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Expression of DNA Methyltransferase Transcripts in The Oocytes and Preimplantation Embryos in Mouse

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생쥐 난자와 착상전 초기배아에서 DNA 메틸전이효소 전사물의 발현

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ABSTRACT : DNA methylation seems to play an important regulatory role in gene expression and cell differentiation during postimplantation embryonic development. However, the significance of DNA methylation which is maintained by the DNA MTase during preimplantation embryonic development, is not fully understood. In order to study the role of DNA methylation in the preimplantation embryos, the expression of DNA MTase transcripts was monitored in the oocytes and preimplantation embryos. The mRNA of DNA MTase was detected in the oocytes and preimplantation embryos. The relative mRNA levels of DNA MTase were high from the stages of GV-oocytes and pronuclear embryos, and thereafter decreased gradually. By the treatment of α-amanitin, it was confirmed that the transcripts presented in pronuclear embryos was derived from the maternal genome. The presence of transcripts of DNA MTase in the oocytes and pronuclear embryos suggests that the maintenance of DNA methylation may be necessary and seems to play an important role in gene expression and cell differentiation during preimplantation embryonic development in mouse.

Key words: Preimplantation embryo, DNA MTase, RT-PCR.

요 약 : 포유류 배아발생 중 DNA 메틸화는 세포분화와 유전자발현에서 중요한 역할을 하는 것으로 알려져 있다. 그러나, 생쥐 착상전 초기배아 발생 중 메틸화효소에 의해 유지되는 DNA 메틸화의 중요성과 자세한 기작은 잘 이해되고 있지 않다. 이 연구에서 DNA 메틸화의 역할에 관하여 알아보기 위하여, 성숙난자와 착상전 초기배아에서 DNA 메틸전이효소의 발현양상을 조사하였다. 이를 위해, DNA 메틸전이효소를 암호화하고 있는 cDNA에서 primer를 고안하였다. Primer의 정확도와 PCR 조건의 적합화를 통하여, DNA MTase 전사물이 성숙난자와 착상전 초기배아에서 검출되었다. DNA MTase의 mRNA량은 성숙난자에서 가장 높으며, 전핵시기까지 비슷한 정도로 유지되었다. 이후 8-세포기까지 지속적으로 감소하다 상실기 배아에서 다시 검출되어 포배기까지 증가하는 양상을 보였다. 그리고, RNA polymerase Ⅱ 억제제를 전핵시기 배아에 처리하여, 난자와 전핵시기 배아에 다량 존재하는 전사물이 모계유래인 것을 확인하였다. 결국, 난자와 전핵시기 배아에 상대적으로 다량 존재하는 DNA 메틸전이효소의 전사물은 아마도 착상전 초기배아에서 DNA 메틸화의 유지에 필요하며, 착상전 초기배아 발생에 있어서 유전자발현과 세포분화에 영향을 줄 것임을 시사하고 있다.

INTRODUCTION

DNA methylation occurs in almost all higher organisms and plays a central role in the control of many genetic functions. CpG methylation is known to be essential for the normal development of the embryos, gene regulation and chromosome inactivation (Cedar, 1988; Tate & Bird, 1993), and is thought to contribute to CpG mutability and carcinogenesis through the ability of 5-methylcytosine to deaminate, forming thymine (Jones et al., 1992). In the genomes of higher eukaryotes, cytosines are methylated in CpG sequences, except in CpG islands, which are found in the promoters of active house-keeping genes (Bird, 1987).

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As a result of the high mutability of m^5-cytosine, CG deficiency is observed in heavily methylated genomes. Apart from normal biological processes, it has been postulated that DNA methylation plays a role in carcinogenesis (Jones & Buckley, 1990), genetic diseases (Cooper & Youssoufian, 1988) and evolution (Sved & Bird, 1990).

The importance of DNA methylation during embryonic development in mice has been well-documented (Li et al., 1992). Analysis of methylation patterns of specific genes during development suggests that patterns established in sperm and oocytes are lost during early development, that regions other than CpG islands become almost fully methylated, and that loss of methylation occures at specific sites in the tissues where a gene is expressed (Jahner & Jaenisch, 1984; Groudine & Conklin, 1985; Cedar & Razin, 1990).

Cytosine methylation is catalyzed by DNA (cytosine-5) methyltransferase, and most m^5C residues occur in the palindrome 5′-CpG-3′, with methylation on both strands (Razin & Riggs, 1980). Hemimethylated DNA produced by DNA replication becomes fully methylated by the action of a maintenance methylase. The key enzymes involved in the first two processes, the DNA methyltransferase, catalyze the transfer of the methyl group from the donor S-adenosyl-methionine to the C5 position of cytosine residues within specific DNA sequence contexts. So far, only one mammalian DNA (cytosine-5) methyltransferase has been identified and characterized (Bestor et al., 1988; Leonhardt & Bestor, 1993).

The DNA methylation plays an important regulatory role in gene expression and cell differentiation during postimplantation embryonic development, nevertheless, the significance of DNA methylation during preimplantation embryonic development is not fully understood. In this experiment, to study the significance of DNA methylation in preimplantation embryos, the transcripts of DNA MTase gene was investigated by RT-PCR.

MATERIALS AND METHODS

1. Collection and culture of oocytes and preimplantation embryos

The GV-oocytes were collected from pregnant mare's serum gonadotropin (PMSG) primed ICR female (Swiss Albino). The ovulated-oocytes were collected from the superovulated mice by flushing the oviduct. The pronuclear, 2-cell, 4-cell, 8-cell, morula, and blastocyst embryos were collected from the superovulated mice that were mated to ICR males at 20, 44, 56, 68, 78, or 96 hr post-human chorionic gonadotropin (hCG) injection by flushing either the oviduct or the uterus.

To verify whether the transcripts of DNA MTase in the pronuclear embryos was originated from maternal genome, the pronuclear embryos were cultured in 0.3% bovine serum albumin (BSA)+ pottasium simplex optimized medium (KSOM, Lawitts & Biggers, 1992) containing with α -amanitin (25 μg/ml) as an inhibitor of RNA polymerase Ⅱ alpha for 24, 36, 48 hr *in vitro*.

2. Design of PCR primers for DNA MTase

Primers for DNA MTase were designed according to the based on the mouse cDNA sequence of Bestor et al. (1988). The position of designed DNA MTase primer binding sites and the consequent cDNA fragment of DNA MTase were represented in Figure 1. The primers were designed to cross the exon and intron regions and produced a diagnostic fragment of 312 bp length. Patial sequence of a cDNA encoding DNA MTase have two restriction enzyme (MspI or AluI) site. The diagnostic fragments were confirmed by restriction enzyme analysis.

3. Total RNA extraction and reverse transcription(RT)

Total RNA was extracted from 30 oocytes/embryos as following. After collection, the oocytes and embryos were washed through 3 drops of phosphate-buffered saline (PBS, Sambrook et al., 1989), counted, and transferred in minimal volume to a chilled 1.5 ml microfuge tube on ice. Each tube was added 300 μl TRIzol (Gibco) and vortexed vigorously on ice. Prior to isolation of the RNA, 0.1 pg rabbit α-globin mRNA (Gibco) was added per oocyte/embryo. This mRNA served as an external marker for RNA recovery and efficiency of the reverse transcription PCR reactions. Total RNA was extracted under the conditions described by the manufacturer and used in experiments or stored at −20℃.

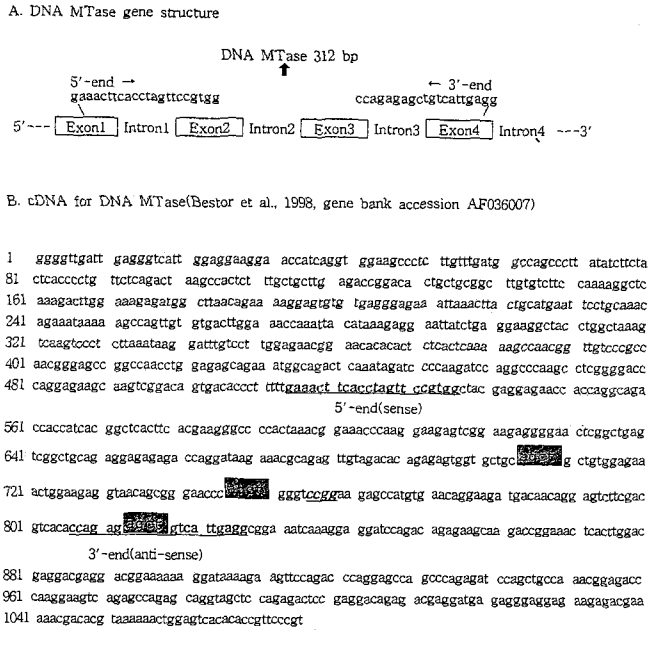

Fig. 1. A representative diagram to illustrate the position of designed DNA MTase primer binding sites (duplicated and underlined) and the consequent cDNA (Bestor et al., 1998) fragment of DNA MTase (Fig. 1A). Partial sequence of a cDNA encoding DNA MTase have two restriction enzyme site of MspI (underlined Italic) and AluI (shadow boxed)(Fig. 1B). The primers were designed to cross the exon and intron regions. The size of PCR product using DNA MTase primers is shown in base pairs (bp).

Total RNA extracted from 30 oocytes/embryos, equivalents, was reverse transcribed to cDNA in a MP480 thermocycler (Takara, Tokyo, Japan). The reactions were carried out in 40 μl of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 5 mM $MgCl_2$ containing 1 mM each of 4 dNTPs, 25 unit of RNasin, and 2.5 pmol of oligo dT-M4 adaptor primer. The tubes were incubated at 37℃ for 2 min, 200 units of reverse transcriptase (AMV reverse transcriptase XL, Takara) were added, and the tubes were transferred to a MP480 thermocycler. Reverse transcription was conducted for 1 hr at 42℃. The samples were then heated for 5 min at 99℃ and then placed on ice. At this point, the samples were either used directly for PCR or stored at −20℃. The AMV reverse transcript XL, reaction buffer, RNasin, and oligo(dT) primer were obtained from the Takara Corporation.

4. Polymerase chain reaction(PCR)

The 5′ and 3′ primers for DNA MTase were 5′-GAAACTTCACCTAGTTCCGT-GG-3′ and 5′-CCTCAATGATAGCTCTCTGG-3′, respectively. The 5′ and 3′ primers for β-actin were 5′-GTGGGCCGCTCTAGGCACCAA-3′ and 5′-CTCTTTGATGTCACG-CATGCCTTTC-3′, respectively (Rappolee et al., 1992). The 5′ and 3′ primers for rabbit α-globin were 5′-GCAGCCACGGTGGCGAGTAT-3′ and 5′-GTGGGACAGGAGC-TTGAAAT-3′, respectively (Cheng et al., 1986). The DNA MTase, β-actin, and α-globin primers gave rise to diagnostic fragments of 312 bp, 539 bp, and 257 bp, respectively.

All PCR reactions were performed in a Takara DNA Thermal Cycler Model 480, in 40 μl volumes covered with 50 μl of mineral oil. Optimal conditions for amplification of the DNA MTase gene marker were determined empirically in the presence of pooled embryos cDNA. The PCR reactions were performed in 40 μl of 10 mM Tris-HCl, pH 8.3, containing 50 mM KCl, 2.0 mM $MgCl_2$, 0.25 mM each of the 4 dNTPs, 2.5 units Taq polymerase (Takara), 25 pmol each of the appropriate 3′ and 5′ primers, and 5 μl of the reverse transcription reaction. When DNA MTase, β-actin, and α-globin were amplified, this volume was equivalent to 3 oocytes/embryos, respectively. The basic PCR program used was incubation at 95℃ for 3 min, followed by a cycle program of 95℃ for 30 sec, 60℃ 30 sec, and 72℃ 30sec. The last cycle was concluded with a 72℃ incubation for 10 min extension. The number of cycles was 35 for α-globin, and 40 for DNA MTase and β-actin. The PCR products were resolved in a 2.5% agarose gel at 100 volts for 40 min, followed by the staining in ethidium bromide (0.5 g/ml) for 30 min and destaining in deionized water for 30 min. Agarose gel was exposed to ultraviolet light and photographed with an Polaroid Camera System.

5. Identification of PCR products

After optimization of a PCR system, the identity of amplified product was verified by restriction enzyme digest. After the RT-PCR with 30 oocytes, the PCR products were removed and either digested or not digested with 5 units of Msp I and Alu I (Boehringer Mannheim) for 1 hr at 37℃ under the conditions described by the manufacturer. The amplified PCR product digested by MspI yielded the

two predicted fragments of approximately 242 and 70 bp, while the second digest with AluI yielded the four expected fragments of approximately 192, 66, 41, and 13 bp. Following electrophoresis on a 2.5% agarose gel, the gels were photographed under UV light and verified the identity of DNA MTase amplicon.

6. Scanning densitometry

The amplified PCR products were visualized in 2.5% agarose gels and stained simultaneously in ethidium bromide as described above, and a photograph was taken. The intensity and area of the amplified signals in the photographs were analyzed by scanning densitometry with a BIO-RAD GS-700 Imaging Densitometer and processed with Quantity One Software (BIO-RAD). Relative gene expression for oocytes or embryos was calculated as a percentage of rabbit α-globin.

RESULTS

1. Fidelity of the PCR primers and identification of the PCR products for DNA MTase

We are constructed the primers for DNA MTase from the cDNA (Bestor et al., 1988). In GV-oocytes, the mRNA was easily detected by RT-PCR, and the diagnostic fragment was identified by MspI and AluI restriction enzyme digest (Fig. 2). The condition of PCR was optimized with cDNA of 3 GV-oocytes equvilant (Fig. 3).

2. Relative levels of the mRNA for DNA MTase in the oocytes and embryos

Using the primers for DNA MTase, the transcripts were easily detected from GV-oocytes to blastocyst. The mRNA

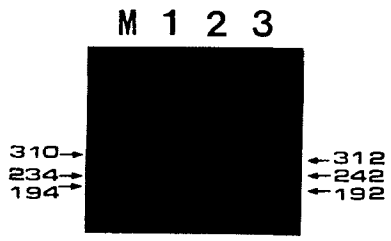

Fig. 2. Identification of amplified DNA MTase transcript by restriction enzyme analysis. M, ϕX-174 HaeⅢ marker. Lane 1, amplified and undigested sample; the major band is 312 bp in length. Lane 2, amplified and digested sample by MspI; a major band of 242 bp and a minor band of 70 bp. Lane 3, amplified and digested sample by AluI; a major band of 192 bp and a minor band of 66, 41, and 13 bp.

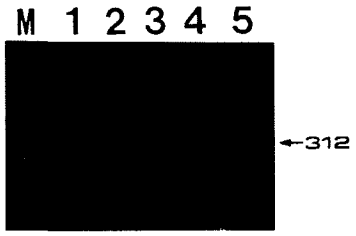

Fig. 3. Optimization of RT-PCR procedure with regard to number of PCR cycles for DNA MTase. The amplified samples were electrophoresed in 2.5% agarose gel, stained with ethidium bromide, and photographed under UV-light. M, ϕX-174 HaeⅢ marker. Lane 1, 25 cycle; Lane 2, 30 cycle; Lane 3, 35 cycle; Lane 4, 40 cycle; Lane 5, 45 cycle.

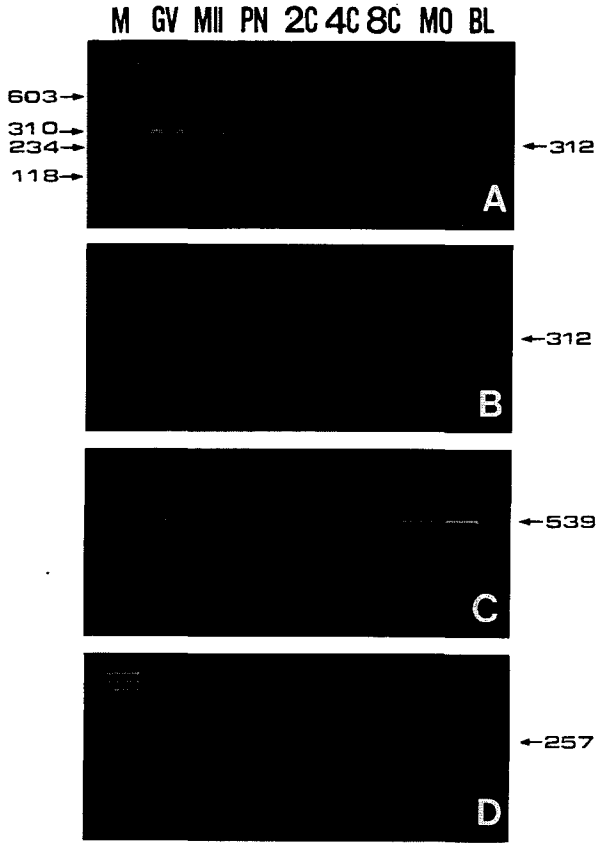

Fig. 4. Expression pattern of DNA MTase mRNA in oocytes and preimplantation embryos. A: DNA MTase. B: negative control for DNA MTase. C: β-actin. D: rabbit α-globin. M, ϕX174/HaeIII marker; GV, GV-oocytes; MII, ovulated-oocytes; PN, pronuclear embryos; 2C, 2-cell embryos; 4C, 4-cell embryos; 8C, 8-cell embryos; Mo, morulae; BL, blastocyst.

levels of DNA MTase were higher in GV-oocytes than other stages, after then gradually decrased until 8-cell stage, and thereafter increased upto blastocyst (Fig. 4A; Fig. 5). The possibility of DNA contamination was confirmed that the 312 bp fragment was not observed in RNA preparations that were not subjected to RT, but were subjected to PCR (Fig. 4B). It is likely reflected that these changes in the mRNA levels is present at the different stages since the assumption that equal amounts of recovered RNA were subjected to RT-PCR was verified by the β-actin served as endogenous control (Fig. 4C) and the relatively uniform rabbit α-globin mRNA diagnostic signal obtained from the different samples (Fig. 4D).

3. Effect of the treatment of α-amanitin

Although the mRNAs of DNA MTase were higher in GV-oocytes and early embryos, whether the transcription of DNA MTase is occured onward 2-cell stage that is unclear. To monitor the transcriptional events, α-amanitin as RNA polymerase II alpha inhibitor was treated at pronuclear embryos for 24, 36, or 48 h. At the end of 24 h culture, the mRNA levels are not different in comparison with that the control embryos. But, the mRNA levels are

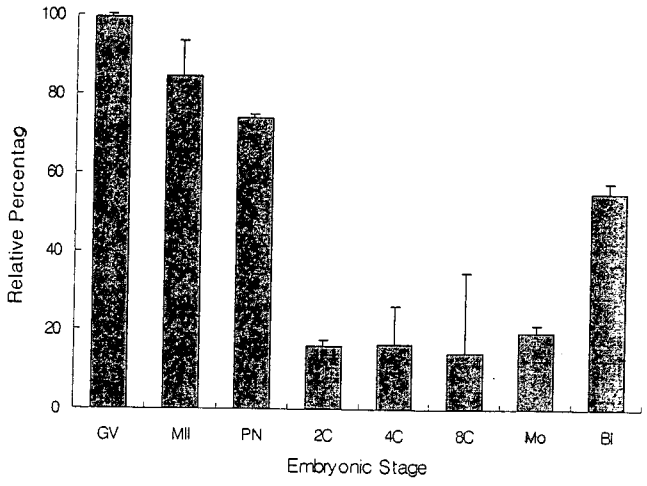

Fig. 5. Diagrammatic representation of the steady-state levels of mRNA in mouse oocytes and preimplantation embryos, as determined by semi-quantitative RT-PCR assays. The results from two experiments were pooled and the data are expressed as the mean+S.D. in which the ratio of target /rabbit α-globin in the GV-oocyte was taken as 100 %. The calculated ratios at the other stages of development were then determined by dividing this ratio by that obtained for the GV-oocyte.

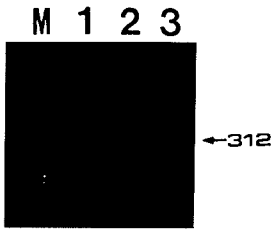

Fig. 6. The change of mRNA levels for DNA MTase following the treatment of α-amanitin for 24 (Lane 1), 36 (Lane 2), or 48 (Lane 3) hr culture. Total RNA isolated from 30 embryos was subjected to reverse transcription-PCR, analyzed for DNA MTase expression. M, ϕX174 -HaeIII marker.

gradually decreased and not detected onward 48 h culture (Fig. 6).

DISCUSSION

In this experiment, the transcripts of DNA MTase gene were detected in the oocytes and preimplantation embryos in mouse. The temporal pattern of the transcripts from GV-oocytes to 8-cell embryos was comparable to the patterns of enzyme activity previously reported (Monk et al., 1991). This pattern is consistent with the temporal pattern of β-actin transcripts in the oocytes and preimplantation embryos, which decreases at first and then increases (Bachvarova et al., 1989). In generally, the decrease is likely to be a consequence of the general degradation of maternal RNA that is initiated during oocyte maturation and is essentially terminated by the late 2-cell stage (Bachvarova et al., 1989). Following zygotic gene activation, which occurs during the 2-cell stage in mouse (Bensaude et al., 1983; Flach et al., 1982), these maternal transcripts are replaced by zygotic ones. In fact, the oocyte accumulates a stockpile of gene transcripts and translation products that are used to direct the initial stages of embryogenesis (Wassarman & Kinloch, 1992). Whereas, some oogenetic products can persist and continue to function beyond the time when embryonic transcription is activated (Kidder, 1992). In accordance with it, by the treatment of α-amanitin, it was confirmed that the transcripts presented in pronuclear embryos is drived from maternal genome and the onset of zygotic

DNA MTase transcription is delayed relative to the general activation of the embryonic genome.

The presence of mRNA of DNA MTase does not provide direct evidence of involvement of DNA methylation, it was assumed that the maintenance of DNA methylation may be necessary in the preimplantation mouse embryos. In addition, the higher levels of transcripts in oocytes and pronuclear embryos may be an important role in the early stages of embryogenesis such as the regulation of genomic function. It was known that genomic methylation patterns are established in the oocyte and in the sperm during gametogenesis and subjected to dramatic changes during the preimplantation period in mouse (Monk et al., 1987; Razin & Cedar, 1993). Following implantation, there is a surge of *de novo* DNA methylation affecting the entire embryo (Monk et al., 1987). In that period, its importance is emphasized from the report that mice with a targeted disruption of the gene coding for the DNA MTase are severely affected in postimplantation embryonic development (Li et al., 1992). Also, the introduction of foreign cellular and viral genomes into early mouse embryos confirmed that the DNA methylation is maintained at the preimplantation stages of mouse embryos (Jahner et al., 1982; Palmiter et al., 1982). Although, in this experiment, the significance of DNA methylation are not fully understood, the DNA methylation seems to play an important role in the regulation of genomic function in preimplantaiton embryonic development.

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REFERENCES

Bachvarova R, Cohen E, Deleon V, Tokunage K, Sakiyama S, and Paynton BV (1989) Amounts and modulation of actin mRNAs in mouse oocytes and embryos. Development 106:561-565.

Bensaude O, Babinet C, Morange M, and Jacob F (1983) Heat shock proteins, first major products of zygotic gene activity in mouse embryo. Nature 305, 331-333.

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

Bird A (1987) CpG islands as gene markers in the vertebrate nucleus. Trend Genet 3:342-347.

Cedar H (1988) DNA methylation and gene activity. Cell 53:3-4.

Cedar H and Razin A (1990) DNA methylation and development. Biochim Biophys Acta 1094:1-8.

Cheng, JF, Raid L, and Hardison RC (1986) Isolation and nucleotide sequences of the rabbit globin gene cluster: Absence of a pair of α-globin genes evolving in concert. J. Biol. Chem. 261:839-898.

Comb M and Goodman HW (1990) CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res 18:3975-3982.

Cooper DN and Youssoufian H (1988) The CpG dinucleotide and human genetic disease. Hum Genet 78:151-155.

Flach G, Johnson MH, Braude PR, Taylor RAS, and Bolton VN (1982) The transition from maternal to embryonic control in the two-cell mouse embryo. EMBO J 1:681-686.

Groudine, M and Conkin KF (1985) Chromatin structure and de novo methylation of sperm DNA: implications for activation of the paternal genome. Science, 228: 1061-1068.

Jahner D, Stuhlmann H, Stewart CL, Harbers K, Lohler J, Simon I, and Jaenisch R (1982) De novo methylation and expression of retroviral genomes during mouse embryogenesis. Nature 298:623-628.

Jahner D and Jaenisch R (1984) In DNA Methylation: Biochemistry and Biological Significance, eds. Razin A, Cedar H, and Riggs AD (Springer, New York), pp. 188-219.

Jones P, Rideout W, Shen J-C, Spruck C, and Tsai Y (1992) Methylation, mutation and cancer. Bioessays 14:

33-36.

Jones PA and Buckley JD (1990) The role of DNA methylation in cancer. Cancer Res 54:1-23.

Kidder GM (1992): Genes involved in cleavage, compaction, and blastocyst formation. In RBL Gwatkin (ed); "Genes in Mammalian Reproduction." New York: Wiley and Sona.

Lawitts, JA, and Biggers JD (1992) Joint effect of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture media. Mol Reprod Dev 31: 189-194.

Leonhardt H and Bestor TH (1993) Structure, function and regulation of mammalian DNA methyltransferase. In DNA Methylation: Molecular Biology and Biological Significance, J.P.Jost and H.P.Saluz, eds. (Basel, Switzerland: Birkhauser Verlag), pp. 109-119.

Li En, Bestor TH, and Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69:915-926.

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

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.

Palmiter RD, Chen HY, and Brinster, RL (1982) Differential regulation of metallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. Cell 29:701-710.

Rappolee DA, Sturm KS, Behrendtsen O, Schultz GA, Pedersen RA, and Werb Z (1992) Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in early mouse embryos. Genes Dev 6:939-952.

Razin A and Cedar H (1993) in DNA methylation and embryogenesis, in DNA methylation: Molecular biology and Biological significance, pp. 343-357, Birkhausser-Verlag, Basel.

Razin A and Riggs AD (1980) DNA methylation and gene function. Science 210:604-610.

Sambrook, J, Fritsch EF, and Maniatis T (1989) Molecular cloning. A laboratory manual. New York: Cold Spring Harbor Laboratory, Press.

Sved J and Bird AP (1990) The expected equilibrium of the CpG dinucleotide in vertebrate genomes under a mutation model. Proc Natl Acad Sci USA 87:4692-4696.

Tate P and Bird A (1993) Effects of DNA methylation on DNA-binding proteins and gene zexpression. Curr Opin Genet Dev 3:226-231.

Wassarman PM and Kinloch RA (1992) Gene expression during oogenesis in mice. Mutat Res 296:3-15.