naturally once every four days and releases 8-12 eggs; consequently, superovulation at 7 weeks of age with pregnant mare's serum gonadotropin (PMSG) followed by human chorionic gonadotropin (hCG) releases 20-60 eggs, depending on the mouse strain (Hogan et al., 1986).

There are several fascinating aspects of oocyte growth and maturation. In contrast to the male, the sexually mature female possesses a finite stock of oocytes that is drawn upon throughout her reproductive life, and as such, there is no possibility of addition or replacement afterwards because oogonial stem cells have disappeared (Mandl and Zuckerman, 1951; Borum, 1966). Unlike somatic and testicular cells, oocytes have developmental potential and this property is, in part, due to their ability to synthesize and accumulate large stores of molecules and organelles required for preimplantation development. Moreover, oocytes have evolved specialized mechanisms for arresting the machinery of the meiotic cell cycle twice: the first time, they remain suspended in prophase I while growth occurs, and the second time, at metaphase II while awaiting fertilization. Many of these aspects and others remain to be studied and clarified at the molecular level.

1.1.5 Summary and Outlook

In summary, the timing of gametogenesis and the structure and ultrastructure of the testis and ovary have been extensively researched in many mammalian species; the various stages of gamete development can be isolated and analyzed biochemically; the growth and hormonal requirements have been established; and with the recent advances in molecular biology, many gamete-specific genes have been isolated, cloned and genetically disrupted to understand their physiological roles. The major limitation in gametogenesis is that it has proved extremely difficult to devise a culture system that will support development all the way from spermatogonia to spermatozoa and oogonia to ova. Few studies have demonstrated the successful, although limited, progression of germ cell development *in vitro* using coculture with Sertoli (Rassoulzadegen et al., 1993) or follicular (Taketo

and Koide, 1981; Eppig and O'Brien, 1996) supporting cells. With recent advances in cloning, preimplantation diagnosis for certain genetic diseases, and other reproductive technologies including cryopreservation of gametes and intracytoplasmic sperm injection (ICSI) as a treatment for infertility, it has become critical to gain a better knowledge of male and female gametogenesis.

Despite all the scientific advances made over the last century to characterize and understand mammalian gametogenesis, the role of DNA methylation in this process has been poorly understood. Sex- and sequence-specific patterns of DNA methylation are thought to originate in the germline and differ markedly between mature oocytes and sperm. DNA methylation has been implicated in the differential expression of imprinted genes, where for example, the imprinted allele inherited from the mother is silenced and its duplicate copy inherited from the father is expressed or vice versa. Recently, concern has been raised over the use of immature germ cells in assisted reproductive technologies since it is not known when or how gametic imprints are fully established (Tycko et al., 1997). Failure to completely establish an imprint in germ cells may lead to developmental consequences in the offspring. Therefore, studies that investigate the timing and mechanisms of the establishment and maintenance of sex-specific DNA methylation patterns in the germline are warranted. The following section will provide an overview of DNA methylation, the enzymes involved in this process, and their roles in the embryo-germline axis.

1.2 DNA Methylation

Among the different mechanisms of epigenetic control that have been postulated to play a role in regulating genome function such as replication timing and chromatin structure, DNA methylation remains the best studied (Razin and Riggs, 1980). Methylation of the mammalian genome is catalyzed by DNA (cytosine-5) methyltransferase, Dnmt, at the 5 position of cytosine, predominantly within 5'-CpG-3' dinucleotides. DNA methylation has been

implicated in several biological functions including the global control of gene regulation and development (Li et al., 1992; Lei et al., 1996) and in specialized biochemical functions such as X-chromosome inactivation, genomic imprinting and host defense (Li et al., 1993; Beard et al., 1995; Walsh et al., 1998; Walsh and Bestor, 1999). Much of the early literature demonstrated a correlation between methylation and gene expression. However, it was a targeted disruption in the predominant DNA methyltransferase *Dnmt1* gene that provided the proof that DNA methylation is critical for normal mammalian development (Li et al., 1992; Lei et al., 1996). Evidence presented below, including DNA methylation differences between male and female gametes and the highly regulated expression of Dnmt1 during spermatogenesis, suggests that regulating DNA methylation in the germline may be important for proper gamete and embryo development.

1.2.1 Creation and Transcriptional Regulation

In mammals, wide-spread methylation occurs non-randomly along the DNA and within cellular genes. The haploid genome contains approximately 5 X 10^7 CpG sites (Schwartz et al., 1962), and of these about 60% are methylated (Bestor et al., 1984). The distribution of 5-methylcytosine is site- and sequence-specific resulting in many unique patterns of DNA methylation that are tissue-specific. Changes in the patterns of DNA methylation involve demethylation and *de novo* methylation, and it is the interplay between these two opposite processes which provides the basis for forming the correct methylation patterns. Once established, methylation patterns can be maintained by clonal inheritance to daughter cells during semi-conservative DNA replication (Bird, 1978; Stein et al., 1982), or lost either by a passive demethylation process whereby methylation does not occur during replication (Rougier et al., 1998), or through a poorly understood active demethylation process (Bhattacharya et al., 1999). How gamete-specific *de novo* methylation patterns are established in the germline is unclear but are likely to involve complex interactions of DNA methyltransferase(s), DNA-binding

proteins or other co-factors, *cis*-acting sequences, and alterations in chromatin structure.

The covalent modification of DNA provides a powerful mechanism to regulate gene expression during development (Holliday and Pugh, 1975; Riggs, 1975). Cytosine methylation in the promoter region of a number of loci has been associated with transcriptional repression and provides a means of compartmentalizing large genomes into expressed and unexpressed sequences (reviewed by Eden and Cedar, 1994; Kass et al., 1997; Ng and Bird, 1999). Much of our understanding of gene regulation and cellular differentiation is derived from studies using cytidine analogs, such as 5-azacytidine, which becomes incorporated into DNA during replication and inhibits DNA methylation (Jones and Taylor, 1980). Although the molecular mechanisms of transcriptional repression are not well defined, it has been postulated that DNA methylation could prevent the binding of the basal transcriptional machinery and of ubiquitous transcription factors to promoters (Becker et al., 1987; Iguchi-Ariga and Schaffner, 1989). Another possibility is that recruitment of histone deacetylase and other specific transcriptional factors that recognize 5-methylcytosine, such as methyl-CpG-binding proteins, could turn transcription off (Jones et al., 1998; Nan et al., 1998). For many of these mechanisms, DNA methylation may not act alone in silencing gene transcription, as other studies have indicated that DNA methylation induces alterations in chromatin structure too (Davey et al., 1997). Together, these studies suggest that tissue-specific gene expression depends on reversible sequence-specific methylation and has been termed the methylation-development hypothesis.

An alternate theory regarding the function of genomic methylation patterns is the host defense hypothesis. Similar to genome defense in bacteria, whereby invading viral sequences are targeted for methylation and then inactivated, a role for cytosine methylation in protecting mammalian DNA against 'parasitic' sequences has been proposed (Barlow, 1993; Yoder et al., 1997a; Bestor, 1998a). Repeat sequences and retrotransposons make up an

estimated 35% of the entire genome and pose a threat to its structure and regulated expression (Yoder et al., 1997a; Bestor, 1998a). Insertion mutations, translocations and other gene re-arrangements, or the production of chimeric mRNAs are possible consequences of transposon action in the mammalian genome (Yoder et al., 1997a). Consistent with this hypothesis is the observation that retroviruses are inactivated by *de novo* methylation during early mouse embryogenesis (Jähner et al., 1982). Moreover, a reduction in methylation levels by a disruption in *Dnmt1* is associated with the over-transcription of intracisternal A particle (*IAP*) retroviruses in mutant embryos (Walsh et al., 1998), arguing for a role of cytosine methylation in limiting the spread of parasitic sequences during development. Certain endogenous genes, such as imprinted genes, may be targets for methylation due to their viral-like structure, with small direct repeat sequences and few or no introns (Barlow, 1993; Neumann et al., 1995; Hurst et al., 1996).

A recent report by Walsh and Bestor (1999) challenges the dogma that tissue-specific gene expression depends on reversible promoter-specific methylation and demethylation. They provide convincing data that promoter methylation of several tissue-specific genes in non-expressing tissues does not correlate with their transcriptional activities in *Dnmt1*-deficient mouse embryos; however, for imprinted genes and retroviruses, expression-methylation correlations do hold. Thus, a new model has been proposed under which cytosine methylation is necessary for the specialized functions of allele-specific gene expression and the transcriptional silencing of parasitic sequence elements, whereas cellular differentiation is controlled by regulatory networks that do not depend on cytosine methylation (Walsh and Bestor, 1999). This model fits well with the notion that DNA methylation plays a role in the germline, where it is believed to set down sex-specific methylation patterns on imprinted genes.

1.2.2 The Family of DNA Methyltransferases

DNA methylation is catalyzed by a family of conserved DNA methyltransferases and is widespread among protists, plants, fungi and animals. However, not all organisms such as the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* display detectable DNA methylation (Regev et al., 1998). In bacteria, at least 30 methyltransferases exist and each type is responsible for methylating a specific cytosine residue of a particular restriction enzyme site (Lauster et al., 1989). In mammals, a few DNA methyltransferases or putative forms of the enzyme have been identified and cloned (Bestor et al., 1988; Okano et al., 1998; Yoder and Bestor, 1998), but unlike bacteria, only one has demonstrated methylating activity for many CpG dinucleotides regardless of their position at enzyme restriction sites (Yoder et al., 1997b; Warnecke et al., 1998). It is postulated that DNA methylation has been conserved during the evolution of eukaryotic organisms because it provides unique possibilities for setting up various types of specialized functions (Colot and Rossignol, 1999).

The predominant form of DNA (cytosine-5)-methyltransferase in mammals is Dnmt1 (Bestor, 1988; Yen et al., 1992; Tucker et al., 1996a; Yoder et al., 1996; Ramchandani et al., 1998). This enzyme catalyzes the transfer of a methyl group from the donor S-adenosyl methionine to the 5 position of cytosine giving rise to 5-methylcytosine. The mouse *Dnmt1* gene encodes a monomeric protein in ES and somatic cells with an apparent M_r of 190,000 that is derived from a 5.2 kb transcript (Bestor et al., 1988; Tucker et al., 1996a; Yoder et al., 1996). The organization of functional domains and sequence motifs within Dnmt1 is shown in Figure 3 (Yoder et al., 1997b). This enzyme is composed of a 1000 amino acid N-terminal regulatory domain and a 500 amino acid C-terminal catalytic domain closely related to bacterial C5-specific restriction methyltransferases (Bestor et al., 1988). Since the cloning and sequencing of the murine cDNA (Bestor et al., 1988), an additional 118 amino acids at the N-terminus has been described (Tucker et al. 1996a; Yoder et al., 1996). The intact Dnmt1 enzyme exhibits *de novo*

and maintenance activities but prefers hemimethylated DNA substrates (Gruenbaum et al., 1982; Bestor and Ingram, 1983). Removal of the regulatory domain induces *de novo* methylation (Bestor, 1992). Sequences within the N-terminus direct Dnmt1 to the nucleus and to replication foci during S-phase of the cell cycle (Leonhardt et al., 1992; Liu et al., 1998). A zinc binding site in the regulatory region has been implicated in the ability of Dnmt1 to distinguish methylated and hemimethylated DNA (Bestor, 1992). In addition, there are two regions within the N-terminus that are closely related to vertebrate homologues of proteins known to be involved in the clonal transmission of states of gene expression in Drosophila; these are the All1/Hrx/Mll motif (Ma et al., 1993) and the Polybromo-1 motif (Bestor, 1996). The catalytic domain of all known methyltransferases shares ten conserved motifs of which motif IV contains the Pro-Cys catalytic centre essential for methyl transfer (Lauster et al., 1989; Posfai et al., 1989; Kumar et al., 1994).

Until recently, the marked diversity of methylation patterns in the vertebrate genome has intrigued molecular and cellular biologists. How can one gene encoding DNA methyltransferase activity be responsible for generating a variety of DNA methylation patterns? Current evidence supports the existence of multiple isoforms that are encoded from the same *Dnmt1* gene as well as distinct *de novo* methyltransferases. Production of Dnmt1 proteins with different N-terminal (Gaudet et al., 1998) and C-terminal (Deng and Szyf, 1998) extensions through alternative splicing of the Dnmt1 mRNA has been documented. Moreover, ES cells homozygous for a null mutation of Dnmt1 still show signs of *de novo* methylation on integrated proviral DNA, indicating the existence of alternate DNA methyltransferases (Lei et al., 1996). Recently, three novel murine DNA methyltransferases have been cloned and characterized including Dnmt2, Dnmt3α and Dnmt3β (Yoder and Bestor, 1998; Okano et al. 1998a; Okano et al., 1998b). Disruption of the catalytic centre in Dnmt2 did not affect *de novo* or maintenance methylation of endogenous viral DNA, indicating that Dnmt2 is not a functional methyltransferase enzyme (Okano et al., 1998a). The *in vivo* functions of

Dnmt3α and Dnmt3β have not yet been determined. Until now, the only mammalian enzyme with significant methylating activity and function *in vivo* is Dnmt1 (Carlson et al., 1992; Li et al., 1992; Lei et al., 1996; Yoder et al., 1997b). More recently, a *bone fide* demethylase enzyme with specific activity for murine CpG sites has been characterized and may play a role in the plasticity of methylation patterns during development and cancer formation (Bhattacharya et al., 1999).

1.2.3 Mammalian Development

Studies in the mouse indicate that DNA methylation patterns do not change rapidly in adult somatic cells but are subject to sweeping changes in the embryo-germline axis (Jähner and Jaenisch, 1984; Razin and Cedar, 1993). Sex- and sequence-specific patterns of DNA methylation are thought to be established in the germline and are later modified in the early embryo (Jähner et al., 1982; Monk et al., 1987; Sanford et al., 1987; Chaillet et al., 1991; Kafri et al., 1992). PGCs are globally undermethylated, but during gametogenesis, methylation levels increase so that the mature spermatozoa and oocytes become highly methylated cells, with the paternal DNA having acquired more global methylation than the maternal DNA (Monk et al., 1987). Following fertilization, the preimplantation embryo undergoes genome-wide demethylation prior to the morula stage and remains in this unmodified state through blastulation (Monk et al., 1987; Kafri et al., 1992; Howlett and Reik, 1991). This process erases DNA methylation patterns inherited from the parental gametes and allows reformatting of the embryonic genome (Razin and Cedar, 1993). However, imprinted loci maintain gamete-specific methylation differences during this period (Tremblay et al., 1997; Shemer et al., 1997). The final adult-like genomic methylation pattern is achieved, in part, by a massive wave of *de novo* methylation after implantation (Kafri et al., 1992). Although changes in DNA methylation during development has been examined, little is known of the mechanisms by which specific sequences are

designated for *de novo* methylation in the germline or precisely when they are established.

Perturbations in DNA methylation patterns by way of targeted gene disruption, antisense, or treatment with hypomethylating agents, are associated with abnormal development in several eukaryotic organisms. For example, disruption of the murine *Dnmt1* produces an embryo lethal phenotype in homozygotes (Li et al., 1992; Lei et al., 1996), as discussed in more detail below. In *Ascobolus*, defects in sexual differentiation and fertility were seen in the offspring from crosses between parents harbouring a disruption in *masc1*, a gene whose product encodes a protein that shares homology with the catalytic domain of eukaryotic DNA methyltransferases (Malagnac et a., 1997). *Arabidopsis* plants that are homozygous for a mutation called *ddm* (decrease in DNA methylation) developed morphological abnormalities in floral and vegetative components after several generations of self-pollination (Kakutani et al., 1995). In transgenic *Arabidopsis* plants that express antisense constructs of a native DNA methyltransferase gene, *MET1*, significant hypomethylation of single-copy and repetitive DNA sequences were observed in association with major phenotypic abnormalities (Finnegan et al., 1996; Ronemus et al., 1996). Pharmacological treatment of zebrafish embryos with the hypomethylating cytosine analogues, 5-azacytidine and 5-aza-2'-deoxycytidine, showed abnormal gastrulation and patterning of the dorsal mesoderm (Martin et al., 1999). In the mouse, administration of 5-aza-2'-deoxycytidine prior to the onset of active spermatogenesis inhibited differentiation of spermatogonia into spermatocytes (Raman and Narayan, 1995). The chronic treatment of male rats with 5-azacytidine resulted in an increase in preimplantation loss, abnormal progeny outcome and a decrease in fertility (Doerksen and Trasler, 1996). These studies and others indicate that DNA methylation is an important component of epigenetic regulation in eukaryotes.

Several lines of evidence indicate that Dnmt1 is essential for normal mammalian development. To date, there are three different knock-outs of

Dnmt1, all of which significantly reduce global methylation levels and perturb development in mutant embryos. The N-allele of *Dnmt1* is a partial loss-of-function mutation that reduces Dnmt1 protein levels by 95% and allows homozygous embryos to develop to 10.5 dpc (Li et al., 1992). These mutant embryos contain 30% of wild type global methylation levels and display a relatively normal morphology but die as a result of wide-spread apoptosis in all tissues (Li et al., 1992). The S- and C-alleles of *Dnmt1* are severe or null mutations that stop development at 8.5 dpc or earlier and show more severe developmental asynchrony (Li et al., 1993a; Lei et al., 1996). Accordingly, global methylation levels are about 5% of wild type in $Dnmt1^S$ and $Dnmt1^C$ homozygotes (Li et al., 1993a; Lei et al., 1996), and thus, in none of the mutants do methylation levels reach zero. $Dnmt1^N$ homozygotes also display abnormal expression of the X-chromosome, imprinted genes and retroviral sequences (Li et al., 1993a; Beard et al., 1995; Walsh et al., 1998; Walsh and Bestor, 1999). Based on the significant role of Dnmt1 in normal embryo development, we predict that this enzyme is also important for germ cell development.

1.2.4 DNA Methylation and Dnmt1 Expression in Gametogenesis

DNA methylation differences between male and female gametes have been examined at various times of development for certain types of genes. It should be kept in mind that the data are limited, since many germ cell stages have not been included and in most cases, methylation at very few CpG sites were tested. Experimentally, the status of DNA methylation can be assayed globally or at the level of the individual gene using either methylation-sensitive restriction enzymes that contain CpGs in their recognition site or by the technique of bisulfite genomic sequencing. With restriction enzyme studies, the methylation status of about 20% of CpGs within a given region can be assessed whereas with genomic sequencing, all CpGs for a gene of interest as defined by the primers can be tested for methylation.

In early work, methylation of single, low copy, repeat sequences and imprinted genes have been compared between male and female germ cells at various times of development. For example, the mouse interspersed family (*MIF*) repeat sequence was unmethylated in PGCs at 12.5 and 14.5 dpc and methylated at 16.5 dpc exclusively in male germ cells (Monk et al., 1989). *L1*, *IAP* and major urinary protein (*MUP*) sequences were all unmethylated in DNA from diplotene oocytes, while the same sequences were highly methylated in DNA from pachytene spermatocytes, round spermatids and epididymal sperm (Sanford et al., 1987). In contrast, a re-examination of *IAP* indicated that PGCs and non-growing diplotene oocytes were unmethylated whereas ovulated oocytes were nearly fully methylated; in the male germline, *de novo* methylation of *IAP* occurred before the onset of spermatogenesis, perhaps in prospermatogonia or earlier (Walsh and Bestor, 1998). Centromeric satellite DNA was for the most part unmethylated in mature gametes of both sexes, and in contrast, highly methylated in somatic cells (Ponzetto-Zimmerman and Wolgemuth, 1984; Sanford et al., 1984). Certain tissue-specific genes showed little methylation at 12.5 dpc, underwent methylation in both germlines between days 15.5 to 18.5 dpc and remained methylated through gametogenesis (Kafri et al., 1992). Interestingly, while methylation on protamines 1 and 2 increased during spermatogenesis, transition protein 1 became progressively less methylated (Trasler et al., 1990). One study has even suggested that DNA methylation of some testis-specific genes increases as sperm pass through the epididymis (Ariel et al., 1994). Together, these studies provide some insight into the timing of erasure and establishment of DNA methylation patterns during gametogenesis. DNA methylation at imprinted loci is considered in more detail in another section.

Evidence of Dnmt1 expression and regulation throughout gametogenesis is limited. In comparison to other tissues of the adult mouse, levels of Dnmt1 mRNA are highest in the ovary and testis (Trasler et al., 1992). In particular, mature oocytes show very high levels of Dnmt1 protein

and enzymatic activity (Carlson et al., 1992). In the male, available data indicate that Dnmt1 is developmentally regulated during spermatogenesis at the level of mRNA, protein, and enzyme activity (Benoit and Trasler, 1994; Numata et al., 1994). Dnmt1 mRNA and protein were detected in enriched populations of isolated mouse and rat germ cells (Trasler et al., 1992; Jue et al., 1995a; Jue et al., 1995b). These data indicated the presence of a 5.2 kb Dnmt1 transcript in types A and B spermatogonia, preleptotene and leptotene/zygotene spermatocytes and round spermatids, corresponding with a M_r 190,000 Dnmt1 protein (Jue et al., 1995a). Pachytene spermatocytes were devoid of Dnmt1 protein, but interestingly, a novel 6.0-6.2 kb Dnmt1 message was detected in this cell type of the mouse (Trasler et al., 1992; Jue et al., 1995a) but not the rat (Jue et al., 1995b). The mechanism by which this alternate transcript is regulated between species and even within a species, as the mouse, has yet to be determined. The localization of Dnmt1 in the nucleus of early meiotic germ cells, especially in distinct foci of some leptotene/zygotene spermatocytes, suggests that it may function in the establishment or maintenance of paternal imprints or possibly in DNA repair or X-chromosome inactivation (Jue et al., 1995a). While much of the work on Dnmt1 has focused on spermatogenesis, little has been done on oogenesis or fetal germ cell development of both sexes.

Characterization of Dnmt1 expression has been extended from the germline into early embryo development. In spite of its role as a replication factor, Dnmt1 protein is concentrated in the cytoplasm of all preimplantation embryos, except for the eight-cell stage where it is also detected in the nuclei (Carlson et al., 1992). Following implantation, this enzyme is exclusively found in the nuclei of proliferating cells in the developing embryo (Trasler et al., 1996), where it is postulated to play an important role in the maintenance of methylation patterns. Other histological studies which have examined Dnmt1 mRNA localization during various stages of embryo and postnatal development support the notion that this enzyme is involved in cellular proliferation (Goto et al., 1994; Niederreither et al., 1998), except in mature

neurons where it may have a function unrelated to DNA replication (Goto et al., 1994). Thus, following fertilization, Dnmt1 undergoes dynamic changes in its subcellular localization and distribution, possibly to facilitate the formation of unique developmental methylation patterns.

1.2.5 Genomic Imprinting

Genomic imprinting defies Mendelian genetics because it represents the differential expression of an allele depending on the parental origin (Barlow, 1993). In the 1980s, nuclear transplantation experiments pioneered by Solter and Surani were the first to suggest that the mammalian genome harbours imprinted genes. Diploid androgenetic embryos derived from two male pronuclei or diploid gynogenetic embryos derived from two female pronuclei failed to develop to term (McGrath and Solter, 1983; McGrath and Solter, 1984; Surani et al., 1984; Surani et al., 1986). Additional evidence for nonequivalence of maternal and paternal genomes came from experiments using mice with certain chromosomal translocations that produced uniparental disomies (reviewed by Cattanach and Jones, 1994). The first evidence of an association between allele-specific DNA methylation and genomic imprinting came from studies using transgenic mice. In a number of experiments, the methylation of individual transgenes was found to correlate with the sex of the transmitting parent; the transgene DNA was differentially methylated, so that the more heavily methylated allele was generally not expressed (Reik et al., 1987; Sapienza et al., 1987; Swain et al., 1987). In a landmark study in the early 1990s, the first imprinted gene, insulin-like growth factor 2 (*Igf2*), was identified. Targeted disruption of *Igf2* resulted in growth-deficient offspring when this gene was inherited from the father but not the mother (DeChiara et al., 1991). Together, these studies laid down the groundwork for the field of genomic imprinting.

In recent years, DNA methylation has been implicated as the mark which differentiates between the maternal and paternal alleles of imprinted genes. Characteristics of DNA methylation that satisfy the criteria for genomic

imprinting include: initiation in the germline before fertilization, association with transcriptional silencing of genes, stable transmission through mitotic divisions in somatic cells and reversibility on passage through the germline of the parent of the opposite sex (Tycko et al., 1997). As an example, Figure 4 illustrates the acquisition of a biochemical imprint on the maternal allele during gametogenesis when the two parental chromosomes are separate and leads to the differential expression and function of one of the parental alleles in the somatic lineages (Hoffman and Vu, 1996). Of the 20 or so imprinted genes that have been identified to date (Figure 5), a majority of them exhibit sex-specific methylation (Bartolomei and Tilghman, 1997; Tilghman, 1999), yet this observation merely suggests a correlative relationship between DNA methylation and genomic imprinting. Convincing evidence that DNA methylation plays a role in genomic imprinting comes from studies on Dnmt1-deficient embryos. These embryos have an impairment in functional imprinting and show abnormal expression of at least four imprinted genes, with biallelic expression of a differentiation-related fetal RNA (*H19*), silencing of expression of insulin-like growth factor 2 (*Igf2*) and Igf2/mannose 6-phosphate receptor (*Igf2r*), and ectopic expression of the X-chromosome mediated by *Xist* (Li et al., 1993a; Li et al., 1993b; Beard et al., 1995; Panning and Jaenisch 1996). Furthermore, germline transmission appears to be required for proper imprinting, as Dnmt1 -/- ES cells fail to show monoallelic expression of imprinted genes when rescued by a knock-in of a Dnmt1 minigene, unless they pass through gametogenesis (Tucker et al., 1996b).

Allele-specific differences in methylation have been described for several transgenes and endogenous genes in the embryo-germline axis. The patterns of gamete-specific methylation indicate that imprints are erased in PGCs, re-established during gametogenesis and maintained in the early embryo, despite the genome-wide decrease in methylation levels (Chaillet, 1994). For one imprinted transgene, termed *RSVIgmyc*, methylation was not detected in PGCs but emerged during gametogenesis with the mature oocyte having more methylation than the sperm; following fertilization, the paternally

inherited transgene underwent further modification (Chaillet et al., 1991). The imprinting behaviour of this transgene has been linked to *cis*-acting sequences within the construct itself (Chaillet et al., 1995). This and other *cis*-acting signals, such as repetitive elements, may attract DNA methyltransferase (Turker and Bestor, 1997). Unfortunately, transgenes that randomly insert into the genome and are viewed as foreign by the host may not have relevance to methylation in normal development, and therefore, it is important to examine the methylation of endogenous imprinted genes. One example of a well-characterized differentially methylated endogenous gene is *H19*. Using bisulfite genomic sequencing, it was determined that a 2-kb region in the 5' end of *H19* was fully methylated in sperm DNA but unmethylated in oocyte DNA, and this difference in methylation between the parental alleles was maintained during preimplantation development (Tremblay et al., 1997). Several other endogenous imprinted genes, such as *Snrpn* (Shemer et al., 1997) and *Igf2r* (Stöger et al., 1993) also follow a similar developmental pattern of methylation, whereby the methylation status is different between sperm and eggs and this difference is maintained during early embryogenesis when most DNA methylation is lost. While most of the previous literature focuses on the maintenance aspect of imprinting during early embryogenesis, little has been done to examine the establishment of these sex-specific patterns in the germline.

The timing of when gametic imprints are erased and re-established is, as yet, unknown. Some recent findings have identified specific developmental windows during gametogenesis in which demethylation and *de novo* methylation might create allele-specific methylation patterns at imprinted loci. The erasure of pre-existing methylation patterns is thought to occur in PGCs and is supported by the observation that fusion of an embryonic germ (EG) cell to a somatic cell leads to massive demethylation of the somatic-cell chromosomes, reactivation of its X-chromosome and a loss of the monoallelic expression of *Peg1/Mest* (Tada et al., 1997). Accordingly, *H19*, *Igf2*, *Igf2r* and *Snrpn* show biallelic expression in PGCs, indicating an erasure of the

imprints, and re-establishment of monoallelic expression later in gametogenesis (Szabó and Mann, 1995). Exciting new evidence suggests that maternal-specific methylation of *Igf2r* is established during oocyte growth (Stöger et al., 1993; Kono et al., 1996). The extended parthenogenetic development to 13.5 dpc of an embryo derived from a non-growing oocyte fused with a fully grown oocyte appears to be due to a disruption in the imprinting process thought to occur during oocyte growth (Kono et al., 1996; Obata et al. 1998). In the male germline, it is not clear when the paternal imprint is established but it may occur after 16.5 dpc, since transplantation of a gonocyte nucleus of this age into an enucleated oocyte supported development of a small embryo lacking genomic imprints to day 9.5 of gestation (Kato et al., 1999). Recent examination of the paternally-methylated *H19* gene indicates that the methylation mark occurs differentially on the parental alleles; the paternal allele is fully methylated prenatally prior to the onset of spermatogenesis while the maternal allele acquires methylation during the early events of spermatogenesis (Davies et al., 1999). These studies point to a relatively early erasure of the previous epigenetic mark, and a relatively late re-establishment in the gametes. Nonetheless, the timing of when specific imprints are established in the germline is not well defined and the mechanism(s) by which this happens is (are) unknown.

There are several possibilities that could explain how the monoallelic expression of an imprinted gene is established and maintained. There may be sperm- and oocyte-specific *de novo* methyltransferases, each capable of recognizing the differences between the male- and female-specific gametic imprint regions. There may be different isoforms of the predominant Dnmt1 which is developmentally regulated to perform both *de novo* and maintenance methylation in a sex-specific fashion at different stages of germ cell development. Another possibility is that germline-specific modifiers or inhibitors may facilitate or block *de novo* methylation, or that specific sequences in *cis* may attract or repel DNA methyltransferases. Not all imprinted genes show marked allele-specific methylation, such as *Mash2*,

suggesting that other mechanisms, such as 'long-range' mechanisms of gene regulation within 'imprinted domains' could be involved (Tycko, 1997; Tilghman, 1999). Imprinted genes are often found in clusters on at least three chromosomal regions in mouse and man. In a few cases, promoter-enhancer competition models have been used to explain why some imprinted genes within a cluster are expressed while others are silenced. For example, *H19* is in promoter-enhancer competition with *Igf2*, and its expression causes repression of *Igf2* in *cis* (Leighton et al., 1995a; Leighton et al., 1995b). Differential chromatin accessibility in imprinted domains in male and female gametes may allow the two alleles of an imprinted gene to be marked differently during gametogenesis.

1.3 Thesis Proposal

A few studies have attempted to identify developmental windows in the germline during which *de novo* methylation might create allele-specific methylation patterns at imprinted loci (Kono et al., 1996; Obata et al., 1998; Kato et al., 1999). However, there has been no study to date that has examined the role of Dnmt1 in the establishment or maintenance of gametic imprints during development. A targeted disruption in *Dnmt1* produces an embryo lethal phenotype that is associated with an impairment in functional imprinting, and provides evidence that cytosine methylation marks endogenous imprinted genes (Li et al., 1992; Li et al., 1993a; Li et al., 1993b; Lei et al., 1996). Despite evidence for the existence of alternate *de novo* methyltransferases (Okano et al., 1998a; Okano et al., 1998b; Yoder and Bestor, 1998), the only mammalian enzyme with demonstrated methylating activity and function *in vivo* is Dnmt1 (Carlson et al., 1992; Li et al., 1992; Lei et al., 1996; Yoder et al.,1997b).

The purpose of this dissertation is to test the hypothesis that **Dnmt1 plays an essential role in the establishment and maintenance of DNA methylation patterns in the germline.** To test this hypothesis, the following questions have been asked:

1. Are there differences in the expression of Dnmt1 in the male and female germlines?
2. What are the mechanisms that regulate the sex-specific expression of Dnmt1 in the germlines?
3. What is the role of Dnmt1 during oocyte growth and maturation?
4. What forms of Dnmt1 are present in early embryos?

To address the first question, the expression and localization of Dnmt1 at the level of mRNA and protein in the fetal, prepubertal and mature testis and ovary were compared using Northern blotting and immunocytochemistry in cytological and histological preparations, respectively. Specific markers to identify PGCs and the stages of meiotic prophase were also used. Both light and confocal microscopy techniques were employed for single, and double-antibody labelling experiments, respectively.

Multiple techniques were used to answer the second question including RNase H mapping, 5' RACE cloning and RT-PCR to identify and map novel exons in the 5' end of *Dnmt1*. Immunocytochemistry and confocal microscopy of testicular and ovarian sections were used to examine the dynamics of Dnmt1 localization in germ cells. Northern blotting on enriched populations of male germ cells and Western analysis on ovarian lysates were performed to characterize alternate Dnmt1 messages and protein isoforms, respectively.

To address the third question, I examined the expression of the different forms of Dnmt1 mRNA and protein in populations of isolated oocytes at different stages of development. Methylation of the maternally-imprinted *Snrpn* gene was assessed in isolated oocytes using bisulfite genomic sequencing. Among the various imprinted genes that are known to be methylated on the maternal allele (see Figure 5), the *Snrpn* gene was a favourable gene to assay specific CpG methylation. The genomic structure of the murine *Snrpn* gene has been characterized and contains a maternally-methylated region at the 5' end, termed *differentially-methylated region 1*

(DMR1), that is maintained during preimplantation development (Shemer et al., 1997), shows biallelic expression in Dnmt1-deficient embryos (Shemer et al., 1997), and is homologous to the human *SNRPN* imprinting locus which is used as diagnostic test for Prader-Willi and Angelman syndromes (Zeschnigk et al., 1997). The timing of the establishment of methylation at the *Snrpn DMR1* locus has not been examined previously.

To determine which forms of Dnmt1 mRNA and protein are present in isolated preimplantation mouse embryos, at a time when genome-wide demethylation occurs (Monk et al., 1987), I used RT-PCR, Western blotting, and immunocytochemistry techniques.

Together, these studies provide important information on the distribution and localization of Dnmt1 throughout the germline of both sexes, the molecular mechanisms that regulate Dnmt1 during gametogenesis, and give insight into the establishment and propagation of the maternally-imprinted *Snrpn* gene.

Figure 1. Schematic diagram of spermatogenesis in the prepubertal and adult mouse testis showing the characteristic morphology of the respective cell types. This complex process occurs in three phases: the mitotic proliferation of spermatogonia (ascending axis); meiosis with its prolonged meiotic prophase (horizontal axis) and two reductive divisions which yield 2N secondary spermatocytes and then 1N haploid round spermatids; and spermiogenesis (descending axis) which culminates in the formation of spermatozoa. Modified from Bellvé et al., 1977; copyright permission granted from The Rockefeller University Press.

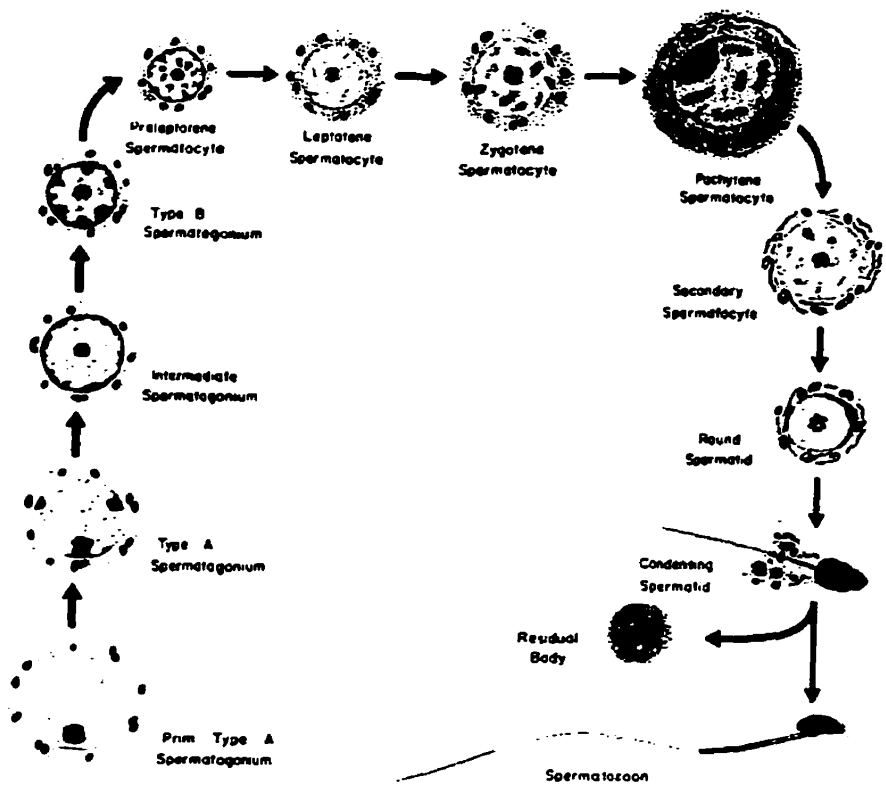

Preleptotene Spermatocyte
Leptotene Spermatocyte
Zygotene Spermatocyte
Pachytene Spermatocyte
Type B Spermatogonium
Secondary Spermatocyte
Intermediate Spermatogonium
Round Spermatid
Type A Spermatogonium
Condensing Spermatid
Residual Body
Prim Type A Spermatogonium
Spermatozoon

Figure 2. Diagram of oocyte meiosis. For simplicity, only three pairs of chromosomes are depicted. 1-4, Prophase stages of the first meiotic division which occur in most mammals during fetal life. The meiotic process is arrested at the diplotene stage ('first meiotic arrest') and the oocyte enters the dictyate stages (5-6). When meiosis is resumed, the first maturation division is completed (7-11). Ovulation occurs usually at the metaphase II stage (11) and the second meiotic division (12-14) takes place in the oviduct following sperm penetration. From Tsafriri, 1978; copyright permission granted from Plenum Publishing Corporation.

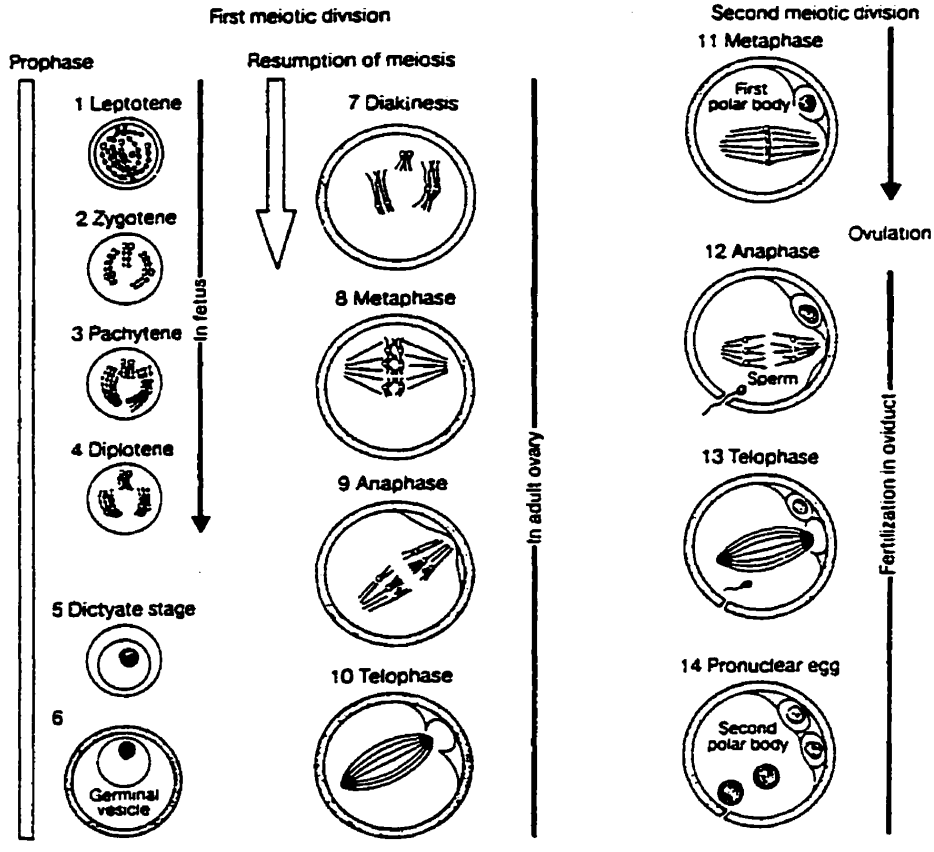

First meiotic division
Second meiotic division
Prophase
Resumption of meiosis
1 Leptotene
2 Zygotene
3 Pachytene
4 Diplotene
5 Dictyate stage
6
Germinal vesicle
7 Diakinesis
8 Metaphase
9 Anaphase
10 Telophase
11 Metaphase
First polar body
12 Anaphase
Sperm
13 Telophase
14 Pronuclear egg
Second polar body
Ovulation
In fetus
In adult ovary
Fertilization in oviduct

Figure 3. Functional domains and sequence motifs in the mammalian DNA methyltransferase, Dnmt1. The protein comprises two domains: a large N-terminal domain that has several regulatory roles and a C-terminal catalytic domain that is closely related to bacterial restriction methyltransferases. Roles of the N-terminus include discrimination of unmethylated and hemimethylated CpG sites, nuclear localization and cell cycle-dependent association with replication foci. The N-terminus shares sequence motifs (All-1/Hrx/Mll and Polybromo-1) with proteins known to be involved in the clonal transmission of states of gene expression in *Drosophila*. From Yoder et al., 1997b; copyright permission granted from Academic Press Limited.

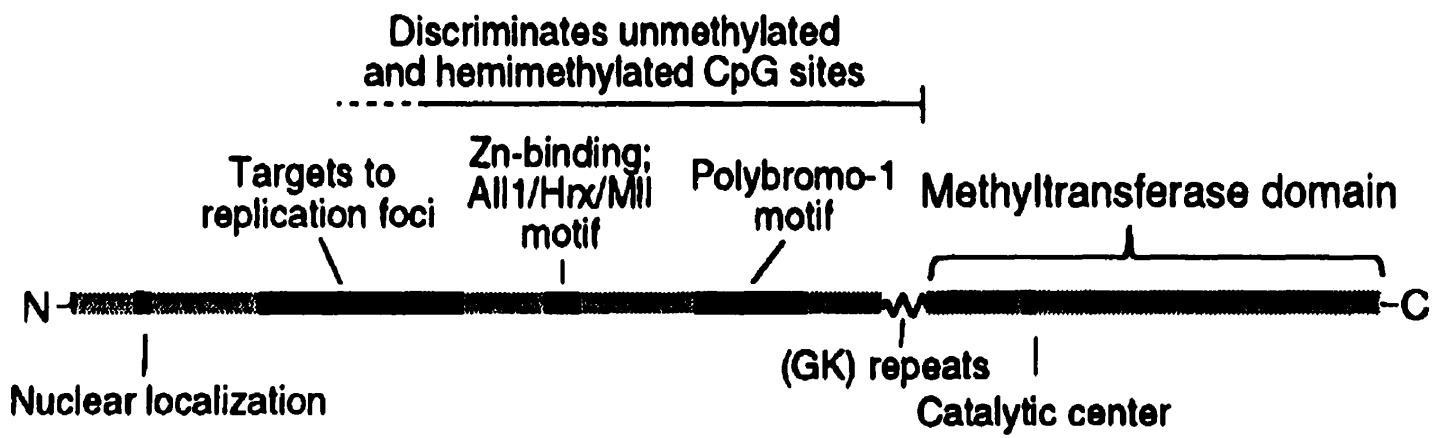
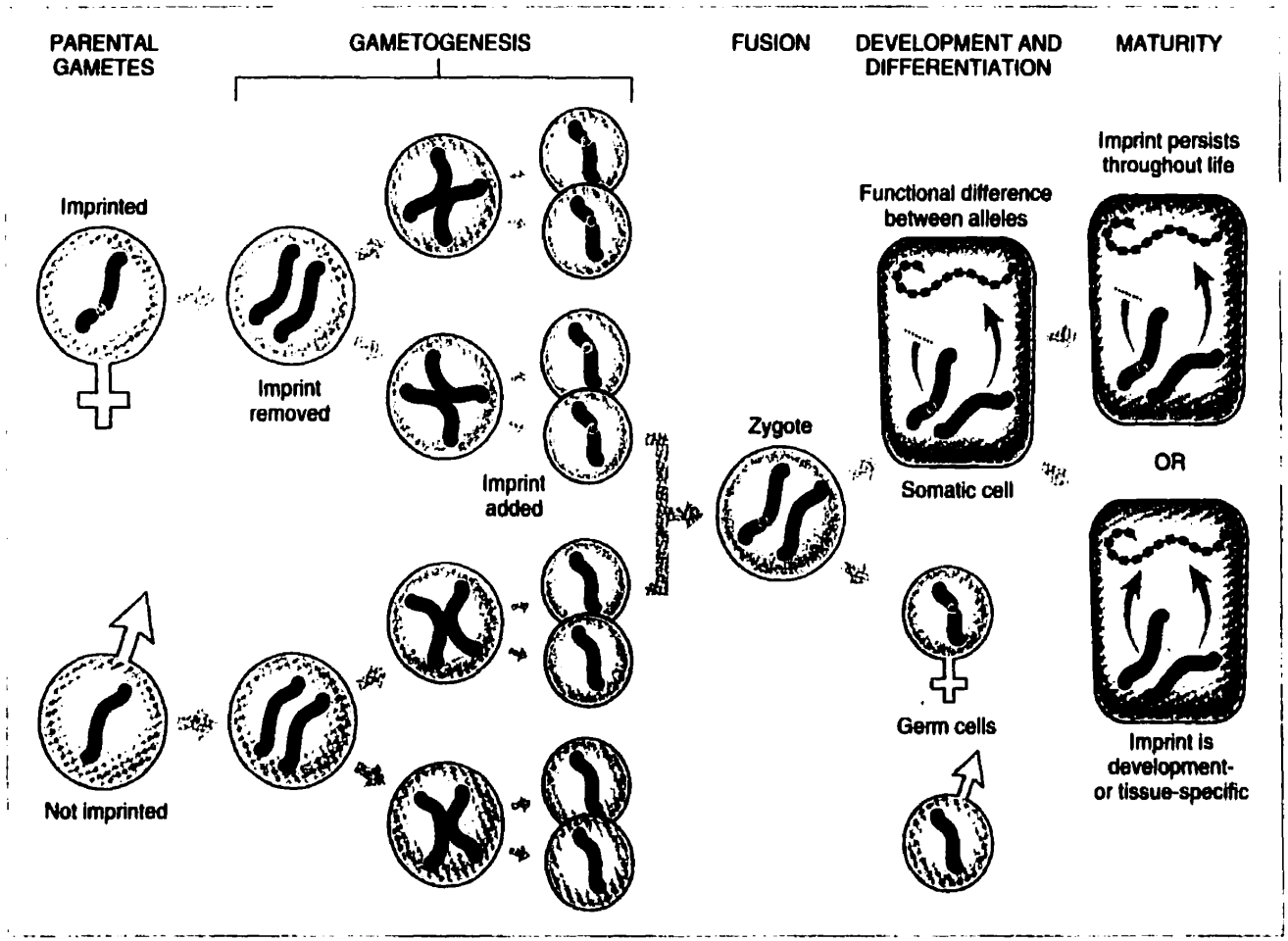

Discriminates unmethylated
and hemimethylated CpG sites
Targets to
replication foci
Zn-binding;
All1/Hrx/Mll
motif
Polybromo-1
motif
Methyltransferase domain
N
C
Nuclear localization
(GK) repeats
Catalytic center

Figure 4. Diagram of genomic imprinting. The biochemical imprint must occur when the two parental alleles are separated, probably during gametogenesis. The imprint must cause a functional difference between the parental alleles. The imprint must also be erased in the diploid gametic precursors. In general, it is thought that the imprinting process remains throughout development, but there are developmentally-specific and tissue-specific instances of imprinting as well. From Hoffman and Vu, 1996; copyright permission granted from Scientific American, Science & Medicine.



JAMES A. PERKINS

Figure 5. List of paternally and maternally imprinted genes in mammals. Imprinted genes are associated with allele-specific DNA methylation of CpG residues. For the moment, this list comprises about 20 imprinted genes, but it is certain that more genes are likely to be identified.

Imprinted Genes

Paternal Expression (Maternal Imprint)	Maternal Expression (Paternal Imprint)
Igf2	*Igf2r*
U2afbp-rs	*H19*
Snrpn	*Mash2*
Xist	*p57 KIP2*
Ipw	*K_VLQT1*
Ins1	
Ins2	
Mas	
Znf 127	
Peg1/Mest	
Peg3/Apoc2	
Grf1	
Gnas	

CHAPTER TWO

DNA Methyltransferase Expression Marks Putative Windows for Sex-Specific Methylation in the Germline

Carmen Mertineit, Teruko Taketo, Peter B. Moens, Suleiman A. Igdoura, Timothy H. Bestor, and Jacquetta M. Trasler

Abstract

The epigenetic modification of DNA by methylation at cytosine nucleotides is initiated in the germline and is required for normal mammalian development. To identify putative windows in which the predominant DNA methyltransferase, Dnmt1, might create sex-specific methylation patterns in the germline, we compared the expression and localization of the enzyme throughout prenatal and postnatal germ cell development in the male and female mouse. The distribution of Dnmt1 appeared to be cell cycle-dependent in replicating germ cells; it was predominantly nuclear during S-phase and surrounded condensed mitotic chromosomes during M-phase. Dnmt1 expression was detected as early as embryonic day 11.5 in primordial germ cells of XX and XY embryos. In the prenatal ovary, Dnmt1 colocalization with meiotic markers revealed expression of the enzyme in early meiotic oocytes, followed by down-regulation at pachytene; Dnmt1 was subsequently re-expressed during oocyte growth in postnatal ovaries. In the testis, Dnmt1 expression coincided with the replication of primordial germ cells/gonocytes in the prenatal gonad, was subsequently down-regulated in mitotic-arresting gonocytes, and was only re-expressed in the dividing primitive and mature spermatogonia after birth. As in the female, Dnmt1 expression in the male germline persisted through early meiosis and was down-regulated at pachytene; however, unlike the female, there was no detectable expression of Dnmt1 after pachytene at later steps of male germ cell development. The down-regulation of Dnmt1 at pachytene was associated with the expression of a 6.0 kb mRNA transcript in the male but not in the female. The timing of Dnmt1 expression in male and female germ cells corresponds to all stages of development when DNA methylation has been reported to occur. Our results suggest distinct, sex-specific developmental times when cytosine methylation of the genome and imprinted genes can be established and maintained by Dnmt1. We propose that DNA methylation in male germ cells is completed prior to the pachytene stage of

meiosis, whereas methylation of DNA in female germ cells can occur both prior to pachytene and at later stages, during oocyte growth.

Introduction

Methylation of DNA in mammals is catalyzed by DNA (cytosine-5)-methyltransferase (Dnmt) and occurs throughout the genome at the five position of cytosine, predominantly within CpG dinucleotides. Methylation of cytosines has been implicated in the control of gene expression (Holliday and Pugh, 1975; Riggs, 1975) and in several normal and abnormal biological processes including development (Li et al., 1992; Lei et al., 1996), X chromosome inactivation (Beard et al., 1995; Panning and Jaenisch, 1996), genomic imprinting (Li et al., 1993a), genome defense (Yoder et al., 1997a) and carcinogenesis (Laird and Jaenisch, 1994). DNA methylation patterns are initially established in the germline and differ markedly for male and female gametes (Sanford et al., 1987; Monk et al., 1987; Driscoll and Migeon, 1990; reviewed by Yoder et al., 1997b). These methylation differences are particularly striking at imprinted loci where they have important implications for allele-specific gene expression in the offspring (reviewed by Bartolomei and Tilghman, 1997; Tycko, 1997; Constância et al., 1998). Gamete-specific methylation differences at other loci such as germ cell specific genes (Trasler et al., 1990) and exogenously derived sequence elements (Sanford et al., 1987; Walsh et al., 1998) have been postulated to play a role in normal germ cell development. Although further modification of methylation patterns occurs after fertilization (Monk et al., 1987; Kafri et al., 1992; Howlett and Reik, 1991), some sequences, such as imprinted loci (Tremblay et al., 1997; Olek and Walter, 1997; Shemer et al., 1997), retain their original differences, arguing for the importance of germline DNA methylation events. Furthermore, it appears that germline transmission is required for proper imprinting. In cells homozygous for a targeted disruption in the DNA methyltransferase gene *Dnmt1*, imprinted gene expression can be rescued, by a knock-in of a *Dnmt1* minigene, but only when the imprinted genes are transmitted through gametogenesis (Tucker et al., 1996b).

The methylation of different types of sequences has been examined in developing male and female germ cells and gives some clues to the timing of

DNA methylation events during germline development. In early studies that assessed overall methylation at CCGG sites, low levels of DNA methylation were found in male and female primordial germ cells (embryonic days 12.5-14.5); during gametogenesis, sperm DNA becomes more methylated than oocyte DNA (Monk et al., 1987; Sanford et al., 1987). Methylation increases in embryonic germ cells of male mice between 14.5 and 18.5 days for a number of sequences including an imprinted transgene *RSVIgmyc* (Chaillet et al., 1991); single copy sequences (Kafri et al., 1992) and *IAPs* (Walsh et al. 1998). Methylation has also been demonstrated to occur postnatally in the male prior to and during early meiotic prophase for sequences such as mouse protamines 1 and 2 (Trasler et al., 1990) and *RSVIgmyc* (Chaillet et al., 1991), and in the female during oocyte development for the transgenes *RSVIgmyc* and *MPA434* (Chaillet et al., 1991; Ueda et al., 1992), *IAPs* (Walsh et al, 1998) and endogenous imprinted genes (Brandeis et al., 1993; Stöger et al., 1993; Kono et al., 1996).

The predominant mammalian DNA (cytosine-5)-methyltransferase, Dnmt1 consists of a large amino-terminal regulatory domain and a smaller carboxy-terminal catalytic domain with homology to bacterial cytosine-5-specific restriction methyltransferases (Bestor et al., 1988). The intact Dnmt1 enzyme exhibits *de novo* and maintenance activities but prefers hemimethylated DNA substrates (Gruenbaum et al., 1982; Bestor and Ingram, 1983). Sequences within the amino terminus direct Dnmt1 to the nucleus and to replication foci at S-phase in somatic cells (Leonhardt et al., 1992; Liu et al., 1998). DNA methylation is essential for normal embryonic development since a targeted disruption of the *Dnmt1* gene results in embryonic death at mid-gestation (Li et al., 1992; Lei et al., 1996). Embryonic stem (ES) cells lacking Dnmt1 exhibit *de novo* methylating activity, indicating the existence of additional DNA methyltransferases (Lei et al., 1996). Recently three new DNA methyltransferases, *Dnmt2* (Yoder and Bestor, 1998; Okano et al., 1998a; Van den Wyngaert et al., 1998), *Dnmt3α* and *Dnmt3ß* (Okano et al., 1998b) have been cloned and characterized.

However, Dnmt1 still remains the only mammalian enzyme with demonstrated DNA methyltransferase activity and function *in vivo* (Carlson et al., 1992; Li et al., 1992; Lei et al., 1996; Yoder et al., 1997b).

Since Dnmt1 is the best characterized component of the DNA methylating system, the expression, localization and regulation of this enzyme in the germline may provide some clues to the mechanisms underlying the establishment and maintenance of DNA methylation patterns during development. Previous studies have demonstrated that Dnmt1 is expressed in the postnatal testis (Trasler et al., 1992; Numata et al., 1994; Jue et al., 1995a; Mertineit et al., 1998) and ovary (Mertineit et al., 1998). At the molecular level, an oocyte-specific 5' exon in the *Dnmt1* gene is associated with the production of a smaller Dnmt1 protein that accumulates to high levels during oocyte growth and maturation, while a pachytene spermatocyte-specific 5' exon gives rise to a non-translated Dnmt1 mRNA in the testis (Mertineit et al., 1998). In the present study, in order to identify the developmental windows during which Dnmt1 might create allele-specific methylation patterns, we compared the expression of Dnmt1 throughout prenatal and postnatal germ cell development in the male and female mouse. We show that the enzyme is present at all developmental times when increases in DNA methylation have been demonstrated to occur. Based on these findings, we suggest that Dnmt1 plays an important role in the initiation and maintenance of DNA methylation patterns in the germline.

Materials and Methods

Animals and Gonad Isolation

Male and female CD-1 mice from Charles River Canada, Inc. (St. Constant, PQ, Canada) were used to obtain whole gonads and isolated germ cells. Noon of the day on which the vaginal plug was found was designated embryonic day 0.5 and the day of delivery as postnatal day 1. Timed pregnant females were killed by cervical dislocation and fetuses removed from the uterine horns and dissected free of extraembryonic membranes. Testes and ovaries could be distinguished in fetuses on embryonic day 12.5 and later by the presence or absence of seminiferous cords, respectively (Hogan et al., 1986). At embryonic day 11.5, male versus female indifferent gonads were distinguished by determining the genetic sex of the embryos using RT-PCR and primers specific for the *Zfy1* and *Zfy2* genes which are located on the Y chromosome (Nagamine et al., 1989). Pairs of urogenital complexes were dissected from fetuses in Eagle's MEM containing Hank's salts and 25 mM Hepes buffer, pH 7.3 (Gibco BRL, Life Technologies, Gaithersburg, MD) and gonads were separated from adjacent mesonephric tissues.

For RNA extraction, fetal and postnatal ovaries and testes were rinsed in sterile DEPC-saline, pooled and frozen in liquid nitrogen. Adult gonads were frozen between slabs of dry ice. All tissues were stored at -70°C until use.

Preparation of Gonads and Germ Cells

Intact genital ridges were fixed by immersion in Ste. Marie's fixative (95% ethanol/glacial acetic acid, 99:1). Alternatively, genital ridges were pooled, washed briefly in Ca^{2+}-Mg^{2+}-free PBS, incubated in 0.2% EDTA-PBS solution for 20 min, transferred to MEM, and mechanically disrupted by pipetting to release primordial germ cells. The cell suspension was fixed for 15 min in 3.7% formaldehyde in PBS and processed for immunocytochemistry.

Fetuses from embryonic days 13.5-18.5 and postnatal mice were perfused through the heart with physiological saline followed by Ste. Marie's fixative. The ovaries and testes were removed, post-fixed in the same fixative for 1 h on ice, dehydrated through graded ethanols, cleared in xylene, and embedded in paraffin. The gonads were sectioned at 5 μm thickness, placed on slides coated with 0.5% gelatin, baked at 60°C for 1 h, and stored at 4°C prior to use.

Oocytes in meiotic prophase were obtained from female ovaries at embryonic days 15.5-17.5. Ovaries were pooled, digested with 0.05% collagenase for 12 min at 37°C, washed in MEM, and further digested with 2.5% trypsin in Rinaldini's solution (Rinaldini, 1959) for 12 min at 37°C. The ovaries were suspended in 10% fetal bovine serum in MEM, washed twice in MEM alone, transferred to Ca^{2+}-Mg^{2+}-free PBS and dissociated by pipetting, centrifuged at 2000 rpm for 3 min, and resuspended in a small volume of MEM. The ovarian cell suspension was applied to 0.5% NaCl droplets on glass multi-spot slides (Shandon Inc., Pittsburgh, P.A.) and allowed to settle for 15 min. The slides were fixed twice for 3 min each in 2% paraformaldehyde, pH 8.2, rinsed three times in 0.4% Kodak Photo-Flo wetting agent (Eastman Kodak Company, Rochester, N.Y.), pH 8.0, air-dried, and stored at -20°C.

Immunocytochemistry

All solutions for immunocytochemistry of Dnmt1 were prepared in PBS, pH 7.2, and procedures were carried out at room temperature, unless specified otherwise. The rabbit polyclonal affinity-purified anti-Dnmt1 antibody (anti-pATH52) has been described previously (Bestor, 1992; Li et al., 1992). Immunoperoxidase staining of Dnmt1 was performed on gonadal sections using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Sections were deparaffinized, rehydrated through graded ethanols, and the endogenous peroxidase activity was quenched with 0.5% H_2O_2 in PBS for 5 min. The sections were subsequently blocked for 30 min in blocking buffer

(0.2% cold-water fish skin gelatin (Sigma Chemical Co., St. Louis, MO), 5% goat serum (Gibco) and 0.2% Tween 20) and incubated with a 1:200 dilution of anti-pATH52 or preimmune serum in blocking buffer overnight at 4°C in a humidified chamber. The slides were rinsed in 0.2% Tween 20 PBS, incubated with a biotinylated goat anti-rabbit antibody (Vector Laboratories) diluted to 1:200 in PBS for 45 min, rinsed, and incubated with the ABC reagent (10 µl/ml avidin DH and 10 µl/ml biotinylated horseradish peroxidase H in PBS) for 45 min. The final reaction was achieved by incubating the sections with 0.01% H_2O_2, 0.05% diaminobenzidine tetrahydrochloride (Sigma), and 5% of 0.2 M imidazole in 25 mM Tris buffered saline, pH 7.6, for 10 min. The sections were counterstained with 0.1% methylene blue, dehydrated through ethanol, cleared in xylene and mounted with Permount (Sigma).

By immunofluorescence, Dnmt1 expression was examined in primordial germ cells and in meiotic prophase oocytes. The mouse monoclonal anti-stage-specific embryonic antigen-1 antibody (anti-SSEA-1, Solter and Knowles, 1978), which recogizes a cell surface glycoprotein on primordial germ cells, was a gift of Dr. T. Feizi (MRC, Harrow, UK). The mouse monoclonal anti-synaptonemal complex antibody, anti-SC, which labels the chromosomal components of germ cells in meiotic prophase has been described previously (Moens et al., 1987; Dobson et al., 1994). Germ cell preparations were incubated with 1:500 anti-pATH52 and either 1:1000 anti-SSEA-1 or 1:1000 anti-SC overnight, washed, and then incubated for 1 hour with a 1:500 dilution of FITC and Texas Red secondary antibodies (Vector Laboratories). Fixed prenatal gonad cell suspensions were permeabilized in 0.2% Triton X-100 for 12 min, immunostained in suspension as described (Cooke et al., 1993), and mounted on electrostatically charged slides. The procedures used for the immunostaining of germ cells in meiotic prophase have been reported previously (Moens et al., 1987). Slides were mounted with Prolong antifade reagent (Molecular Probes, Eugene, OR) containing 0.4 µg/ml DAPI (Molecular Probes). Slides were examined with a

Zeiss Axiophot or a Zeiss LSM410 confocal microscope as described (Laird et al., 1995).

RNA Extraction and Northern Blotting

Total RNA was extracted from tissues using the one-step RNA isolation method with TRIZOL reagent (Gibco BRL), electrophoresed on 1.5% agarose-formaldehyde gels, and transferred to Zetabind nylon membranes (CUNO, Meriden, CT). Blots were hybridized under the conditions of Church and Gilbert (1984) with a Dnmt1 cDNA probe (pR5) (Bestor et al., 1988) or a cDNA probe to exon 1p (Mertineit et al., 1998) labelled to specific activities of 5×10^8 to 8×10^8 cpm/μg of DNA by the random priming method (Feinberg and Vogelstein, 1984). An end-labelled oligonucleotide probe for 18S rRNA was used to assess RNA loading (Benoit and Trasler, 1994). Blots were analyzed using a phosphorimager (Fuji Medical Systems USA, Inc., Stamford, CT).

Results

Localization of Dnmt1 during Fetal Germ Cell Development

DNA methylation patterns are initiated during prenatal gonad development. To clarify the role of Dnmt1 in this process, we examined the expression and localization of Dnmt1 in primordial germ cells of the sexually indifferent gonad at embryonic day 11.5 and following sexual differentiation, from embryonic day 12.5-18.5, in the immature testis and ovary. The cell surface marker SSEA-1 was used to identify primordial germ cells (Solter and Knowles, 1987). Paraffin sections of the genital ridge, of both XX and XY embryos, double-labelled with anti-pATH52 and anti-SSEA-1 antibodies showed strong expression of Dnmt1 in many cells including SSEA-1-positive primordial germ cells (Fig. 1A). In dissociated genital ridge preparations, a more detailed examination of Dnmt1 immunostaining was conducted using laser-scanning confocal microscopy and z-sectioning. SSEA-1 clearly marked the cell surface of isolated primordial germ cells (Fig. 1C,1D,1F,1G), whereas Dnmt1 was strongly expressed throughout these cells (Fig. 1B,1D,1E, 1G). Z-sectioning through the entire depth of many different primordial germ cells confirmed the presence of Dnmt1 in the nucleus with some staining in the cytoplasm. During division of germ cells (Fig. 1E) and somatic cells (Fig. 1H), Dnmt1 surrounded the condensed chromosomes at metaphase, anaphase and telophase. When rabbit preimmune serum was used as a negative control for Dnmt1, SSEA-1-labelled primordial germ cells showed no staining, nor did any of the somatic cells (data not shown).

Following sexual differentiation, Dnmt1 exhibited marked changes in expression in the developing fetal testis and ovary. Two ages, embryonic days 13.5 and 18.5, representing times before and after the DNA of prenatal gonocytes becomes methylated, respectively, are shown in Fig. 2. In contrast to the preimmune control which showed no specific immunoreactivity in the embryonic day 13.5 testis (Fig. 2A), histological sections of the same age incubated with the anti-Dnmt1 antibody showed strong expression of Dnmt1 in proliferating germ and somatic cells (Fig. 2B). Mitotic gonocytes within the

testicular cords revealed both intense Dnmt1 nuclear staining as well as staining around the chromosomes which appeared during M-phase (Fig. 2B). Throughout mid to late embryogenesis, Dnmt1 levels in gonocytes of embryonic days 14.5-15.5 gradually decreased, and by 18.5 days, the enzyme was no longer detected in the gonocytes but was abundant in the supporting cells along the base of the tubules (Fig. 2C). Similar results were found when sections were examined using immunofluorescence rather than immunoperoxidase (data not shown). The down-regulation of Dnmt1 in testicular gonocytes between embryonic days 15.5 and 18.5 coincides with the mitotic-arrest of germ cells at this time in development.

In the female mouse, germ cells actively proliferate by mitosis until embryonic days 13.5-14.5 and then enter and complete much of meiotic prophase between day 14.5 and birth. At embryonic day 13.5, fetal ovaries incubated with rabbit preimmune serum remained negative (Fig. 2D), whereas sections incubated with the anti-Dnmt1 antibody showed strong expression of the enzyme in germ cells (Fig. 2E). Dnmt1 staining of germ cells gradually decreased between days 14.5 and 17.5 (data not shown), and by day 18.5 when the majority of oocytes were at the pachytene- or diplotene-stages of meiotic prophase, oocytes were devoid of Dnmt1 while various supporting cells were positively stained (Fig. 2F). Delineation of the timing during meiosis of the down-regulation of Dnmt1 in the female germline was only possible using markers for the specific stages of meiotic prophase (see below).

Localization of Dnmt1 in Meiotic Prophase Oocytes

To examine the expression of Dnmt1 in meiotic prophase oocytes at leptotene, zygotene and pachytene, we double-labelled dissociated fetal ovary preparations with anti-Dnmt1 and anti-synaptonemal complex antibodies; the latter served as a marker to identify oocytes in the various phases of meiotic prophase I according to the state of synapsis between homologous chromosomes (Moens et al., 1987; Dobson et al., 1994; Fig. 3).

During the leptotene phase, the chromosome cores appeared as fine threads (Fig. 3A), whereas Dnmt1 staining was intense and largely diffuse throughout the nucleus in this class of oocytes (Fig. 3B). At zygotene, which was characterized by sites of chromosome pairing (Fig. 3C), Dnmt1 was moderately expressed throughout the nucleoplasm (Fig. 3D). Pachytene ooctyes were identified by the complete formation of the synaptonemal complex between pairs of homologous chromosomes (Fig. 3E and 3G). In early pachytene oocytes Dnmt1 staining was weak (Fig. 3F), but in late pachytene oocytes Dnmt1 staining was dramatically reduced (Fig. 3H). Taken together, the data in Figs. 2 and 3 show that Dnmt1 staining was gradually down-regulated during meiotic prophase in the female. In contrast to germ cells, somatic cells in all stages of fetal ovary development were identified by their lack of staining for the synaptonemal complex (Fig. 3I) and many exhibited bright punctate foci with diffuse nucleoplasmic staining for Dnmt1 (Fig. 3J).

Sexually Dimorphic Expression of Dnmt1 during Postnatal Germ Cell Development

After birth, DNA methylation patterns continue to be established and are completed by the time the gametes have matured. Gonocytes arrested in the G1 phase of the cell cycle remained negative for Dnmt1 in comparison to neighbouring Sertoli cells and some interstitial cells which were positively stained in the testis of one day old mice (Fig. 4A). Upon resuming their mitotic activity, gonocytes move to the basal aspect of the seminiferous epithelium and give rise to primitive type A spermatogonia; these germ cells were highly reactive for Dnmt1 at day 4 (Fig. 4B). In the day 7 testis, the enzyme was highly expressed in spermatogonia located along the basement membrane (Fig. 4C).

In the seminiferous epithelium of adult mice, Dnmt1 was expressed in the nuclei of mitotic and early meiotic germ cells in a stage-specific manner (Fig. 5). The mouse has 12 distinct stages of the cycle of the seminiferous

epithelium in which specific germ cell types are coordinately arranged and associated with one another via the supporting Sertoli cells (Oakberg, 1956a). The stages were identified according to the criteria of Oakberg (1956b) and Russell (1990). Consistent with its role in DNA replication, Dnmt1 was expressed at high levels in spermatogonia, including type A, intermediate and type B (Fig. 5A and 5B). For instance, two heavily stained spermatogonia are clearly visible at the base of the seminiferous epithelium of the Stage IV tubule in Fig. 5B; these cells are likely to be type B spermatogonia that undergo mitosis at this stage (Oakberg, 1956b). Dnmt1 was also abundant in the nuclei of preleptotene spermatocytes that are found only at stages VII and VIII (Fig. 5C) and undergo the last cycle of DNA replication prior to meiosis. During meiotic prophase I, moderate staining of Dnmt1 was observed in leptotene spermatocytes found at stage IX and X (Figure 5D) and gradually decreased in zygotene spermatocytes at stage XI and XII (Figure 5E). Weak staining was detected in early pachytene spermatocytes between stages I to IV (Fig. 1A), but by mid to late pachytene (stages VI to XI, panels B-D), Dnmt1 was no longer detected and remained absent in round and elongating spermatids (Fig. 5A-E, cells in the epithelium above the dotted line) in all 12 stages. Unlike mitotic metaphase germ cells (e.g. Fig. 1E-G), Dnmt1 was not detected in meiotic metaphase cells found at stage XII (Fig. 5E). As in the female, Dnmt1 staining was gradually down-regulated during meiotic prophase in the male.

In the postnatal ovary, Dnmt1 was re-expressed in diplotene-arrested oocytes and was dependant on the stage of oocyte growth. In the ovary of 7 day old females, the small non-growing oocytes, like their precursors the prenatal diplotene oocytes, were devoid of Dnmt1 staining (Fig. 5F). Larger growing oocytes had positive nuclei but the bulk of Dnmt1 appeared concentrated in the oocyte cytoplasm, whereas surrounding granulosa and stromal cells contained low levels of Dnmt1 (Fig. 5F). In the adult ovary, oocytes in primary (Fig. 5G) and secondary follicles (data not shown) showed very intense and uniform Dnmt1 staining and were surrounded by highly

reactive granulosa cells. Many granulosa cells in these follicles exhibited punctate nuclear foci characteristic of replicating cells (inset in Fig. 5G). Staining was detected in the corpora lutea; however, this was non-specific as it was also observed in ovarian sections incubated with preimmune serum (data not shown). Thus, while in the male Dnmt1 was not detectable in cells beyond the pachytene phase of meiosis, in the female the enzyme was re-expressed in diplotene oocytes in the postnatal ovary.

Dnmt1 mRNA Levels in Ovaries and Testes

In previous studies, we identified a 6.0 kb Dnmt1 mRNA that was expressed during male meiosis and was associated with the down-regulation of Dnmt1 at pachytene (Trasler et al., 1992; Mertineit et al., 1998). To determine if a similar mRNA was associated with the down-regulation of Dnmt1 at female pachytene, mRNAs from prenatal and postnatal ovaries were characterized on Northern blots. The ubiquitous 5.2 kb mRNA was present in all samples but was highly abundant in the postnatal ovary at 3 and 7 days of age in comparison to the fetal mesonephros and ovaries and the testis at any time of development (Fig. 6A). As described previously, in addition to the 5.2 kb transcript, a second transcript of 6.0 kb was detected in the testis; this more slowly migrating Dnmt1 transcript is due to the alternative splicing of a 5' first exon, exon 1p (Mertineit et al., 1998). To determine if pachytene stage oocytes also express the 6.0 kb Dnmt1 transcript, we stripped and rehybridized the same RNA blot with a cDNA probe to exon 1p. Despite the fact that more than 50% of oocytes in embryonic days 16.5 and 17.5 ovaries are at the pachytene stage of meiosis (Speed et al., 1987; O'Keefe et al., 1997), we failed to detect the 6.0 kb message in these samples; in contrast, the 6.0 kb mRNA was present in testes that contain pachytene germ cells (postnatal day 20 and adult) but not present in testes that do not contain pachytene cells (prenatal and postnatal day 6). These results suggest that the appearance of the 6.0 kb transcript at the pachytene stage of meiosis is unique to male gametogenesis.

Discussion

In this study, we compared the expression and localization of Dnmt1 throughout germ cell development in the male and female mouse in order to identify potential windows in which Dnmt1 might create sex-specific methylation patterns. Based on our results (summarized in Table 1) and data in the literature on the timing of DNA methylation in the germline, we propose that methylation of DNA is first initiated differentially in the prenatal gonad in both sexes, and then continues postnatally up until the pachytene stage of meiosis in the male but, in contrast, occurs only after pachytene, during oocyte growth (diplotene) in the female.

We show immunocytochemically that Dnmt1 is highly abundant in the day 11.5 genital ridge and in primordial germ cells labelled with the anti-SSEA1 marker. Upon sexual differentiation, intense Dnmt1 immunoreactivity was maintained in fetal germ cells between days 12.5 and 14.5 in both sexes. Our results are consistent with the notion that Dnmt1 is the predominant DNA methyltransferase in the genital ridge, as all detectable enzymatic activity in lysates of day 12.5 genital ridges was attributed to Dnmt1 (Yoder et al., 1997b). Yet, primordial germ cells escape the wave of *de novo* methylation that occurs in the postimplantation embryo. Monk et al. (1987) have shown that DNA from male and female germ cells at 12.5 and 14.5 days of gestation is globally undermethylated compared to the DNA of somatic tissues. In addition, almost all sequences that have been examined to date with the exception of *Xist* (Ariel at al., 1995; Razin and Shemer, 1995), encompassing a number of single copy or repetitive sequences including the mouse interspersed family repetitive sequence (*MIF*; Monk et al., 1987), the transgenes *RSVIgmyc* (Chaillet et al., 1991) and *MPA434* (Ueda et al., 1992), tissue-specific and imprinted genes (Kafri et al., 1992; Brandeis et al., 1993, Shemer et al., 1997; Tada et al, 1998), and *IAP* retroviruses (Walsh et al., 1998) are undermethylated in DNA from primordial germ cells. Moreover, the fusion of embryonic germ cells to somatic cells was shown to induce marked demethylation of several imprinted and non-imprinted genes (Tada et al.,

1997). Unexpectedly, the nuclear localization and high levels of Dnmt1 expression in primordial germ cells in the present study do not correlate with the unmethylated status of the DNA of these cells. This indicates that other factors may be involved in protecting the DNA from Dnmt1, that the enzyme is biologically non-functional in these primordial germ cells or that there is an active overriding demethylating activity.

The distribution of Dnmt1 changes dynamically in a cell cycle-dependent manner in mitotic germ cells. We examined gonocytes in the fetal and prepubertal testis to address the nature of Dnmt1 expression during the mitotic cell cycle. In general, we observed three different patterns of Dnmt1 staining in mitotic gonocytes: intense nuclear staining that appeared to be associated with S-phase; staining throughout the cell which appeared during M-phase and was often seen in cells at metaphase; or no detectable staining which was associated with gonocytes arrested in early G1-phase. Our results are consistent with other reports that have examined the intracellular distribution of DNMT1 during the cell cycle of somatic cells (Vogel et al., 1988). In synchronous proliferating human carcinoma cells, DNMT1 was absent in early G1 phase, appeared in late G1, and accumulated and persisted throughout S and G2 phases of the cell cycle (Vogel et al., 1988). It is well established that Dnmt1 is closely tied to cell proliferation and is strongly attracted to the hemimethylated CpG sites that are produced by semiconservative DNA replication (Bestor, 1992). In somatic cells, Dnmt1 showed a diffuse distribution during early S-phase and was localized to distinct nuclear toroidal structures during mid to late S-phase (Leonhardt et al., 1992; Liu et al., 1998). We show here that the localization of Dnmt1 in mitotic germ cells is predominantly nuclear, which is consistent with its role as a replication factor. Our results are consistent with previous findings on enriched populations of isolated type A and type B spermatogonia which also exhibited nuclear foci of Dnmt1 (Jue et al., 1995a).

The nuclear expression and localization of Dnmt1 during prenatal and postnatal germ cell development appeared to coincide with all times at which increases in DNA methylation have been reported to occur. Germ cell differentiation differs markedly in the male and female fetal gonads. In the male, gonocytes divide actively until embryonic day 14.5 when they enter a phase of mitotic arrest. In contrast, in the female the oogonia divide until about day 14.5 and then enter meiotic prophase. Dnmt1 was expressed in germ cells in male fetuses between embryonic days 12.5 and 15.5. This expression coincides with the timing of initiation of methylation between 13.5 and 18.5 days of gestation for a number of sequences including *MIF* (Monk et al., 1987); the imprinted transgene *RSVIgmyc* (Chaillet et al., 1991), *IAP* (Walsh et al., 1998) and some single copy, tissue-specific and imprinted genes (Kafri et al., 1992; Brandeis et al., 1993). Interestingly, transplantation of a gonocyte nucleus at mitotic-arrest (embryonic days 14.5-16.5) into an enucleated oocyte supported development of a small embryo with an abnormal placenta and lacking genomic imprints to day 9.5 of gestation (Kato et al., 1999). These recent findings suggest that epigenetic modifications to the paternal genome must occur after embryonic day 16.5, possibly during early spermatogenesis, which is in agreement with the re-expression of Dnmt1 in actively proliferating postnatal spermatogonia and early meiotic germ cells. In the female fetus, we show that Dnmt1 is expressed in oogonia and early meiotic cells until pachytene. The role of the enzyme in these cells is unclear at present since, although a few sites in a small number of genes have been reported to be methylated in the female fetal gonad (Kafri et al., 1992; Brandeis et al., 1993), as described below, many of the sequences that have been tested appear to become methylated in the postnatal period during oocyte growth.

After birth, male germ cells resume mitosis, followed by meiotic and postmeiotic development; the mitotic, meiotic and postmeiotic phases then continue in a cyclical fashion, the seminiferous cycle, throughout life. We show here that the expression of Dnmt1 is stage-specific in the seminiferous

epithelium and that its highest level of expression is found in spermatogonia and preleptotene germ cells in tubule stages VI to VIII. Our stage-specific localization of Dnmt1 protein in mitotic spermatogonia and early meiotic spermatocytes is consistent with the distribution of Dnmt1 mRNA performed by *in situ* hybridization (Numata et al., 1994) and is predicted to play a role in maintaining the methylation patterns initially established in the fetal germ cells. There is also limited evidence that methylation increases during meiotic prophase in the male for mouse protamines 1 and 2 (Trasler et al., 1990) and the imprinted gene *H19* (Davies et al., 1999).

In female germ cells, mitotic divisions and early meiotic development until diplotene are completed before birth. After birth, diplotene oocytes are recruited into a cycle of growth and maturation, followed by ovulation of metaphase II oocytes and the completion of meiosis at the time of fertilization. Unlike the male, Dnmt1 is re-expressed following the pachytene stage of meiosis in the female, during oocyte growth. The nuclear localization of Dnmt1 during oocyte growth coincides with the timing of methylation of a number of different types of sequences, notably that of imprinted genes. Examination of the imprinted gene *Igf2r* (Brandeis et al., 1993; Stöger et al., 1993, Kono et al., 1996), the *RSVIgmyc* and *MPA434* transgenes (Chaillet et al, 1991; Ueda et al., 1992) and *IAP* and *MUP* retroviral sequences (Howlett and Reik, 1991; Walsh et al., 1998), indicates that *de novo* methylation occurs in a window marking the transformation of non-growing oocytes to fully grown oocytes. For imprinted genes, support for the functional importance of methylation during oocyte growth comes from nuclear transplantation studies where nuclei from non-growing oocytes and fully grown oocytes have been shown to differ in their developmental potential, suggesting that fully grown oocytes have acquired maternal germline specific imprints not present in non-growing oocytes (Kono et al., 1996). Following the initial period of oocyte growth, the accumulation of large amounts of Dnmt1 in the cytoplasm of oocytes may play an additional role and function as a maternal store for early embryo development. The shorter oocyte-specific Dnmt1 isoform is active

toward both hemimethylated and unmethylated substrates (Carlson et al., 1992) and is functional *in vivo* (Gaudet et al., 1998).

We observed a gradual down-regulated expression of Dnmt1 in early meiotic prophase germ cells that was similar in both sexes. This down-regulation had been noted in the male in previous studies (Jue et al., 1995a; Mertineit et al., 1998). Here, by using the synaptonemal complex proteins as a marker to identify oocytes in various phases of meiotic prophase, we were able to demonstrate a similar down-regulation of Dnmt1 expression from leptotene to pachytene in the female. The process of crossing-over and strand exchange is a unique feature of meiosis that is associated with the pachytene stage. Several types of DNA structures including four-way junctions, mismatches and hemimethylated sites appear during this process and are highly preferred substrates for Dnmt1 (Bestor and Tycko, 1996). Therefore, eliminating the expression of Dnmt1 at pachytene in both sexes may provide a mechanism to protect meiotic recombination intermediates from ectopic *de novo* methylation. Interestingly, the disappearance of Dnmt1 protein in the male occurs via a unique process that involves the production of a 6.0 kb, non-translated Dnmt1 pachytene-specific mRNA (Jue et al., 1995a; Mertineit et al., 1998). Here, we found no evidence of a 6.0 kb Dnmt1 transcript in the prenatal ovary despite the presence of significant amounts of pachytene stage oocytes. We proposed that the 6.0 kb Dnmt1 mRNA interferes with translation and thereby prevents the production of enzyme during later stages of spermatogenesis. The absence of this mRNA in prenatal pachytene oocytes may allow Dnmt1 to become re-expressed in postnatal growing diplotene oocytes.

The findings in the present studies are of particular importance for sex-specific timing of establishment of genomic imprinting. The methylation of cytosine residues present at CpG sites has emerged as the imprinting mark candidate due to the differential methylation that occurs on the two parental alleles of many imprinted genes (Bartolomei and Tilghman, 1997; Bestor, 1998). As Dnmt1 is considered to be the predominant mammalian DNA

methylating enzyme with significant *de novo* and maintenance activities (Yoder et al., 1997b), we suggest that it may be involved in setting up and propagating sex-specific methylation patterns at imprinted loci at specific times in germ cell development. The expression profile of Dnmt1 described here (summarized in Table 1) suggests that paternally imprinted genes may undergo *de novo* methylation prior to the pachytene stage of spermatogenesis, whereas maternally imprinted genes may undergo *de novo* methylation in the growing oocyte. These predictions can now be tested by interfering with expression of Dnmt1 in the germline and carefully examining changes in methylation of a few imprinted genes in DNA from purified populations of germ cells at different stages of development.

Acknowledgements

We thank Guylaine Benoit for her excellent technical assistance and Dr. Y. Clermont for his help in staging the mouse seminiferous epithelium. This work was supported by grants from The Medical Research Council of Canada (MRC) to J.M.T. and to T.T., an NSERC grant to P.B.M. and NIH grants T.H.B. C.M. is a recipient of the Eileen Peters McGill Major Fellowship. J.M.T. is an MRC Scientist and a Scholar of the Fonds de la Recherche en Santé du Québec.

Figure 1. Immunocytological localization of Dnmt1 in the genital ridge. Embryonic day 11.5 genital ridge preparations were double-labelled with anti-pATH52 to detect Dnmt1 (green) and anti-SSEA-1 to identify primordial germ cells (red) and counterstained with DAPI to visualize the nuclei (blue). (A) Longitudinal section of an embryonic testis. Examples of primordial germ cells positive for Dnmt1 immunoreactivity are indicated by the open arrows. (B-D) High magnification of an isolated primordial germ cell imaged in the same z-plane via confocal microscopy. (B) Dnmt1 immunoreactivity. (C) SSEA-1 immunoreactivity. (D) Superimposed confocal images of Dnmt1 and SSEA-1 in the same germ cell. (E-G) High magnification of a primordial germ cell during M-phase of the cell cycle imaged by confocal microscopy. (E) Dnmt1 immunoreactivity surrounds condensed chromosomes. (F) SSEA-1 immunoreactivity. (G) Superimposed confocal images of Dnmt1 and SSEA-1. (H-J) High magnification of a somatic cell at metaphase photographed by conventional epifluorescence microscopy. (H) Dnmt1 immunoreactivity. (I) DAPI staining of condensed metaphase chromosomes. (J) Double exposure of Dnmt1 and DAPI. Magnification: (A) X450; (B-J) X4000.

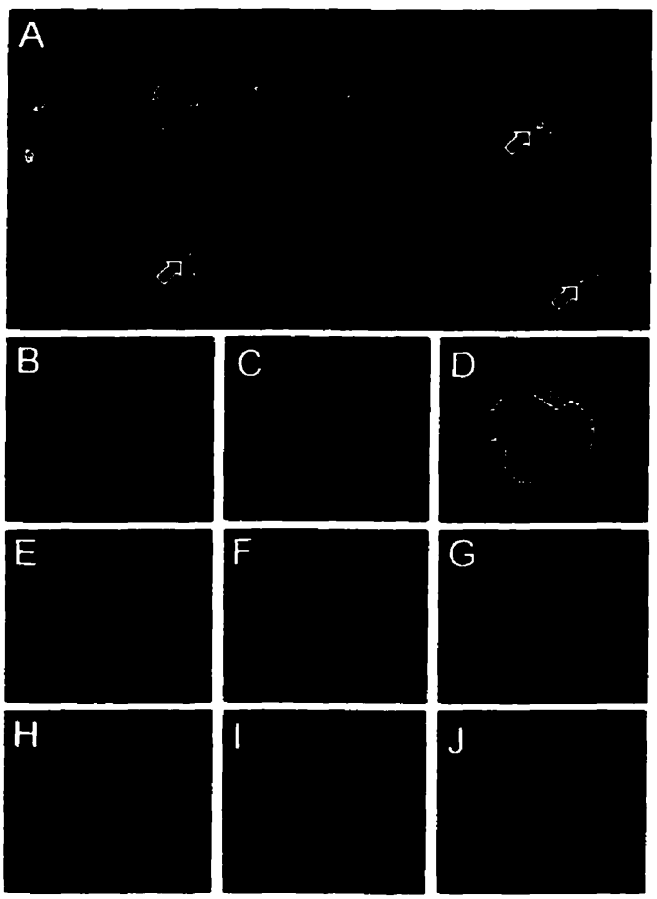

A
B
C
D
E
F
G
H
I
J

Figure 2. Immunohistological localization of Dnmt1 in the fetal mouse testis and ovary. (A and B), Embryonic day 13.5 testis; (C), embryonic day 18.5 testis; (D and E), embryonic day 13.5 ovary; (F), embryonic day 18.5 ovary. Paraffin-embedded fetal gonads were stained with immunoperoxidase using anti-pATH52 to detect Dnmt1 (B, C, E, F) or rabbit preimmune serum (A and D). The sections were counterstained with methylene blue. Immunoperoxidase staining is brown. Examples of dividing cells are indicated by thick, curved arrows. Representative cell types are indicated as follows: D, diplotene-stage oocyte; GC, germ cell; P, pachytene-stage oocyte; S, Sertoli cell. Magnification: (A–F), X630.

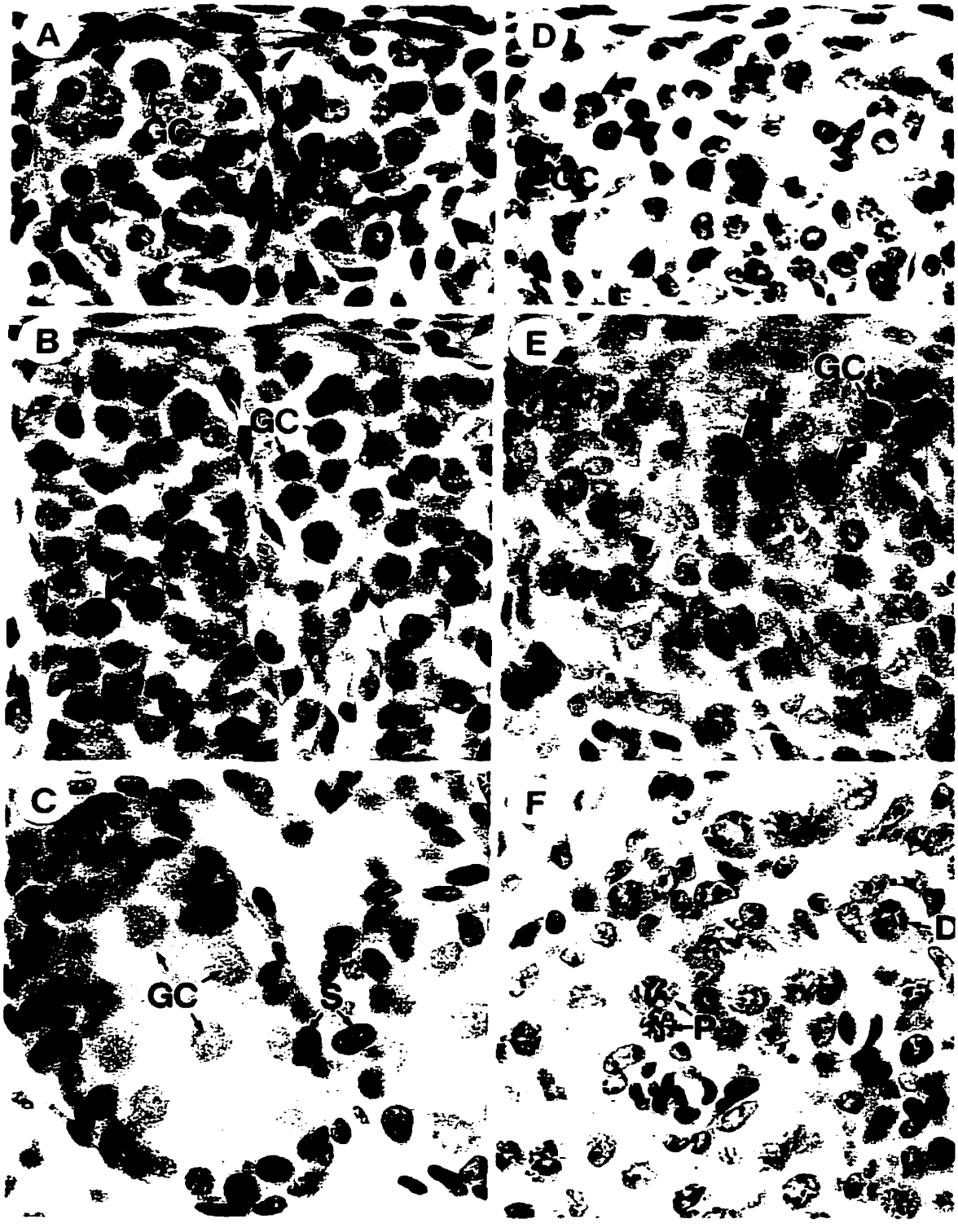

A
GC
D
GC
B
GC
E
GC
C
GC
S
F
D
P

Figure 3. Dnmt1 expression in meiotic prophase oocytes. Fetal ovary preparations were double-labelled with anti-pATH52 to detect Dnmt1 (red) and anti-SC (green) to identify the various stages of meiotic prophase based on the state of synapsis between homologous chromosomes. (A and B), leptotene; (C and D), zygotene; (E and F), early pachytene; (G and H); late pachytene; (I and J), somatic cell. Note that Dnmt1 staining is gradually down-regulated during meiotic prophase in the female mouse. Magnification: (A-J), X4500.

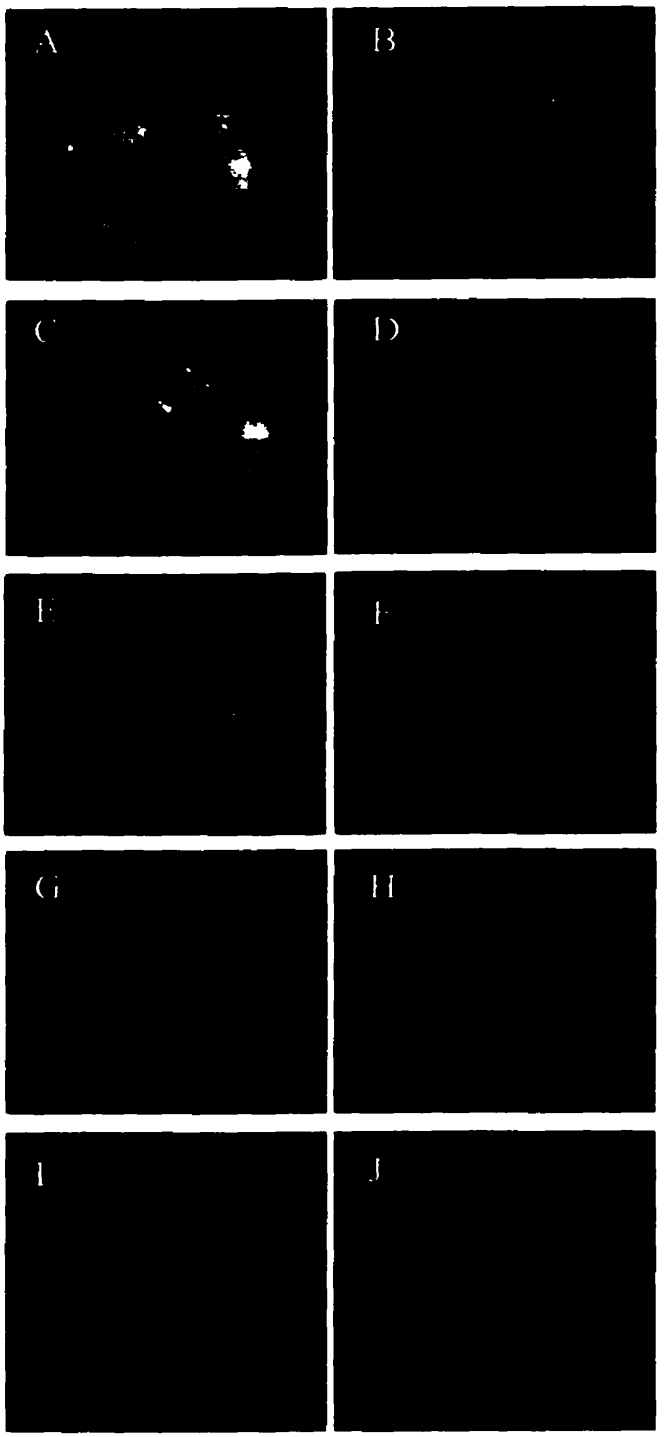

A
B
C
D
E
F
G
H
I
J

Figure 4. Immunohistological localization of Dnmt1 in the postnatal mouse testis. (A), Day 1 testis; (B), day 4 testis; (C), day 7 testis. Paraffin-embedded testis sections were stained with immunoperoxidase using anti-pATH52 to detect Dnmt1. The sections were counterstained with methylene blue. Immunoperoxidase staining is brown. Examples of dividing germ cells intensely stained for Dnmt1 are indicated by large arrows and positively staining interstitial cells by open arrows. Representative cell types are indicated as follows: GC, germ cell; S, Sertoli cell. Note that germ cells in the center of the tubules are unreactive at day 1 but become highly reactive by day 4 as they descend towards the basement membrane and resume mitotis. Magnification: (A–C), X630.

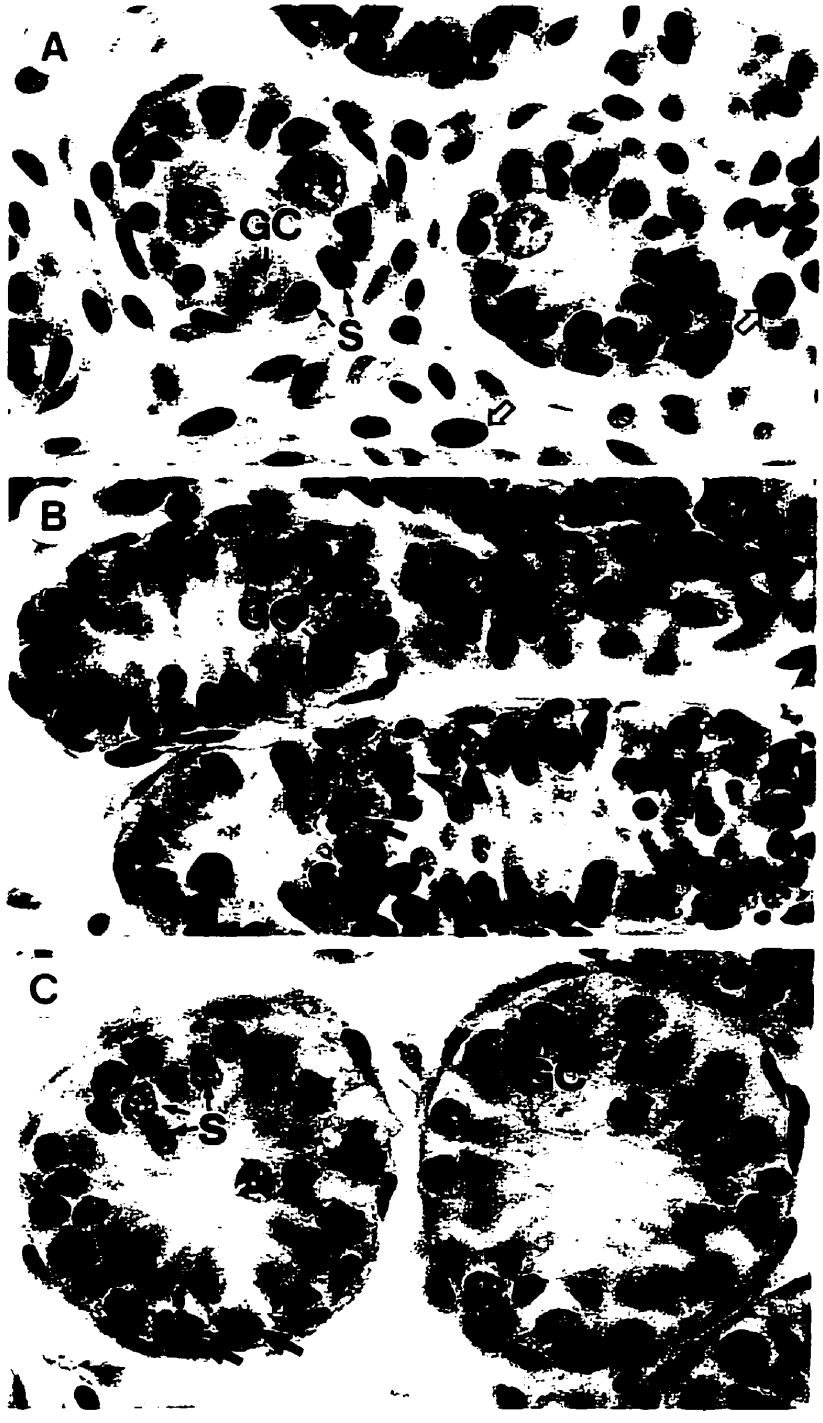

A
GC
S
B
C
S

Figure 5. Sexually dimorphic expression of Dnmt1 during postnatal gametogenesis. Paraffin-embedded mouse testis and ovary sections were stained with immunoperoxidase using anti-pATH52 to detect Dnmt1. The sections were counterstained with methylene blue. Immunoperoxidase staining is brown. Staging of the tubules was according to Oakberg (1956b) and Russell (1990). (A) Stage IV-V shows a reactive spermatogonium (S) on the basement membrane. Early pachytene (EP) spermatocytes exhibit low levels of Dnmt1 staining (B) Stage VI shows intense Dnmt1 immunoreactivity in spermatogonia (S) and low levels of staining in mid pachytene (MP) spermatocytes. (C) Stage VIII shows intense Dnmt1 immunoreactivity in preleptotene (PL) germ cells. (D) Stage X-XI shows moderate Dnmt1 staining in leptotene (L) spermatocytes. (E) Stage XII shows low levels of Dnmt1 staining in zygotene (Z) spermatocytes. Unreactive late pachytene spermatocytes are marked by asterisks. Germ cells in meiotic metaphase (stage XII) in which Dnmt1 is undetectable are indicated by open arrows. In (A) through (E) Dnmt1 was at undetectable levels in all haploid round and elongating spermatids, located in the area of the seminiferous epithelium above the dotted lines. (F) Day 7 ovary section shows Dnmt1 immunostaining in growing oocytes. Unreactive non-growing oocytes are indicated by the large arrows. (G) An adult ovary section shows a reactive growing oocyte surrounded by reactive granulosa cells. (G, inset) Punctate, nuclear foci in the granulosa cells are marked by small arrows. Note that Dnmt1 staining is stage-specific and is gradually down-regulated during meiotic prophase in the mouse testis and not expressed at or after diplotene. In contrast, diplotene growing oocytes show a re-expression of Dnmt1. Magnification: (A-E), X630; (F and G) X400; (G, inset), X630.

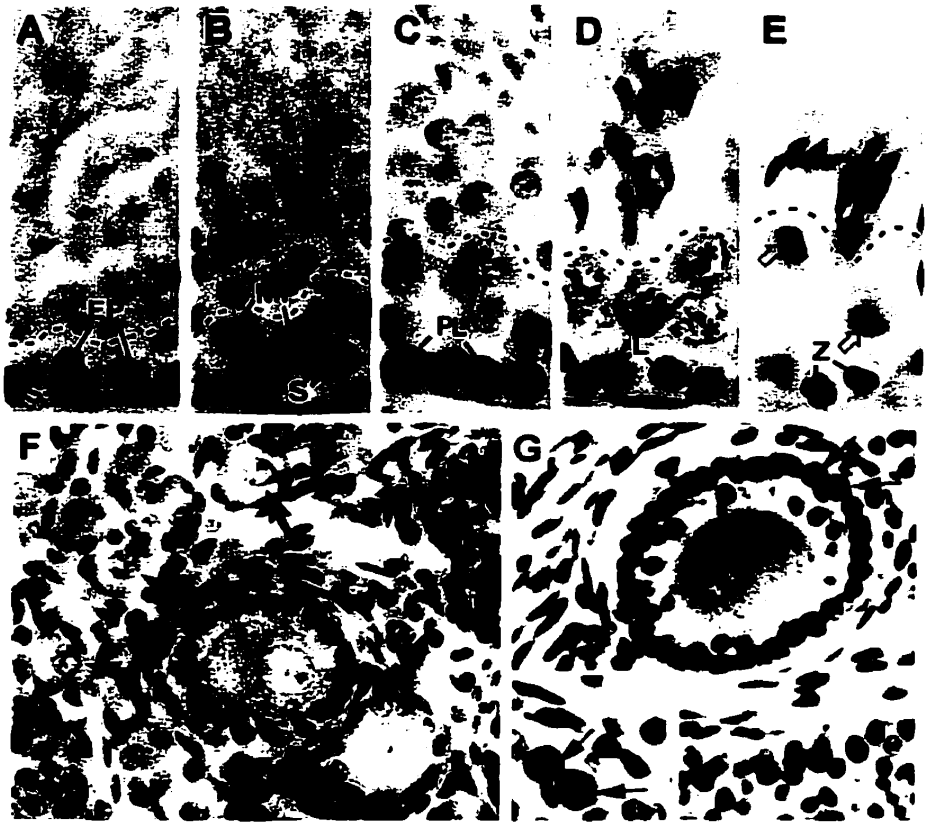

A
B
C
D
E
F
G
Z

Figure 6. Northern blot analysis of Dnmt1 mRNA in the developing mouse ovary and testis. Total RNA was extracted from embryonic day 16.5 mesonephros (lane 1), embryonic day 16.5 ovary (lane 2), embryonic day 17.5 ovary (lane 3), day 3 postnatal ovary (lane 4), day 7 ovary (lane 5), day 70 adult ovary (lane 6), embryonic day 16.5 testis (lane 7), day 6 postnatal testis (lane 8), day 20 testis (lane 9), and day 70 adult testis (lane 10). Ten-microgram aliquots of total RNA were loaded into each lane of a 1.5% agarose-formaldehyde gel and transferred to a nylon membrane. (A) Membrane probed with a ^{32}P-labelled Dnmt1 cDNA probe (pR5) (Bestor et al., 1988). (B) Membrane shown in (A) was stripped and rehybridized with a ^{32}P-labelled cDNA probe to the pachytene 1p 5' exon (Mertineit et al., 1998). (C) Equal loadings were confirmed by hybridizing the same blot with a ^{32}P-labelled oligonucleotide complementary to 18S rRNA.

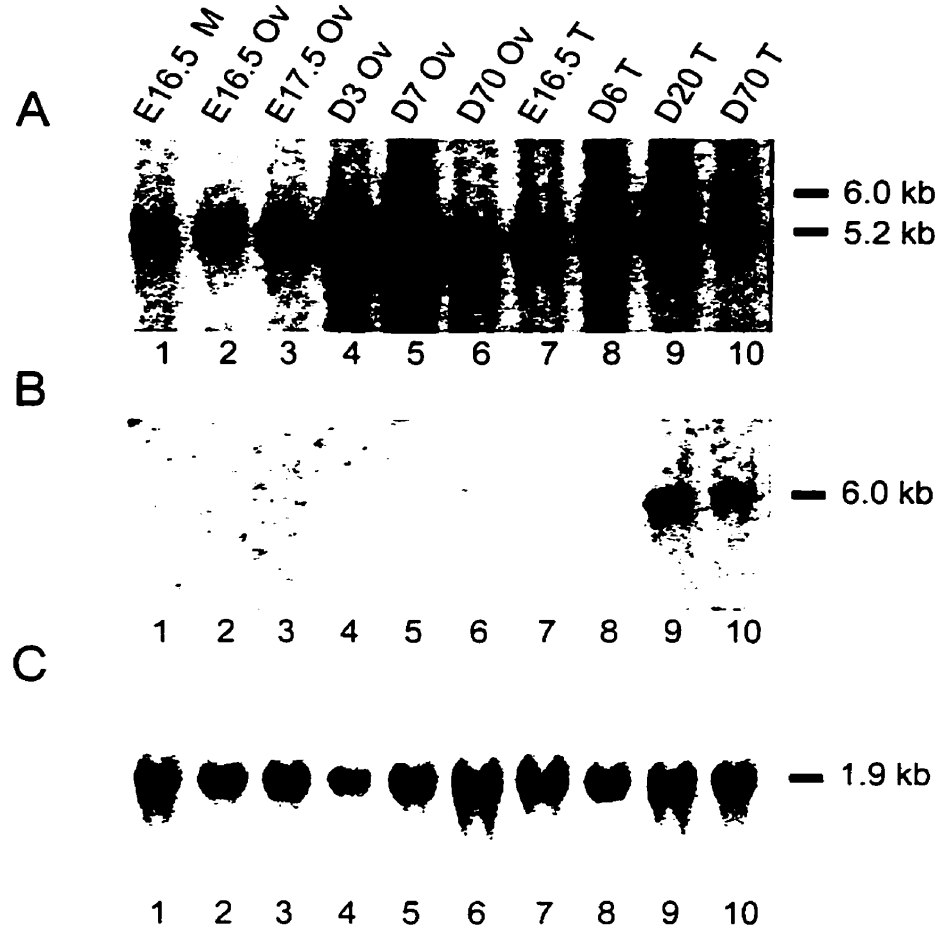
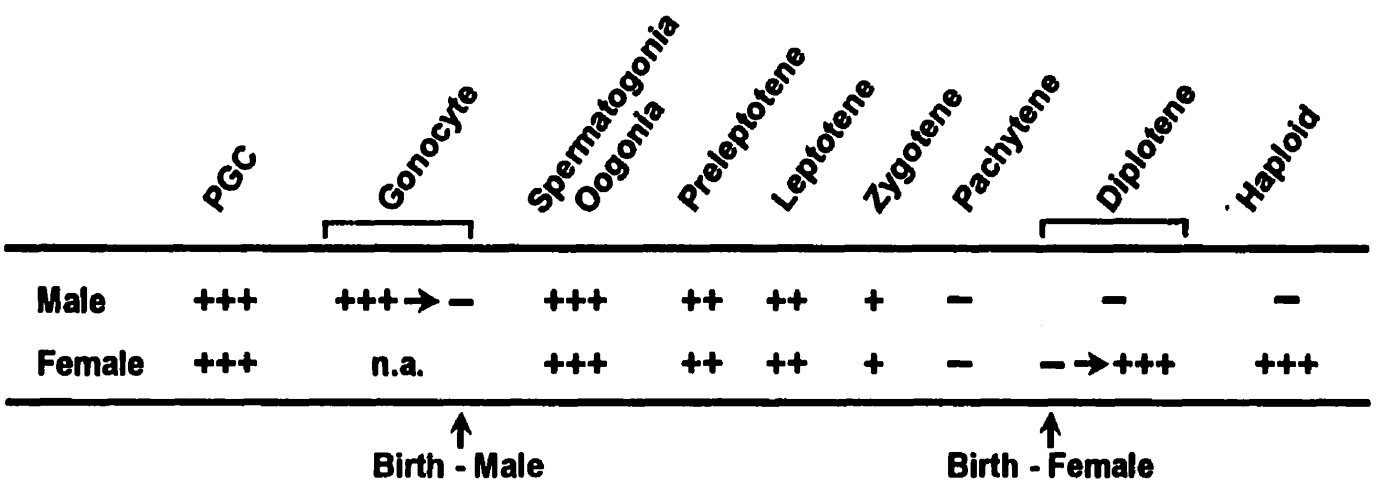

A
E16.5 M
E16.5 Ov
E17.5 Ov
D3 Ov
D7 Ov
D70 Ov
E16.5 T
D6 T
D20 T
D70 T
6.0 kb
5.2 kb
1 2 3 4 5 6 7 8 9 10
B
6.0 kb
1 2 3 4 5 6 7 8 9 10
C
1.9 kb
1 2 3 4 5 6 7 8 9 10

Figure 7. Dnmt1 protein in male and female prenatal and postnatal gametogenesis. Relative levels of Dnmt1 expression in the different germ cell types (+ to +++) or absence (-) are indicated. Much of meiotic prophase occurs before birth in the female, while it occurs after birth in the male (depicted by vertical arrows). PGC, primordial germ cell; n.a., not applicable.


PGC
Gonocyte
Spermatogonia
Oogonia
Preleptotene
Leptotene
Zygotene
Pachytene
Diplotene
Haploid
Male
+++
+++→–
+++
++
++
+
–
–
–
Female
+++
n.a.
+++
++
++
+
–
–→+++
+++
Birth - Male
Birth - Female

Connecting Text

In Chapter Two, the primary objective of the study was to identify the developmental windows in which Dnmt1 might create sex-specific methylation patterns. To address this objective, we compared the expression and localization of Dnmt1 at the level of mRNA and protein in the fetal, prepubertal and mature testis and ovary of the mouse. Our data show differential expression of Dnmt1 mRNA and protein localization in the male and female germlines and suggest distinct, sex-specific developmental times when cytosine methylation of the genome and imprinted genes can be established and maintained by Dnmt1. More importantly, however, the differential expression of Dnmt1 in the male and female germlines indicates that the regulation of this enzyme may differ between sexes and from somatic cells. To determine the molecular mechanisms that control Dnmt1 in the germlines, we searched for additional exons in the 5' end of the *Dnmt1* gene. The following chapter describes the characterization of novel 5' exons that are sex-specific and control the production and localization of enzyme during specific developmental stages of gametogenesis.

CHAPTER THREE

Sex-Specific Exons Control DNA Methyltransferase in Mammalian Germ Cells

Carmen Mertineit, Jeffrey A. Yoder, Teruko Taketo, Dale W. Laird, Jacquetta M. Trasler, Timothy H. Bestor

Status: published in *Development* 125, 889-897 (1998)

Abstract

The spermatozoon and oocyte genomes bear sex-specific methylation patterns that are established during gametogenesis and are required for the allele-specific expression of imprinted genes in somatic tissues. The mRNA for Dnmt1, the predominant maintenance and *de novo* DNA (cytosine-5)-methyltransferase in mammals, is present at high levels in postmitotic murine germ cells but undergoes alternative splicing of sex-specific 5' exons which controls the production and localization of enzyme during specific stages of gametogenesis. An oocyte-specific 5' exon is associated with the production of very large amounts of active Dnmt1 protein that is truncated at the N-terminus and sequestered in the cytoplasm during the later stages of oocyte growth, while a spermatocyte-specific 5' exon interferes with translation and prevents production of Dnmt1 during the prolonged crossing-over stage of male meiosis. During the course of postnatal oogenesis Dnmt1 is present at high levels in nuclei only in growing dictyate oocytes, a stage during which gynogenetic developmental potential is lost and biparental developmental potential is gained.

Introduction

The genomes of the male and female gametes contain very different methylation patterns that are established during gametogenesis (Sanford et al., 1987; Monk et al., 1987; Driscoll and Migeon, 1990; reviewed by Yoder et al., 1997a). Sex-specific differences in methylation patterns are most prominent at imprinted loci (Tremblay et al., 1997) and parasitic sequence elements (the location of most genomic m^5C; Yoder et al., 1997a). Gametic methylation patterns undergo some alterations after fertilization (Sanford et al., 1987; Monk et al., 1987) but imprinted loci retain characteristic methylation differences throughout development (Tremblay et al., 1997; Olek and Walter, 1997).

The predominant form of DNA (cytosine-5)-methyltransferase in mammals is Dnmt1, which is composed of a C-terminal domain closely related to bacterial C5-specific restriction methyltransferases and a large N-terminal domain that has multiple regulatory functions (Bestor et al., 1988; Bestor and Verdine, 1994; Bestor, 1996). Homozygous mutations of the *Dnmt1* gene in mice lead to severe abnormalities in genomic imprinting and X inactivation (Li et al., 1993a; Li et al., 1993b; Panning and Jaenisch, 1996), and mutant embryos die prior to midgestation (Li et al., 1992). There is also genetic evidence for a second specialized DNA methyltransferase that participates in the silencing of newly-integrated retroviral DNA (Lei et al., 1996; Lengauer et al., 1997), and a candidate DNA methyltransferase that may serve this function has been identified (Yoder and Bestor, 1998). However, Dnmt1 is the predominant *de novo* and maintenance DNA methyltransferase in all tissues and cell types examined to date (Yoder et al, 1997b). Early predictions (Holliday and Pugh, 1975; Jähner and Jaenisch, 1984) of distinct classes of *de novo* and maintenance DNA methyltransferases have not been confirmed, and the extant data indicate that Dnmt1 is the major *de novo* and maintenance DNA methyltransferase in mammals (Yoder et al., 1997b).

Dnmt1 mRNA has been reported to be expressed at high levels in mitotic and postmitotic male germ cells (Singer-Sam et al., 1990; Trasler et al., 1992; Numata et al., 1994) and Dnmt1 protein has been found in large amounts in mature oocytes and early embryos (Monk et al., 1991; Howlett and Reik, 1991; Carlson et al., 1992). We report here that Dnmt1 is subject to unusual transcriptional and post-transcriptional regulatory mechanisms in germ cells that depend on alternative splicing of sex-specific 5' exons. Dnmt1 protein in oocytes within antral follicles is almost exclusively cytoplasmic and is truncated at the N-terminus due to alternative splicing of a 5' exon, while a different 5' exon in pachytene spermatocytes leads to the production of a non-translated mRNA. The control of Dnmt1 expression in germ cells shows a number of unusual features: alternative splicing of sex-specific exons, the use of fundamentally different mechanisms (cytoplasmic sequestration in oocytes versus translational down-regulation in pachytene spermatocytes) to reduce the amount of Dnmt1 in the vicinity of chromosomes in male and female germ cells, and large changes in amount, size, and localization of the enzyme in postmitotic germs cells and in early embryos.

Experimental Procedures

Isolation of Gonads and Germ Cells

CD-1 mice were obtained from Charles River Canada, Inc. (St. Constant, Quebec, Canada). Genital ridges were isolated from day 11 mouse embryos. Ovaries and testes were distinguished in embryos of day 12.5 postcoitum and later by the presence of seminiferous cords in the testes. Purified populations of male germ cells were obtained from enzymatically-dissociated mouse testes by sedimentation at unit gravity on 2-4% BSA gradients (Bellvé, 1993). Isolated type A spermatogonia (average purity=86%, n=2 cell separations) and type B spermatogonia (85% pure, n=2) were obtained from the testes of 8-day-old mice. Preleptotene spermatocytes (87% pure, n=3), leptotene/zygotene spermatocytes (90% pure, n=3), and prepubertal pachytene spermatocytes (82% pure, n=3) were obtained from the testes of 17-day-old mice. Pachytene spermatocytes (83% pure, n=3) were obtained from 70-day-old mice. Female mice were superovulated and oocytes recovered from oviducts by established methods (Hogan et al., 1986).

Immunocytochemistry

Mice were perfused through the heart with physiological saline followed by Ste. Marie's fixative (95% ethanol/glacial acetic acid, 99:1). Ovaries and testes were dissected, post-fixed for 1 h on ice, dehydrated through graded ethanols, cleared in xylene and embedded in paraffin. Gonads were sectioned at 5 μm thickness, placed on slides coated with 0.5% gelatin, heated to 60°C for 1 h, and stored at 4°C prior to use. All solutions for immunofluorescence of Dnmt1 were prepared in phosphate buffered saline (PBS), pH 7.2, and procedures were carried out at room temperature, unless otherwise specified. The polyclonal rabbit anti-Dnmt1 antibody (anti-pATH52) has been described previously (Bestor, 1992; Li et al., 1992). Sections were deparaffinized, rehydrated through graded ethanols, blocked for 30 min in blocking buffer (0.2% cold-water fish skin gelatin (Sigma Chemical Co., St. Louis, MO), 5% goat serum (Gibco) and 0.2% Tween 20) and incubated with

a 1:200 dilution of anti-pATH52 or preimmune serum in blocking buffer overnight at 4°C in a humidified chamber, then incubated for 1 h with a 1:200 dilution of fluorescein-conjugated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA). Slides were counterstained with 0.5 µg/ml propidium iodide (Molecular Probes, Eugene, OR) for 5 min, washed three times in PBS and mounted with an antifade solution containing 0.025% 1,4-diazobicyclo-(2,2,2)-octane (DABCO, Sigma) in 90% glycerol (Laird et al., 1995). The specimens were examined with a Zeiss Axiophot or Zeiss LSM410 confocal microscope.

RNA Blot Analysis

Total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987), electrophoresed on 1.5% agarose formaldehyde gels and transferred to Zetabind nylon membranes (CUNO, Meriden, CT). Blots were hybridized under the conditions of Church and Gilbert (1984) with Dnmt1 cDNA probes (Bestor et al., 1988) or cDNA probes for exon 1p, labelled to specific activities of $5x10^8$ to $8x10^8$ cpm/µg of DNA by random priming (Feinberg and Vogelstein, 1984). An end-labelled oligonucleotide probe for 18S rRNA was used to assess RNA loading. Blots were analyzed by a phosphorimager (Fuji Medical Systems USA, Inc., Stamford, CT).

Immunoblot Analysis

Lysates of mouse ovaries, superovulated oocytes and purified male germ cells were prepared by homogenization in 0.15 M NaCl, 0.05 M Tris Cl (pH 7.5), 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 100 µg/ml PMSF. Proteins (20 µg except where noted) were denatured by heating at 100° C in 2% SDS, separated by electrophoresis on SDS-5% polyacrylamide gels, and transferred to nitrocellulose membranes (Protran; Schleicher & Schuell). Blots were stained with Ponceau S to confirm equal loadings and probed with anti-pATH52 as described (Li et al., 1992).

RNase H Mapping, 5' RACE Cloning, and RT-PCR Analysis

The oligonucleotides used for RNase H mapping were oligo 1 (5' TTGGCGGGACAACCGTTGG 3'), oligo 2 (5' AATTTCTCCCTCACACAC 3') and oligo 3 (5' ATCCCTCACTCCTCGAA 3'). Oligonucleotides (250 pmol) were annealed to 20 µg of total RNA. RNase H treatment, electrophoresis, blotting and probing were as described (Yoder et al., 1996).

RACE cloning was based on techniques described by Troutt (1992), Frohman (1993) and Edwards et al. (1991). The Dnmt1-specific oligos 1 or 5 (5' GCAGGAATTCATGCAGTAAG 3') were annealed to 1.0 µg of total RNA in 10 mM Tris, pH 8.3. cDNA synthesis was performed with 10 units of AMV-Reverse Transcriptase (Promega) in 50 mM Tris pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT and 0.3 mM dNTP. The reaction was incubated at 42° C for 20 min and then at 50° C for 60 min. RNA was degraded with 0.5 N NaOH for 10 min at 37°C. The cDNA was precipitated with ethanol and resuspended in 10 µl RNA ligase buffer (10 mM Tris, pH 8.0, 25% polyethylene glycol-8000, 1 mM hexamine cobalt chloride, 10mM $MgCl_2$, 1 mg/ml BSA and 10 mM ATP) supplemented with an anchor oligo that was blocked at the 3' end (Durand et al., 1990) and phosphorylated at the 5' end. The sequence of the anchor oligo was 5' CACGAATTCACTATCGATTCTGGAACCTTCAGAGG 3'. Ligation involved 10 units T4 RNA ligase (New England Biolabs) and incubation for 16 hr at 22°C. A portion (0.5 µl) of the mixture was added to a 25 µl primary PCR reaction (10 mM Tris pH 9.0, 50 mM KCl, 0.1% Triton X-100, 0.2 mM dNTP, 2.5 units Taq polymerase (Promega) and 1 mM Anchor Primer-1 (5' GAAGGTTCCAGAATCGATAG 3') and Dnmt1 oligo 5. Incubation conditions were 93°C for 15 sec, 55°C for 15 sec, 72°C for 15 sec for 30 cycles. A final extension at 72°C was performed for 10 min. A portion (0.5 µl) of the primary reaction was added to a 25 µl secondary nested PCR. The reaction conditions were as for the primary PCR, except anchor primer-2 (5'

GAATCGATAGTGAATTCGTG 3') and nested Dnmt1 oligos 2 or 4 (5' ACACAAGCCGCAGCAGTGT 3') were used.

The complete sequence of exon 1o was obtained by analysis of multiple RACE clones from ovaries of 7 day and adult animals. 427 nucleotides of exon 1p were identified by RACE. The remaining sequence was deduced by RT-PCR analysis of testis RNA with primers complementary to genomic sequence.

The Superscript One-Step RT-PCR System (Life Technologies) was used to analyze splicing variants of Dnmt1. Reactions were performed as directed by the manufacturer. One µg of total RNA was seeded into a 50 µl RT-PCR reaction with oligo 5 as the RT primer. Oligo 6 (5'-GGGTCTCGTTCAGAGCTG-3') was used to amplify the somatic transcript and oligo 7 (5'-GGTTGATTGAGGGTCATT-3') was used to amplify the oocyte transcript. Reverse transcription was allowed to proceed for 10 min at 50° then shifted to 55° for 30 min. PCR involved 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec. A final extension at 72° C was performed for 7 min. An aliquot of the reaction (10 µl) was analyzed by electrophoresis through a 3% wide-range agarose gel (Sigma).

Results

Localization of Dnmt1 in Male and Female Germ Cells

Ovaries and testes were sectioned and stained for Dnmt1 with the antibody pATH52, which is highly specific for Dnmt1 and recognizes multiple epitopes within the region between amino acids 255-753 (Bestor 1992; Li et al., 1992; Leonhardt et al., 1992). Spermatogenesis occurs in synchronous waves that pass down the seminiferous tubule; the distinctive morphology of the haploid spermatids present at a given stage allows accurate identification of cell types *in situ*. Spermatogonia are mitotically-active stem cells that lie at the basal side of the seminiferous epithelium, and migrate towards the lumen of the tubule as they differentiate into spermatocytes, undergo meiosis, and begin spermiogenesis. Meiotic prophase lasts about 12 days in the mouse and is conventionally divided into the leptotene, zygotene, pachytene and diplotene phases according to the state of synapsis of meiotic chromosomes; crossing-over occurs during the pachytene stage, which occupies more than 6 days of meiotic prophase. Figure 1 shows that spermatogonia, preleptotene, and leptotene spermatocytes all express high levels of nuclear Dnmt1, while pachytene spermatocytes are not detectably stained (Figure 1) despite the fact that they have been reported to contain large amounts of Dnmt1 mRNA (Singer-Sam et al., 1990; Trasler et al., 1992; Numata et al., 1994). This suggests that Dnmt1 protein is sharply down-regulated at the pachytene stage of spermatogenesis via post-transcriptional mechanisms.

Male germ cells undergo mitosis and meiosis throughout adult life, but germ cells in ovaries complete the last round of mitosis and most of meiotic prophase prior to birth. The growth of arrested primary oocytes commences after birth, and gonadotropin stimulation causes fully-grown oocytes to resume meiosis. As shown in Figure 2, dramatic changes in the amount and localization of Dnmt1 in oocytes were observed in postnatal ovaries. Non-growing oocytes do not stain detectably (arrows in Figure 2A), while growing oocytes show intense staining of nuclei and substantial cytoplasmic staining (Figure 2B and 2C). As the oocytes continue to grow, Dnmt1 is no longer

detectable in nuclei but accumulates to very high levels in the cytoplasm (Figure 2C and 2D). Late-stage dictyate oocytes in Graafian follicles show very intense and uniform cytoplasmic staining (Figure 2D). At the time of ovulation all Dnmt1 staining is cytoplasmic and is associated with the oocyte cortex (Figure 2E); this indicates that Dnmt1 is actively retained near the oocyte cortex, as exclusion from the nucleus or the vicinity of the spindle would produce a uniform cytoplasmic distribution. Mature oocytes and early preimplantation embryos contain very high levels of Dnmt1 protein: at least 10,000-fold more per cell than does a cycling somatic cell (Carlson et al. 1992).

In summary, immunolocalization data reveal that pachytene spermatocytes contain no detectable Dnmt1 protein, but other studies have shown that they contain large amounts of Dnmt1 mRNA. The growing oocyte contains very large amounts of Dnmt1 protein, which during the growth phase largely disappears from the nucleus and comes to be localized first throughout the cytoplasm and, at ovulation, in a shell just within the oocyte cortex.

Sizes and Amounts of Dnmt1 Protein and mRNA in Male and Female Germ Cells

Male germ cells at specific stages of spermatogenesis were isolated from enzymatically-dissociated testes by sedimentation at unit gravity in gradients of bovine serum albumin as described (Bellvé, 1993). As shown in Figure 3A, Dnmt1 protein is abundant in Type A and Type B spermatogonia, easily detectable in preleptotene and leptotene/zygotene spermatocytes, but undetectable in pachytene spermatocytes. These findings are consistent with the immunocytochemical data of Figure 1.

As shown in Figure 3B, the total amount of Dnmt1 mRNA does not vary more than two-fold during prophase of meiosis I, while the amount of protein decreases from high in spermatogonia to undetectable in pachytene spermatocytes (Figure 3A). A larger form of Dnmt1 mRNA is present in

pachytene spermatocytes (Figure 3B) and has been observed only in these cells (Trasler et al., 1992). Data that will be described later indicate that the larger 6.0 kb mRNA is the result of sex-specific alternative splicing of a 5' exon.

Analysis of RNA purified from pre- and post-natal ovaries showed only a band of ~5.2 kb (data not shown), which is characteristic of the somatic form of Dnmt1 (Bestor et al., 1988; Yoder et al., 1996). However, immunoblot analysis revealed that ovaries contain a species of Dnmt1 of M_r 190,000 and a second species of M_r 175,000; this latter species was the only detectable form in ovulated oocytes but was undetectable in ovaries of newborn mice, which contain predominantly non-growing oocytes (Figure 4A). Only the M_r 175,000 form is detectable in isolated oocytes, which indicates that the M_r 190,000 form is derived from supporting cells in the ovary. These data identify a smaller, oocyte-specific form of the Dnmt1 protein and agree with the immunolocalization data of Figure 2, where growing oocytes were seen to stain intensely with Dnmt1 antibodies while non-growing oocytes were unstained.

Complex Alternative Splicing of Sex-specific Exons in Dnmt1 mRNA

The discovery of additional 5' sequences in the somatic Dnmt1 mRNA that had been refractory to cloning (Tucker et al., 1996; Yoder et al., 1996; Glickman 1997), together with evidence of a larger Dnmt1 mRNA in testis and a smaller protein in oocytes, prompted a search for alternatively spliced exons in male and female germ cells. RNase H mapping (Hake and Hecht, 1993) identified alternative exons at the 5' end of both male (Figures 3D) and female (Figure 4C) germ cells. The mRNA in pachytene spermatocytes was found to have about 800 additional nucleotides at the 5' end (Figure 3D), and the oocyte-specific form was found to be about 100 nucleotides shorter than the somatic mRNA (Figure 4C). RACE (rapid amplification of cDNA ends; Frohman, 1993) was used to clone the germ cell-specific sequences.

Comparison of pachytene spermatocyte-specific exon sequences with the sequence of a clone of genomic DNA spanning the first exon of the somatic Dnmt1 mRNA (the gift of K. L. Tucker and R. Jaenisch) revealed that the pachytene spermatocyte-specific sequences lie in a single exon of ~780 nucleotides whose 5' boundary is ~80 nucleotides downstream of the 3' end of exon 1 of the somatic mRNA (Figures 3C and 5A). The TSSG and TSSW algorithms of the Gene finder suite (http://defrag.bcm.tmc.edu:9503/gene-finder/gf.html) identified a high-probability promoter immediately 5' of the spermatocyte-specific exon. The pachytene-specific exon was named exon 1p, and the first exon of the somatic form was named exon 1s (Figure 5A). These were concluded to represent the first exons as there were no splice acceptor consensus sequences within the range delimited by RNase H mapping of 5' ends (Figure 3 and Yoder et al., 1997b).

Exon 1p contains 7 ATG codons (Figure 5B), 5 of which are in a favorable context for translation initiation (Kozak, 1996). The first ATG that can yield active Dnmt1 is the tenth in the sequence, and according to Kozak (1996) initiation from such codons does not occur or is very inefficient. The pachytene spermatocyte form of Dnmt1 mRNA is only weakly associated with polysomes (Trasler et al., 1992), and all data indicate that the form of Dnmt1 mRNA present in pachytene spermatocytes is not actively translated. The inactive mRNA appears to be as stable as the productive mRNA, as their steady-state levels are similar (Figure 3B). Untranslated mRNA has been observed to be stable in male germ cells in other cases (reviewed by Kleene, 1996).

RACE cloning and cDNA library screens were used to identify the oocyte-specific 5' sequences. These were found to reside in a single exon (1o) of 157 nucleotides that lies ~6 kb 5' of the exon 1s (Figure 5A). No splice acceptor sites were within the 300 nucleotides of genomic sequence upstream of the 5' end of the cDNA, and exon 1o is concluded to represent an oocyte-specific 5' exon. The first in-frame ATG codon is in exon 4 (Figure 5A); it had been found earlier that *in vitro* transcription-translation from that

ATG codon yields a protein that precisely comigrates with Dnmt1 protein from oocytes upon SDS-polyacrylamide gel electrophoresis (Carlson et al., 1992). There are 3 additional ATG codons upstream of the one that can yield a protein of the size observed in oocytes (Figure 5A and 5B), but as will be described later the likelihood that this mRNA is translated is much greater than for the male pachytene mRNA. As shown in Figure 4B, exon 1o is found only when the truncated form of Dnmt1 protein is also present.

Developmental Regulation of Dnmt1 Localization in Germ Cells and Early Embryos

Figure 6 summarizes expression and localization data for the Dnmt1 protein and mRNA during gametogenesis and early development. Dnmt1 is present at high levels in spermatogonia and spermatocytes until the pachytene stage, where it falls to undetectable levels (Jue et al., 1995a). The transient drop at the pachytene stage coincides with the disappearance of the 5.2 kb Dnmt1 mRNA and the accumulation of a larger 6.0 kb Dnmt1 mRNA, which is not translated.

Oocytes accumulate very large amounts of Dnmt1 protein during the growth phase; this enzyme is nuclear in growing oocytes (the only point during postnatal oogenesis where nuclear Dnmt1 has been observed) but becomes cytoplasmic towards the end of the growth phase and is confined to a shell near the oocyte cortex in ovulated oocytes (Carlson et al., 1992, and this report). The cytoplasmic Dnmt1 is encoded by an alternatively spliced mRNA that has a different 5' exon and which is lacking N-terminal sequences present in the somatic enzyme. Dnmt1 remains cytoplasmic in the preimplantation embryo (except for a brief period at the eight-cell stage; Carlson et al., 1992) until implantation. After implantation Dnmt1 is exclusively nuclear in all tissue types and cell lines examined to date (Trasler et al., 1996).

Discussion

The Dnmt1 mRNA in germ cells undergoes alternative splicing of sex-specific exons which controls production and localization of Dnmt1 protein, and fundamentally different mechanisms (cytoplasmic sequestration versus translational down-regulation) act to reduce the amount of nuclear Dnmt1 protein at specific stages of gametogenesis. There are also large changes in the total amount of Dnmt1 and repeated reversals of the ratio of protein in nucleus versus cytoplasm in oocytes and early embryos.

Alternative Splicing of Sex-specific Exons

Three alternative 5' exons have been identified in the Dnmt1 gene: one is specific to the growing oocyte, one to the pachytene spermatocyte, and one to all somatic cells and other germ cell types of both sexes. Extensive characterization of the 5' region of the Dnmt1 mRNA of somatic cells has failed to show evidence of alternative splicing or multiple transcriptional start sites in this region (Yoder et al., 1996; Tucker et al., 1996a). While alternative splicing and alternative transcriptional start sites in mammalian germ cells have been observed in many cases (reviewed by Kleene, 1996), this is the first case in which each sex has been found to use a different 5' exon. It is also unusual that one 5' splice site should accept 3 different 3' splice sites; this had previously been documented in only a few cases (Rotwein and Hall, 1991; Bermingham and Scott, 1993).

Cytoplasmic Sequestration vs. Translational Repression

Dnmt1 behaves in somatic cells as a replication factor: it is nucleoplasmic through most of the cell cycle and associates with replication foci during S-phase (Leonhardt et al., 1992), as do other replication factors (Spector, 1993). Cytoplasmic Dnmt1 has not been observed in any somatic cell during interphase (Trasler et al., 1996). The situation in oocytes and early embryos is quite different. As summarized in Figure 6, Dnmt1 shows a nuclear distribution in early growing oocytes, a lack of nuclear staining and a

uniform cytoplasmic distribution in later growing oocytes, and a subcortical distribution in ovulated oocytes. Maternal stores of Dnmt1 protein in mature oocytes are very large (Carlson et al., 1992). Staining remains cytoplasmic in embryos until the eight-cell stage, where nuclei stain intensely (Carlson et al., 1992). Nuclei in the blastocyst are again free of Dnmt1, but after implantation all staining is found in nuclei (Trasler et al., 1996).

Dnmt1 contains a functional nuclear localization sequence (NLS) near the N-terminus. This NLS is not deleted from the oocyte-specific Dnmt1, and the cytoplasmic localization of the truncated Dnmt1 found in oocytes cannot be explained by a lack of the appropriate nuclear import signal. Furthermore, the truncated form of Dnmt1 has full enzymatic activity and is capable of entering nuclei as shown by the fact that it briefly does so in eight-cell embryos (Carlson et al., 1992). Dnmt1 appears to be actively retained near the cortex of the mature oocyte, as the enzyme is confined to a shell just under the surface of the oocyte. It is of interest that the extreme N-terminus of the somatic form of Dnmt1 was identified as a factor that binds to annexin V with high affinity (Ohsawa et al., 1996). Annexin V or a related membrane-associated protein may be actively involved in the cytoplasmic sequestration of truncated Dnmt1 in the oocyte. The complete cytoplasmic sequestration of a replication factor appears to be unprecedented. It is also of interest that the amount of Dnmt1 per cell in oocytes and early embryos is several thousand-fold higher than in any somatic cell type, and the possibility of a cytoplasmic function unrelated to DNA methylation cannot be excluded.

Translational Repression of Dnmt1 in Pachytene Spermatocytes

Translation of many mRNAs depends on a ribosome scanning mechanism: the ATG closest to the 5' cap site is the sole initiation codon, if it conforms to the RXXATGG consensus (Kozak, 1996). However, there are numerous exceptions to this rule; short upstream open reading frames inhibit translation in some cases but not others. The Drosophila Antp mRNA is translated despite 15 short upstream open reading frames (Ye et al., 1997),

and the BiP and FGF2 mRNAs are also translated from mRNAs that contain upstream ORFs (reviewed by Geballe and Morris, 1994; Morris, 1997). In some cases, notably that of S-adenosyl L-methionine decarboxylase, tissue-specific translation depends on the presence of an upstream ORF (reviewed by Morris, 1997).

Dnmt1 mRNA is expressed at high levels in pachytene spermatocytes (Singer-Sam et al., 1990; Trasler et al., 1992; Numata et al., 1994), but there is no detectable Dnmt1 protein at this stage. It was found that pachytene spermatocyte Dnmt1 mRNA has an alternative 5' exon that contains 7 ATG codons, 5 of which conform to the consensus for favorable translation (Kozak, 1996). Additional small ORFs in exons 2 and 3 cause the ATG of the first ORF that can yield active enzyme to be the 10th in the sequence. The multiple upstream ORFs appear to strongly inhibit translation, as the mRNA is only weakly associated with ribosomes in pachytene spermatocytes (Trasler et al., 1992) and there is no detectable Dnmt1 protein at stages where the pachytene-specific exon is present (figures 1B and 3A). It is concluded that the pachytene-specific exon interferes with translation of the downstream ORF that can produce enzymatically active Dnmt1. The oocyte-specific exon contains a single ORF of 14 codons, and there is an ORF of 3 codons in exon 2 and another of 8 codons in exon three before the long ORF. Because the oocyte contains very large amounts of truncated Dnmt1 protein, the upstream ORFs in the oocyte-specific mRNA are clearly compatible with translation (reviewed by Gabelle and Morris, 1994; Morris, 1997).

Sex-specific Exons and Cytosine Methylation in Gametogenesis

It has been suggested that genomic methylation patterns might be established by a family of sequence-specific *de novo* methyltransferases during gametogenesis and early development, and that a maintenance methyltransferase dependent on hemimethylated sites might perpetuate the resulting methylation patterns in somatic cells (Holliday and Pugh, 1975; Jähner and Jaenisch, 1984). However, no specialized *de novo*

methyltransferase has been identified, there is no discernible sequence context that distinguishes methylated and unmethylated sites in genomic DNA (Tremblay et al., 1997; Olek and Walter, 1997), and while Dnmt1 does prefer hemimethylated substrates by a factor of 10- to 30-fold it is the predominant *de novo* methyltransferase in extracts of all cell types and tissues examined (Yoder et al., 1997b). The *de novo* methylation activity of Dnmt1 is cued by factors other than direct digital sequence recognition (Bestor and Tycko, 1996; Yoder et al., 1997b). There are high levels of Dnmt1 protein in nuclei of postmitotic germ cells at specific stages, and we suggest that this may identify the stages at which *de novo* methylation occurs in germ cells.

While little is known of the timing of *de novo* methylation during gametogenesis, nuclear transplantation studies have shown that the nuclei of non-growing oocytes can support the development of gynogenetic mouse embryos to day 13.5, while gynogenetic embryos containing nuclei of later oocytes do not develop past day 9 (Kono et al., 1996). However, nuclei of more mature oocytes were much more effective in supporting the development of biparental embryos than were nuclei of non-growing oocytes (Kono et al., 1996). This indicates that gynogenetic developmental potential is lost and biparental developmental potential gained during oocyte growth. As diagrammed in Figure 6, oocyte growth is the only stage of oogenesis in which nuclei contain large amounts of Dnmt1 protein, and this finding indicates that the regulatory regions of imprinted genes may undergo *de novo* methylation at this time. The large amounts of truncated, cytoplasmic Dnmt1 protein appear to function as a maternal store of DNA methyltransferase during early development, as homozygous mutant embryos develop to day 8.5, while embryonic stem cells of the same genotype (which do not have large stores of Dnmt1) promptly undergo apoptosis when induced to differentiate (Lei et al., 1996).

Meiotic chromosomes present several features that make them vulnerable to *de novo* methylation during crossing over. Structural features associated with recombination are preferred targets of Dnmt1 (Bestor and

Tycko, 1996), and DNA in perichiasmate regions is exposed to diffusible proteins (Narayan and Raman, 1995). Germ cells may protect their meiotic DNA from dysregulated *de novo* methylation by excluding Dnmt1 from the vicinity of meiotic chromosomes at all stages except for the growing oocyte, where it is suggested that imprinted genes undergo *de novo* methylation. The concentration of Dnmt1 into large nuclear foci during an interval in the development of leptotene/zygotene spermatocytes suggests that *de novo* methylation of paternally imprinted loci might occur at this time (Trasler et al., 1990; Jue et al., 1995a).

Acknowledgements

We thank Guylaine Benoit for technical assistance, Jurrien Dean for oocyte cDNA libraries, J. Richard Chaillet, Rudolf Jaenisch and Kerry Lee Tucker for genomic clones of fragments of the *Dnmt1* locus, and Hugh Clarke, J. Richard Chaillet, Louis Hermo, Suleiman Igdoura and Colum Walsh for discussions. Supported by NIH grants GM00616 and CA60610 (to T.H.B.), and by MRC (Canada) grants MT-11362 (to J.M.T.), MT-10684 (to T.T.) and MT-12241 (to D.W.L.). J.M.T. is a Scholar of the Fonds de la Recherche en Santé du Québec.

Figure 1. Dnmt1 in spermatogenesis. Pseudocolor confocal laser scanning micrographs of a sector of a seminiferous tubule viewed down the long axis of the tubule; A and C are stained for DNA (blue) and B and D for Dnmt1 (red). In B, preleptotene spermatocytes stain intensely for Dnmt1, while pachytene spermatocytes show no detectable staining. C and D show that type A spermatogonia and leptotene spermatocytes contain large amounts of Dnmt1 while later pachytene spermatocytes are free of Dnmt1 staining.

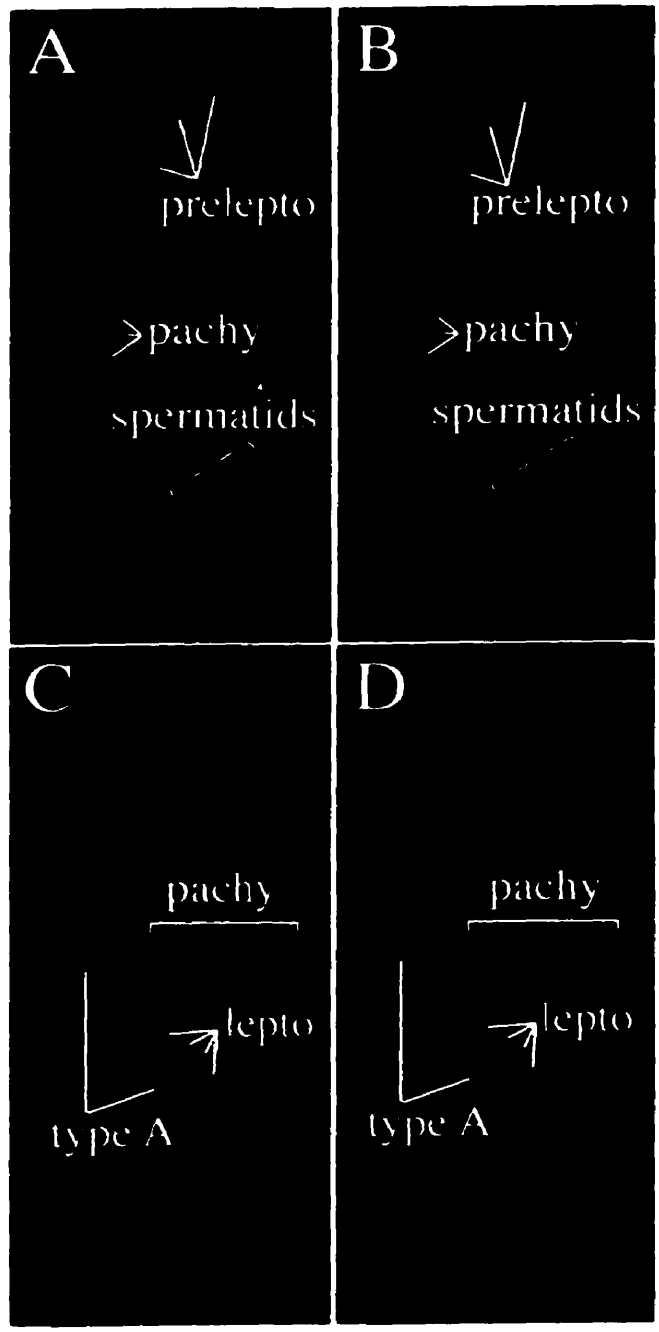

A
prelepto
pachy
spermatids
B
prelepto
pachy
spermatids
C
pachy
lepto
type A
D
pachy
lepto
type A

Figure 2. Dnmt1 in oogenesis. As in Figure 1, blue is DNA and red is Dnmt1. In A, non-growing oocytes in the ovary of a newborn mouse show no sign of Dnmt1 staining, although neighboring supporting cells show significant staining. The growing oocyte in B stains intensely in both nucleus and cytoplasm; in C the growing oocytes at the top of the field show predominantly cytoplasmic staining, while the oocyte at the bottom of the field shows staining in both nucleus and cytoplasm. Oocytes from B and C are from a 7 day old animal. In D, a fully-grown oocyte in a Graafian follicle from a 70 day adult has intense, uniform cytoplasmic staining. The optical sections in E show that Dnmt1 is associated with the oocyte cortex in an ovulated oocyte arrested in metaphase of meiosis II. DNA in nuclei of oocytes stains faintly with intercalating dyes, in part because the nuclei are large and DNA concentration low. Late oocytes contain such large amounts of Dnmt1 protein that staining of somatic cells is not apparent at this exposure.

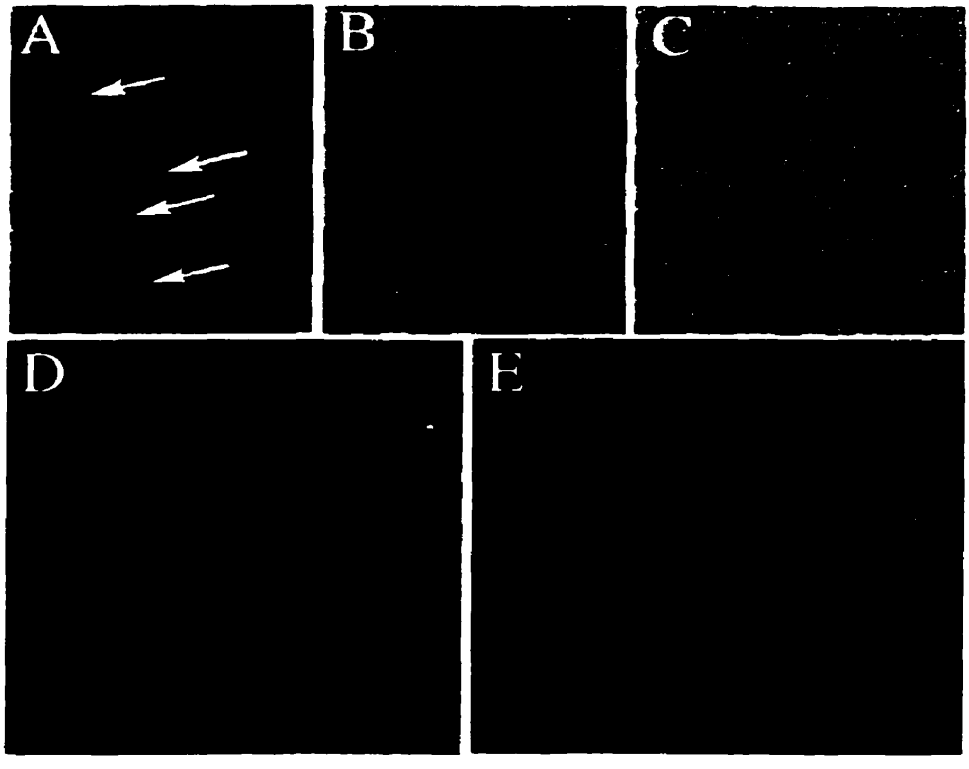
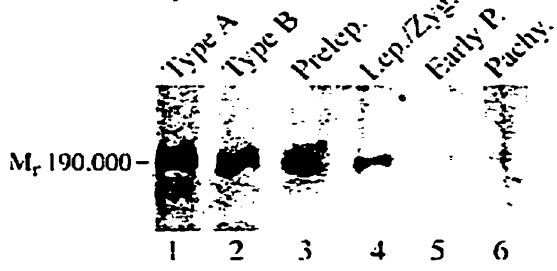

A
B
C
D
E

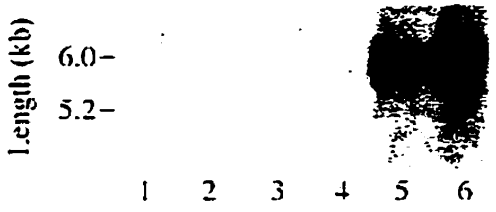
Figure 3. Sizes and amounts of Dnmt1 protein and mRNA in spermatogenic cells. Male germ cells were fractionated by unit-gravity sedimentation in gradients of bovine serum albumin prior to analysis. In A-C lanes 1 and 2 contained type A and type B spermatogonia, respectively; lane 3 contained preleptotene spermatocytes, lane 4 contained leptotene and zygotene spermatocytes, lane 5 contained early pachytene spermatocytes from testes of prepubertal 17 day old mice, and lane 6 contained pachytene spermatocytes from a 70 day old animal. In A, an immunoblot stained with the anti-Dnmt1 antiserum pATH52 demonstrates the presence of Dnmt1 in postmitotic leptotene/zygotene spermatocytes and the absence of Dnmt1 in pachytene spermatocytes. Protein quantities were determined by BCA assay (Pierce) and equal amounts of total protein were loaded onto each lane. The RNA blot in B shows that the total amount of Dnmt1 mRNA is nearly constant within the stages examined, but the mRNA in pachytene spermatocytes can be seen to contain additional sequences, which were localized to the 5' end and cloned; C shows the blot in B stripped and probed with the alternative 5' sequences. Equal loadings were confirmed by rehybridization of the blot with a ^{32}P-labled oligonucleotide complementary to 18S ribosomal RNA (Trasler et al., 1992). D, RNase H mapping of the 5' sequences. Oligos 2 and 3 (figure 5 and Experimental Procedures) targeted RNase cleavage to a site near the splice junction and indicated that the pachytene-specific sequences total ~770 nucleotides. RNA was from testes of 20 day old animals.

A. Dnmt1 protein



B. Dnmt1 mRNA

C. Pachytene-specific exon 1p



D. RNAaseH mapping

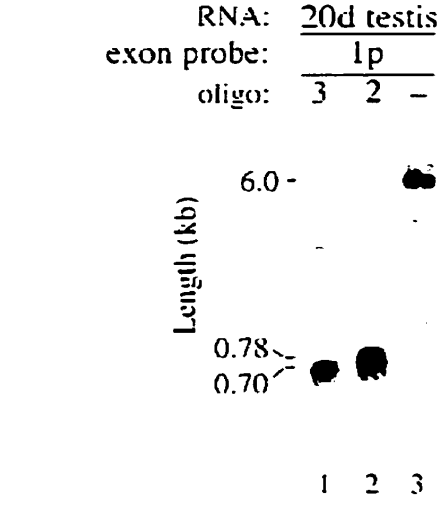
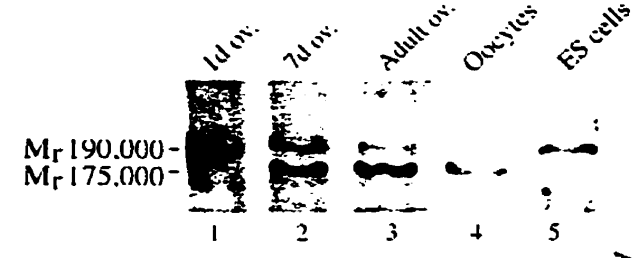
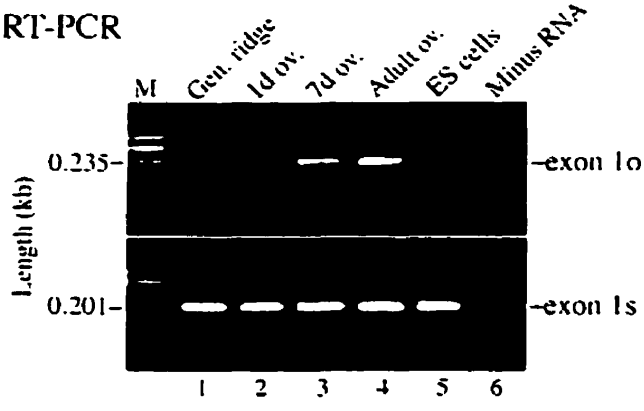


Figure 4. Size, distribution, and amount of Dnmt1 mRNA and protein in ovaries and oocytes. A, immunoblot analysis of Dnmt1 in ovaries and oocytes. A smaller (M_r 175,000) form of Dnmt1 appears in ovaries at the time when oocyte growth has begun (lane 2) and is the sole form in ovulated oocytes (lane 4). The oocyte contains very large amounts of Dnmt1; lanes 4 and 5 have similar band intensities, but lane 4 contained 30 oocytes, and lane 5 contained ~10^5 embryonic stem cells. In B, RT-PCR analysis of an oocyte-specific 5' exon reveals that oocyte-specific sequences are present only at stages where the truncated protein is also found. The oligos were 5 and 7 (Figure 5 and Experimental Procedures). The lower panel in B shows that oligos specific to the somatic form of the Dnmt1 mRNA (oligos 5 and 6 in Figure 5 and Experimental Procedures) gave a PCR product with RNA from all tested sources. In C, RNase mapping of the oocyte-specific exon 1o and somatic exon 1s. Oligo 1 was used to target RNase H cleavage to exon 4; the probes used are indicated. Exon 1o can be seen to be present at high levels only in ovaries from 7 day mice, when most oocytes are in the growth phase.

A. Dnmt1 immunoblot



B. RT-PCR



C. RNAaseH mapping

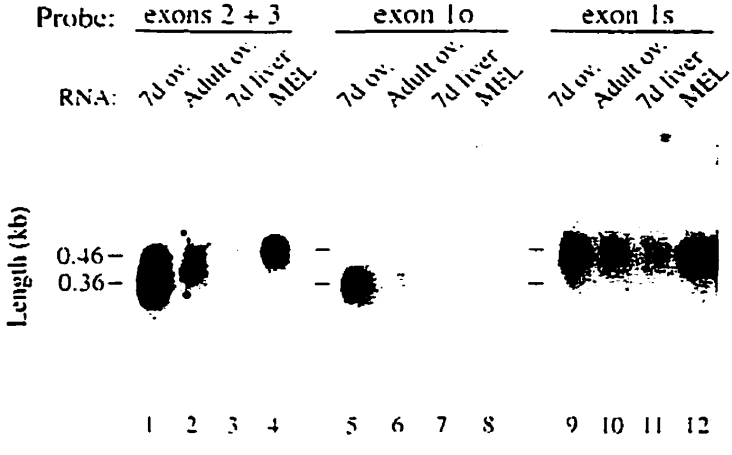
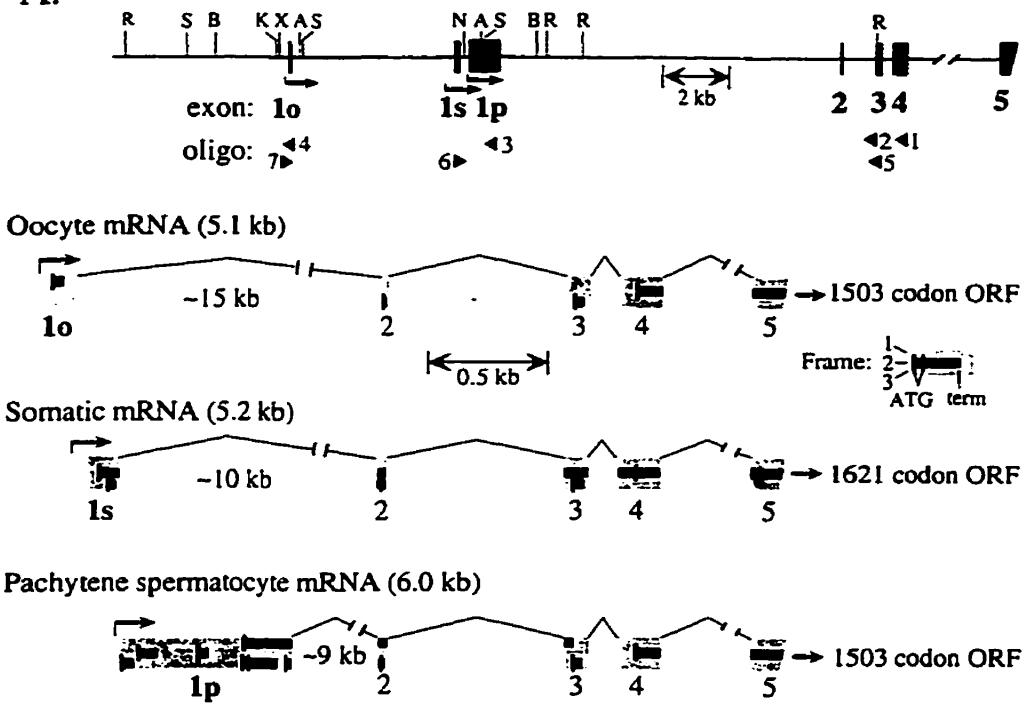


Figure 5. Organization of Dnmt1 exons in genomic DNA and mRNA in somatic cells, pachytene spermatocytes, and oocytes. A. The positions of exons 1o, 1s, and 1p in the *Dnmt1* genomic locus on proximal mouse chromosome 9 are shown at top; splicing patterns and distribution of open reading frames are shown below. Sequences of the alternatively-spliced exons are shown in B; exon 2, which is common to all three transcripts, is underlined, and ATG codons are boxed. The restriction endonuclease sites shown at the top of A are: R, EcoRI; S, SacI; B, BamHI; K, KpnI; X, XbaI; A, AvrII; and N, NotI.

A.



B.

Exons 1o and 2

GGGGTTGATTGAGGGTCATTGGAGGAAGGAACCATCAGGTGGAAGCCCTCTTGTTTGATG
GCCAGCCCTTATATCTTCTACTCACCCCTGTTCTCAGACTAAGCCACTCTTTGCTGCTTG
AGACCGGACACTGCTGCGGCTTGTGTCTTCCAAAAGGCTCAAAGACTTGGAAAGAGATGG
CTTAACAGAAAAG

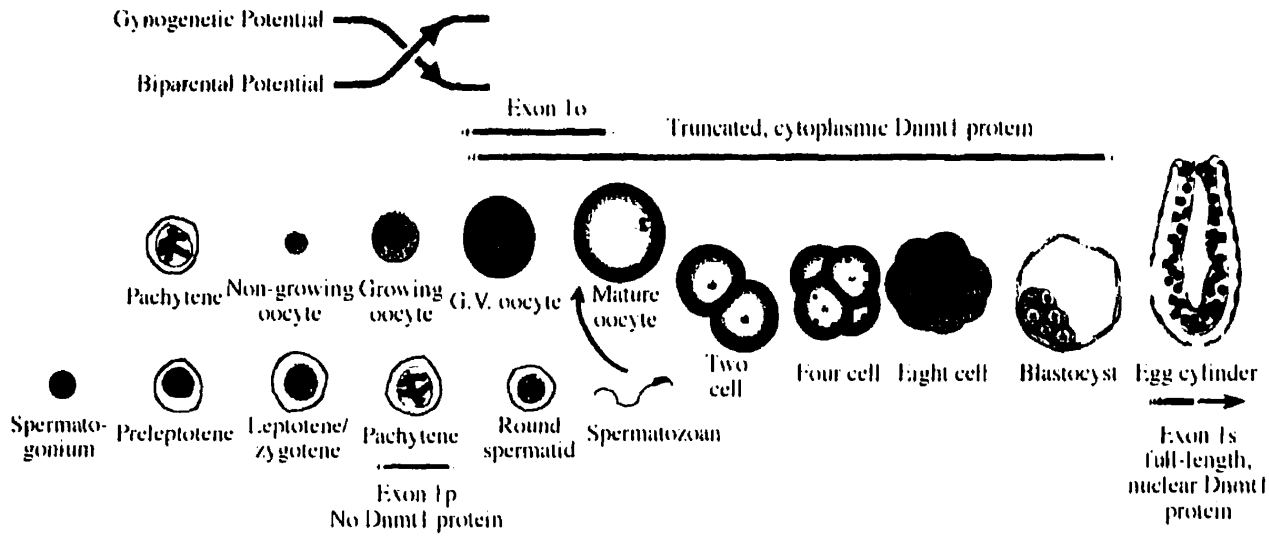
Exons 1s and 2

GCCCCTCCCAATTGGTTTCCGCGCGCGCGAAAAAGCCGGGGTCTCGTTCAGAGCTGTTCT
GTCGTCTGCAACCTGCAAGATGCCAGCGCGAACAGCTCCAGCCCGAGTGCCTGCGCTTGC
CTCCCCGGCAGGCTCGCTCCCGGACCATGTCCGCAGGCGGCTCAAAGACTTGGAAAGAGA
TGGCTTAACAGAAAAG

Exons 1p and 2

CTGACAGCCAGGGCGCGCATGCGCGGGGCAGCGTTTGGCGCGGGCTGTGCTTCCTGTCGC
GCGTGCCCTTAGCTGTTGGCGCCAAAATGGGTGGGGGCCGGGGTGCTGGCAAGCCCATTG
ACTGGGAGCTTGAGGGAGCAAGGGGATCAGCCCTCTGAAATAGAACAAGCTTGGGGACAC
CCTCAGGGGTTCCCCTCTTTGGTCAGCCAGGCTCCTGGGTGTGCAGGAAGTGCCCCTCTC
CCGGCCTGTGGACACCCTGAAGCCCCCACTCCGCTCCAGCCTCCACTTCCGGTGCCCTGC
AGATCCCGCCCGCGCCCACCCGAGGGTCCCTCCGCCGGTTCCCCTGGCCCTGGTTCAGAAG
GGCCCTCCTTCCCAGCCCCGCTGTCAAGTCCTAGGACCTTTTCTCTCTCATCCTGCCGGG
CCGGGCCTGGGCAGCTTGAAGCCCTCGTGGGATTTTGTTTTGTTTTCATCCTAACGTAAA
GGAGCTCCTGCGATCCTGGAGCTATCAAAGAGACTCTGCCTGGAAAGAGCTGGTGCTGCA
GAATGGGCCCTCCACTTTGGCAGTGACATAACTCTGTCCCGAGATCCCAGAGCAATCGAG
GAAACCTTTAACTCCATTCAAAGTGTTTATCAGGGGGAAGGAAAGATGAATGGTAGTGAG
ATGTCTCGGCCCCCGCCCTATTATTTTAGCCCCTGTAAACCAGTTTTTTCGGTCCTGGCG
CCATCGAGGAAGCTTTTTACATTGTCCAGTGTTTATTTCGAGGAGTGAGGGATGGCAGGC
TCAAAGACTTGGAAAGAGATGGCTTAACAGAAAAG

Figure 6. Dnmt1 protein and mRNA in male and female germ cells and in early mouse embryos. The amount and location of Dnmt1 is represented by the intensity of red shading; nuclei are represented in blue when free of detectable Dnmt1 staining. Dnmt1 is present in postnatal oocyte nuclei only during the growth phase, during which time nuclei lose gynogenetic developmental potential and gain biparental potential (Kono et al., 1996). The accumulation of large amounts of truncated cytoplasmic Dnmt1 coincides with the appearance of the alternative 5' exon. Nuclear foci which stain intensely for Dnmt1 appear briefly in leptotene/zygotene spermatocytes; staining of nucleoplasm during this stage is very faint outside of the foci (Jue et al., 1995a). Dnmt1 is not detectable in pachytene spermatocytes, in which another alternative exon prevents translation of Dnmt1 mRNA. Dnmt1 is briefly in nuclei at the eight-cell stage but is found in the cytoplasm of blastocysts. After implantation Dnmt1 protein is localized to nuclei in all somatic cell types.


Gynogenetic Potential
Biparental Potential
Exon 1o
Truncated, cytoplasmic Dnmt1 protein
Pachytene
Non-growing oocyte
Growing oocyte
G.V. oocyte
Mature oocyte
Two cell
Four cell
Eight cell
Blastocyst
Egg cylinder
Spermato-gonium
Preleptotene
Leptotene/ zygotene
Pachytene
Round spermatid
Spermatozoan
Exon 1p
No Dnmt1 protein
Exon 1s
full-length, nuclear Dnmt1 protein

Connecting Text

In Chapter Three, the primary objective of the study was to determine the molecular mechanisms that regulate the differential expression of Dnmt1 mRNA and protein localization in the male and female germlines. We identified and characterized two novel exons in the 5' end of the *Dnmt1* gene that are sex-specific. By alternative splicing, one of these 5' exons gives rise to a larger, non-translated transcript specific to pachytene spermatocytes of the male, while the second is associated with the production of a smaller, active protein in postnatal ovaries and oocytes of the female. The finding that Dnmt1 is present in oocyte nuclei during the growth phase correlates with the time that nuclear transplantation studies have identified as the critical window for the establishment of maternal-specific methylation imprints. In the subsequent chapter, we examine the dynamics and role of the oocyte-specific Dnmt1 during postnatal oocyte development and its association with the methylation status of the maternally-imprinted *Snrpn* gene. We also investigate the different forms of Dnmt1 in preimplantation embryos.

CHAPTER FOUR

Expression of an Oocyte-Specific Form of DNA Methyltransferase-1 Coincides with the Acquisition of Maternal Methylation Imprints on *Snrpn* During Oocyte Growth and Maturation

Carmen Mertineit, Guylaine Benoit, Carina Y. Howell, Feng Ding, Richard J. Chaillet, Timothy H. Bestor, Hugh J. Clarke, Jacquetta M. Trasler

Abstract

The epigenetic modification of DNA by methylation at cytosine in CpG dinucleotides is initiated in the germline and is required for normal mammalian development. Previously we showed that a unique isoform of the predominant mammalian DNA methyltransferase (Dnmt), *Dnmt1,* of M_r 175,000 is produced during postnatal oogenesis from an oocyte-specific first exon, exon 1o, and differs by 118 amino acids at the N-terminus from the full-length somatic form of M_r 190,000, originating from first exon 1s. Our aim in the current study was to determine what Dnmt1 isoforms are present during oocyte growth and preimplantation development, times when the methylation of imprinted genes and other sequences such as endogenous retroviruses are established and maintained. RT-PCR, Western analysis and immunocytochemistry with isoform-specific antibodies were used to determine the forms of Dnmt1 in isolated populations of pure non-growing, growing and metaphase II oocytes and early embryos. Whereas both 1o- and 1s-derived transcripts of Dnmt1 were found in growing and mature oocytes, only the 1s splice variant was seen after the 2-cell stage. The M_r 175,000 Dnmt1 protein was detected during postnatal oocyte and preimplantation development and was followed by a switch to the M_r 190,000 somatic form in the postimplantation embryo, suggesting that the smaller isoform is the only active Dnmt1 in the window of oocyte-preimplantation embryo development. This form of the enzyme undergoes dynamic changes in the amount and subcellular localization where it is first detected predominantly in the nucleus and later accumulates to high levels in the cytoplasm during oocyte growth. Following ovulation, the M_r 175,000 form of Dnmt1 resides largely in the cytoplasm of preimplantation embryos except at the eight-cell stage where it is also detected in the nuclei. To determine whether the expression of the oocyte-specific form of Dnmt1 correlates with the establishment of maternal-specific methylation imprints during oocyte growth and maturation, we examined the methylation status of 16 CpG sites in the 5' end of the *Snrpn* gene using bisulfite genomic sequencing. While the DNA of non-growing

oocytes was largely unmethylated, a mosaic pattern of allelic methylation was observed in mid-size growing oocytes and the full acquisition of the methylation-imprint was completed by metaphase II. We propose that the oocyte-specific Dnmt1 may play a role in the establishment of maternal-specific methylation imprints in the maturing oocyte and may be important for the maintenance of the imprints in the preimplantation embryo.

Introduction

DNA methylation patterns are important for regulating genome function and are determined by the enzymatic processes of methylation and demethylation. Methylation of DNA occurs at the 5 position of cytosine, predominantly within CpG dinucleotides, by a family of mammalian DNA methyltransferases (Bestor et al., 1988; Okano et al., 1998b). Cytosine methylation has been implicated in specialized biochemical functions of large genomes such as allele-specific gene expression and the heritable transcriptional silencing of parasitic sequence elements (Li et al., 1993a; Walsh et al., 1998; Walsh and Bestor, 1999). During development, sex- and sequence-specific patterns of DNA methylation are established in the germline and later modified in the embryo (Monk et al., 1987; Sanford et al., 1987; Kafri et al., 1992). Many imprinted genes show parent-of-origin derived methylation differences that are set down in a sex-specific manner in developing germ cells and are maintained throughout development in the somatic lineage (Tremblay et al., 1997; Olek and Walter, 1997).

The predominant form of DNA (cytosine-5)-methyltransferase in mammals is Dnmt1, which is composed of a large amino-terminal regulatory domain and a smaller carboxy-terminal catalytic domain closely related to bacterial C5-specific restriction methyltransferases (Bestor et al., 1988). Sequences within the N-terminus direct Dnmt1 to the nucleus and to replication foci during S-phase (Leonhardt et al., 1992; Liu et al., 1998). The intact Dnmt1 enzyme exhibits *de novo* and maintenance activities but prefers hemimethylated DNA substrates (Gruenbaum et al., 1982; Bestor and Ingram, 1983). Targeted disruption of the *Dnmt1* gene is associated with a significant decrease in global DNA methylation, abnormal expression of X-linked genes, imprinted genes and retroviral sequences, with embryonic death occurring by mid-gestation in homozygous mutant embryos (Li et al., 1992; Li et al., 1993a; Beard et al., 1995; Lei et al., 1996; Walsh et al., 1998). Embryonic stem (ES) cells deficient in Dnmt1 still show signs of *de novo* methylation on integrated proviral DNA, indicating the existence of alternate

DNA methyltransferases *in vivo* (Lei et al., 1996). Recently, three novel murine DNA methyltransferases have been cloned and characterized including Dnmt2, Dnmt3α and Dnmt3β (Yoder and Bestor, 1998; Okano et al. 1998b). Until now, however, the only mammalian enzyme with significant methylating activity and function *in vivo* is Dnmt1 (Carlson et al., 1992; Yoder et al., 1997b; Li et al., 1992).

It is unclear how the heritable monoallelic expression pattern of imprinted genes is established. At present, there is compelling evidence to suggest that Dnmt1 may be involved in the initiation of genomic imprinting. Sex-specific 5' exons are employed to control the production and localization of Dnmt1 during specific stages of gametogenesis, so that the enzyme is present at all developmental times when increases in DNA methylation have been demonstrated to occur (Mertineit et al., 1998; Mertineit et al., 1999). Thus, Dnmt1 was localized in the nucleus of early growing oocytes (Mertineit et al., 1998) when, for example, the *Igf2r* intronic region became methylated (Stöger et al., 1993; Kono et al., 1996). In the male germline, Dnmt1 was detected in discrete nuclear foci of mitotic and early meiotic spermatocytes (Jue et al., 1995a) at a time when the 5' region of *H19* acquired its full methylation imprint (Davis et al., 1999). It appears that passage of imprinted genes through the germline is necessary for proper allelic expression. In ES cells lacking Dnmt1, imprinted gene expression and methylation were restored by a knock-in of a *Dnmt1* minigene upon passage through the germline (Tucker et al., 1996b). Also, methylation at the 5' end of *H19* in Dnmt1 -/- ES cells and in cells rescued with the *Dnmt1* minigene was low in contrast to wild-type cells which were largely methylated, further suggesting that germline transmission is necessary for the acquisition of the *H19* methylation imprint (Warnecke et al., 1998).

In previous studies, we identified an isoform of Dnmt1 of M_r 175,000 in ovarian lysates (Mertineit et al., 1998) and preimplantation embryos (Carlson et al., 1992). We determined that this smaller isoform of Dnmt1 was produced from a different first exon, exon 1o, than the M_r 190,000 somatic

form of the enzyme, produced from exon 1s (Mertineit et al., 1998). Here, we provide evidence that the M_r 175,000 isoform of Dnmt1 is the only form of the enzyme present in isolated oocytes and preimplantation embryos. The tightly regulated expression of the oocyte-derived Dnmt1 isoform coincides with the acquisition of the maternal methylation imprint on *Snrpn*.

Materials and Methods

Animals

All tissues, oocytes and embryos were obtained from CD-1 mice (Charles River Canada, St. Constant, QC). Noon of the day on which a vaginal plug was found was designated embryonic (E) day 0.5 and the day of delivery as post-natal day (D) 1. Timed pregnant females were killed by cervical dislocation and embryos were dissected out of the uterine horns at E7.5 and at E17.5-18.5. Testes and ovaries could be distinguished in embryos at E12.5 and later by the presence or absence of seminiferous cords, respectively (Hogan et al., 1986).

Collection of Oocytes and Embryos

Immature oocytes in prophase I of meiosis were collected at E17.5-18.5, day 1 (at birth), day 5, day 10, and day 15 using techniques developed by Eppig and colleagues (see Eppig and Telfer, 1993). Ovaries from 3 or 4 mice were dissected in phosphate buffered saline (PBS), pH 7.2, torn into smaller pieces, and transferred into a screw-top conical tube containing 2 ml of PBS, 2 mg/ml collagenase (Sigma Chemical Company, St. Louis, MO), 0.025% trypsin (Gibco BRL, Burlington, ON) and 0.02 mg/ml DNase (Sigma). The tube was capped tightly and shaken at high speed for 10 minutes at 37°C, and the contents were subsequently diluted by half with Hepes-buffered minimal essential medium, MEM-H (Gibco BRL), modified as described (Schroeder and Eppig, 1984; Clarke et al., 1988).

Fully grown, germinal vesicle (GV)-stage oocytes were obtained by puncture of the ovarian follicles of 21 to 35 day-old females in MEM-H supplemented with 100 µg/ml of dibutyryl cyclic AMP, dbcAMP (Sigma), as previously described (Clarke et al., 1992). The dbcAMP prevents meiotically competent oocytes from undergoing germinal vesicle breakdown (GVBD) in culture (Cho et al., 1974). GVBD oocytes were derived from GV oocytes that were isolated in MEM-H free of dbcAMP and incubated in 5 µl microdrops of

bicarbonate-buffered MEM under oil at 37°C in an atmosphere of 5% CO_2 in air for 2-4 hours.

Metaphase II (MII) oocytes were collected from 7-week-old females that were superovulated by injection of 7.5 IU of pregnant mares' serum gonadotropin, PMSG (Ayerst Vetlabs), followed 44-48 hours later by 5 IU of human chorionic gonadotropin, hCG (Ayerst Vetlabs). These oocytes were recovered from the oviducts 20 hours post-hCG and the cumulus cells were dispersed with 1 mg/ml hyaluronidase (Roche Diagnostics, Laval, QC), as described (Hogan et al., 1986).

Preimplantation embryos were obtained as previously described (Clarke et al., 1992). Superovulated females were caged individually with stud males overnight, and examined for the presence of a vaginal plug the next morning. One-cell embryos were recovered from the oviducts at E0.5 in defined Hepes-buffered KSOM medium (Erbach et al., 1994) and the cumulus mass was dispersed as described above. Two-cell embryos were obtained by flushing the oviducts of females killed 1.5 days post-hCG. Four-cell, eight-cell, morula and blastocyst-stage embryos were obtained by culturing two-cell embryos in 5 µl microdrops of bicarbonate-buffered KSOM under oil at 37°C in an atmosphere of 5% CO_2 in air. Postimplantation embryos were dissected out of the uterine horns at E7.5.

For all isolation procedures, healthy-looking oocytes and preimplantation embryos were collected in a 35 mm petri dish using a mouth-controlled micropipette and washed free of any adhering somatic cells by transfer through two dishes of culture medium. Oocytes and embryos were either processed immediately for immunofluorescence, or pooled and stored at -80°C in 10 µl of DEPC-PBS for RNA extraction, or in lysis buffer for immunoblotting, or in culture medium for DNA isolation.

RNA Isolation and RT-PCR

Total RNA was extracted from homogenized tissues using the Trizol reagent (Gibco BRL) according to the manufacturer's instructions. For

isolated oocytes and preimplantation embryos (n = 80 per tube), 10 µg of glycogen carrier protein (Roche Diagnostics) was added to the Trizol and cellular RNA was recovered as described previously (Clarke et al., 1997). The RNA pellets obtained from these isolated cells were dissolved in 20 µl of DEPC-treated water. The SuperScript One-Step RT-PCR System (Gibco BRL) was used to analyze the oocyte and somatic transcripts produced by the alternative splicing of exons 1o and 1s of *Dnmt1*, respectively (Mertineit et al., 1998). Reactions were performed as directed by the manufacturer. For tissues, 0.5 µg of total RNA was seeded into a 25 µl RT-PCR reaction. For isolated cells, 10 µl of the dissolved RNA pellet was seeded into the 25 µl RT-PCR reaction. Oligo 5 (Mertineit et al., 1998), 5'-GCAGGAATTCATGCAGTAAG-3', was used as the RT primer for the amplification of both transcripts. Oligo 6 (5'-GGGTCTCGTTCAGAGCTG-3') was used to amplify the somatic transcript and oligo 7 (5'-GGTTGATTGAGGGTCATT-3') was used to amplify the oocyte transcript as described previously (Mertineit et al., 1998). Reverse transcription was allowed to proceed for 10 minutes at 50°C then shifted to 55°C for 30 minutes. PCR involved 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds. A final extension at 72°C was performed for 7 minutes. A portion of the reaction (8 or 10 µl) was analyzed by electrophoresis through 12% polyacrylamide gels.

Western Blotting

Oocytes and preimplantation embryos were pooled (n = 30, 50, 200 or 400) and transferred to 10 µl of 2X sample buffer (10% SDS, 5% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue, 1 M Tris, pH 6.8). Lysates of whole gonads, accessory reproductive tissues, and postimplantation embryos were prepared by homogenization in suspension buffer (0.15 M NaCl, 0.05 M Tris, pH 7.5, 2 µg/ml leupeptin, 2 µg/ml aprotinin and 100 mM PMSF), centrifuged, and the supernatant was added to an equal volume of 2X sample buffer. The amount of protein in the lysates was quantified using the amido

black protein spot assay, as described (Dieckmann-Schuppert and Schnittler, 1997). All samples were denatured by heating at 95°C for 5 minutes, separated by electrophoresis on SDS-5% polyacrylamide gels and transferred to Hybond ECL nitrocellulose membranes (Amersham, Oakville, ON). The gels were stained in Coomassie Blue to examine the efficiency of protein transfer and membranes were stained with Ponceau S to confirm the presence of protein in all lanes. Membranes were blocked in blocking buffer (5% Carnation skim milk in 10 mM Tris, 140 mM NaCl, pH 8.0) for a least one hour and probed with a novel polyclonal affinity-purified rabbit anti-UPT82 antibody directed against the 118 amino acid terminus of Dnmt1 (Yoder et al., 1996; Mertineit et al., 1998) or preimmune serum diluted to 1:3,000 in blocking buffer overnight. Following three washes of 5 minutes each in 0.1% Tween-20 TBS, the membrane was incubated for one hour at room temperature in biotinylated donkey anti-rabbit IgG (Jackson Research Labs) diluted to 1:5,000 in blocking buffer. The membrane was washed as above, incubated for 30 minutes in 1:1,000 streptavidin-horseradish peroxidase conjugate (Amersham) and washed as above. Specific antibody labelling was detected using the chemiluminescence method (ECL Plus, Amersham). The same membranes were reprobed with 1:15,000 anti-pATH52 antibody (Li et al., 1992).

Immunocytochemistry

Denuded oocytes and preimplantation embryos were freed of the zona pellucida using acidified (pH 2.5) Tyrode's medium (Hogan et al., 1986), and fixed for 10 to 15 minutes at room temperature in 3.7% formaldehyde in PBS. Alternatively, small ovarian follicles were fixed intact. All solutions for immunofluorescence of Dnmt1 were prepared in PBS and procedures were carried out at room temperature, unless specified otherwise. The fixed cells were blocked for at least one hour in blocking buffer (3% BSA, 0.1% Triton X-100) and then incubated in either 1:100 preimmune serum, 1:100 anti-UPT82 or 1:500 anti-pATH52 antibodies diluted in the same blocking buffer overnight

at 4°C in a humidified chamber. The cells were washed 3 times for 5 minutes each in blocking buffer, then incubated in 1:250 of goat-anti-rabbit Bodipy-TMRX secondary antibody (Molecular Probes, Eugene, OR) for one hour, and washed as before. To mount the cells for viewing, a drop of Vectashield mounting medium (Vector Laboratories, Burlingame, CA) supplemented with 0.4 µg/ml of the DNA-binding dye DAPI (Roche Diagnostics) was placed on a glass microscope slide and the cells were carefully transferred by mouth-pipette into the drop. A cover slip was gently placed on the drop to spread the mounting medium and flatten the cells, and was subsequently sealed with nail polish. Immunofluorescence was visualized using a Zeiss Axiophot or Zeiss LSM410 confocal microscope, as described (Laird et al., 1995).

DNA Isolation and Bisulfite Modification

Using the DNA Stat-60 isolation method (TEL TEST "B" Inc, Friendswood, TX), genomic DNA was extracted from 500 or more oocytes at three different stages of development: postnatal days 1 and 10 and MII. Genomic DNA was ethanol-precipitated overnight at -80°C with 40 µg of muscle glycogen (Roche Diagnostics) and 2 volumes of absolute ethanol. The DNA was pelleted by centrifugation, washed once with 70% ethanol, resuspended in 80 µl of deionized water and digested with HindIII (Gibco BRL). Following phenol chloroform extraction, precipitation and suspension in 25 µl TE (10mM Tris-HCl, pH 7.5, 1mM EDTA), the DNA was denatured with a final concentration of 0.3 M NaOH for 30 minutes at 42°C.

Bisulfite modification of the denatured DNA was initiated by adding 255 µl of freshly made 40.5% sodium bisulfite (Sigma), and 15 µl of 10 mM hydroquinone (Sigma). The reaction mixture was covered with paraffin oil, and incubated at 55°C for 16-18 hours in the dark. After incubation, the DNA was recovered using Geneclean II (Intermountain Scientific Corporation, La Jolla, CA). The DNA was resuspended in 25 µl of TE buffer. Denaturation was performed in 0.3 M NaOH at 37°C for 15 minutes. The solution was neutralized with ammonium acetate, pH 7.0, to a final concentration of 3 M

and the DNA was ethanol-precipitated in the presence of 40 µg of muscle glycogen at -80°C for 30 minutes. Following centrifugation at 14,000 rpm for 30 minutes and a 70% ethanol wash, the DNA was resuspended in 25 µl of TE and stored in the dark at –20°C. A treated sample was used immediately and was never kept longer than two weeks.

PCR Amplification, Cloning, and Sequencing

The primers used to generate products from bisulfite-altered DNA are specific for the top strand of mutagenized DNA. For the 5' region of the mouse *Snrpn* gene, the sequences of the primers with the nucleotide position of the first base indicated in parentheses (*Snrpn* GenBank accession number AF081460) are as follows: outside forward, 5'-TATGTAATATGATATAGTTTAGAAATTAG-3'(nt 2073); outside reverse, 5'-AATAAACCCAAATCTAAAATATTTTAATC-3' (nt 2601); inside forward, 5'-AATTTGTGTGATGTTTGTAATTATTTGG-3' (nt 2151); inside reverse, 5'-ATAAAATACACTTTCACTACTAAAATCC-3' (nt 2570). In order to assess possible somatic cell contamination in the oocyte samples and to determine the efficiency of cytosine deamination by the bisulfite treatment, we also examined the methylation profile of the 5' region of *H19* (Tremblay et al., 1997). The primer sequences of *H19* (GenBank accession number U19619) are those used by Tremblay et al. (1997): outside forward, BMsp2t1, 5'-GAGTATTTAGGAGGTATAAGAATT-3' (nt 1278); outside reverse, BHha1t3, 5'-ATCAAAAACTAACATAAACCCCT-3' (nt 1751); inside forward, BMsp2t2, 5'-GTAAGGAGATTATGTTTATTTTTGG-3' (nt 1304); inside reverse, BHha1t4, 5'-CCTCATTAATCCCATAACTAT-3' (nt 1726). To generate the *Snrpn* and *H19* 5' products, two rounds of PCR were performed with fully nested primer pairs. The first-round PCR utilized the outside primer pairs, whereas the second-round PCR utilized the inside primer pairs. Each 25 µl PCR reaction contained 4 µl of bisulfite-treated oocyte DNA, 25 ng of each primer, 2.5 µl (100 µM) dNTPs (Invitrogen,Carlsbad, CA), 5 µl of 5X PCR Buffer (for *H19*: 300 mM Tris-HCl, 75 mM ammonium sulfate, 7.5 mM MgCl2,

pH 8.5; for *Snrpn*: 300 mM Tris-HCl, 7.5 mM ammonium sulfate, 12.5 mM MgCl2, pH8.5) and 1.25 U of DNA Taq Polymerase (Gibco BRL). First-round PCR was performed under the following conditions: 4 minutes at 94°C, 2 minutes at 55°C, and 2 minutes at 72°C for two cycles. Thirty-five cycles of PCR were then performed for 1 minute at 94°C, 2 minutes at 55°C, and 2 minutes at 72°C. For the second round of PCR, 4 µl of the first-round sample was used and the conditions for the PCR were the same, except that the first two cycles were omitted.

To confirm the amplification of the *Snrpn* and *H19* PCR products, 20 µl of each PCR sample was electrophoresed on a 1% agarose gel, and the bands were excised and purified with the GeneClean II kit. The DNA was subcloned using the TOPO TA Cloning kit, version F (Invitrogen). Resulting plasmid DNA was isolated using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI) and analyzed by gel-electrophoresis following EcoR I (Gibco BRL) digestion at 37°C for 2 hours. Clones containing the *Snrpn* and *H19* inserts were sequenced using the T7 Sequenase DNA sequencing kit, version 2.0 (Amersham Life Science Inc., Cleveland, OH). The methylation status of 16 CpG sites was examined in the 5' region of *Snrpn* and 15 sites were examined in *H19*.

Results

Expression and Localization of Dnmt1 mRNA Splice Variants and Protein Isoforms in Tissues and Isolated Oocytes

Alternative splicing of somatic exon 1s and oocyte exon 1o in the 5' region of *Dnmt1* is associated with the production of 5.2 kb and 5.1 kb transcripts, respectively (Mertineit et al., 1998). Both of these transcripts generate active proteins by using different in-frame ATG translation initiation start sites in exon 1s to produce the full-length form of the enzyme and in exon 4 to produce a truncated isoform (Carlson et al., 1992; Yoder et al., 1996; Mertineit et al., 1998; Gaudet et al., 1998). Using RT-PCR analysis, we examined multiple tissues in the mouse to determine the expression profile of the somatic and oocyte transcripts. The somatic Dnmt1 transcript was present in all adult tissues (Figure 1A). A 1o containing mRNA was detected exclusively in the adult ovary (Figure 1A) and was associated with postnatal ovary development (Figure 1B). The presence of the 1o transcript in post-natal ovaries suggested that it may be associated with the growth and maturation of oocytes and prompted a careful examination of the Dnmt1 mRNA splice variants in isolated oocytes at different stages of development. As shown in Figure 1C, the 1s transcript was detected in fetal oocytes obtained at 17.5 to 18.5 days of gestation and persisted through oocyte growth and maturation to metaphase II. The 1o transcript was detected in isolated oocytes as early as postnatal day 5 and was maintained in all classes of growing oocytes through to metaphase II. The presence of the 1o transcript in newborn ovaries (day 1) but not in oocytes isolated at this time can be explained by the fact that the oocytes selected for the RT-PCR assay were non-growing and were fewer in number (n=80) than those found in a whole ovary.

The detection of both the 1s and 1o transcripts in growing oocytes suggested that two forms of the Dnmt1 protein could potentially exist in these germ cells. Previous immunoblot analysis with the pATH52 antibody, which is highly specific for Dnmt1 and recognizes multiple epitopes within the region

between amino acids 255-753 (Bestor, 1992; Li et al., 1992; Leonhardt et al., 1992), revealed that ovaries from prepubertal and adult mice contain two species of Dnmt1: a full-length form of M_r 190,000 and a truncated species of M_r 175,000 (Mertineit et. al, 1999). However, it was not determined whether one or both protein species were present in different classes of growing oocytes. In order to determine which forms of Dnmt1 protein were present in oocytes at various stages of development, an antibody (UPT82) was raised against amino acids 1-118 of the amino terminus of Dnmt1 to identify exclusively the full-length somatic form. There are 118 additional amino acids in the amino-terminal regulatory domain of the full-length form of Dnmt1 that are lacking in the truncated isoform. While the pATH52 antibody recognizes both species of Dnmt1, the UPT82 antibody recognizes only the full-length form (See Figure 2A).

In this study, an immunoblot analysis of oocytes at different stages of growth showed no signs of the somatic form of Dnmt1 after probing with the UPT82 antibody (Figure 2B). In contrast, tissue lysates prepared from a day 70 testis, a day 10 uterus/oviduct, a day 10 ovary and a day 49 ovary showed evidence of the M_r 190,000 Dnmt1 species. The same membrane was reprobed with the pATH52 antibody and revealed the M_r 175,000 form of Dnmt1 in all classes of growing oocytes, with the exception of day 1 oocytes (Figure 2C). The truncated protein was also detected in the day 10 and day 49 ovaries (Figure 2C). Thus, the presence of both Dnmt1 species in the ovaries appears to be due to the full-length form in the proliferating somatic cells and the truncated isoform in the growing oocytes (Mertineit et al. 1998). More importantly, the appearance of the truncated form of Dnmt1 beginning in day 5 oocytes is consistent with the appearance of the 1o transcript, suggesting that this message may be preferentially translated in growing oocytes.

The immunoblotting data presented here do not exclude the possibility that low levels of the full-length form of Dnmt1 may reside within the growing oocytes. We previously reported dynamic changes in the amount and

subcellular localization of Dnmt1 in oocytes of postnatal ovaries immunostained with the pATH52 antibody (Mertineit et al., 1998; Mertineit et al., 1999). Using the same antibody, we show here in isolated day 5 oocytes intense nuclear and lower levels of cytoplasmic staining of Dnmt1 (Figure 3A; a, b, b'). In day 10 oocytes, the nucleus stained moderately while the cytoplasm stained intensely (Figure 3A; c, d, d'). During oocyte growth, the enzyme appeared to accumulate to high levels in the cytoplasm of larger day 15 (Figure 3A; e, f, f') and fully grown germinal vesicle stage oocytes (Figure 3A; g, h, h'). Overall, the nuclear staining was weaker in larger oocytes which may be linked to the increase in volume of the nucleus during the growth phase. These results suggest that oocytes may have both the full-length and truncated forms of the enzyme and that these forms may be differentially compartmentalized. To test the possibility that the full-length form of Dnmt1 is present in oocytes at levels that are not detected on immunoblots and to determine its subcellular localization, we immunostained small follicles and larger oocyte-granulosa cell complexes with the UPT82 antibody. Confocal z-sections at 5 μm intervals through an entire follicle showed no signs of the full-length form of Dnmt1 within the oocyte, whereas surrounding somatic cells were highly reactive (Figure 3B). Even fully grown germinal vesicle stage oocytes were unreactive in contrast to adhering granulosa cells (data not shown). These data indicate that the full-length form of Dnmt1 is not present in growing oocytes and further suggest that there are mechanisms which regulate the subcellular localization of the truncated form of Dnmt1.

In summary, the data provide evidence of a smaller, oocyte-specific form of Dnmt1 protein that is apparently translated from the 1o transcript during oocyte growth and maturation. This alternate form of Dnmt1 may have a unique function in oocytes.

Methylation Analysis of *Snrpn* during Oocyte Growth

To determine if there is an association between the expression of the oocyte-derived isoform of Dnmt1 and the acquisition of methylation imprints

during the oocyte growth phase, we examined the methylation status of the *Snrpn* gene using the genomic bisulfite sequencing assay. The *Snrpn* genomic structure, shown in Figure 4A, contains a maternally-methylated region at the 5' end (*DMR1*), which correlates inversely with the Snrpn paternal expression, and a paternally-methylated region at the 3' end (*DMR2*) (Shemer et al., 1997). The time in which this maternal methylation mark becomes established in the germline is not known. Based on the tightly regulated expression of the oocyte isoform of Dnmt1, we hypothesized that the methylation in *DMR1* may be established during oocyte growth. Therefore, we examined 16 CpG sites between nt2151-nt2570 in *DMR1* which covers a part of the sequence upstream from the transcription start site and exon 1 (Figures 4A and 4B). This target sequence in the mouse corresponds to the human *SNRPN* imprinting locus and differences in DNA methylation patterns within this region are used in the diagnosis of Prader-Willi and Angelman syndromes (Zeschnigk et al., 1997).

The bisulfite mutagenesis method was used to examine the methylation status of the *Snrpn DMR1*. In this method, sodium bisulfite treatment of DNA converts cytosine residues to uracil but leaves 5-methylcytosine unchanged. Following mutagenesis, the DNA is PCR-amplified, cloned and sequenced. Results of the methylation analysis of the *Snrpn DMR1* are presented for three stages of postnatal oocyte development in Figure 5. Two separate batches of oocyte DNA were bisulfite-treated and the 5' regions of *Snrpn* (Shemer et al., 1997) and *H19* (Tremblay et al., 1997) were PCR-amplified and subcloned. For both genes, between 10-25 clones were sequenced from each batch of oocyte DNA obtained from day 1, day 10 and metaphase II arrested oocytes. The methylation data of clones derived from both batches of oocytes were combined in Figure 5. Day 1 oocytes from newborn pups were largely unmethylated at all sites examined with the exception of CpG site 2279 which had an average percent methylation of 25%, and CpG sites 2400, 2416, 2418 and 2470 which were 15% methylated (Figure 5A). In day 10 growing oocytes, all 16 CpG sites showed signs of methylation ranging

from 8% to 24% on average with site 2279 having the highest percentage of methylation at 50% (Figure 5B). Metaphase II arrested oocytes were highly methylated at all 16 CpG sites from all clones examined (Figure 5C). As an internal control to assess the extent of cytosine deamination following bisulfite mutagenesis and to determine if there was any somatic cell contamination during the oocyte isolation procedures, the methylation profile of the 5' end of *H19* (Tremblay et al., 1997) was examined. For all batches of isolated oocytes, the expressed maternal allele of *H19* was unmethylated at all 15 CpG sites in all clones (data not shown).

A summary of the methylation status in individual clones of *Snrpn DMR1* is shown in Figure 6. During postnatal oogenesis, we observed dynamic changes in the distribution of methylated cytosines that were unique to each stage of oocyte development. Overall, in day 1 oocytes 60% of the clones were completely unmethylated while 40% were methylated randomly at a single CpG site (nt 2279, batch 1) or at 4 to 5 centrally located CpG sites (batch 2)(Figure 6A). In day 10 oocytes, a mosaic pattern of cytosine methylation was observed in which 50% of the clones were completely unmethylated, 26% were methylated at the single CpG site 2279, and 24% were methylated at 11 or more CpG sites (Figure 6B). Interestingly, those clones that were highly methylated in the day 10 oocytes showed complete or almost complete methylation within the body of the target sequence and were often lacking methylation at the distal and proximal ends. However, there were a few clones that were 100% methylated at this time of development. It appears that most of the methylation in oocytes occurs between the mid-growth stage at day 10 and metaphase II arrest, as all clones obtained from metaphase II oocytes were fully methylated at all sites (Figure 6C).

These data indicate that there is a correlation between the expression of the oocyte-specific Dnmt1 and the establishment of the methylation imprint on the *Snrpn DMR1* during postnatal oocyte development.

Forms of Dnmt1 during Preimplantation Development

During mouse preimplantation development, there is a genome-wide decrease in cytosine methylation which erases DNA methylation patterns inherited from the parental gametes and allows reformatting of the embryonic genome (Monk et al., 1987; Kafri et al., 1992). However, imprinted loci maintain gamete-specific methylation differences during this period (Tremblay et al., 1997; Shemer et al., 1997). To determine which forms of Dnmt1 may play a role in the maintenance of these sex-specific methylation differences, we examined the mRNA splice variants and protein isoforms of Dnmt1 during early embryo development. As shown in Figure 7A, RT-PCR analysis of pre- and post-implantation embryos revealed the somatic mRNA of Dnmt1 in all stages of embryo development, whereas the oocyte-specific mRNA was detected only in the fertilized one-cell embryo. Interestingly, immunoblot analysis of early embryos with the UPT82 antibody provided evidence of the M_r 190,000 Dnmt1 protein in the E7.5 embryo but not in any of the preimplantation embryos (Figure 7B, top panel). When the same nitrocellulose membrane was reprobed with the pATH52 antibody, which detects both the M_r 190,000 and M_r 175,000 species of Dnmt1, only the smaller isoform was observed in all preimplantation embryos (Figure 7B, bottom panel). The data show a switch from the smaller isoform in preimplantation embryos to the full-length enzyme in postimplantation embryos. Consistent with the immunoblotting results, the full-length Dnmt1 enzyme was not detected by immunofluorescence in preimplantation embryos at any stage. In particular, at the eight-cell stage, the only time during preimplantation development when Dnmt1 is found in the cytoplasm and the nuclei of blastomeres (Carlson et al., 1992; and Fig. 7C), immunostaining with the UPT82 antibody followed by confocal z-sectioning did not detect the presence of the somatic isoform of Dnmt1 in either compartment.

Despite the production of the somatic-specific Dnmt1 transcript in the preimplantation period, it appears that the smaller Dnmt1 protein derived from

the growing oocyte is the only form of the enzyme that is present in the early embryo.

Discussion

In this study, we provide evidence for the production of an oocyte-specific form of Dnmt1 during postnatal oocyte development that may be involved in the establishment and maintenance of maternal-specific methylation imprints.

Developmental Regulation of Dnmt1 during Oogenesis

The discovery of three alternative 5' exons in the mouse *Dnmt1* gene provides a mechanism to regulate the production of tissue-specific and sex-specific forms of the enzyme during development. While exon 1s is associated with the production of a 5.2 kb message and a M_r 190,000 functional protein that is characteristic of all somatic cells (Yoder et al., 1996; Yoder et al., 1997), exons 1o and 1p are linked to the production of gamete-specific Dnmt1 variants in the ovary and testis, respectively (Mertineit et al., 1998). In oocytes, a 5.1 kb message with an alternate in-frame ATG translation initiation site in exon 4 leads to the synthesis of a smaller isoform of about M_r 175,000 that is fully functional *in vivo* (Gaudet et al., 1998; Mertineit et al., 1998). A larger 6.0 kb testis-specific transcript remains untranslated in pachytene spermatocytes (Mertineit et al., 1998). Based on these data, there are two functional Dnmt1 proteins that are expressed from the same gene but differ by 118 amino acids in the N-terminus due to different transcriptional and translational regulatory mechanisms (Yoder et al., 1996; Mertineit et al., 1998; Gaudet et al., 1998). Both proteins contain a nuclear localization signal, sequences for targeting to replication foci (Leonhardt et al., 1992; Liu et al., 1998), and a C-terminus containing the catalytic domain (Bestor et al., 1988). The shorter isoform of Dnmt1, like the full-length Dnmt1, is active toward both unmethylated and hemimethylated substrates (Carlson et al., 1992).

Our results indicate that an oocyte-specific Dnmt1 transcript appears at a time when dictyate stage oocytes, arrested in prophase I of meiosis, are recruited into the growth phase and is associated with the synthesis and

accumulation of a smaller Dnmt1 protein isoform. Consistent with our findings, small oocytes in primordial follicles are relatively quiescent compared to the burst of transcription and translation which occurs upon commencing the growth phase. In contrast to a proliferating somatic cell, a mature oocyte contains at least 10,000-fold more Dnmt1 protein per cell (Carlson et al., 1992).

A somatic-specific Dnmt1 transcript, which was detected in all of our isolated oocyte samples and was apparently not translated into the full-length protein, may be translationally repressed or may represent pre-spliced mRNA. Several mechanisms of translational control operate in oocytes and early embryos. For example, changes in the length of the poly (A) tail of several maternal and zygotic mRNAs occur in a developmentally regulated manner and these changes regulate the translation of these mRNAs (Bachvarova, 1992; Salles et al., 1992; Wickens, 1992). Furthermore, there is a proposal that precocious activation of the maternal mRNA is prevented by association with specialized proteins, which package the RNA in such a way as to exclude the ribosomes from gaining access to the regulatory elements (Meric et al., 1996). Despite the presence of four potential in-frame ATGs in the 5' end of *Dnmt1*, all of which fulfill the criteria of a good translation initiation site (Kozak, 1996), ATG_3 which lies in exon 1s is preferentially used by somatic cells (Gaudet et al., 1998). In the absence of ATG_3, ATG_4 in exon 4 is utilized (Gaudet et al., 1998), as seen here in growing oocytes. Further investigation is required to determine why the oocyte-specific Dnmt1 transcript is preferentially translated over its somatic counterpart.

The full-length Dnmt1, although capable of *de novo* methylation, prefers hemimethylated DNA substrates and is targeted to the nucleus in replicating cells. In mitotic somatic cells and germ cells, this enzyme is nucleoplasmic during interphase, associates with replication foci during S-phase, and surrounds condensed chromosomes following nuclear envelope breakdown during M-phase (Leonhardt et al., 1992; Trasler et al., 1996; Mertineit et al., 1999). Early meiotic germ cells show nucleoplasmic staining until the

pachytene stage in both sexes (Mertineit et al., 1999). Compared to the full-length Dnmt1, the localization of the smaller Dnmt1 is quite different and is developmentally restricted to growing oocytes and preimplantation embryos. This isoform is prominent in the nucleus of small growing oocytes and accumulates to high levels in the cytoplasm as the oocytes enlarge and mature. Due to the increase in nuclear volume during the growth phase, Dnmt1 staining in the nucleus of large oocytes appears less dramatic in comparison to its intense staining in the cytoplasm. These results are consistent with a recent observation of both nuclear and cytoplasmic Dnmt1 staining in xenopus oocytes (Kimura et al., 1999). We suggest separate functions for the nuclear and cytoplasmic distributions of this smaller Dnmt1 isoform in the mouse. While the large cytoplasmic maternal store may support early preimplantation development, the nuclear presence of the enzyme may be involved in the establishment of maternal-specific methylation imprints.

Establishment of Maternal-specific Methylation Imprints

The timing of when parental imprints are set down in the germline is not well defined. The methylation of cytosine residues in CpG dinucleotides has emerged as the imprinting mark candidate due to the differential methylation patterns observed on the parental alleles of many imprinted genes. DNA methylation is a heritable modification that is stably propagated during DNA replication and is reversible so that the methylation imprint can be switched in the germline to reflect the sex of the parent (Surani, 1991). Evidence that Dnmt1 plays a role in maintaining genomic methylation patterns and is important for parent-of-origin-specific gene expression is supported by a targeted disruption of the *Dnmt1* gene where homozygous mutant embryos show perturbations in the expression of several imprinted genes (Li et al., 1993; Shemer et al., 1997).

The nuclear localization of Dnmt1 during oocyte growth coincides with the timing of methylation for a number of different types of sequences, notably

that of imprinted genes. Examination of the imprinted gene *Igf2r* (Stöger et al., 1993, Brandeis et al., 1993; Kono et al., 1996), the *RSVIgmyc* and *MPA434* transgenes (Chaillet et al., 1991; Ueda et al., 1992) and the *IAP* retroviral sequence (Walsh et al., 1998), indicate that *de novo* methylation occurs in a window marking the transformation of non-growing oocytes to metaphase II oocytes. However, these findings were largely based on methylation-sensitive restriction enzyme assays of a limited number of cytosine residues. For imprinted genes, support for the functional importance of methylation during oocyte growth comes from nuclear manipulation studies in which parthenogenetic embryos containing one genome from a neonate-derived non-growing oocyte and the other from a fully grown oocyte develop three days longer than normal parthenogenotes (Kono et al., 1996). These findings suggest that oocytes at different stages of growth differ in their developmental potentials and that primary imprinting markers may be set down during the oocyte growth phase. Furthermore, these parthenogenetic embryos exhibited a dysregulation in the expression of several paternally and maternally imprinted genes, including *Snrpn*, further supporting the notion that primary imprinting may occur during oocyte growth (Obata et al., 1998).

Our methylation analysis of the maternally imprinted *Snrpn* region indicates dynamic changes in the distribution of methylated cytosines during oocyte growth and maturation. For many alleles, partial methylation was observed at one or a few CpG sites in newborn oocytes, increased to 11 or more CpG sites in day 10 oocytes, and was completed on all alleles by metaphase II. The diversity in partially methylated and completely unmethylated alleles seen in day 1 and 10 oocytes may be due to heterogeneity within the population of isolated oocytes or may, in part, represent a group of oocytes that will not acquire meiotic competence and are destined to die. Alternatively, it is conceivable that the methylation on *Snrpn DMR1* occurs differentially on the parental alleles during postnatal oogenesis. In this study, the clones with signs of CpG methylation may represent the maternal allele, whereas the unmethylated clones may represent the paternal

allele. As reported in a previous study (Davis et al., 1999), the acquisition of the *H19* methylation imprint occurred on the paternal allele prior to birth, preceeding the onset of spermatogenesis whereas the maternal allele became fully methylated during the early stages of spermatogenesis. Further investigation is required to elucidate the mechanisms underlying the mosaic pattern of cytosine methylation on *Snrpn* during oocyte growth and to determine whether other maternally imprinted genes acquire a methylation-imprint with similar dynamics in the same period of oocyte development.

Developmental Regulation of Dnmt1 in Early Embryos

In the mouse, maternal messages are degraded prior to the two-cell stage and there is a transition from transcription of maternal genes to activation and transcription of zygotic genes. While the oocyte-specific transcript disappears after the one-cell embryo stage, the two-cell and later embryos produce the somatic-specific Dnmt1 transcript. At the protein level, however, the only functional Dnmt1 detected in the preimplantation embryos is the smaller form derived from the mature oocyte, indicating that there may be translational repression of the somatic-specific transcript in early embryos. Previous reports have noted that mouse preimplantation embryos contain very high levels of Dnmt1 protein and activity both of which decline between the eight-cell to the blastocyst stages (Monk et al., 1991; Carlson et al., 1992).

Aside from its distribution in the nucleus and cytoplasm in growing oocytes, this smaller Dnmt1 undergoes dynamic changes in its subcellular localization in preimplantation embryos. It is concentrated in the peripheral cytoplasm from the oocyte stage to the four-cell stage, enters the nuclei for a short time at the eight-cell stage, and remains at lower levels in the cytoplasm until the blastocyst stage (Carlson et al., 1992). Following implantation, there is a switch from the smaller form to the full-length form that is associated with intense nuclear localization characteristic of cycling somatic cells (Trasler et al., 1996). Although Dnmt1 behaves as a replication factor, its cytoplasmic

sequestration in oocytes and early embryos appears to be unprecedented and the possibility of a cytoplasmic function unrelated to DNA methylation cannot be excluded.

Genomic methylation patterns undergo a dramatic decrease following fertilization (Sanford et al., 1987; Monk et al., 1987; Kafri et al., 1992) but imprinted loci retain characteristic methylation differences during preimplantation development (Tremblay et al., 1997; Olek and Walter, 1997; Shemer et al., 1997). While the nuclear association of Dnmt1 in growing oocytes may have a role in the establishment of maternal-specific methylation imprints, this isoform may similarly play a maintenance role for the consolidation of imprints at the eight-cell stage. Alternatively, this oocyte-specific Dnmt1 could play a host defense role by inactivating retroviral sequences during oocyte growth and preimplantation development (Walsh et al., 1998).

In summary, we provide evidence of an oocyte-specific isoform of Dnmt1 whose expression is regulated during postnatal oocyte development and may be linked to the establishment of maternal-specific methylation imprints at this time. To date, this smaller Dnmt1 is the only functional DNA methyltransferase enzyme in oocytes and preimplantation embryos, where it may serve a maintenance function for the consolidation of gametic imprints at the eight-cell stage. To explore the potential connection between the oocyte-specific Dnmt1 and its possible function in establishing and maintaining methylation patterns during early development, it will be important to examine the developmental consequences in mice lacking this form of Dnmt1. Alternatively, substituting the oocyte-specific Dnmt1 with the somatic Dnmt1 may have developmental repercussions as well.

Acknowledgments

This work was supported by the Medical Research Council of Canada (to H.J.C. and to J.M.T.) and the National Institute of Health (to T.H.B. and R.C.). C.M. is a recipient of the Eileen Peters McGill Major Fellowship and J.M.T. is a Medical Research Council of Canada Scientist and a F.R.S.Q Chercheur-Boursier.

Figure 1. RT-PCR analyses of Dnmt1 mRNA splice variants in tissues, ovaries and oocytes. A somatic- (201 bp) and an oocyte-specific Dnmt1 transcript (235 bp) were reverse transcribed and PCR-amplified from the same source of total RNA and subjected to electrophoresis on 12% polyacrylamide gels. (A) A 1s transcript was detected in all tissues from a day 70 male and female mouse, while a 1o transcript was exclusively seen in the ovary. (B) The 1s transcript was present in all developing ovaries, whereas the 1o transcript was detected in postnatal day 1 to day 70 ovaries. (C) Total RNA from 80 isolated oocytes of each class of development was divided into two portions for the RT-PCR analyses of the 1s and 1o transcripts. All classes of isolated oocytes showed signs of the 1s transcript whereas the 1o transcript became apparent as early as postnatal day 5 and was maintained in growing oocytes through to metaphase II. Shown is a representative gel from 1 of 3 independent experiments.

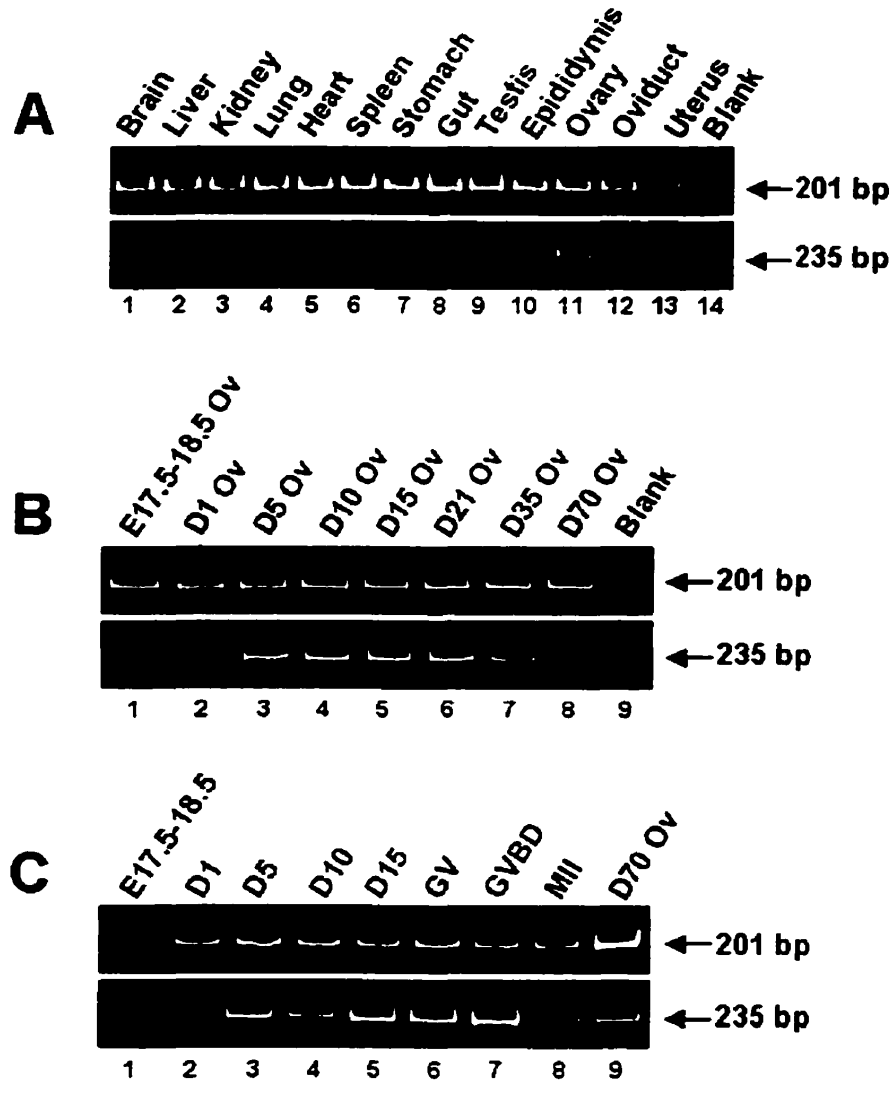

A
Brain
Liver
Kidney
Lung
Heart
Spleen
Stomach
Gut
Testis
Epididymis
Ovary
Oviduct
Uterus
Blank
201 bp
235 bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
E17.5-18.5 Ov
D1 Ov
D5 Ov
D10 Ov
D15 Ov
D21 Ov
D35 Ov
D70 Ov
Blank
201 bp
235 bp
1 2 3 4 5 6 7 8 9
C
E17.5-18.5
D1
D5
D10
D15
GV
GVBD
MII
D70 Ov
201 bp
235 bp
1 2 3 4 5 6 7 8 9

Figure 2. Dnmt1-specific antibodies and immunoblot analyses of Dnmt1 isoforms in oocytes. (A) Schematic of Dnmt1 protein identifying N-terminal sequences for the affinity-purified UPT82 and pATH52 antibodies. The UPT82 antibody recognizes various epitopes between amino acids 1-118 to solely identify the M_r 190,000 Dnmt1 species, while the pATH52 antibody recognizes multiple epitopes between 255-753 (Bestor, 1992; Li et al., 1992; Leonhardt et al., 1992) to detect the M_r 190,000 form and a smaller Mr 175,000 variant (Mertineit et al., 1998). NLS, nuclear localization signal; PBHD, Polybromo-1 homology domain; TRF; sequence for targetting to replication foci; ZnB, zinc binding domain. (B) Immunoblot probed with the UPT82 antibody shows the M_r 190,000 form of Dnmt1 in tissue lysates but not in isolated oocytes. (C) Same blot as in (B) reprobed with the pATH52 antibody reveals the M_r 175,000 variant in all classes of isolated oocytes except for day 1 oocytes, in addition to the full-length Dnmt1 in ovary lysates. Lane 1, day 70 testis (20 µg); Lane 2, day 10 uterus/oviduct (10 µg); Lane 3, day 10 ovary (10 µg); Lane 4, n=400 day 1 oocytes; Lane 5, n=400 day 5 oocytes; Lane 6, n=200 day 10 oocytes; Lane 7, n=200 day 15 oocytes; Lane 8, n=50 GV oocytes; Lane 9, n=50 MII oocytes; Lane 10, day 10 ovary (10 µg); Lane 11, day 49 ovary (15 µg); Lane 12, day 70 testis (20 µg). The presence of protein in all lanes was verified by staining with Ponceau S. Shown is a representative blot from 2 independent experiments.

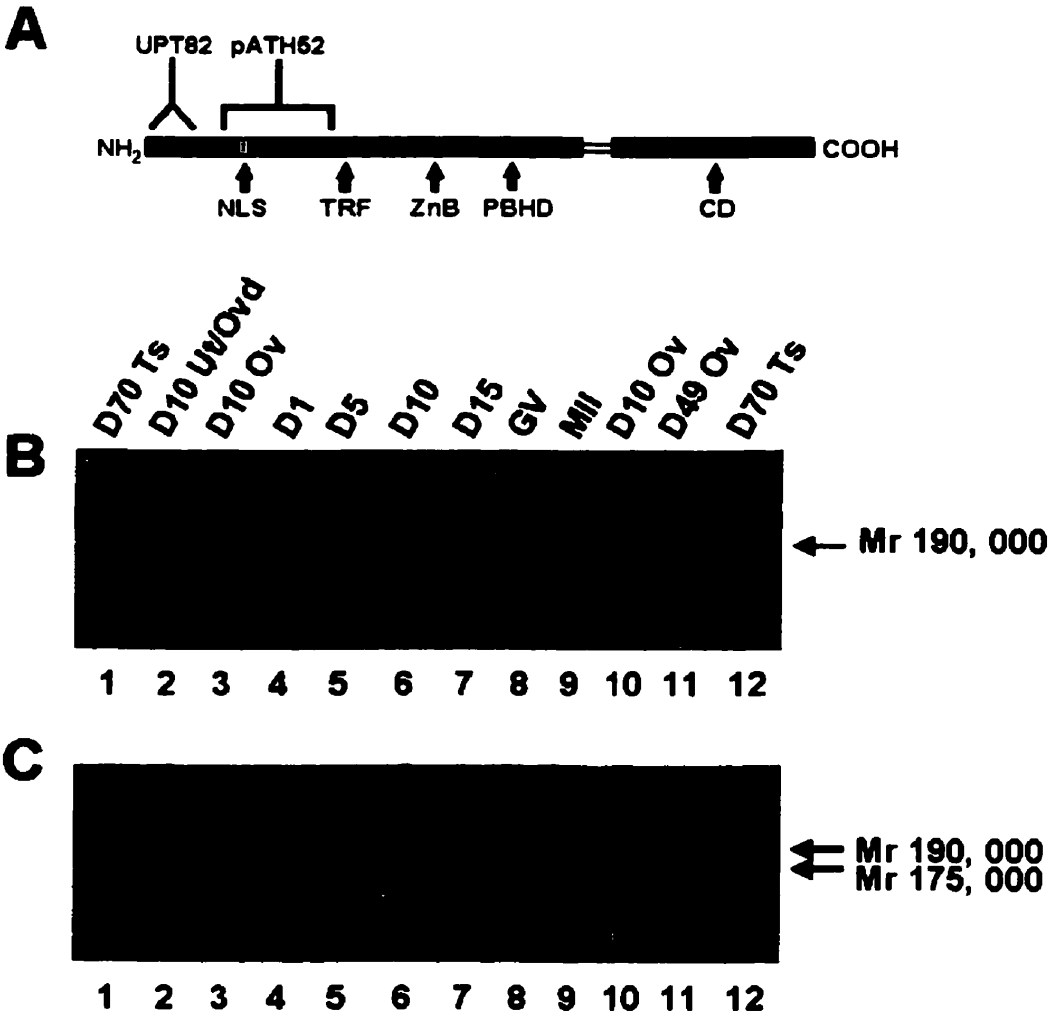

A
UPT82
pATH52
NH2
COOH
NLS
TRF
ZnB
PBHD
CD
B
D70 Ts
D10 UvOvd
D10 Ov
D1
D5
D10
D15
GV
MII
D10 Ov
D49 Ov
D70 Ts
Mr 190, 000
1 2 3 4 5 6 7 8 9 10 11 12
C
Mr 190, 000
Mr 175, 000
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3. Localization of Dnmt1 isoforms in growing oocytes. Isolated oocytes (A) and small ovarian follicles (B) were fixed in 3.7% formaldehyde in PBS and processed immunocytochemically for the detection of either the full-length or truncated Dnmt1 isoforms (See Materials and Methods). The DNA is marked by DAPI in blue and Dnmt1 is represented in red. Panel (A) is immunostaining with pATH52 to localize the full-length and truncated Dnmt1 isoforms, whereas panel (B) is immunostaining with UPT82 to localize only the full-length Dnmt1. For panel (A): (a,b,b') day 5 oocyte; (c,d,d') day 10 oocyte; (e,f,f') day 15 oocyte; (g,h,h') fully grown GV oocyte. Note that (a-h) were obtained by epifluorescent microscopy while (b'-h') represent an optical slice through the z-plane by confocal microscopy. In (B), serial sections of a small follicle by confocal microscopy show signs of the full-length Dnmt1 in surrounding somatic cells but not in the oocyte. Together, these data indicate that only the smaller Dnmt1 isoform is present in growing oocytes.

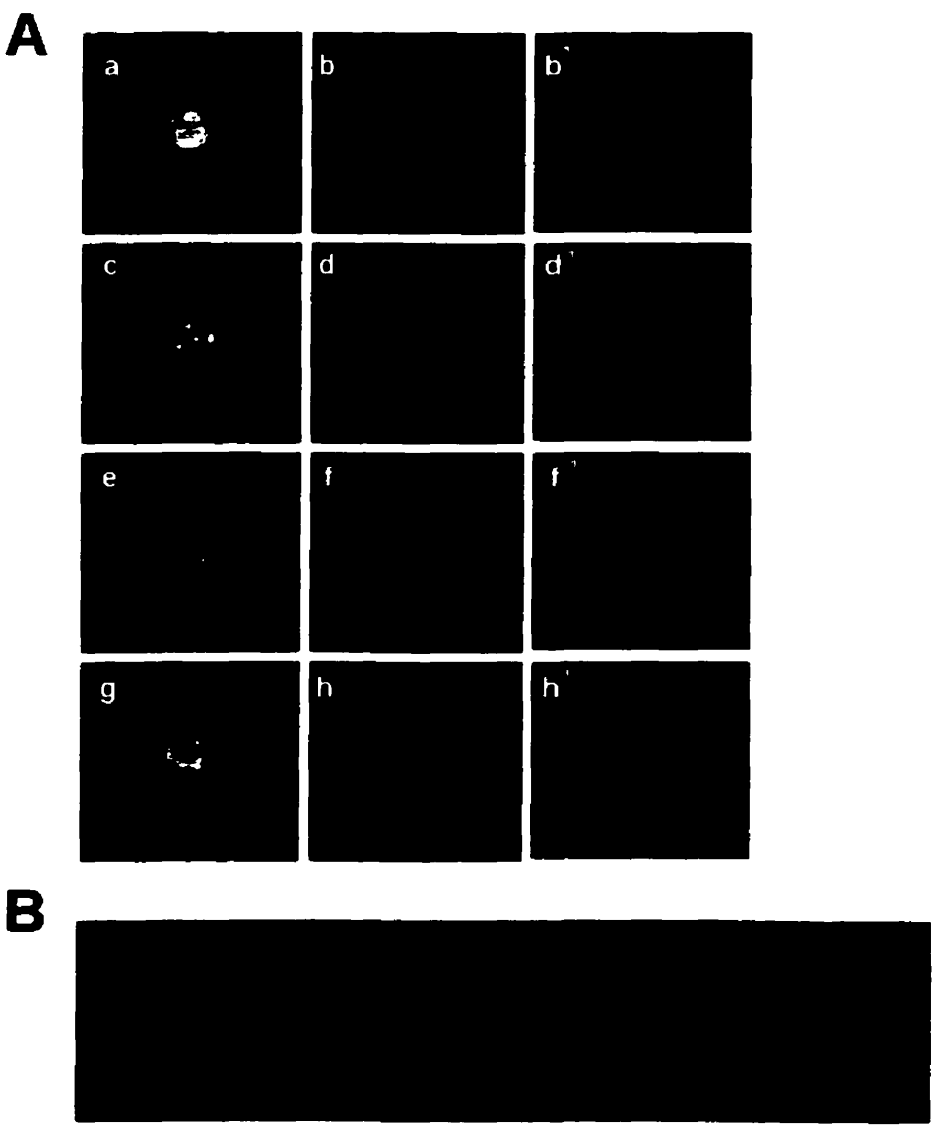
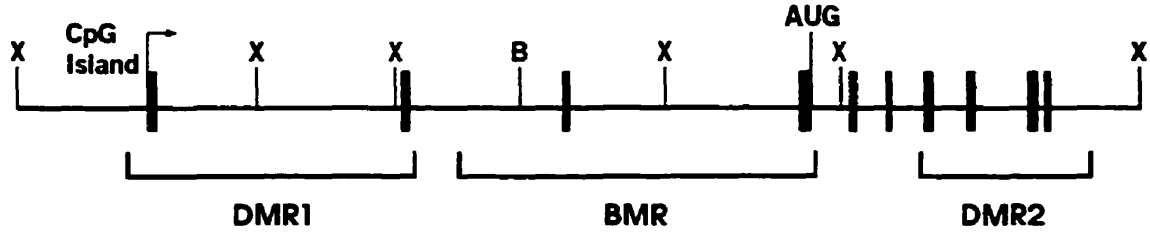

A
a
b
b'
c
d
d'
e
f
f'
g
h
h'
B

Figure 4. Genomic structure of the 5' end of the mouse *Snrpn* gene. (A) Schematic diagram of the 5' *Snrpn* locus showing the CpG island upstream of the transcription start site and exon 1. The *Srnpn* gene contains two differentially methylated regions (*DMRs*): a maternally-methylated region at the 5' end (*DMR1*), which correlates inversely with the Snrpn paternal expression, and a paternally methylated region at the 3' end (*DMR2*) (Shemer et al., 1997); *BMR*, biallelic methylated region. The restriction endonuclease sites shown are: X, *Xba*I; B, *Bam*HI (B) Location of CpG dinucleotides and regions subjected to bisulfite mutagenesis in the upstream region of the *Snrpn* gene. Primers used to amplify a 420 bp *Snrpn* product are underlined. CpG sites are numbered and boxed. The transcription start site is indicated by a right-handed arrow.

A.



B.

```
2041  5' -  TGTGTGTGTG  TGTGTGTGTG  TGTGTGATGC  ACTATGTAAC  ATGATATAGC  CTAGAAACCA
2101        GTCTTCCTCA  TATTGGAGAT  CAAACCTTTT  TTCCTCTCCC  ACATAGTAAA  AATCTGTGTG
2161        ATGCTTGCAA  TCACTTGGGA  ACAATTTTTT  AAAAAAATTA  AATGTATTTA  GTAATAGGCA
2221        ATTATATCCA  TTATTCCAGA  CTGACAGTGA  TTTTTTTTTA  AATACACGCT  CAAATTTCCG
2281        TAGTAGGAAT  GTTCAAGCAT  TCCTTTTGGT  AGCTGCCTTT  TGGCAGGACA  TTCCGGTCAA
2341        AGGGACATAG  ACCCCTGCAT  TGCCGCAAAA  ATGTGCGCAT  GTGCAGCCAT  TGCCTGGGAC
2401        GCATGCGTAG  GGAGCCGCGC  GACAAACCTG  AGCCATTGCG  GCAAGACTAG  CGCAGAGAGG
2461        AGAGGGAGCC  GGAGATGCCA  GACGCTTGGT  TCTGAGGAGT  GATTTGCAAC  GCAATGGAGC
2521        GAGGAAGGTC  AGCTGGGCTT  GTGGATTCTA  GTAGTGAAAG  TGCATCCTAT  TTGACCAAAA
2581        CATTCTAGAT  TTGGGCTTAT  TAAGATTTTT  GACAACCCAG  ATACCTTTAT  TTTTGAGAAT  - 3'
```

Figure 5. Profile of *Snrpn* methylation during postnatal oocyte development. A total of 16 CpG sites in the *Snrpn DMR1* were assayed by the bisulfite mutagenesis procedure as described in the Materials and Methods. (A) Day 1 oocytes. (B) Day 10 oocytes. (C) Metaphase II oocytes. The data indicate a gradual increase in cytosine methylation during oocyte growth and the full acquisition of the methylation imprint by metaphase II.

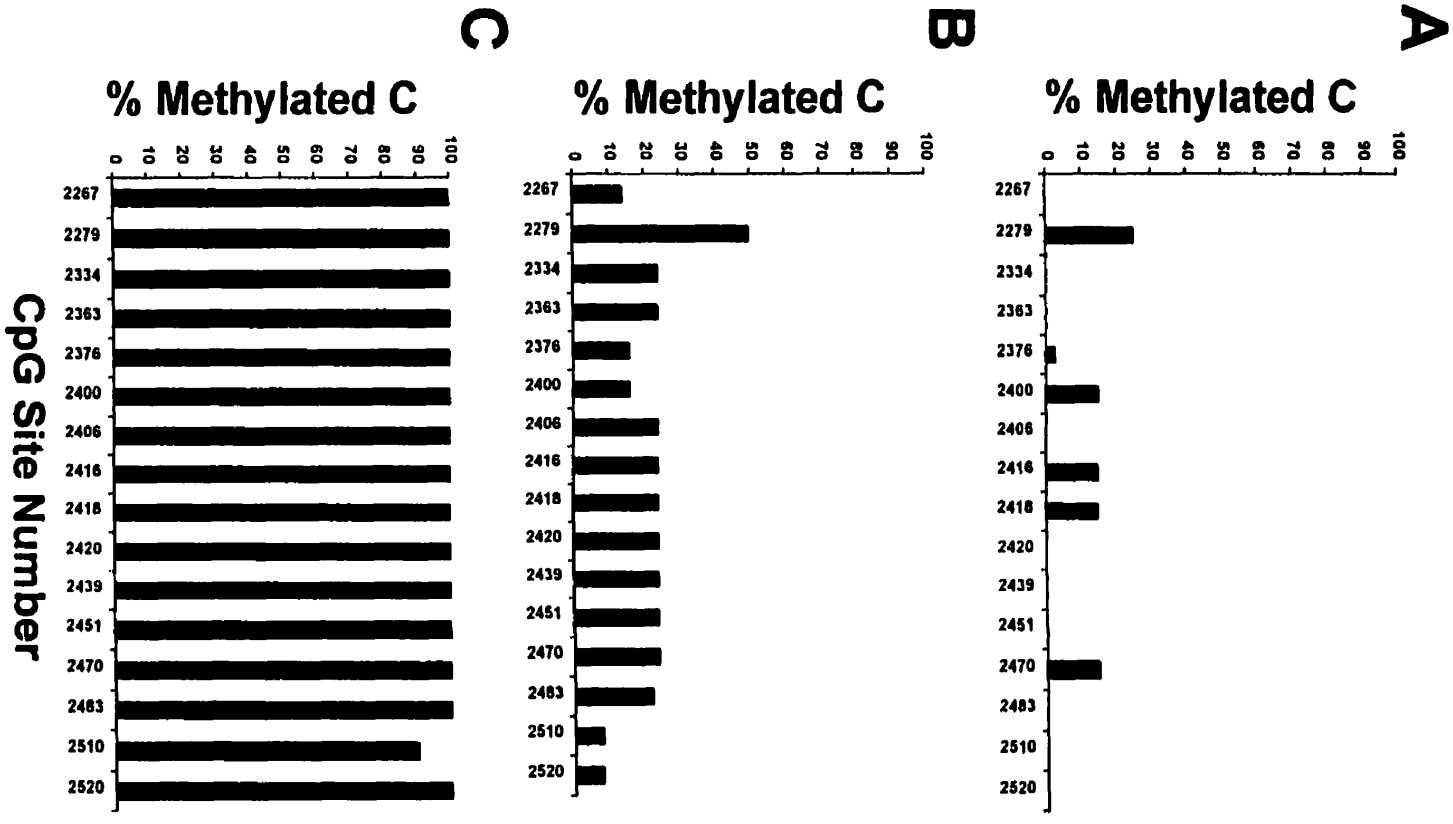

C
% Methylated C
0 10 20 30 40 50 60 70 80 90 100
2267
2279
2334
2363
2376
2400
2406
2416
2418
2420
2439
2451
2470
2483
2510
2520
CpG Site Number
B
% Methylated C
0 10 20 30 40 50 60 70 80 90 100
2267
2279
2334
2363
2376
2400
2406
2416
2418
2420
2439
2451
2470
2483
2510
2520
A
% Methylated C
0 10 20 30 40 50 60 70 80 90 100
2267
2279
2334
2363
2376
2400
2406
2416
2418
2420
2439
2451
2470
2483
2510
2520

Figure 6. Summary of *Snrpn* methylation data for individual clones. The same data displayed in Figure 5 are shown here for individual clones to illustrate the unique mosaic pattern of specific cytosine methylation during postnatal oocyte development. Two batches of 10-25 clones each were assayed in day 1 (A), day 10 (B) and metaphase II arrested oocytes (C). Each line corresponds to an individual strand of DNA. Sixteen CpG dinucleotides are represented (shown 5' to 3') by the lollipops perpendicular to the line. A filled circle corresponds to a methylated cytosine, and an open circle to an unmethylated cytosine. Those cytosines that could not be read on the sequencing gels are represented as the stick of the lollipop.

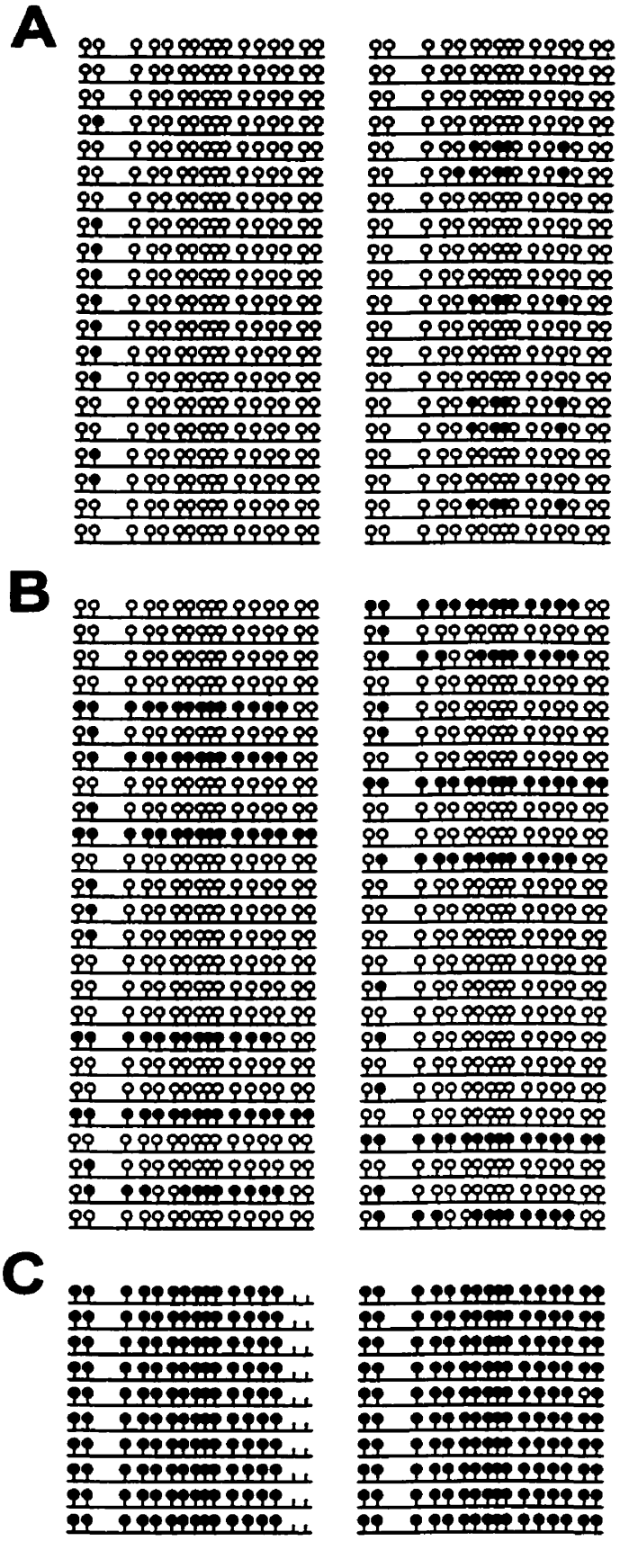
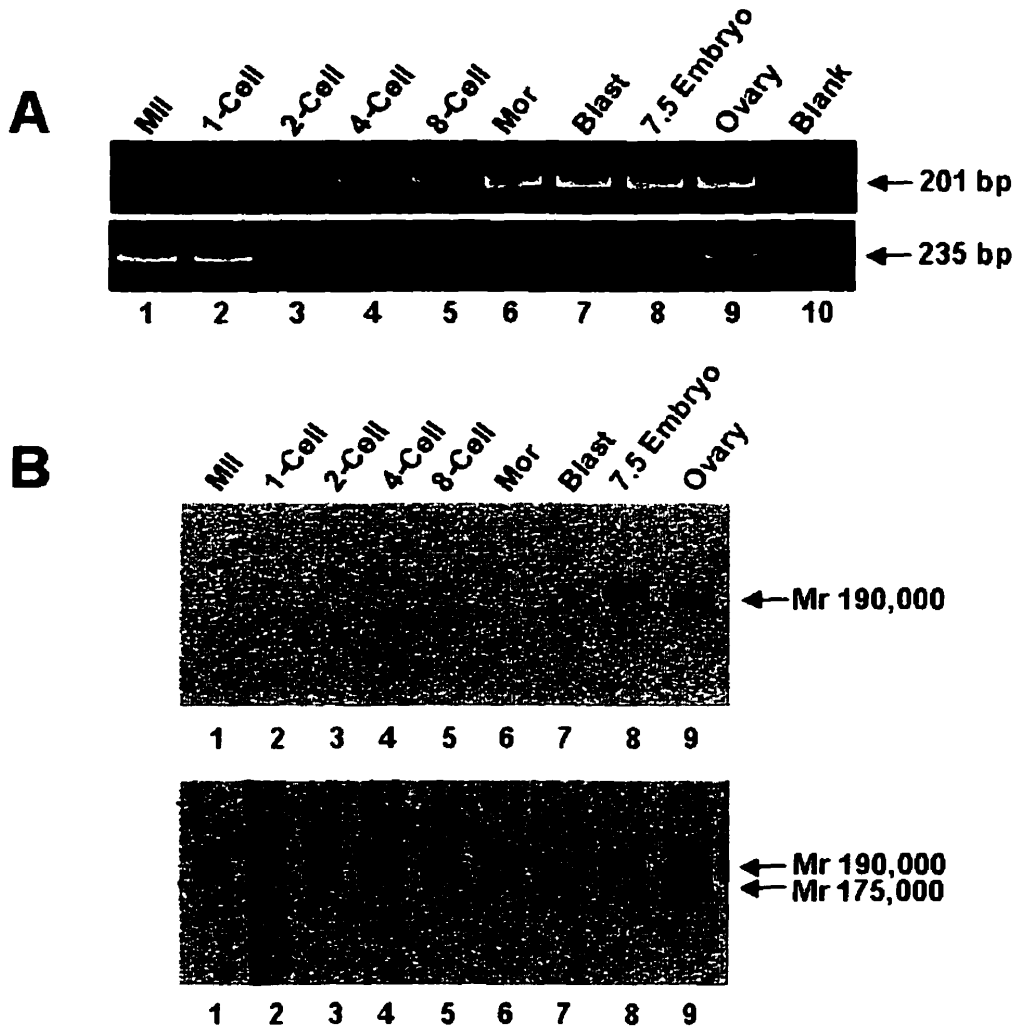

A
B
C

Figure 7. Expression and localization of Dnmt1 variants in early embryos. (A) RT-PCR analyses of the 1s and 1o Dnmt1 transcripts in early mouse embryos were performed as described in Materials and Methods. Top panel, 1s Dnmt1 splice variant; Bottom panel, 1o Dnmt1 splice variant (B) Immunoblot analyses of Dnmt1 isoforms in early embryos. Top panel, UPT82; Bottom panel, pATH52. A total of 30 mature oocytes and preimplantation embryos were loaded in lanes 1-7; Lane 8, postimplanted embryo at 7.5 dpc (15 µg); Lane 9, day 49 ovary (15 µg). (C) Immunolocalization and confocal microscopy of the full-length and smaller Dnmt1 isoforms in 8-cell embryos. (1) UPT82 shows no signs of the full-length Dnmt1 in the 8-cell embryo. (2) Immunostaining with pATH52 shows uniform nuclear (except for nucleoli) and granular staining in the cytoplasm only at the 8-cell stage, as reported previously (Carlson et al., 1992). These data suggest that the smaller Dnmt1 isoform in preimplantation embryos is the only Dnmt1 species present at this time, as the 1s transcript was apparently not translated.


A
MII
1-Cell
2-Cell
4-Cell
8-Cell
Mor
Blast
7.5 Embryo
Ovary
Blank
201 bp
235 bp
1 2 3 4 5 6 7 8 9 10
B
MII
1-Cell
2-Cell
4-Cell
8-Cell
Mor
Blast
7.5 Embryo
Ovary
Mr 190,000
1 2 3 4 5 6 7 8 9
Mr 190,000
Mr 175,000
1 2 3 4 5 6 7 8 9
C
1
2

CHAPTER FIVE

Discussion

Discussion

In this dissertation, I have examined the expression and regulation of Dnmt1 during gametogenesis in order to understand the potential role of the enzyme in the establishment and maintenance of DNA methylation patterns. Here I will provide a general overview of the major findings and suggest future directions in which this project could proceed. I will speculate upon the mechanisms that underlie the establishment and propagation of gametic imprints during development and discuss the implications of my work toward assisted reproduction, disease and cloning.

5.1 Overview of Major Findings

This project was initiated by undertaking a comparison between the expression profile of Dnmt1 in male as compared to female germ cell development in an attempt to identify differences in the amount, size, or localization of Dnmt1 mRNA and protein (see Chapter Two). The pattern of expression for this enzyme was similar in mitotic oogonia and spermatogonia and gradually decreased to lower levels during meiotic prophase until pachytene, where it was no longer detected. Surprisingly, Dnmt1 was re-expressed following pachytene in the female but not the male. Of interest, the down-regulation of Dnmt1 at pachytene was associated with the production of a larger mRNA transcript in the male but not the female. These findings suggested that the transcriptional and post-transcriptional regulation of this enzyme could differ between sexes and from its previously characterized behaviour in fibroblast and ES cells (Leonhardt et al., 1992; Yoder et al., 1996). Combined with germ cell-specific methylation data in the literature, this comparative approach led to the identification of putative windows in gametogenesis during which Dnmt1 may set down sex- and sequence-specific DNA methylation patterns. I propose that *de novo* methylation is completed prior to the pachytene stage of meiosis in the male germline and during oocyte growth in the female germline.

In order to determine the molecular mechanisms that regulate the differential expression of Dnmt1 mRNA and protein localization between male and female germ cells, a search for alternatively spliced exons in the 5' end of *Dnmt1* was conducted (see Chapter Three). Two novel exons were identified and characterized. One of these 5' exons (exon 1o) was associated with the production of a smaller functional protein in growing oocytes of the female, while the second (exon 1p) gave rise to a larger, non-translated mRNA specific to pachytene spermatocytes of the male. These data are the first to show transcriptional and post-transcriptional regulatory mechanisms of Dnmt1 in germ cells that depend on the alternative splicing of female- and male-specific exons. Of great importance is the finding that Dnmt1 was present in oocyte nuclei during the growth phase at a time when DNA replication does not occur, suggesting that it may have a *de novo* methylation function in growing oocytes. This hypothesis is also supported by nuclear transplantation studies which have identified the oocyte growth phase as a critical window for the establishment of maternal-specific methylation imprints (Kono et al., 1996; Obata et al., 1998). In addition, the accumulation of large quantities of Dnmt1 protein in the cytoplasm of growing oocytes is unusual for a replication factor and indicates that it may play an additional role in preimplantation development, (as discussed in more detail below). Prior to this study, there was evidence of only one functional mammalian Dnmt1 of M_r 190,000 (Leonhardt et al., 1992; Li et al., 1992) that was associated with the alternative splicing of the 5' 1s exon (Yoder et al., 1996). The molecular basis of the smaller isoform originally reported by Carlson et al. (1992) was determined by the work in this thesis (Mertineit et al., 1998) and by the later functional studies of Gaudet et al. (1998). What was not clear in these studies, however, was whether both the somatic- (M_r 190,000) and oocyte- (M_r 175,000) species of Dnmt1 were present in growing oocytes or whether there was only one predominant active species.

The purpose of the studies in the Fourth Chapter was to focus on the dynamics of Dnmt1 expression and regulation in the window of postnatal

oocyte development and to determine its potential function(s) in oocytes and early embryos. The somatic- and oocyte-forms of Dnmt1 mRNA and protein were characterized in isolated populations of oocytes obtained at different times of development and correlated with the methylation status of the maternally-imprinted *Snrpn* gene. The data provide compelling evidence of a highly regulated smaller Dnmt1 isoform that is produced by the alternative splicing of exon 1o in the 5' region of *Dnmt1* during oocyte growth and maturation. Of significance, the timing of *de novo* methylation on *Snrpn* during postnatal oogenesis is consistent with the 'trafficking' behaviour of Dnmt1 (Chapter Four and Mertineit et al., 1998) and with the substantial *de novo* methyltransferase activity reported previously in mature oocytes (Carlson et al., 1992). An interesting observation is that the methylation of *Snrpn* was not acquired in a clonal fashion on all alleles but exhibited a mosaic pattern of differentially methylated alleles during the growth phase and was fully established on all alleles in metaphase II oocytes. Examination of the different forms of Dnmt1 protein in early embryos indicated that the smaller oocyte-derived isoform was apparently the only functional Dnmt1 species during preimplantation development and was followed by a switch to the larger somatic form during postimplantation development. Surprisingly, the appearance of the somatic-specific Dnmt1 mRNA but not its corresponding protein during oocyte and preimplantation development could be due to either translational repression or 'leaky' transcriptional regulation of *Dnmt1*. Based on the present findings and those of others (Carlson et al., 1992; Gaudet et al., 1998), I propose that the oocyte-specific Dnmt1 plays a role in the establishment of maternal methylation imprints during postnatal oogenesis and is important for the propagation of parental imprints in the early embryo. Although the data indicate a correlation between the appearance of the oocyte-specific Dnmt1 and the establishment of the methylation imprint on *Snrpn* during postnatal oocyte development, the ultimate proof requires that the inactivation or removal of this enzyme species

leads to the loss of methylation on *Snrpn* and other maternally methylated genes (as discussed below).

5.2 Future Directions

In this thesis, several novel observations were presented with respect to the expression, localization and regulation of Dnmt1 throughout male and female gametogenesis. I will discuss a few key observations that I believe, if followed up, will have significant impact on our understanding of the erasure and establishment of gametic imprints, as well as the role of Dnmt1 and DNA methylation in gametogenesis. I will also suggest other avenues of exploration.

A) Primordial germ cells

Primordial germ cells (PGCs) are unique because maternal and paternal genomes are epigenetically equivalent only at this stage of development (Kato et al., 1999). Despite evidence that PGCs are globally demethylated and potentially imprint-free (Monk et al., 1987; Tada et al., 1997; Tada et al., 1998), we detected Dnmt1 at high levels in these cells in our studies (Chapter Two). This finding indicates that other factors may be involved in protecting the DNA from *de novo* methylation by Dnmt1, that Dnmt1 is biologically nonfunctional at this time of development, or that there is an active overriding demethylase activity.

To address these possibilities, large quantities of isolated PGCs or well-characterized cell lines, such as the EG cell line (totipotent permanent cell line derived from PGCs; Matsui et al., 1992) are required. Techniques that permit the isolation of viable PGCs have been described previously (Buehr and McLaren, 1993), and require positive cell identification with PGC-specific antibodies and/or cell sorting, but these isolation procedures are laborious and the purity of the PGCs may not be absolute. Alternatively, it should be easier and more efficient to isolate populations of PGCs of higher purity from a transgenic line that utilizes an endogenous promoter of a PGC-

specific gene (*Oct3/4*) to drive the expression of a fluorescent biomarker, such as green fluorescent protein (GFP) (Chris Wylie, personal communication). With these techniques in mind, let us consider the approaches that could be used to address the above scenarios.

One factor that may be involved in the protection of PGC DNA from ectopic *de novo* methylation is alterations in chromatin structure. To my knowledge, little work has been done to address the role of chromatin structure in regulating global or specific gene expression in PGCs. Studies that probe chromatin structure by assessing its sensitivity to nuclease digestion could be performed on DNA isolated from a large population of PGCs or on EG cells. Using the nuclease hypersensitivity assay, the unmethylated maternal allele of *H19* was shown to have an alteration in chromatin conformation that is mediated by protein-DNA interaction rather than DNA methylation in somatic cells (Hark and Tilghman, 1998).

To determine whether Dnmt1 is active in PGCs requires enzyme activity assays. Previous reports have indicated that Dnmt1 is active towards both unmethylated and hemimethylated substrates in mature oocytes (Carlson et al., 1992) and developing testes (Benoit and Trasler, 1994), and is the predominant *de novo* methyltransferase in genital ridges and embryos screened with a FdC oligonucleotide (Yoder et al., 1997b). Similar approaches which measure either the incorporation of radiolabelled methyl groups onto unmethylated and hemimethylated substrates or utilize a universal mechanism-based probe to screen for new species of DNA methyltransferases could be used in isolated PGCs or EG cells to assess the activity and forms of DNA methyltransferases. Alternatively, the role of Dnmt1 in the origin of the germline could be determined by performing a conditional knock-out of the enzyme with the Cre recombinase system driven by a PGC specific promoter to selectively inactivate Dnmt1 in these early germ cells.

With the recent identification of a *bone fide* demethylase enzyme (Bhattacharya et al., 1999), it would be interesting to examine its role in the

demethylation process in PGCs and at other times in development when global methylation levels are known to decrease, such as during preimplantation development. Molecular tools that characterize the demethylase mRNA and its activity at CpG sites are presently available (Bhattacharya et al., 1999). However, the generation of a specific demethylase antibody would facilitate further characterization at the protein level, where it could be used in Western blots and even in cytological preparations of PGCs and preimplantation embryos to determine its subcellular localization at these stages of development.

Together, these studies may help shed light on the mechanisms of erasure of methylation patterns in PGCs.

B) Dnmt1 expression in early meiosis

The finding of Dnmt1 nuclear expression in early meiotic cells of both sexes indicates that this enzyme may have an alternate function that is distinct from its maintenance methylation role in replicating cells. To determine the possible role of Dnmt1 in early meiotic germ cell development, the testis provides an ideal system for analysis, in which spermatogenesis is an ongoing process and the early meiotic events can be studied in a stage-specific fashion *in situ* (Oakberg, 1956b) or in enriched populations of isolated germ cells (Bellvé et al., 1977). It has been suggested that Dnmt1 may play a role in the establishment or maintenance of paternal imprints, or possibly in DNA repair or X-chromosome inactivation in early testicular spermatocytes (Jue et al., 1995a). Recently, the timing of the full acquisition of the methylation imprint on the *H19* gene (Davies et al., 1999) was found to correlate with the localization of Dnmt1 in discrete nuclear foci of early meiotic leptotene/zygotene spermatocytes (Jue et al., 1995a). To elucidate a role for Dnmt1 during early meiosis, a conditional knock-out of Dnmt1 in early meiotic spermatocytes could be produced using a previously established testicular Cre recombinase system (Vidal et al., 1998). In this case, the Cre enzyme driven by promoter sequences of the synaptonemal complex protein (Sycp1)

clips out the selected *Dnmt1* sequence that has been lox P flanked, and thus inactivates Dnmt1 only in leptotene/zygotene spermatocytes where the Sycp1 promoter is active. Ideally, it would be better to inactivate Dnmt1 even earlier, such as in spermatogonia or preleptotene spermatocytes; however, specific promoters for these germ cell types have not been identified yet. These conditional knock-out mice could be used to examine the methylation and expression of paternally imprinted and X-linked genes. It is also possible that Dnmt1 is expressed in early meiotic cells to methylate and subsequently inactivate genes not needed at later stages of germ cell development. If this is the case, a lack of Dnmt1 expression in meiotic cells would be expected to lead to ectopic expression of genes in spermatocytes and could result in abnormalities in germ cell development and testicular morphology. Studies that could address the role of Dnmt1 in repair include co-localization experiments with repair enzymes in cytological and histological preparations. Alternatively, repair could be induced with DNA damaging agents and the levels of expression and cellular localization of Dnmt1 and DNA repair enzymes could be determined.

C) Down-regulation of Dnmt1 protein at pachytene

A gradual down-regulated expression of Dnmt1 in early meiotic germ cells was observed in both the male and female, and may provide a mechanism to protect meiotic recombination intermediates from ectopic *de novo* methylation. Ectopic *de novo* methylation would be expected to interfere with gene expression during germ cell development and potentially promote germline mutations. Several types of DNA structures including four-way junctions, mismatches and hemimethylated sites appear during meiotic recombination and are highly preferred substrates for Dnmt1 that could allow CpG sites that are not normally methylated to become vulnerable to ectopic methylation (Bestor and Tycko, 1996). Of interest, the 6.0 kb message that appears during pachytene in the male mouse was not detected during the down-regulation of Dnmt1 protein in mouse pachytene oocytes (Chapter Two)

or rat pachytene spermatocytes (Jue et al., 1995b), suggesting that this mechanism of protection is sex-specific within the mouse species and perhaps also species-specific. Based on the genomic structure of the 5' end of the murine *Dnmt1* gene, it is postulated that exon 1p interferes with translation and prevents production of a functional Dnmt1 protein; however, the role of the 6.0 kb pachytene spermatocyte-specific Dnmt1 mRNA is unknown (Mertineit et al., 1998). Nevertheless, Dnmt1 protein is not detected in late pachytene germ cells of male or female mice or male rats. To determine if meiotic chromosomes are protected from ectopic methylation by the down-regulation of Dnmt1, transgenic mice could be produced that over-express Dnmt1 under a testis-specific pachytene promoter, like phosphoglycerate kinase-2 (PGK2), or an earlier leptotene/zygotene germ cell promoter, like Sycp1. These transgenic males could then be used to examine germ cell development and subsequent progeny outcome, in addition to the methylation status of critical genes, such as tumour suppressor genes. The use of the murine PGK2 promoter in transgenic mice has previously been used to successfully over-express *c-mos* in pachytene spermatocytes (Higgy et al., 1995).

D) The use of alternate first exons in Dnmt1

Controversy surrounding the vast diversity of methylation patterns in the vertebrate genome has raised the question of whether there may be different functional isoforms of the predominant Dnmt1 or alternate *de novo* methyltransferases. In this thesis, we show that a unique Dnmt1 isoform of M_r 175,000 is produced during postnatal oogenesis from an oocyte-specific first exon, exon 1o, and differs by 118 amino acids at the N-terminus from the full-length somatic form of M_r 190,000, originating from first exon 1s (Mertineit et al., 1998). A larger testis-specific Dnmt1 transcript that is associated with exon 1p does not appear to be translated and may not contribute to the creation of genomic methylation patterns (Trasler et al., 1992; Mertineit et al., 1998). Recent evidence for the existence of alternate Dnmt1 isoforms with

different N-terminal (Gaudet et al., 1998) and C-terminal (Deng and Szyf, 1998) extensions has been documented. However, functional data for only the M_r 175,000 (Carlson et al., 1992; Gaudet et al., 1998) and M_r 190,000 (Leonhardt et al., 1992; Li et al., 1992; Yoder et al., 1997b; Lui et al. 1998) Dnmt1 species have been reported to date.

The finding of sex-specific *Dnmt1* exons, 1o and 1p, indicates that there may be sex-specific promoters. The 1o and 1p specific promoters could be potentially useful for transgenic studies aimed at over- or under-expressing genes during oocyte growth in the female or in pachytene spermatocytes of the male, respectively. Characterization of the main regulatory elements of the sex-specific promoters is warranted and requires further cloning and sequencing of the genomic region upstream of exons 1o and 1p. Future studies that characterize the sequences to which transcription factors bind the 1o and 1p promoter elements using gel shift assays with nuclear extracts from isolated oocytes and fractionated pachytene spermatocytes would provide valuable information on how the female and male germ cell-specific Dnmt1 mRNAs are transcriptionally regulated. Furthermore, the use of transgenic mice that express different deletion constructs of the promoter regulatory elements linked to a reporter gene would identify sequences in the promoter that are required for germ cell-specific expression *in vivo*.

E) Role of the oocyte-specific Dnmt1 isoform

The tight correlation between the timing of expression of the oocyte-specific Dnmt1 and the acquisition of the methylation imprint on *Snrpn* during oocyte growth and maturation is potentially of great interest for our understanding of the mechanisms and timing of establishment of methylation imprints in the female germline (Chapter Four). One approach to determining if there is a link between these two events, is to produce a targeted disruption of exon 1o in order to abolish the production of the M_r 175,000 Dnmt1 isoform during oocyte growth. This experimental approach seems reasonable as

exon 1o lies ~5 kb upstream of exon 1s; thus, a knock-out of exon 1o is predicted not to interfere with the regulation of the somatic form of Dnmt1.

In the ideal experiment, not only should a disruption of exon 1o fail to produce any functional oocyte-specific Dnmt1 protein, but the somatic form of Dnmt1 should not be able to substitute for the oocyte form, so that the disruption of Dnmt1 in postnatal oogenesis is a null mutation rather than a partial loss-of-function mutation. Our results and those of Carlson et al. (1992) indicate that the oocyte-specific Dnmt1 is the only functional protein present during postnatal oocyte development since the 1s transcript appeared not to be translated. As a first step, it will be important to determine if oocytes develop normally in the absence of the M_r 175,000 form of Dnmt1 in the Dnmt1^{1o} -/- females. If oocyte development is perturbed, then it will be of interest to determine the role of the oocyte-specific Dnmt1 and DNA methylation in oocyte growth and maturation. However, if the Dnmt1^{1o} -/- oocytes develop normally to metaphase II, then it will be possible to assess the role of the oocyte-specific Dnmt1 in the establishment of maternal methylation imprints. Analyses of the methylation on *Snrpn* or any other maternally imprinted gene, such as *Peg1/Mest*, *Peg3* or *Igf2r* would be expected to be decreased or abolished in mature metaphase II oocytes derived from the Dnmt1^{1o} -/- females. Overall, these studies will determine whether the oocyte-specific Dnmt1 is critical for the establishment of maternal methylation imprints in the window of oocyte growth and maturation.

Follow-up studies of the forms of Dnmt1 mRNA and protein during preimplantation embryo development indicated that the only active enzyme species at this time of development is the maternal store of the smaller Dnmt1 isoform (Chapter Four; Carlson et al., 1992). Previous reports have noted that early mouse embryos contain very high levels of Dnmt1 protein and activity, both of which decline after the eight-cell stage (Monk et al., 1991; Carlson et al., 1992). Potential roles of the maternally-derived Dnmt1 isoform during preimplantation development include the propagation of gametic imprints and/or the silencing of endogenous retroviral sequences, particularly

at the 8-cell stage when this isoform was localized to the nuclei. Embryos produced from matings between Dnmt1^{1o} -/- males and females could be examined for developmental defects, perturbations in the methylation and expression of imprinted and X-linked genes, and the over-expression of endogenous retroviral sequences. Alternatively, the offspring produced by such a cross may be embryo lethal at an earlier time in development than the Dnmt1^{N}, Dnmt1^{S} and Dnmt1^{C} knock-outs reported previously (Li et al., 1992; Lei et al., 1996). In fact, the latter three knock-outs were probably able to survive to 8.5-10.5 dpc due to the large cytoplasmic stores of enzyme that were present in the oocyte at the time of fertilization.

If the knock-out of the M_r 175,000 Dnmt1 isoform is indeed a null mutation, but methylation imprints remain unchanged in oocytes and embryos, then it could suggest that other *de novo* DNA methyltransferases could be involved in the establishment and maintenance of gametic imprints. At present, good candidates for *de novo* methylation in oocytes and early embryos include *Dnmt3α* and *Dnmt3β*, which have recently been cloned and characterized at the level of the gene and mRNA (Okano et al., 1998b). Knock-outs of these two *de novo* methyltransferases are currently being produced. It would also be useful to generate specific antibodies in order to examine the expression of the two enzymes in developing oocytes and early embryogenesis.

Many exciting possibilities remain to be explored in the direction of the oocyte-specific Dnmt1 in order to gain a better understanding of its biological function. Other functional assays that could be done include substitution experiments using transgenic technology, in which the oocyte form of Dnmt1 replaces the somatic form in somatic cells, or the somatic form replaces the oocyte form in oocytes. Alternatively, over-expression of the oocyte-specific Dnmt1 may have developmental consequences where it may lead to hypermethylation of specific sequences and promote cancer.

F) Establishment of methylation imprints

The timing of when methylation imprints are established in the germline is not well defined and the mechanisms by which they are acquired remains even more of a mystery. My studies (Chapter Four) and those of others (Stöger et al., 1993; Kono et al., 1996) indicate that maternal imprints may be established during oocyte growth and maturation. To further understand the dynamics of how methylation imprints are established in the female germline, it will be important to examine the methylation status of multiple CpG sites for other maternally imprinted genes such as *Peg1/Mest*, *Peg3* and *Igf2r* at different stages of oocyte development. Does methylation occur at the same time during oocyte growth for all maternally imprinted genes or are they imprinted at different stages of oocyte development? Seeking the answers to such questions will provide key information to the mechanisms of genomic imprinting.

Fascinating new studies by Davies and colleagues (1999) indicate that the maternal and paternal alleles acquire methylation patterns differentially during gametogenesis. Examination of the paternally-methylated *H19* gene indicates that the methylation mark on the paternal allele is completed first prior to the onset of spermatogenesis, while the methylation of the maternal allele lags behind and is not completed until, or shortly prior to, the pachytene stage of spermatogenesis (Davies et al., 1999). In our studies (Chapter Four), the methylation of *Snrpn* exhibited a mosaic pattern of differentially methylated alleles during the oocyte growth phase and was fully established on all alleles by metaphase II, suggesting that a similar phenomenon of differential methylation on parental alleles may occur in the female germline. To test this hypothesis, interspecific mouse hybrids where the maternal and paternal alleles can be distinguished by a polymorphism could be used with the genomic bisulfite sequencing technique to assay allele-specific methylation of *Snrpn* in different classes of oocytes. A similar approach has been used for *H19* in male germ cells and early embryos (Tremblay et al., 1995; Davies et al., 1999). In addition, examination of the methylation status

at the *Snrpn DMR1* locus should be extended to include fetal oocytes prior to birth, in order to determine whether there is complete erasure of methylation. Preliminary data on *H19* indicate that PGCs from midgestation embryos are unmethylated at all CpG sites on the maternally- and paternally-derived parental alleles, and the fact that the paternal allele becomes methylated before the maternal allele indicates that a form of epigenetic 'memory' exists on the paternally-derived allele (Davies et al., 1999). Together, these studies will further define the timing of erasure and establishment of maternal methylation imprints and provide clues regarding the nature of 'memory' on gametic imprints.

G) 'Trafficking' behaviour of Dnmt1 during oocyte and early embryo development

The intracellular 'trafficking' behaviour of the oocyte-specific Dnmt1 during postnatal oocyte and preimplantation development is dynamic and quite different from the 'trafficking' behaviour of the somatic Dnmt1 in proliferating cells. The Dnmt1 isoform showed a nuclear distribution and accumulated to high levels in the cytoplasm during oocyte growth and later showed a subcortical distribution in metaphase II oocytes (Mertineit et al., 1998; Chapter Four). During preimplantation development, this isoform remained concentrated in the peripheral cytoplasm up until the four-cell stage, was present in the cytoplasm and nuclei at the eight-cell stage, after which time it was restricted to the cytoplasm where it remained until the blastocyst stage (Carlson et al., 1992). A search of the literature has not uncovered any other proteins that show similar dynamics in the nuclear and cytoplasmic distributions during oocyte and early embryo development. This unusual 'trafficking' behaviour cannot be attributed to the lack of a critical sequence motif in the N-terminus, as the smaller isoform appears to be identical to the full-length somatic form, except that it lacks the first 118 amino acids. The role of these 118 amino acids is not clear, but expression studies in somatic cells using Dnmt1 deletion constructs tagged with a fluorescent GFP

indicated that both the somatic and oocyte-specific forms were targetted to the nucleus and to replication foci at S-phase (Lui et al., 1998). Based on this observation and our results, I propose that there must be proteins in the oocyte cytoplasm which interact with Dnmt1 to regulate its cytoplasmic localization both in oocytes and early embryos. To test this hypothesis, co-immunoprecipitation studies or the yeast-two hybrid system could be used to identify possible protein players in ovulated oocytes. Co-immunoprecipitation studies of the human Dnmt1 in somatic cells has demonstrated that the enzyme forms a complex with proliferating-cell nuclear antigen (PCNA) and that this complex is a target for $p21^{waf1}$ (Chuang et al., 1997). Such protein-protein interaction studies for Dnmt1 have not been carried out in germ cells, but could yield important information as to why such large quantities of this replication factor are required for either the oocyte or the early embryo. As mentioned previously, it is suspected that the oocyte cytoplasmic stores of Dnmt1 may be what allows development to progress to 8.5-10.5 dpc in the mutant Dnmt1 embryos (Li et al., 1992; Lei et al., 1996).

5.3 Implications

The findings in this thesis may have important implications for new reproductive technologies and certain clinical imprinting syndromes. Since genomic imprinting is inherent to gametogenesis and can affect subsequent somatic development in the offspring, procedures involving the manipulation of gametes should take into account the timing of when imprints are established and erased. For example, with the use of early male germ cells, including round spermatids for intracytoplasmic sperm injection (ICSI) as a treatment for male infertility, it is important to determine when the imprinting process on the paternal allele is complete. Failure to consolidate a paternal imprint prior to fertilization could, at least in theory, lead to tumor susceptibility and developmental abnormalities in the offspring (Tycko et al., 1994). Likewise, a similar situation may apply to oocytes. In addition, certain human diseases such as Prader-Willi, Angelman and Beckwith-Wiedemann arise

from a disruption in normal imprinting and are often associated with dysregulated methylation patterns at imprinted loci (Tycko et al., 1994). In these cases, there are imprinting errors (chromosomal deletion, uniparental disomy or mutation) which lead to either the lack of expression of a given imprinted gene through the biallelic methylation of the parental alleles, or the expression from both alleles due to failure to methylate one allele in the germline. With important epigenetic marks like methylation being established in the adult as oocytes grow and mature, we need to be concerned about drugs or environmental exposures that may disrupt this process.

The exciting new discoveries concerning the cloning of mammals have triggered a need to understand the role of epigenetics in the reprogramming of a somatic cell nucleus. The cloning of a sheep from an adult somatic nucleus derived from the udder resulted in the birth of Dolly (Wilmut et al., 1997) and has been the subject of much controversy (Sgaramella and Zinder, 1998). Shortly afterwards, it was reported that two generations of identical clones (Cumulina and her pups) could be derived from the same cumulus cell nucleus in mice (Wakayama et al., 1998). Despite the recent success of Dolly and Cumulina and her pups, most cloning attempts have led to failures, suggesting that epigenetics may play a role. It is quite possible that failure to reset methylation patterns and epigenetic states, as is predicted to occur during gametogenesis (Tucker et al., 1996b), may affect the development of clonal offspring derived from transplantation of nuclei from differentiated cells into enucleated oocytes. Even if it becomes possible to clone animals with fair efficiency, genomic imprinting and other epigenetic effects might give rise to substantial and unpredictable phenotypic variation among clonal offspring (Bestor, 1998b).

5.4 Summary

Overall, Dnmt1 is highly regulated during gametogenesis at the level of the gene, message and protein. The discovery of a unique oocyte-specific Dnmt1 isoform that is restricted to postnatal oocyte and preimplantation

development suggests that it may play a role in the establishment and maintenance of DNA methylation imprints during development. Many facets of DNA methylation and demethylation in addition to the growing family of DNA methyltransferases remain to be studied during gametogenesis in order to gain a better understanding of the timing and mechanism by which gametic imprints are established and erased.

Original Contributions

1. Dnmt1 is found at high levels in male and female primordial germ cells and its distribution appears to be cell-cycle dependent in mitotically dividing germ cells.

2. In both the female and male germlines, Dnmt1 is expressed in preleptotene, leptotene and zygotene germ cells and is down-regulated at pachytene. While Dnmt1 is not re-expressed at later times of germ cell development in the male, the enzyme is re-expressed in growing diplotene oocytes in the female.

3. Sex-specific alternate first exons 1o and 1p lead to the production of an ovary-specific transcript of 5.1 kb and a testis-specific transcript of 6.0 kb, respectively.

4. An oocyte-specific Dnmt1 isoform exhibits dynamic changes in localization during oocyte growth and maturation. Non-growing oocytes show no signs of Dnmt1 staining; small oocytes show intense nuclear staining which becomes increasingly more cytoplasmic during the oocyte growth phase; ovulated oocytes show intense staining in the cortical region.

5. Characterization of the forms of Dnmt1 mRNA and protein in isolated populations of oocytes and early embryos indicates that the M_r 175,000 oocyte-specific isoform is restricted in expression to the window of oocyte growth and preimplantation embryo development, at a time when the M_r 190,000 form is not expressed.

6. The acquisition of the methylation imprint on *Snrpn* during oocyte growth and maturation correlates temporally with the timing of expression of the oocyte-specific Dnmt1.

CHAPTER SIX

References

References

Albertini, D.F. (1992). Cytoplasmic microtubular dynamics and chromatin organization during mammalian oogenesis and oocyte maturation. *Mut. Res.* **269**, 57-68.

Allen, E. (1922). The oestrous cycle in the mouse. *Am. J. Anat.* **30**, 297-371.

Amann, R.P. (1981). A critical review of methods for evaluation of spermatogenesis from seminal characteristics. *J. Androl.* **2**, 37-60.

Anderson, E. and Albertini, D.F. (1976). Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. *J. Cell Biol.* **71**, 680-686.

Ariel, M., Cedar, H. and McCarrey, J. (1994). Developmental changes in methylation of spermatogenesis-specific genes include reprogramming in the epididymis. *Nature Genet.* **7**, 59-63.

Ariel, M., Robinson, E., McCarry, J.R. and Cedar, H. (1995). Gamete-specific methylation correlates with imprinting of the murine *Xist* gene. *Nature Genet.* **9**, 312-315.

Bachvarova, R.F. A maternal tail of poly (A) – the long and the short of it. (1992). *Cell* **69**, 895-897.

Bakken, A.H. and McClanahan, M. (1978). Patterns of RNA synthesis in early meiotic prophase oocytes from fetal mouse ovaries. *Chromosoma* **67**, 21-40.

Barlow, D.P., Stöger, R., Herrmann, B.G., Saito, K. and Schweifer, N. (1991). The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature* **349**, 84-87.

Barlow, D.P. (1993). Methylation and imprinting: from host defense to gene regulation? *Science* **260**, 309-310.

Bartolomei, M.S. and Tilghman, S.M. (1997). Genomic imprinting in mammals. *Annu. Rev. Genet.* **31**, 493-525.

Beard, C., Li, E. and Jaenisch, R. (1995). Loss of methylation activates Xist in somatic but not in embryonic cells. *Genes & Dev.* **9**, 2325-2334.

Becker, P.B., Ruppert, S. and Schutz, G. (1987). Genomic footprinting reveals cell type-specific DNA binding of ubiquitous factors. *Cell* **51**, 435-443.

Bellvé, A.R. (1993). Purification, culture, and fractionation of spermatogenic cells. *Methods Enzymol.* **225**, 84-113.

Bellvé, A.R., Cavicchia, J.C., Millette, C.F., O'Brien, D.A., Bhatnagar, Y.M. and Dym, M. (1977). Spermatogenic cells of the prepubertal mouse: isolation and morphological characterization. *J. Cell Biol.* **74**, 68-85.

Benoit, G. and Trasler, J.M. (1994). Developmental expression of DNA methyltransferase messenger ribonucleic acid, protein, and enzyme activity in the mouse testis. *Biol. Reprod.* **50**, 1312-1319.

Bently, K.S. and Working, P.K. (1988). Use of seminiferous tubule segments to study stage specificity of unscheduled DNA synthesis in rat spermatogenic cells. *Environ. Mol. Mutagenesis* **12**, 285-297.

Bermingham, J.R. and Scott, M.P. (1993). Developmentally regulated alternative splicing of transcripts from the Drosophila homeotic gene Antennapedia can produce four different proteins. *EMBO J.* **7**, 3211-3222.

Bestor, T.H. (1992). Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. *EMBO J.* **11**, 2611-2617.

Bestor, T. H. (1996). DNA methyltransferase in mammalian development and host defense. In *Epigenetic Mechanisms of Gene Regulation.* (V. Russo, R. Matienssen, and A. D. Riggs, Eds.) pp. 61-76. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Bestor, T.H. (1998a). The host defense function of genomic methylation patterns. In *Epigenetics, Novartis Foundation Symposium, 214.* pp.187-199. Wiley, Chichester.

Bestor, T.H. (1998b). Cytosine methylation and the unequal developmental potentials of the oocyte and sperm genomes. *Am. J. Hum. Genet.* **62**, 1269-1273.

Bestor, T.H. and Ingram, V.M. (1983). Two DNA methyltransferases from murine erythroleukemia cells: purification, sequence specificity, and mode of interaction with DNA. *Proc. Natl. Acad. Sci. USA* **80**, 5559-5563.

Bestor, T., Hellewell, S.B. and Ingram, V.M. (1984). Differentiation of two mouse cell lines is accompanied by demethylation of their genomes. *Mol. Cell Biol.* **4**, 1800-1806.

Bestor, T. H., Laudano, A., Mattaliano, R. and Ingram, V. M. (1988). Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzyme is related to bacterial restriction methyltransferases. *J. Mol. Biol.* **203**, 971-983.

Bestor, T.H. and Coxon, A. (1993). The pros and cons of DNA methylation. *Curr. Biol.* **3**, 384-386.

Bestor, T. H. and Verdine, G. L. (1994). DNA methyltransferases. *Curr. Opin. Cell Biol.* **6**, 380-389.

Bestor, T. H. and Tycko, B. (1996). Creation of genomic methylation patterns. *Nature Genet.* **12**, 363-367.

Bhattacharya, S.K., Ramchandani, S., Cervoni, N. and Szyf, M. (1999). A mammalian protein with specific demethylase activity for mCpG DNA. *Nature* **397**, 579-583.

Bingel, A.S. and Schwartz, N.B. (1969). Timing of LH release and ovulation in the cyclic mouse. *J. Reprod. Fertil.* **19**, 223-229.

Bird, A.P. (1978). Use of restriction enzymes to study eukaryotic DNA methylation. II. The symmetry of methylated sites supports semiconservative copying of the methylation pattern. *J. Mol. Biol.* **118**, 49-60.

Borum, K. (1961). Oogenesis in the mouse: a study of the meiotic prophase. *Exp. Cell Res.* **24**, 495-507.

Borum, K. (1966). Oogenesis in the mouse: a study of the origin of the mature ova. *Exp. Cell Res.* **45**, 39-47.

Byskov, A.G. (1974). Cell kinetic studies of follicular atresia in the mouse ovary. *J. Reprod. Fertil.* **37**, 277-285.

Brambell, F.W.R. (1927). The development and morphology of the gonads of the mouse. Part I. The morphogenesis of the indifferent gonad and of the ovary. *Proc. Roy. Soc. B* **101**, 391-409.

Brandeis, M., Kafri, I., Ariel, M., Chaillet, J.R., McCarrey, J., Razin, A. and Cedar, H. (1993). The ontogeny of allele-specific methylation associated with imprinted genes in the mouse. *EMBO J.* **12**, 3669-3677.

Buehr, M. and McLaren, A. (1993). Isolation and culture of primordial germ cells. *Methods Enzymol.* **225**, 58-77.

Carlson, L.L., Page, A.W. and Bestor, T.H. (1992). Properties and localization of DNA methyltransferase in preimplantation mouse embryos: implications for genomic imprinting. *Genes Dev.* **6**, 2536-2541.

Cattanach, B.M. and Jones, J. (1994). Genetic imprinting in the mouse: implications for gene regulation. *J. Inher. Metab. Dis.* **17**, 403-420.

Chaillet, J.R. (1994). Genomic imprinting: lessons from mouse transgenes. *Mut. Res.* **307**, 441-449.

Chaillet, J.R., Vogt, T.F., Beier, D.R. and Leder, P. (1991). Parental-specific methylation of an imprinted transgene is established during gametogenesis and progressively changes during embryogenesis. *Cell* **66**, 77-83.

Chaillet, J.R., Bader, D.S. and Leder, P. (1995). Regulation of genomic imprinting by gametic and embryonic processes. *Genes Dev.* **9**, 1177-1187.

Chuang, L.S., Ian, H., Koh, T., Ng, H., Xu, G. and Li, B.F.L. (1997). Human DNA (cytosine-5)-methyltransferase-PCNA complex as a target for $p21^{waf1}$. *Science* **277**, 1996-2000.

DeChiara, T.M., Robertson, E.J. and Efstratiadis, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* **64**, 849-859.

Dollar, J.R., Hand, G.S., Beck, L.R. and Boots, L.R. (1979). The baboon as a primate model for the study of endometrium. *Am. J. Obst. Gyn.* **134**, 305-309.

Chiquoine, A.D. (1954). The identification, origin, and migration of the primordial germ cells in the mouse embryo. *Anat. Rec.* **118**, 135-146.

Cho, W.K., Stern, S. and Biggers, J.D. (1974). Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation *in vitro*. *J. Exp. Zool.* **187**, 383-386.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**,156-159.

Chouinard, L. (1991). A light- and electron-microscrope study of the nucleolus during growth of the oocyte in the prepubertal mouse. *J. Cell Sci.* **9**, 637-663.

Church, G.M. and Gilbert, W. (1984). Genomic sequencing. *Proc. Natl. Acad. Sci. USA* **81**, 1991-1995.

Clarke, H.J., Rossant, J. and Masui, Y. (1988). Suppression of chromosome condensation during meiotic maturation induces parthenogenetic development of mouse oocytes. *Development* **104**, 97-103.

Clarke, H.J., Oblin, C. and Bustin, M. (1992). Developmental regulation of chromatin composition during mouse embryogenesis: somatic histone H1 is first detectable at the 4-cell stage. *Development* **115**, 791-799.

Clarke, H.J., Bustin, M. and Oblin, C. (1997). Chromatin modifications during oogenesis in the mouse: removal of somatic subtypes of histone H1 from oocyte chromatin occurs post-natally through a post-transcriptional mechanism. *J. Cell Sci.* **110**, 477-487.

Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol. Rev.* **52**, 198-236.

Colot, V. and Rossignol, J.-L. (1999). Eukaryotic DNA methylation as an evolutionary device. *BioEssays* **21**, 402-411.

Constância, M. Pickard, B., Kelsey, G. and Reik, W. (1998). Imprinting mechanisms. *Genome Res.* **8**, 881-900.

Cooke, J.E., Godin, I., French-Constant, C., Heasman, J. and Wylie, C.C. (1993). Culture and manipulation of primordial germ cells. *Methods Enzymol.* **225**, 37-58.

Cooke, J., Heasman, J. and Wylie, C. (1996). The role of interleukin 4 in the regulation of mouse primordial germ cell numbers. *Dev. Biol.* **174**, 14-22.

Davey, C., Pennings, S. and Allan, J. (1997). CpG methylation remodels chromatin structure *in vitro*. *J. Mol. Biol.* **267**, 276-288.

Davies, K. (1992). Imprinting and splicing join together. *Nature* **360**, 492.

Davies, T.L., Trasler, J.M., Moss, S.B., Yang, G.J. and Bartolomei, M.S. (1999). Acquisition of the *H19* methylation imprint occurs differentially on the parental alleles during spermatogenesis. *Genomics* **58**, 18-28.

Debey, P., Szollosi, M.S., Szollosi, D., Vautier, D., Girouse, A. and Besombes, D. (1993). Competent mouse oocytes isolated from antral follicles exhibit different chromatin organization and follow different maturation dynamics. *Mol. Reprod. Dev.* **36**, 59-74.

Deng, J. and Szyf, M. (1998). Multiple isoforms of DNA methyltransferase are encoded by the vertebrate cytosine DNA methyltransferase gene. *J. Biol. Chem.* **273**, 22869-22872.

Dieckmann-Schuppert, A. and Schnittler, H.J. (1997). A simple assay for quantification of protein in tissue sections, cell cultures, and cell homogenates, and of protein immobilized on solid surfaces. *Cell & Tissue Res.* **288**, 119-126.

Dietrich, A.J.J. and Mulder, R.J.P. (1983). A light- and electron microscopic analysis of meiotic prophase in female mice. *Chromosoma* **88**, 377-385.

Dobson, M.J., Pearlman, R.E., Karaiskakis, A., Spyropoulos B., Moens, P.B. (1994). Synaptonemal complex proteins: occurrence, epitope mapping and chromosome disjunction. *J. Cell Sci.* **107**, 2749-2760.

Doerksen, T. and Trasler, J.M. (1996). Developmental exposure of male germ cells to 5-azacytidine results in abnormal preimplantation development in rats. *Biol. Reprod.* **55**, 1155-1162.

Donovan, P.J., Stott, D., Godin, I., Heasman, J. and Wylie, C.C. (1987). Studies on the migration of mouse germ cells. *J. Cell Sci Suppl.* **8**, 359-367.

Driscoll, D. J. and Migeon, B. R. (1990). Sex difference in methylation of single-copy genes in human meiotic germ cells: implications for X chromosome inactivation, parental imprinting, and origin of CpG mutations. *Somat. Cell Mol. Genet.* **16**, 267-282.

Ducibella, T., Duffy, P. and Buetow, J. (1994). Quantification and localization of cortical granules during oogenesis in the mouse. *Biol. Reprod.* **50**, 467-473.

Durand, M., Chevrie, K., Chassignol, M., Thuong, N.T. and Marizot, J.C. (1990). Circular dichroism studies of an oligodeoxyribonucleotide containing a hairpin loop made of a hexaethylene glycol chain: conformation and stability. *Nucl. Acids Res.* **18**, 6353-6359.

Dym, M. and Fawcett, D.W. (1970). The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biol. Reprod.* **3**, 308-326.

Dym, M. and Fawcett, D.W. (1971). Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. *Biol. Reprod.* **4**, 195-215.

Eddy, E.M. and Hahnel, A.C. (1983). Establishment of the germ cell line in mammals. In *Current Problems in Germ Cell Differentiation* (A. McLaren and C.C. Wylie, Eds.), pp. 41-70. Cambridge University Press, Cambridge.

Eden, S. and Cedar, H. (1994). Role of DNA methylation in the regulation of transcription. *Curr. Opin. Genet. Devel.* **4**, 255-259.

Edwards, J. B. D. M., Delort, J. and Mallet, J. (1991). Oligodeoxyribonucleotide ligation to single-stranded cDNAs: a new tool for cloning 5' ends of mRNAs and for constructing cDNA libraries by *in vitro* amplification. *Nucl. Acids Res.* **19**, 5227-5232.

Eppig, J.J. and Telfer, E. (1993). Isolation and culture of oocytes. *Methods Enzymol.* **225**, 77-84.

Eppig, J.J. and O'Brien, M.J. (1996). Development *in vitro* of mouse oocytes from primordial follicles. *Biol. Reprod.* **54**, 197-207.

Erbach, G.T., Lawitts, J.A., Papaioannou, V.E. and Biggers, J.D. (1994). Differential growth of the mouse preimplantation embryo in chemically defined media. *Biol. Reprod.* **50**, 1027-1033.

Evans, M.J. and Kaufman, M.H. (1981). Establishment of a culture of pluripotential cells from mouse embryos. *Nature* **292**, 154-156.

Everett, J.W. (1994). Pituitary and hypothalamus: perspective and overview. In *The Physiology of Reproduction, Second Edition* (E. Knobil and J.D. Neill, Eds.) pp. 1509-1526. Raven Press, Ltd., New York.

Fawcett, D.W., Ito, S. and Slautterback, D. (1959). The occurrence of intercellular bridges in groups of cells exhibiting synchronous differentiation. *J. Biophys. Biochem. Cytol.* **5**, 453-460.

Feinberg, A.P. and Vogelstein, B. (1984). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **137**, 266-267.

Finnegan, E.J., Peacock, W.J. and Dennis, E.S. (1996). Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. *Proc. Natl. Acad. Sci. USA* **93**, 8449-8454.

Fritz, I. (1978). Sites of actions of androgens and follicle stimulating hormone on cells of the seminiferous tubule. In *Biochemical Actions of Hormones* (G. Litwack, Ed.) pp. 249-278. Academic Press, New York.

Frohman, M. A. (1993). Rapid amplification of complementary DNA ends for generation of full-length complementary DNAs: thermal RACE. *Methods Enzymol.* **218**, 340-356.

Gabriel, J.M., Gray, T.A., Stubbs, L., Saitoh, S., Ohta, T. and Nicholls, R.D. (1998). Structure and function correlations at the imprinted mouse *Snrpn* locus. *Mam. Genome* **9**, 788-793.

Gaudet, F., Talbot, D., Leonhardt, H. and Jaenisch, R. (1998). A short DNA methyltransferase isoform restores methylation *in vivo*. *J. Biol. Chem.* **272**, 32725-32729.

Geballe, A.P. and Morris, D.R. (1994). Initiation codons within 5'-leaders of mRNAs as regulators of translation. *Trends Biochem.* **19**, 159-164.

Ginsburg, M., Snow, M.H.L. and McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development* **110**, 521-528.

Glickman, J.F., Pavlovich, J.G. and Reich, N.O. (1997). Peptide mapping of the murine DNA methyltransferase reveals a major phosphorylation site and the start of translation. *J. Biol. Chem.* **272**, 17851-17857.

Godin, I. and Wylie, C. (1991). TGF-β1 inhibits proliferation and has a chemotropic effect on mouse primordial germ cells in culture. *Development* **113**, 1451-1457.

Godin, I., Deed, R., Cooke, J., Zsebo, K., Dexter, M. and Wylie, C.C. (1991). Effects of the *Steel* gene product on mouse primordial germ cells in culture. *Nature* **352**, 807-809.

Gomperts, M., Wylie, C. and Heasman, J. (1994a). Primordial germ cell migration. In: *Germline Development, Ciba Foundation Symposium 182*, pp. 121-139. John Wiley & Sons Ltd., Chichester, England.

Gomperts, M., Garcia-Castro, M., Wylie, C. and Heasman, J. (1994b). Interactions between primordial germ cells play a role in their migration in mouse embryos. *Development* **120**, 135-141.

Gosden, R., Krapez, J. and Briggs, D. (1997). Growth and development of the mammalian oocyte. *BioEssays* **19**, 875-882.

Goto, K., Numata, M., Komura, J.-I., Ono, T., Bestor, T.H. and Kondo, H. (1994). Expression of DNA methyltransferase gene in mature and immature neurons as well as proliferating cells in mice. *Differentiation* **56**, 39-44.

Griswold, M.D. (1993). Action of FSH on mammalian Sertoli cells. In *The Sertoli Cell* (M.D. Griswold and L.D. Russell, Eds.), pp. 493-508. Cache River Press, Clearwater.

Gruenbaum, Y., Cedar, H. and Razin, A. (1982). Substrate and sequence specificity of a eukaryotic DNA methylase. *Nature* **295**, 620-622.

Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Munsterberg, A., Vivian, N., Goodfellow, P. and Lovell-Badge, R. (1990). A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature* **346**, 245-250.

Hake, L.E. and Hecht, N.B. (1993). Utilization of an alternative transcription start site of somatic cytochrome c in the mouse produces a testis-specific cytochrome c mRNA. *J. Biol. Chem.* **268**, 4788-4797.

Haqq, C.M. and Donahoe, P.K. (1998). Regulation of sexual dimorphism in mammals. *Physiol. Rev.* **78**, 1-33.

Hark, A.T. and Tilghman, S.M. (1998). Chromatin conformation of the H19 epigenetic mark. *Hum. Mol. Genet.* **7**, 1979-1985.

Hecht, N.B. (1995). The making of a spermatozoon: a molecular perspective. *Dev. Genet.* **16**, 95-103.

Higgy, N.A., Zackson, S.L. and Van der Hoorn, F.A. (1995). Cell interactions in testis development: overexpression of *c-mos* in spermatocytes leads to increased germ cell proliferation. *Dev. Genet.* **16**, 190-200.

Hilscher, B., Hilscher, W., Bülthoff-Ohnholz, B., Krämer, U., Birke, A., Pelzer, H. and Gauss, G. (1974). Kinetics of gametogenesis. I. Comparative histological and autoradiographic studies of oocytes and transitional prospermatogonia during oogenesis and prespermatogenesis. *Cell Tissue Res.* **154**, 443-470.

Hilscher, W. (1991). The genetic control and germ cell kinetics of the female and male germ line in mammals including man. *Hum. Reprod.* **6**, 1416-1425.

Hogan, B., Constantini, F. and Lacy E. (1986). *Manipulating the Mouse Embryo: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Holliday, R. and Pugh, J.E. (1975). DNA modification mechanisms and gene activity during development. *Science* **187**, 226-232.

Howlett, S.K. and Reik, W. (1991). Methylation levels of maternal and paternal genomes during preimplantation development. *Development* **113**, 119-127.

Hurst, L.D., McVean, G. and Moore, T. (1996). Imprinted genes have few and small introns. *Nature Genet.* **12**, 234-237.

Iguchi-Ariga, S.M.M. and Schaffner, W. (1989). CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation. *Genes Dev.* **3**, 612-619.

Jähner, D., Stuhlmann, H., Stewart, C.L., Harbers, K., Löhler, J., Simon, I. and Jaenisch, R. (1982). De novo methylation and expression of retroviral genomes during mouse embryogenesis. *Nature* **298**, 623-628.

Jähner, D. and Jaenisch, R. (1984). DNA methylation in early mammalian development. In: *DNA methylation*, (A. Razin, H. Cedar and A. Riggs, eds.), pp. 189-219, Springer-Verlag, Berlin.

Jones, P.A. (1996). DNA methylation errors and cancer. *Cancer Res.* **56**, 2463-2467.

Jones, P.A. and Taylor, S.M. (1980). Cellular differentiation, cytidine analogs and DNA methylation. *Cell* **20**, 85-93.

Jones, P.L., Veenstra, G.J., Wade, P.A., Vermaak, D., Kass, S.U., Landsberger, N., Strouboulis, J. and Wolffe, A.P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nature Genet.* **19**, 187-191.

Jost, A. (1953). Studies on sex differentiation in mammals. *Recent Prog. Horm. Res.* **8**, 379-418.

Jost, A., Vigier, B., Prepin, J. and Perchellet, J. (1973). Studies on sex differentiation in mammals. *Recent Prog. Horm. Res.* **29**, 1-41.

Jue K, Bestor TH, and Trasler JM. (1995a). Regulated synthesis and localization of DNA methyltransferase during spermatogenesis. *Biol. Reprod.* **53**, 561-569.

Jue, K., Benoit, G., Alcivar, A.A. and Trasler, J.M. (1995b). Developmental and hormonal regulation of DNA methyltransferase in the rat testis. *Biol. Reprod.* **52**, 1364-1371.

Kafri, T., Ariel, M., Brandeis, M., Shemer, R., Urven, L., McCarrey, J., Cedar, H. and Razin A. (1992). Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. *Genes Dev.* **6**, 705-714.

Kakutani, T., Jeddeloh, J. and Richards, E.J. (1995). Characterization of an *Arabidopsis thaliana* DNA hypomethylation mutant. *Nucl. Acids Res.* **23**, 130-137.

Kass, S.U., Pruss, D. and Wolffe, A.P. (1997a). How does DNA methylation repress transcription? *Trends Genet.* **13**, 444-449.

Kass, S.U., Landsberger, N. and Wolffe, A.P. (1997b). DNA methylation directs a time-dependent repression of transcription. *Curr. Biol.* **7**, 157-165.

Kato, Y., Rideout III, W.M., Hilton, K., Barton, S.C., Tsunoda, Y. and Surani, M.A. (1999). Developmental potential of mouse primordial germ cells. *Development* **126**, 1823-1832.

Kleene, K.C. (1996). Patterns of translational regulation in the mammalian testis. *Mol. Repro. Devel.* **43**, 268-281.

Kluin, P.M. and de Rooij, D.G. (1981). A comparison between the morphology and cell kinetics of gonocytes and adult type undifferentiated spermatogonia in the mouse. *Int. J. Androl.* **4**, 475-493.

Kimura, H., Suetake, I. and Tajima, S. (1999). Xenopus maintenance-type DNA methyltransferase is accumulated and translocated into germinal vesicles of oocytes. *J. BioChem.* **125**, 1175-1182.

Kono, T., Obata, Y., Yoshimzu, T., Nakahara, T. and Carroll, J. (1996). Epigenetic modifications during oocyte growth correlates with extended parthenogenetic development in the mouse. *Nature Genet.* **13**, 91-94.

Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P. and Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for Sry. *Nature* **351**, 117-121.

Kozak M. (1996). Interpreting cDNA sequences: some insights from studies on translation. *Mam. Genome* **7**, 563-574.

Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R.J. and Wilson, G.G. (1994). The DNA (cytosine-5) methyltransferases. *Nucl. Acids Res.* **22**, 1-10.

Laird, D.W., Castillo, M. and Kasprzak, L. (1995). Gap junction turnover, intracellular trafficking, and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor cells. *J. Cell Biol.* **131**, 1193-1203.

Laird, P.W. and Jaenisch, R. (1994). DNA methylation and cancer. *Hum. Mol. Genet.* **3**, 1487-1495.

Lalande, M. (1997). Parental imprinting and human disease. *Ann. Rev. Genet.* **30**, 173-195.

Lauster, R., Trautner, T.A. and Noyer-Widner, M. (1989). Cytosine-specific type II DNA methyltransferases. A conserved enzyme core with variable target-recognizing domains. *J. Mol. Biol.* **206**, 305-312.

Leblond, C.P. and Clermont, Y. (1952a). Spermiogenesis of rat, mouse, hamster and guinea pig as revealed by the "periodic acid-fuchsin sulfurous acid" technique. *Am. J. Anat.* **90**, 167-215.

Leblond, C.P. and Clermont, Y. (1952b). Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann. NY Acad. Sci.* **55**, 548-573.

Lei, H., Oh, S.P., Okano, M., Jüttermann, R., Goss, K.A., Jaenisch, R. and Li, E. (1996). De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development* **122**, 3195-3205.

Leighton, P.A., Saam, J.R., Ingram, R.S. Stewart, C.L. and Tilghman, S.M. (1995a). An enhancer deletion affects both *H19* and *Igf2* expression. *Genes Dev.* **9**, 2079-2089.

Leighton, P.A., Ingram, R.S., Eggenschwiler, J., Efstratiadis, A. and Tilghman, S.M. (1995b). Disruption of imprinting caused by deletion of the *H19* gene region in mice. *Nature* **375**, 34-39.

Lengauer, C., Kinzler, K.W. and Vogelstein, B. (1997). DNA methylation and genetic instability in colorectal cancer cells. *Proc. Natl. Acad. Sci. USA* **92**, 2545-2550.

Leonhardt, H., Page, A.W., Weier, H.-U. and Bestor, T.H. (1992). A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* **71**, 865-873.

Lerchner, W. and Barlow, D.P. (1997). Paternal repression of the imprinted mouse *Igf2r* locus occurs during implantation and is stable in all tissues of the post-implantation mouse embryo. *Mech. Dev.* **61**, 141-149.

Li, E., Bestor, T.H. and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69**, 915-926.

Li, E., Beard, C. and Jaenisch, R. (1993a). Role for DNA methylation in genomic imprinting. *Nature* **366**, 362-365.

Li, E., Beard, C., Forster, A.C., Bestor, T.H. and Jaenisch, R. (1993b). DNA methylation, genomic imprinting, and mammalian development. *Cold Spring Harb. Symp. Quant. Biol.* **LVIII**, 297-305.

Lifschytz, E. and Lindsley, D.L. (1972). The role of X-chromosome inactivation during spermatogenesis. *Proc. Natl. Acad. Sci. USA* **69**, 182-186.

Liu, Y., Oakeley, E.J., Sun, L. and Jost, J.P. (1998). Multiple domains are involved in the targeting of the mouse DNA methyltransferase to the DNA replication foci. *Nucl. Acids Res.* **26**, 1038-1045.

Lyon, M.F. (1961). Gene action in the X-chromosome of the mouse (*Mus musculus L.*). *Nature* **190**, 372-373.

Ma, Q., Alder, H., Nelson, K.K., Chatterjee, D., Gu, Y., Nakamura, T., Canaani, E., Croce, C.M., Siracusa, L.D. and Buchberg, A.M. (1993). Analysis of the murine ALL-1 gene reveals conserved domains with human ALL-

1 and identifies a motif shared with DNA methyltransferases. *Proc. Natl. Acad. Sci. USA* **90**, 6350-6354.

Malagnac, F., Wendel, B., Goyon, C., Faugeron, G., Zickler, D., Rossignol, J.-L., Noyer-Weidner, M., Vollmayr, P., Trautner, T.A. and Walter, J. (1997). A gene essential for de novo methylation and development in Ascobolus reveals a novel type of eukaryotic DNA methyltransferase structure. *Cell* **91**, 281-290.

Mandl, A.M. and Zuckerman, S. (1951). The relation of age to numbers of oocytes. *J. Endocrinol.* **7**, 190-193.

Martin, C.C., Laforest, L., Akimenko, M.A., Ekker, M. (1999). A role for DNA methylation in gastrulation and somite patterning. *Dev. Biol.* **206**, 189-205.

Martin, G.R. (1980). Teratocarcinomas and mammalian embryogenesis. *Science* **209**, 768-776.

Martin, G.R. (1981). Isolation of pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78**, 3585-3588.

Matsui, Y., Toksoz, D., Nishikawa, S., Nishikawa, S.-I., Williams, D., Zsebo, K. and Hogan, B.L.M. (1991). Effect of Steel factor and leukemia inhibitory factor on murine primordial germ cells in culture. *Nature* **353**, 750-752.

Matsui, Y., Zsebo, K. and Hogan, B.L.M. (1992). Derivation of pluripotent embryonic stem cells from murine primordial germ cells in culture. *Cell* **70**, 841-847.

Mattson, B.A. and Albertini, D.F. (1990). Oogenesis: chromatin and microtubule dynamics during meiotic prophase. *Mol. Reprod. Dev.* **25**, 374-383.

McCoshen, J.A. and McCallion, D.J. (1975). A study of the primordial germ cells during their migratory phase in steel mutant mice. *Experientia* **31**, 589-590.

McDonald, L.E., Paterson, C.A. and Kay, G.F. (1998). Bisulfite genomic sequencing-derived methylation profile of the *Xist* gene throughout early mouse development. *Genomics* **54**, 379-386.

McGrath, J. and Solter, D. (1983). Nuclear transplantation in mouse embryos. *J. Exp. Zool.* **228**, 355-362.

McGrath, J. and Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* **37**, 179-183.

McLaren, A. (1983). Primordial germ cells in mice. *Bibl. Anat.* **24**, 59-66.

McLaren, A. (1984). Meiosis and differentiation of mouse germ cells. In *Controlling Events in Meiosis, 38th Symp. Soc. Exp. Biol.* (C.W. Evans and H.G. Dickinson, Eds.) pp. 7-23. Cambridge University Press, Cambridge.

McLaren, A. (1988). The developmental history of female germ cells in mammals. *Oxf. Rev. Reprod. Biol.* **10**, 162-179.

McLaren, A. (1995). Germ cells and germ cell sex. *Philos. Trans. R. Soc. Lond.* **350**, 229-233.

McLaren, A. (1998). Germ cells and germ cell transplantation. *Int. J. Dev. Biol.* **42**, 855-860.

McMahon, A., Fosten, M. and Monk, M. (1981). Random X-chromosome inactivation in female primordial germ cells in the mouse. *J. Embryol. Exp. Morph.* **64**, 251-258.

Meehan, R., Lewis, J., Cross, S., Nan, X., Jeppesen P. and Bird, A. (1992). Transcriptional repression by methylation of CpG. *J. Cell Sci. Suppl.* **16**, 9-14.

Meric, F., Searfoss, A.N., Wormington, M. and Wolffe, A.P. (1996). Masking and unmasking maternal mRNA: the role of polyadenylation, transcription, splicing and nuclear history. *J. Biol. Chem.* **271**, 30804-30810.

Mertineit, C., Yoder, J.A., Taketo, T., Laird, D.W., Trasler, J.M. and Bestor, T.H. (1998). Sex-specific exons control DNA methyltransferase in mammalian germ cells. *Development* **125**, 889-897.

Mertineit, C., Taketo, T., Moens, P.B., Igdoura, S.A., Bestor, T.H. and Trasler, J.M. (1999). DNA methyltransferase expression marks putative windows for sex-specific methylation in germline. Submitted.

Migeon, B.R. (1994). X-chromosome inactivation: molecular mechanisms and genetic consequences. *Trends Genet.* **10**, 230-235.

Moens, P.B., Heyting, C., Dietrich, A.J.J. van Raamsdonk, W. and Chen, Q. (1987). Synaptonemal complex antigen location and conservation. *J. Cell Biol.* **105**, 93-103.

Monesi, V. (1962). Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis using tritiated thymidine. *J. Cell Biol.* **14**, 1-18.

Monk, M. and Harper, M.I. (1979). Sequential X chromosome inactivation coupled with cellular differentiation in early mouse embryos. *Nature* **281**, 311-313.

Monk, M. and McLaren, A. (1981). X chromosome activity in foetal germ cells of the mouse. *J. Embryol. Exp. Morph.* **63**, 75-84.

Monk, M., Boubelik, M. and Lehnert, S. (1987). Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Development* **99**, 371-382.

Monk, M., Adams, R. L. P. and Rinaldi, A. (1991). Decrease in DNA methylase activity during preimplantation development in the mouse. *Development* **112**, 189-192.

Morris, D.R. (1997). *Cis*-acting mRNA stuctures in gene-specific translational control. In *mRNA Metabolism and Post-transcriptional Gene Regulation.* (J.B. Harford and D.R. Morris, Eds.), pp. 165-180, Wiley-Liss, New York.

Motro, B., van der Kooy, D., Rossant, J., Reith, A. and Bernstein, A. (1991). Contiguous patterns of c-kit and steel expression: analysis of mutations at the *W* and *Sl* loci. *Development* **113**, 1207-1222.

Nagamine, C.M., Chan, K., Kozak, C.A. and Lau, Y.-F. (1989). Chromosome mapping and expression of a putative testis-determining gene in mouse. *Science* **243**, 80-83.

Nan, X., Ng, H.H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N. and Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393**, 386-389.

Narayan, G. and Raman, R. (1995). Cytological evaluation of global DNA methylation in mouse testicular genome. *Hereditas* **123**, 275-283.

Nebel, B.R., Amarose, A.P. and Hackett, E.M. (1961). Calendar of gametogenic development in the prepubertal male mouse. *Science* **134**, 832-833.

Ng, H.-H. and Bird, A. (1999). DNA methylation and chromatin modification. *Curr. Opin. Genet. Dev.* **9**, 158-163.

Niederreither, K., Harbers, M., Chambon, P. and Dollé, P. (1998). Expression of T:G mismatch-specific thymidine-DNA glycosylase and DNA methyltransferase genes during development and turmorigenesis. *Oncogene* **17**, 1577-1585.

Neumann, B., Kubicka, P., Barlow, D.P. (1995). Characteristics of imprinted genes. *Nat. Genet.* **9**, 12-13.

Numata, M., Ono, T. and Iseki, S. (1994). Expression and localization of the mRNA for DNA (cytosine-5)-methyltransferase in mouse seminiferous tubules. *J. Histochem. Cytochem.* **42**, 1271-1276.

Oakberg, E.F. (1956a). Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. *Am. J. Anat.* **99**, 507-516.

Oakberg, E.F. (1956b). A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am. J. Anat.* **99**, 391-413.

Obata, Y., Kaneko-Ishino, T., Koide, T., Takai, Y., Ueda, T., Domeki, I., Shiroishi, T., Ishino, F. and Kono T. (1998). Disruption in primary imprinting

during oocyte growth leads to the modified expression of imprinted genes during embryogenesis. *Development* **125**, 1553-1560.

Ogawa, O., Eccles, M.R., Szeto, J., McNoe, L.A., Yun, K., Maw, M.A., Smith, P.J. and Reeve, A.E. (1993). Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumor. *Nature* **362**, 749-751.

Ohsawa, K., Imai, Y., Ito, D. and Kohsaka, S. (1996). Molecular cloning and characterization of Annexin V-binding proteins with highly hydrophilic peptide structure. *J. Neurochem.* **67**, 89-97.

Okano, M., Xie, S. and Li, E. (1998a). Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. *Nucl. Acids Res.* **26**, 2536-2540.

Okano, M., Xie, S. and Li, E. (1998b). Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nature Genet. **19**, 219-220.

O'Keefe, C., Hulten, M.A. and Tease, C. (1997). Analysis of proximal X chromosome pairing in early mouse meiosis. *Chromosoma* **106**, 276-283.

Oko R.J., Jando, V., Wagner, C.L., Kistler, W.S. and Hermo, L.S. (1996). Chromatin reorganization in rat spermatids during the disappearance of testis-specific histone, H1t, and the appearance of transition proteins TP1 and TP2. *Biol. Reprod.* **54**, 1141-1157.

Olek, A. and Walter, J. (1997). The pre-implantation ontogeny of the *H19* methylation imprint. *Nature Genet.* **17**, 275-276.

Orlando, P., Grippo, P. and Geremia, R. (1984). DNA repair synthesis-related enzymes during spermatogenesis in the mouse. *Exp. Cell Res.* **153**, 499-506.

Orr-Utreger, A., Avivi, A., Zimmer, Y., Givol, D., Yarden, Y. and Lonai, P. (1990). Developmental expression of *c-kit*, a proto-oncogene encoded by the *W* locus. *Development* **109**, 911-923.

Palmer, S.J. and Burgoyne, P.S. (1991). The *Mus musculus domesticus Tdy* allele acts later than the *Mus musculus musculus Tdy* allele: a basis for XY sex-reversal in C57BL/6-YPOS mice. *Development* **113**, 709-714.

Panning, B. and Jaenisch, R. (1996). DNA hypomethylation can activate *Xist* expression and silence X-linked genes. *Genes Dev.* **15**, 1991-2002.

Parker, K.L., Schedl, A. and Schimmer, B.P. (1999). Gene interactions in gonadal development. *Annu. Rev. Physiol.* **61**, 417-433.

Pedersen, T. and Peters, H. (1968). Proposal for a classification of oocytes and follicles in the ovary of the mouse. *J. Reprod. Fert.* **17**, 555-557.

Pellas, T.C., Ramachandran, B., Duncan, M., Pan, S.S., Marone, M. and Chada, K. (1991). Germ-cell deficient (gcd), and insertional mutation manifested as infertility in transgenic mice. *Proc. Natl. Acad. Sci. USA* **88**, 8787-8791.

Peters, H. (1969). The development of the mouse ovary from birth to maturity. *Acta Endocrinologica* **62**, 98-116.

Philpott, C.C., Ringuette, M.J. and Dean, J. (1987). Oocyte-specific expression and developmental regulation of ZP3, the sperm receptor of the mouse zona pellucida. *Dev. Biol.* **121**, 568-575.

Ponzetto-Zimmerman, C. and Wolgemuth, D.J. (1984). Methylation of satellite sequences in mouse spermatogenic and somatic DNAs. *Nucl. Acids Res.* **12**, 2807-2822.

Posfai, J., Bhagwat, A.S., Posfai, G. and Roberts, R.J. (1989). Predictive motifs derived from cytosine methyltransferases. *Nucl. Acids Res.* **17**, 2421-2435.

Rainier, S., Johnson, L.A., Dobry, C.J., Ping, A.J., Grundy, P.E. and Feinberg, A.P. (1993). Relaxation of imprinted genes in human cancer. *Nature* **362**, 747-749.

Raman, R. and Narayan, G. (1995). 5-Aza deoxycytidine-induced inhibition of differentiation of spermatogonia into spermatocytes in the mouse. *Mol. Reprod. Dev.* **42**, 284-290.

Ramchandani, S., Bigey, P. and Szyf, M. (1998). Genomic structure of the human DNA methyltransferase gene. *Biol. Chem.* **379**, 535-540.

Rassoulzadegan, M., Paquis-Flucklinger, V., Bertino, B., Sage, J., Jasin, M., Miyagawa, K., Van Heyningen, V., Besmer, P. and Cuzin, F. (1993). Transmeiotic differentiation of male germ cells in culture. *Cell* **75**, 997-1006.

Razin, A. and Riggs, A.D. (1980). DNA methylation and gene function. *Science* **210**, 604-610.

Razin, A. and Cedar, H. (1993). DNA methylation and embryogenesis. In DNA Methylation: Molecular Biology and Biological Significance (J.P. Jost and H.P. Saluz) pp. 343-357. Birkhäuser Verlag, Basel, Switzerland.

Razin, A. and Cedar, H. (1994). DNA methylation and genomic imprinting. *Cell* **77**, 473-476.

Razin, A. and Shemer, R. (1995). DNA methylation in early development. *Hum. Mol. Genet.* **4**, 1751-1755.

Regev, A., Lamb, M.J. and Jablonka, E. (1998). The role of DNA methylation in invertebrates: developmental regulation or genome defense? *Mol. Biol. Evol.* **15**, 880-891.

Reik, W., Collick, A., Norris, M.L., Barton, S.C. and Surani, M.A. (1987). Genomic imprinting determines methylation of parental alleles in transgenic mice. *Nature* **328**, 248-251.

Resnick, J.L., Bixler, L.S., Cheng, L. and Donovan, P.J. (1992). Long-term proliferation of mouse primordial germ cells in culture. *Nature* **359**, 550-551.

Riggs, A.D. (1975). X-inactivation, differentiation, and DNA methylation. *Cytogenet. Cell Genet.* **14**, 9-25.

Rinaldini, L.M. (1959). An improved method for the isolation and quantitative cultivation of embryonic cells. *Experimental Cell Res.* **16**, 477-505.

Robaire, B. and Hermo, L. (1988). Efferent ducts, epididymis, and vas deferens: structure, functions and their regulation. In *The Physiology of Reproduction.* (E. Knobil and J. Neill, Eds.). pp 999-1080. Raven Press Ltd., New York.

Roller R.J., Kinloch, R.A., Hiraoka, B.Y., Li, S.S.-L. and Wassarman, P.M. (1989). Gene expression during mammalian oogenesis and early embryogenesis: quantification of three messenger RNAs abundant in fully grown mouse oocytes. *Development* **106**, 251-261.

Ronemus, M.J., Galbiati, M., Ticknor, C., Chen, J. and Dellaporta, S.L. (1996). Demethylation-induced developmental pleiotropy in *Arabidopsis*. *Science* **273**, 654-657.

De Rooij, D.G. and Kramer, M.F. (1967). Spermatogonial stem cell renewal in rats and mice. *Z. Zellforsch.* **85**, 206-209.

Rotwein, P. and Hall, L.J. (1991). Evolution of insulin-like growth factor II: characterization of the mouse Igf-II gene and identification of two pseudo-exons. *DNA* **9**, 725-735.

Rougier, N., Bourc'his, D., Gomes, D.M., Niveleau, A., Plachot, M., Paldi, A. and Viegas-Pequignot, E. (1998). Chromosome methylation patterns during mammalian preimplantation development. *Genes Dev.* **12**, 2108-2113.

Russell, L.D., Ettlin, R.A., Hikim, A.P.S. and Clegg, E.D. (1990). *Histological and Histopathological Evaluation of the Testis*. Cache River Press, Clearwater, Florida.

Salles, F.J., Darrow, A.L., O'Connell, M.L. and Strickland, S. (1992). Isolation of novel maternal mRNAs regulated by cytoplasmic polyadenylation. *Genes Dev.* **6**, 1202-1212.

Sanford, J., Forrester, L. and Chapman, V. (1984). Methylation patterns of repetitive DNA sequences in germ cells of Mus musculus. *Nucl. Acids Res.* **12**, 2823-2836.

Sanford, J.P., Clark, H.J., Chapman, V.M. and Rossant, J. (1987). Differences in DNA methylation during oogenesis and spermatogenesis and their

persistence during early embryogenesis in the mouse. *Genes Dev.* **1**, 1039-1046.

Sapienza, C., Peterson, A.C., Rossant, J. and Balling, R. (1987). Degree of methylation of transgenes is dependent on gamete of origin. *Nature* **328**, 251-254.

Sapsford, C.S. (1962). Changes of the cells of the sex cords and seminiferous tubules during the development of the testis of the rat and mouse. *Austr. J. Zool.* **10**, 178-192.

Sassone-Corsai, P. (1997). Transcriptional checkpoints determining the fate of male germ cells. *Cell* **88**, 163-166.

Schroeder, A.C. and Eppig, J.J. (1984). The developmental capacity of mouse oocytes that matured spontaneously *in vitro* is normal. *Dev. Biol.* **104**, 831-840.

Schäfer M., Nayernia, K., Engel, W. and Schäfer, U. (1995). Translational control in spermatogenesis. *Dev. Biol.* **172**, 344-352.

Schultz, R.M. (1986). Molecular aspects of mammalian oocyte growth and maturation. In *Experimental Approaches to Mammalian Embryonic Development* (J. Rossant and R.A. Pedersen, Eds), pp. 195-237, Cambridge University Press, Cambridge.

Schwartz, M.N., Trautner, T.A. and Kornberg, A. (1962). Enzymatic synthesis of DNA. *J. Biol. Chem.* **237**, 1961-1967.

Schwartz, N.B. (1974). The role of FSH and LH and of their antibodies on follicle growth and on ovulation. *Biol. Reprod.* **10**, 236-272.

Sgaramella, V. and Zinder, N.D. (1998). Dolly confirmation. *Science* **279**, 635.

Sharpe, R.M. (1994). Regulation of spermatogenesis. In *The Physiology of Reproduction, Second Edition* (E. Knobil and J.D. Neill, Eds.), pp. 1363-1434, Raven Press, Ltd., New York.

Shemer, R., Birger, Y., Riggs, A.D. and Razin, A. (1997). Structure of the imprinted mouse *Snrpn* gene and establishment of its parental-specific methylation pattern. *Proc. Natl. Acad. Sci. USA* **94**, 10267-10272.

Singer-Sam, J., Robinson, M.O., Bellvé, A.R., Simon, M.I. and Riggs, A.D. (1990). Measurement by quantitative PCR of changes in HPRT, PGK-1, PGK-2, APRT, MTase and Zfy gene transcripts during mouse spermatogenesis. *Nucl. Acids Res.* **18**, 1255-1259.

Solter, D. and Knowles, B.B. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. Natl. Acad. Sci. USA* **75**, 5565-5569.

Sorensen, R.A. and Wassarman, P.M. (1976). Relationship between growth and meiotic maturation of the mouse oocyte. *Dev. Biol.* **50**, 531-536.

Spector, D.L. (1993). Macromolecular domains within the cell nucleus. *Annu. Rev. Cell Biol.* **9**, 265-315.

Speed, R.M. (1982). Meiosis in the foetal mouse ovary. I. An analysis at the light microscope level using surface-spreading. *Chromosoma* **85**, 427-437.

Stein, R., Gruenbaum, Y., Pollack, Y., Razin, A. and Cedar, H. (1982). Clonal inheritance of the pattern of DNA methylation in mouse cells. *Proc. Natl. Acad. Sci. USA* **79**, 61-65.

Stewart, C.L., Gadi, I. and Bhatt, H. (1994). Stem cells from primordial germ cells can re-enter the germ line. *Dev. Biol.* **161**, 626-628.

Stöger, R., Kubicka, P., Liu, C.-G., Kafri, T., Razin, A., Cedar, H. and Barlow, D.P. (1993). Maternal-specific methylation of the imprinted mouse *Igf2r* locus identifies the expressed locus as carrying the imprinting signal. *Cell* **73**, 61-71.

Surani, M.A. (1991). Genomic imprinting: developmental significance and molecular mechanism. *Curr. Opin. Genet. Dev.* **1**, 241-246.

Surani, M.A.H., Barton, S.C. and Norris, M.L. (1984). Development of reconstituted mouse eggs suggest imprinting of the genome during gametogenesis. *Nature* **308**, 548-550.

Surani, M.A.H., Barton, S.C. and Norris, M.L. (1986). Nuclear transplantation in the mouse: heritable differences between parental genomes after activation of the embyronic genome. *Cell* **45**, 127-136.

Swain, J.L., Stewart, T.A. and Leder, P. (1987). Parental legacy determines methylation and expression of an autosomal transgene: a molecular mechanism for parental imprinting. *Cell* **50**, 719-727.

Szabó, P.E. and Mann, J.R. (1995). Biallelic expression of imprinted genes in the mouse germ line: implications for erasure, establishment, and mechanisms of genomic imprinting. *Genes Dev.* **9**, 1857-1868.

Szybek, K. (1972). *In vitro* maturation of oocytes from sexually immature mice. *J. Endocrinol.* **54**, 527-528.

Tada, M., Tada, T., Lefebvre, L., Barton, S.C. and Surani, M.A. (1997). Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. *EMBO J.* **16**, 6510-6520.

Tada, T., Tada, M., Hilton, L., Barton, S.C., Sado, T., Takagi, N. and Surani, M.A. (1998). Epigenotype switching of imprintable loci in embryonic germ cells. *Dev. Genes Evol.* **207**, 551-561.

Taketo, T. and Koide, S.S. (1981). *In vitro* development of testis and ovary from indifferent fetal mouse gonads. *Dev. Biol.* **84**, 61-66.

Taketo-Hosotani, T., Nishioka, Y., Nagamine, C.M., Villalpando, I. and Merchant-Larios, H. (1989). Development and fertility of ovaries in the B6.YDOM sex-reversed female mouse. *Development* **107**, 95-105.

Tam, P.P.L. and Snow, M.H.L. (1981). Proliferation and migration of primordial germ cells during compensatory growth in the mouse embryo. *J. Embryol. Exp. Morph.* **64**, 133-147.

Tilghman, S.M. (1999). The sins of the fathers and mothers: genomic imprinting in mammalian development. *Cell* **96**, 185-193.

Trasler, J.M., Hake, L.E., Johnson, P.A., Alcivar, A.A. and Hecht, N.B. (1990). DNA methylation and demethylation events during meiotic prophase in the mouse testis. *Mol. Cell. Biol.* **10**, 1828-1834.

Trasler, J.M., Alcivar, A.A., Hake, L.E., Bestor, T.H. and Hecht, N.B. (1992). DNA methyltransferase is developmentally expressed in replicating and non-replicating male germ cells. *Nucl. Acids Res.* **20**, 2541-2545.

Trasler, J.M., Trasler, D.G., Bestor, T.H., Li, E. and Ghibu, F. (1996). DNA methyltransferase in normal and *Dnmt^{n}/Dnmt^{n}* mouse embryos. *Dev. Dynamics* **206**, 239-247.

Tremblay, K.D., Duran, K.L. and Bartolomei, M.S. (1997). A 5' 2-kilobase-pair region of the imprinted mouse *H19* gene exhibits exclusive paternal methylation throughout development. *Mol. Cell Biol.* **17**, 4322-4329.

Troutt, A.B., McHeyzer-Williams, M.G., Pulendran, B. and Nossal, G.J. (1992). Ligation-anchored PCR: a simple amplification technique with single-sided specificity. *Proc. Natl. Acad. Sci. USA* **89**, 9823-9825.

Tsafriri, A. (1978). Oocyte maturation in mammals. In *The Vertebrate Ovary* (R.E. Jones, Ed.) pp. 409-442. Plenum Publishing Corp., New York.

Tucker, K.L., Talbot, D., Lee, M.A., Leonhardt, H. and Jaenisch, R. (1996a). Complementation of methylation deficiency in embryonic stem cells by a DNA methyltransferase minigene. *Proc. Natl. Acad. Sci. USA* **93**, 12920-12925.

Tucker, K.L., Beard, C., Dausman, J., Jackson-Grusby, L., Laird, P.W., Lei, H., Li, E. and Jaenisch, R. (1996b). Germ-line passage is required for establishment of methylation and expression patterns of imprinted but not of nonimprinted genes. *Genes Dev.* **10**, 1008-1020.

Turker, M.S. and Bestor, T.H. (1997). Formation of methylation patterns in the mammalian genome. *Mut. Res.* **386**, 119-130.

Tycko, B. (1997). DNA methylation in genomic imprinting. *Mut. Res.* **386**, 131-140.

Ueda, T., Yamazaki, K., Suzuki, R., Fujimoto, H., Sasaki, H., Sakaki, Y. and Higashinakagawa, T. (1992). Parental methylation patterns of a transgenic locus in adult somatic tissues are imprinted during gametogenesis. *Development* **116**, 831-839.

Van den Wyngaert, I., Sprengel, J., Kass, S.U. and Luyten, W.H. (1998). Cloning and analysis of a novel human putative DNA methyltransferase. *FEBS Letts.* **426**, 283-289.

Vidal, F., Sage, J., Cuzin, F. and Rassoulzadegan, M. (1998). Cre expression in primary spermatocytes: a tool for genetic engineering of the germ line. *Mol. Reprod. Dev.* **51**, 274-280.

Vogel, M.C., Papadopoulos, T., Müller-Hermelink, H.K., Drahovshy, D. and Pfeifer, G.P. (1988). Intracellular distribution of DNA methyltransferase during the cell cycle. *FEBS Lett.* **236**, 9-13.

Walsh, C.P., Chaillet, J.R. and Bestor, T.H. (1998). Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nature Genet.* **20**, 116-117.

Walsh, C.P. and Bestor, T.H. (1999). Cytosine methylation and mammalian development. *Genes Dev.* **13**, 26-34.

Wakayama, T., Perry, A.C.F., Zuccotti, M., Johnson, K.R. and Yanagimachi, R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394**, 369-374.

Wassarman, P.M. and Josefowicz, W.J. (1978). Oocyte development in the mouse: an ultrastructural comparison of oocytes isolated at different stages of growth and meiotic competence. *J. Morph.* **156**, 209-236.

Wassarman, P.M. and Albertini, D.F. (1994). The mammalian ovum. In *The Physiology of Reproduction, Second Edition* (E. Knobil and J.D. Neill, Eds.), pp. 79-122. Raven Press, Ltd., New York.

Wickens, M.P. (1992). Forward, backward, how much, when: mechanisms of poly (A) addition and removal and their role in early development. *Semin. Dev. Biol.* **3**, 399-412.

Wickramasinghe, D., Ebert, K.M. and Albertini, D.F. (1991). Meiotic competence acquisition is associated with the appearance of M-phase characteristics in growing mouse oocytes. *Dev. Biol.* **143**, 162-172.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810-813.

Wylie, C.C., Stott, D. and Donovan, P.J. (1986). Primordial germ cell migration. In *Developmental Biology* (L.W. Browder, ed.), pp.433-448. Plenum, New York.

Wylie, C.C. and Heasman, J. (1993). Migration, proliferation, and potency of primordial germ cells. *Semin. Dev. Biol.* **4**, 161-170.

Ye, X., Fong, P., Lizuka, N., Choate, D. and Cavener, D.R. (1997). Ultrabithorax and Antennepedia 5' untranslated regions promote developmentally regulated internal translation initiation. *Mol. Cell. Biol.* **17**, 1714-1721.

Yen, R.-W. C., Vertino, P.M., Nelkin, B.D., Yu, J.J., El-Deiry, W., Cumaraswamy, A., Lennon, G.G., Trask, B.J., Celano, P. and Baylin, S.B.

(1992). Isolation and characterization of the cDNA encoding human DNA methyltransferase. *Nucl. Acids Res.* **20**, 2287-2291.

Yoder, J.A., Chiu Yen, R.-W., Vertino, P.M., Bestor, T.H. and Baylin, S.B. (1996). New 5' regions of the murine and human genes for DNA (cytosine-5)-methyltransferase. *J. Biol. Chem.* **271**, 31092-31097.

Yoder, J.A., Walsh, C.P. and Bestor, T.H. (1997a). Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.* **13**, 335-340.

Yoder, J.A., Soman, N.S., Verdine, G.L. and Bestor, T.H. (1997b). DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. *J. Mol. Biol.* **270**, 385-395.

Yoder, J.A. and Bestor, T.H. (1998). A candidate mammalian DNA methyltransferase related to pmt1p of fission yeast. *Hum. Mol. Genet.* **7**, 279-284.

Zeschnigk, M., Schmitz, B., Dittrich, B., Buiting, K., Horsthemke, B. and Doerfler, W. (1997). Imprinted segments in the human genome: different DNA methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic sequencing method. *Hum. Mol. Genet.* **6**, 387-395.

Zuccotti, M. and Monk, M. (1995). Methylation of the mouse *Xist* gene in sperm and eggs correlates with imprinted *Xist* expression and paternal X-inactivation. *Nature Genet.* **9**, 316-320.

Appendix