MeCP2 Expression and Promoter Methylation of Cyclin D1 Gene Are Associated with Cyclin D1 Expression in Developing Rat Epididymal Duct

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Hypermethylation-dependent silencing of the gene is achieved by recruiting methyl-CpG binding proteins (MeCPs). Among the MeCPs, MeCP2 is the most abundantly and ubiquitously expressed in various types of cells. We first screened the distribution and expression pattern of MeCP2 in adult and developing rat tissues and found strong MeCP2 expression, albeit rather ubiquitously among normal tissues, in ganglion cells and intestinal epithelium in the small intestine, in Purkinje cells and neurons in the brain, in spermatogonia and in epithelial cells in the epididymal duct of the testis. We then assessed the expression and the methylation pattern of the promoter region of cyclin D1 by immunohistochemistry and sodium bisulfite mapping, and found that cyclin D1 expression in the epididymal duct decreased rapidly during rat development: strong in newborn rats and very weak or almost negative in 7-day-old rats. Mirroring the decrease of cyclin D1 expression, methylated cytosine at both CpG and non-CpG loci in the cyclin D1 promoter was frequently observed in the epididymal duct of 7-day-old rats but not in that of newborn rats. Interestingly, MeCP2 expression also increased concomitant with the increase of methylation. Cyclin D1 expression in the epididymal duct may be efficiently regulated by the epigenetic mechanism of the cooperative increase of MeCP2 expression and promoter methylation.

Key words: Cyclin D1, MeCP2, methylation, epididymis, *in situ* hybridization

I. Introduction

After replication, mammalian DNA is marked by the addition of methyl groups to certain cytosine bases that are almost exclusively in sequence with CpG [9]. CpG methylation is involved in the long-term silencing or inactivation of genes during mammalian development, tumorigenesis and aging [14]. The mechanism of gene silencing by methylated cytosine varies among promoters [2, 6]: direct interference of the binding of the transcription factors by methylated cytosine or indirect interference by recruiting methyl-CpG-binding proteins (MeCPs). The precise mechanism of gene silencing by MeCPs in mammalian cells is either by non-specifically binding to the methyl-CpG [15] to prevent the transcriptional factors like Sp1 from DNA binding [11] or by altering the chromatin structure by recruiting histone-modifying enzymes [12, 16]. Among MeCPs, MeCP2, the mutation of which is known to cause Rett's syndrome [1, 13, 20], is most abundantly expressed as a chromosomal protein and requires a single methylated CpG site for preferential binding to DNA [5, 15].

Although MeCP2 is an essential protein ubiquitously transcribed in all tissues [13, 18], we have previously found that the level of MeCP2 mRNA expression is not equal in all colorectal cancer cells [3], and that silencing of E-cadherin expression requires both promoter hypermethylation and a significant amount of MeCP2 expression. The exact physiological role of such cooperative and coordinated change of promoter hypermethylation and MeCP2 expression in nor-

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mal tissues is, however, currently largely unknown.

In this study, to investigate the expression profile of the MeCP2 gene and the epigenetic mechanism that regulates rat cyclin D1 expression in normal rat tissues, we analyzed the level of MeCP2 expression by *in situ* hybridization and the methylation status of the rat cyclin D1 gene promoter with the use of microdissected samples from corresponding cells by sodium bisulfite mapping. We focused especially on CpG methylation of the cyclin D1 gene promoter and MeCP2 expression in the testis of developing rats.

II.   Materials and Methods

Tissue preparation

Sprague-Dawley rats were obtained from our breeding colony. Newborn, 7-day-old and 12-month-old male rats were decapitated, and tissue samples were removed, fixed with 4% paraformaldehyde and embedded in paraffin. All animal experimental procedures were conducted according to the Guidelines for Animal Experimentation at Kobe University School of Medicine.

Immunohistochemistry

Paraffin-embedded tissues were cut and dewaxed through a series of graded alcohol. After antigen retrieval by microwave irradiation (citrate, pH 6) for 10 min, endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 10 min. The specimens were then incubated with 2% non-fat dry milk in phosphate-buffered saline (PBS) for 10 min and with primary antibodies against cyclin D1 (human, rat, and mouse reactive, Zymed Laboratories Inc., South San Francisco, CA, USA) for 15 min. After three 10-min washes with PBS, the specimens were incubated with rabbit anti-mouse IgG antibody preabsorbed with normal rat serum. Finally, the cyclin D1 protein was immunolocalized by the streptavidine-biotin peroxidase complex method.

Microdissection, bisulfite mapping and quantitative real time reverse transcriptase (RT)-polymerase chain reaction (PCR)

The methylation status of the rat cyclin D1 gene promoter was analyzed by sodium bisulfite mapping with the use of microdissected mouse cartilagenous tissue. Seven-mm-thick sections were cut from formalin-fixed and paraffin-embedded thin sections and stained with HE. The laser capture microdissection system (LM200, Olympus, Tokyo, Japan) was used to select cells. Three independent microdissected samples from each part were analyzed by sodium bisulfite mapping. Dissected samples were lysed overnight in 25 ml of lysis solution containing 0.1 mg/ml proteinase K, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1% Tween 20. Low-melting agarose beads (3.2%) were formed, and methylated CpG was analyzed by the sodium bisulfite method as described [4, 8, 17]. Ten ml of low-melting agarose beads containing 0.5 mg genomic DNA derived from each part were formed in mineral oil and denatured with NaOH (final concentration, 0.1 M). The beads were dipped in 1.5 ml of freshly prepared bisulfite solution containing 1 mM hydro-

quinone and 3.5 M of $NaHSO_3$ at pH 5. The liquid surface was sealed with mineral oil, and the samples were incubated for 20 hr at 50°C under light-protected conditions. The beads were then washed with Tris-EDTA, desulfonated with 0.5 N NaOH for 30 min, neutralized with 1 N HCl, and finally washed with Tris-EDTA. Bead fragments containing approximately up to 0.1 mg DNA were analyzed by nested PCR using the following sets of primers to cover the promoter region of the rat cyclin D1 gene:

5'-GTTGATGAAATTGAAAGAAGT-3' (sense) for first PCR;

5'-ACTTTACAACTTCAACAAAACTCCCCT-3', (antisense) for first PCR;

5'-GGAGGATTTATTTGGGGAAAGAAG-3' (sense) for nested PCR;

5'-ACTTTACAACTTCAACAAAACTCCCCT-3', (antisense) for nested PCR.

The PCR condition was as follows: 30 cycles at 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and the elongation step for 5 min at 72°C. The amplified DNA was cloned into the TA vector (Invitrogen, Carlsbad, CA, USA), and at least 10 independent clones were selected and sequenced using the dideoxy termination method (ABI PRISM™ Model 310 genetic analyzer; Applied Biosystems, Foster City, CA, USA).

Preparation of digoxigenin (DIG)-labeled single-stranded MeCP2 DNA probe

The single-stranded antisense DNA probe specific for rat MeCP2 was prepared by polymerase chain reaction (PCR) as described [7]. A 341-bp cDNA fragment from rat MeCP2 was obtained by RT-PCR using the following pair of oligonucleotide primers:

5'-GCAGAGACATCAGAAGGGTC-3': sense;
5'-TTCTTAGGTGGTTTCTGCTC-3': antisense.

The cDNA sequence of purified PCR products was confirmed by the dideoxy termination method. To prepare the DIG-labeled single-stranded antisense DNA probe, the purified PCR product was subjected to unidirectional PCR with the antisense primer alone in the presence of DIG-dUTP (digoxigenin DNA labeling mixture; Boehringer Mannheim, Mannheim, Germany). For negative controls, the DIG-labeled sense probe was similarly prepared with sense primer-primed unidirectional PCR.

In situ hybridization (ISH)

After dewaxing in xylene and hydrating through a series of graded ethanol, the tissue sections were treated with 2 mg/ml proteinase K (SIGMA, St. Louis, MO, USA) at 37°C for 10 min, refixed with 4% PFA, acetylated with 100 mM triethanolamine containing 0.25% acetic acid for 10 min, and dehydrated through a series of graded ethanol. The sections were then incubated in a hybridization medium [10 mM Tris-HCl (pH 7.3), 1 mM EDTA, 600 mM NaCl, 0.25% sodium dodecyl sulfate, 1×Denhardt's medium, 50% (v/v) deionized formamide/1 ng/ml probe DNA, 10% dextran sulfate] at 50°C in a moist chamber for 24 hr.

After hybridization, the slides were washed with 5×SSC, 50% deionized formamide/2×SSC, 2×SSC and 0.2×SSC. To visualize the hybridized probe, the slides were incubated with alkaline phosphatase (ALP)-conjugated anti-DIG antibody (Roche Diagnostics GmbH) for 60 min after blocking with 0.5% non-fat dry milk in 100 mM Tris-HCl (pH 7.5) containing 150 mM NaCl (TS buffer) for 30 min. The specimens were then washed twice with TS buffer for 20 min and immersed briefly in 100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl and 50 mM $MgCl_2$. The colorimetric reaction was done with NBT/BCIP stock solution (Roche Diagnostics GmbH) in the dark for 50 min, then stopped with TE buffer. The slides were mounted with CRYSTAL/MOUNT™ (Biomeda, Foster City, CA, USA) and analyzed under a light microscope using the differential interference contrast system without counterstaining.

Statistical analysis

The intensities of the signal of MeCP2 in epididymal duct, stromal cells and capillary endothelium of normal developing testis of (0- and 7-day-old) rats were analyzed by the one way ANOVA as described [10] to examine whether there were any statistical differences between the two groups. Twenty-five high-power fields for each of the three independent samples were evaluated. Values of $P<0.05$ were considered significant.

III. Results

MeCP2 expression in normal adult rat intestine, cerebellum and testis (Figs. 1 and 2)

Although MeCP2 was ubiquitously expressed in normal adult rat tissues, the characteristic distribution pattern of MeCP2 mRNA was observed in the intestine, central nervous system and testis. In the small intestine (Fig. 1A), besides weak and diffuse expression in almost all kinds of cells, including infiltrating inflammatory cells, MeCP2 was present to a greater extent in bases of the mucosal crypt and mucosal surface epithelial cells (Fig. 1B, arrowheads and asterisks, respectively). Strong MeCP2 expression was observed in ganglion cells in the proper muscle (Fig. 1B, arrows). In the colon (Fig. 1C), weak and diffuse MeCP2 expression was observed in the cytoplasm of goblet cells as well as in infiltrating inflammatory cells throughout the colon mucosa, except in ganglion cells (Fig. 1D, arrows) where the expression was significantly strong. Negative controls prepared by sense probe did not show any significant staining (inserts in Fig. 1B, D). In the adult testis (Fig. 2A), besides weak and diffuse expression in both germ cells (pachytene spermatocytes, elongated spermatids, preleptene spermatocytes) within the seminiferous tubules and in stromal cells, strong MeCP2 expression was observed among spermatogonia lining the periphery of seminiferous
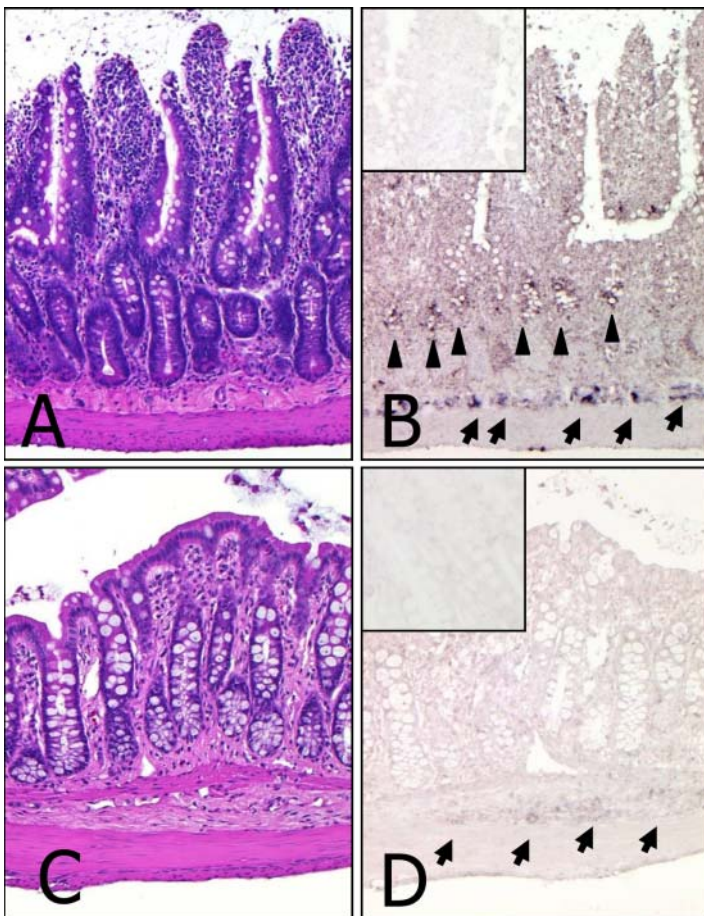
Fig. 1. Distribution of MeCP2 mRNA expression in adult rat gastrointestinal tract demonstrated by ISH. **A.** HE staining of small intestine. **B.** ISH for MeCP2 mRNA expression in small intestine. Besides weak and diffuse expression in almost all kinds of cells, including infiltrating inflammatory cells and epithelial cells of the small intestine, strong MeCP2 expression is observed among cells in transition from proliferating neck cells in the crypt to mature goblet cells (arrowheads). Strong MeCP2 expression is also observed in ganglion cells in the proper muscle (arrows). **C.** HE staining of colon mucosa. **D.** ISH for MeCP2 mRNA expression in the colon. In contrast to the small intestine, only weak MeCP2 expression is observed in the cytoplasm of colon mucosal cells as well as in infiltrating inflammatory cells throughout the colon mucosa, except in ganglion cells (arrows) expressing a significantly high level of MeCP2. Negative controls prepared by sense probe did not show any significant staining (inserts in **B** and **D**)
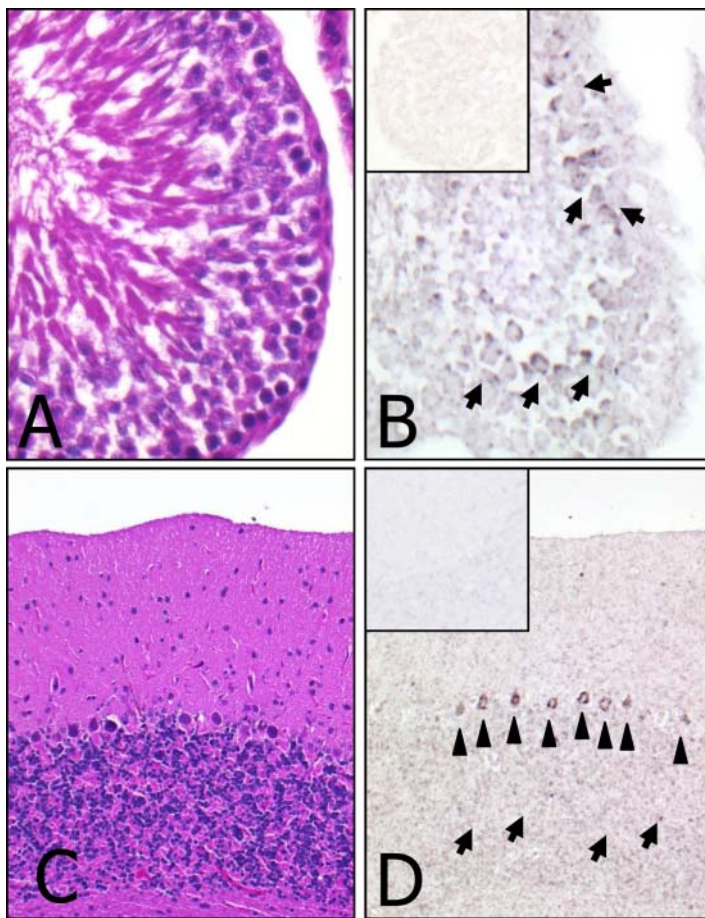
Fig. 2. The distribution of MeCP2 mRNA expression in adult rat testis and cerebellum demonstrated by ISH. **A.** HE staining of the adult rat testis. **B.** ISH for MeCP2 mRNA expression in the testis. Besides weak and diffuse expression in germ cells (pachytene primary spermatocytes, elongated spermatids, preleptene primary spermatocytes) in the seminiferous tubules and in stromal cells, strong MeCP2 expression is observed among spermatogonia lining the periphery of seminiferous tubules (arrows). Weak MeCP2 expression is observed in early and late spermatids. **C.** HE staining of the adult rat cerebellum. **D.** ISH for MeCP2 mRNA expression in the cerebellum. The intensity of MeCP2 mRNA expression varies among cell types in the cerebellum: highest in Purkinje cells (arrowheads), lower in glial cells in the molecular layer (arrows) and the lowest in the cortex. Negative controls prepared by sense probe did not show any significant staining (inserts in **B** and **D**).

tubules (Fig. 2B, arrows). In the cerebellum (Fig. 2C), MeCP2 expression was abundantly distributed throughout the layers with the highest level in Purkinje cells (Fig. 2D, arrowheads), lower in cells in the molecular layer (Fig. 2D, arrows) and the lowest in the cortex. Negative controls sense probe did not show any significant staining (inserts in Fig. 2B, D).

MeCP2 and cyclin D1 expression in seminiferous tubules of normal developing testis of (0- and 7-day-old) rats (Fig. 3)

Starting from the newborn rat testis (Fig. 3A), only very weak staining of cyclin D1 expression was observed in a small fraction of Leydig cells (Fig. 3B, arrow), and was negative in the nuclei or the cytoplasm of primordial germ cells in the seminiferous tubules of the testis. Weak and diffuse MeCP2 expression was observed in the cytoplasm of germ cells in the seminiferous tubules, Leydig stromal (Fig. 3C, arrows) and endothelial cells (Fig. 3C, arrowheads). Similarly, cyclin D1 in the testis of the 7-day-old rat (Fig. 3D) was almost negative except in a few Leydig cells in the stroma (Fig. 3E, arrows). Weak MeCP2 expression was ubiquitously observed in both germ cells and stromal cells (Fig. 3F, arrows). Negative controls prepared by sense probe did not show any significant staining (inserts in Fig. 3C, F). No significant staining was observed in negative controls prepared by replacing the primary antibody with

non-immunized serum for immunohistochemistry (data not shown).

MeCP2 and cyclin D1 expression in epididymal duct of normal developing testis of (0- and 7-day-old) rats (Fig. 4)

In the epididymal duct of the testis of the newborn (0-day) rat (Fig. 4A), strong cyclin D1 expression was observed in the nucleus and cytoplasm of epithelial cells of the duct (Fig. 4B, arrows). ISH disclosed diffuse MeCP2 expression among stromal cells (Fig. 4C, arrows), but weak in the cytoplasm of epithelial cells of the duct. In the testis of the 7-day-old rat (Fig. 4D), cyclin D1 expression was markedly reduced and confined to a few epithelial cells of the duct (Fig. 4E). MeCP2 was strongly expressed in most of the cytoplasm of epithelial cells of the duct (Fig. 4F, arrows). By statistical analysis, while no significant difference of MeCP2 expression was observed in stromal cells and capillary endothelium between 0- and 7-day-old rat, MeCP2 expression was significantly ($P<0.05$) higher in 7-day-old than that of 0-day-old rat. Negative controls prepared by sense probe did not show any significant staining (inserts in Fig. 4C, F). No significant staining was observed in negative controls prepared by replacing the primary antibody with non-immunized serum for immunohistochemistry (data not shown).
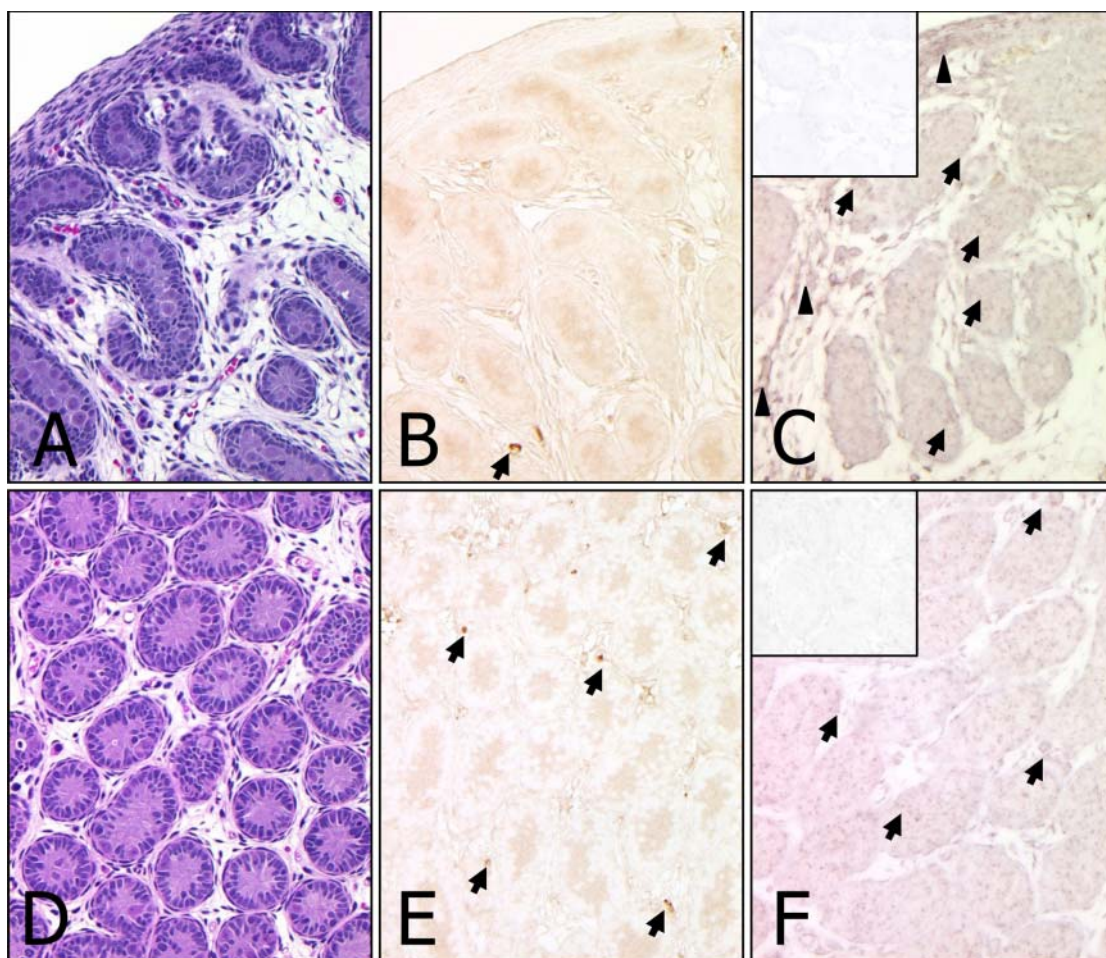
Fig. 3. Light micrograph of portions of seminiferous tubules of newborn (0-day) and 7-day-old rats. **A.** HE staining of seminiferous tubules of the developing testis of the newborn rat. **B.** Immunohistochemistry for cyclin D1. Besides periodic positive immunoreactions in a small fraction of the Leydig cells (arrow), cyclin D1 is negative in the nuclei or the cytoplasm of primordial germ cells in the seminiferous tubules of the testis. **C.** ISH for MeCP2 mRNA expression. Weak and diffuse MeCP2 expression is observed in the cytoplasm of germ cells in the seminiferous tubules, Leydig (arrows) and endothelial cells (arrowheads). **D.** HE staining of seminiferous tubules of the developing testis of the 7-day-old rat. **E.** Immunohistochemistry for cyclin D1. Cyclin D1 expression is almost negative except in a few Leydig cells in the stroma (arrows). **F.** ISH for MeCP2 mRNA expression in the testis of the 7-day-old rat. Weak MeCP2 expression is ubiquitously observed in both germ cells and stromal cells (arrows). Negative controls prepared by sense probe did not show any significant staining (inserts in **C** and **F**).

Mapping methylation status of the rat cyclin D1 promoter by sodium bisulfite modification method (Fig. 5)

Numerous CpG loci (vertical bars in Fig. 5A) constitute a CpG island in the 5'-flanking region of rat cyclin D1 gene. The cyclin D1 promoter was totally unmethylated in micro-dissected samples of the epididymal collecting duct of the newborn rat (Newborn-1, -2 and -3). In the 7-day-old rat, methylated cytosines were frequently observed in both CpG and non-CpG loci around CRE and two continuous Sp1-binding sites (7-days-1, -2 and -3).

IV. Discussion

Transcriptional repression by methyl-CpG is mediated mainly by MeCPs in a sequence-independent process that involves changes in histone acetylation levels and in chro-matin structure [5, 16]. Among the MeCPs, MeCP2 is the most abundantly expressed in the physiologic condition and plays a central role in recruiting transcriptional repressors [5]. Indeed, MeCP2 is expressed rather ubiquitously among normal tissues [13], albeit some cellular heterogeneity in the normal brain is observed. In our current study, we screened the MeCP2 expression status in various normal rat tissues with the use of ISH, and did find that MeCP2 was ubiqui-tously transcribed in all the tissues; however, the intensity of MeCP2 mRNA expression varied among cell types (Figs. 1 and 2). Because MeCP2 expression was high among these cells soon after mitosis and undergoing terminal differentia-tion (goblet cells in the crypt and spermatogonia in the testis) or specially differentiated neurons (ganglion and Purkinje cells), we speculated that MeCP2 expression was enhanced in post mitotic cells where the methylcytosine rapidly accu-
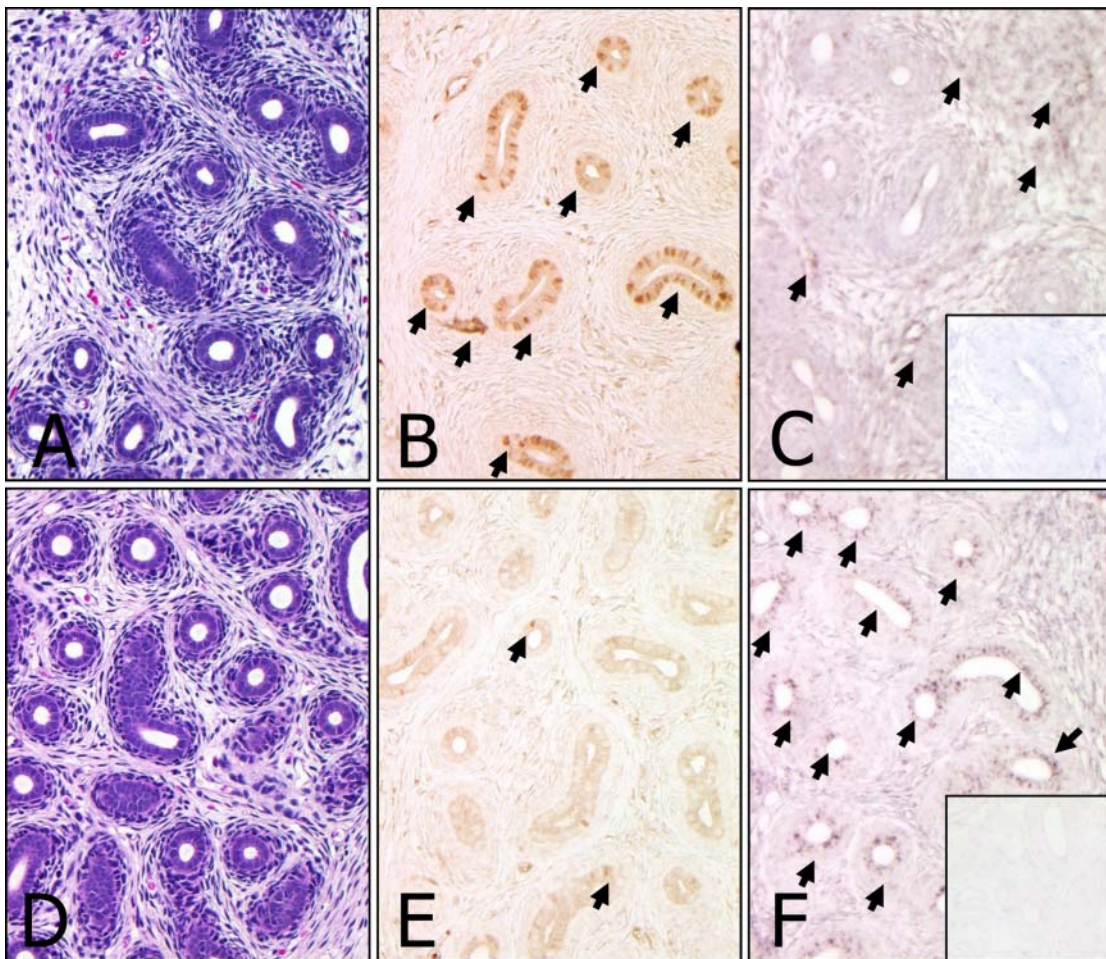
Fig. 4. Light micrograph of portions of the epididymal ducts of newborn (0-day) and 7-day-old rats. **A.** HE staining of the epididymal ducts of the newborn rat. **B.** Immunohistochemistry for cyclin D1. Strong cyclin D1 expression is observed in the nucleus and cytoplasm of epithelial cells of the duct (arrows). **C.** ISH for MeCP2 mRNA expression. MeCP2 expression is diffusely observed among stromal cells (arrows), but is weak in the cytoplasm of epithelial cells of the duct. **D.** HE staining of the epididymal duct of the 7-day-old rat. **E.** Immunohistochemistry for cyclin D1. In the 7-day-old rat, cyclin D1 expression is markedly reduced and confined to a few epithelial cells of the duct (arrows). **F.** ISH for MeCP2 mRNA expression of the 7-day-old rat. MeCP2 is strongly expressed in most of the cytoplasm of epithelial cells in the duct (arrows). Negative controls prepared by sense probe did not show any significant staining (inserts in **C** and **F**).

mulates by *de novo* methyltransferase. Taken together with our previous findings [3] in colorectal cancers that the level of MeCP2 mRNA expression is not equivalent in all colorectal cancer cells, and that silencing E-cadherin expression takes place where both promoter hypermethylation and a significant amount of MeCP2 expression are required, we speculate that a significant amount of MeCP2 expression must also be required for sufficient and efficient epigenetic silencing of the genes during normal post-mitotic differentiation.

The testis provides rare opportunities for studying genes involved in cell cycle regulation, since it contains cells in both mitosis and meiosis as well as differentiated cells with little proliferative activity. In the testis, besides the transition from diploid primordial germ cells to haploid spermatozoa that require genome-wide reprogramming of DNA methylation, stage- and epididymal-specific gene ex-

pression also requires unique epigenetic regulation. The promoter region of the cyclin D1 gene, on the other hand, contains multiple cis-acting elements, including binding sites for activator protein-1 (AP-1), for signal transducers and activators of transcription (STAT), for Sp1, and for activating transcription factor (ATP)/cAMP-responsive element-binding protein (CREB) [19]. The basic promoter structure of the 5'-regulatory region of the cyclin D1 gene has been demonstrated as a TATA-less promoter with a CRE and two continuous Sp1-binding sites through which most of the growth factors exert their cell proliferative stimuli. We have previously found that the steady-state expression of cyclin D1 is epigenetically regulated by the cytosine methylation status in rat leukemia cell lines [6], in that methylation of both CpG and non-CpG loci spanning the two continuous Sp1-binding sites and CRE of the cyclin D1 promoter defines its transcriptional activity. To investigate whether or not a similar
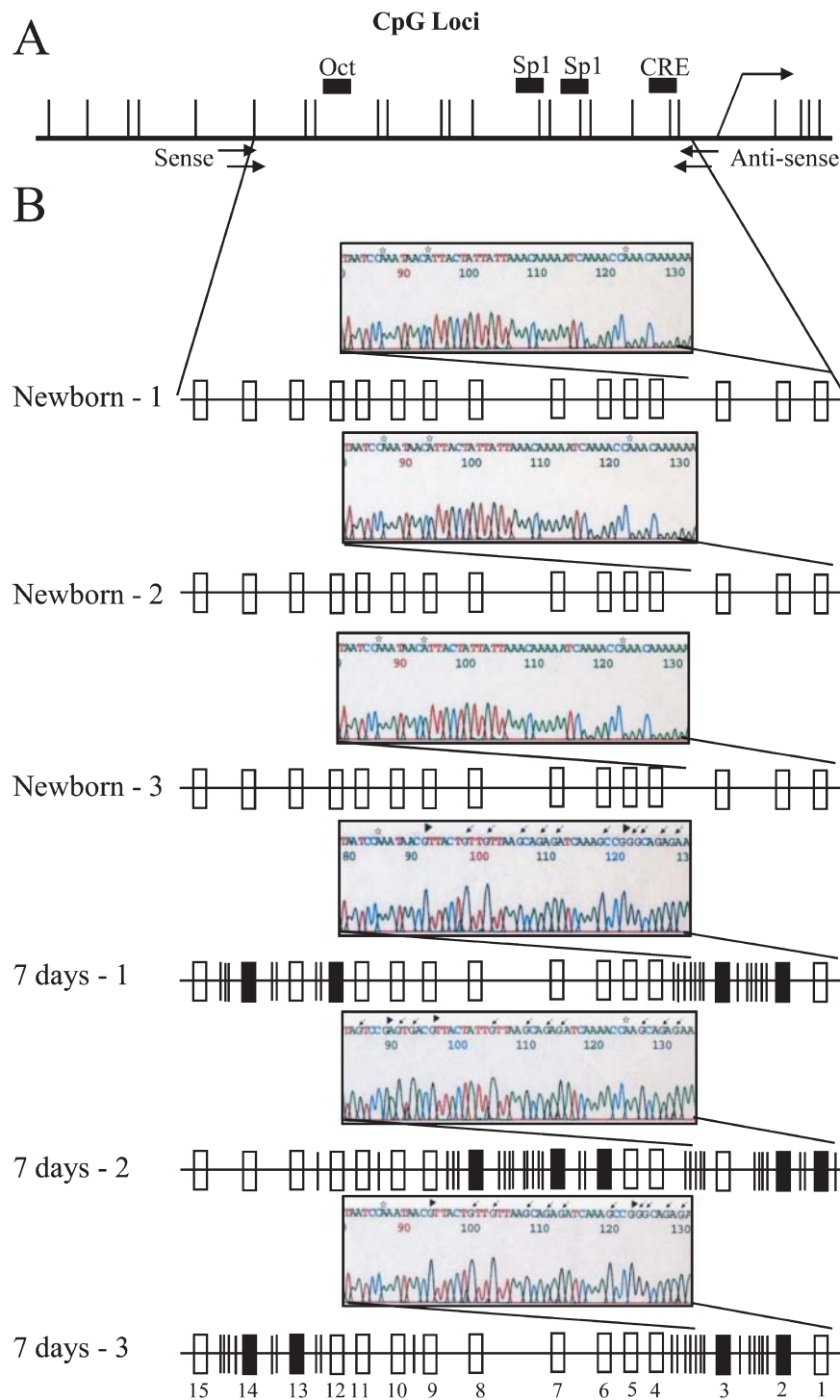
Fig. 5. Mapping methylation status of the epididymal duct of newborn (0-day) and 7-day-old rats by sodium bisulfate modification method. **A.** A schematic structure of the rat cyclin D1 promoter region. Numerous CpG loci (bars) constitute a CpG island in the 5'-flanking region of rat cyclin D1 gene. Sense and antisense primers (arrows) were set to cover the CpG loci located in the basic promoter region. **B.** DNA extracted from the collecting duct of epididymal tissues from newborn and 7-day-old rats were subjected to sodium bisulfite modification before PCR amplification to access more precise information about the methylation status, including the non-CpG locus. All bisulfite sequencing from microdissected tissue samples from the collecting duct of the newborn rat (Newborn-1, -2 and -3) remained unmethylated. In the 7-day-old rat, methylated cytosines are frequently seen at both CpG and non-CpG loci around CRE and two continuous Sp1-binding sites. The methylation status is designated as (black quadrangle) methylated at the CpG locus, (quadrangle) non-methylated at the CpG locus, and (bar) methylated at the non-CpG locus. Processed sequencing data for part of the cyclin D1 gene promoter of the newborn and the 7-day-old rat are inserted.

epigenetic mechanism is involved in cyclin D1 expression during rat testicular tissue development (0- and 7-day-old rats) and spermatogenesis in adult rats, we first checked cyclin D1 protein expression by immunohistochemistry. Unexpectedly, during testicular tissue development, instead of cells of the germ cell lineage expressing only a nominal amount of cyclin D1 protein (Fig. 3), epididymal ductal cells of the newborn rat expressed high levels of cyclin D1 protein, which then rapidly diminished in the 7-day-old rat (Fig. 4). Consequently, we targeted the molecular mechanism that can be linked to cyclin D1 expression in the epididymal duct. Examination of the methylation status of the cyclin D1 promoter from microdissected tissue samples revealed that the silencing of cyclin D1 is correlated with the accumulation of methyl-cytosine at both the CpG and non-CpG loci during the development of the epididymal ducts (Fig. 5). This observation supports our previous observations that the methylation of both CpG and non-CpG around the two continuous Sp1-binding sites and the CRE element of cyclin D1 promoter is an important epigenetic event [6], and that a coordinated significant increase of MeCP2 expression is required for the sufficient and efficient epigenetic silencing of the genes.

In conclusion, our data provide a clue for exploring the regulatory mechanism of MeCP2 expression that significantly accelerated soon after the addition of de novo methylation during post-mitotic terminal differentiation.

V. Acknowledgements

VI. References

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