Involvement of DNA methylation in regulating rat Prop1 gene expression during pituitary organogenesis

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Abstract. PROP1 is a pituitary specific transcription factor that plays a crucial role in pituitary organogenesis. The Prop1 shows varied expression patterns that promptly emerge and then fade during the early embryonic period. However, the regulatory mechanisms governing Prop1 expression remain unclear. Here, we investigated whether Prop1 was under epigenetic regulation by DNA methylation. Bisulfite sequencing was performed on DNA obtained from the pituitary glands and livers of rats on embryonic days (E) 13.5 and E14.5, and postnatal days (P) 4 and P30. The methylation of CpG sites in seven regions from 3-kb upstream of the Prop1 transcription start site through to its second intron were examined. Certain differences in CpG-methylation levels were observed in Region-1 (~2772 b to ~2355 b), Region-4 (~198 b to +286 b), Region-5 (+671 b to +990 b), and Region-6 (+1113 b to +1273 b) based on comparisons between pituitary and liver DNA on E13.5. DNA methylation in pituitary glands on E14.5, P4, and P30 was generally similar to that observed in in the pituitary gland on E13.5, whereas the anterior and intermediate lobes of the pituitary gland on P4 and P30 showed only small differences. These results indicate that Prop1 is under regulation by CpG methylation during the early period of pituitary primordium development around E13.5.

Key words: DNA methylation, Organogenesis, Pituitary, PROP1, Rat

The pituitary gland is a major endocrine tissue that plays important roles in growth, metabolism, reproduction, stress response, and homeostasis for all vertebrates. The adenohypophysis (anterior and intermediate lobes of the pituitary gland) acquires the ability to synthesize and secrete many hormones through the differentiation of progenitor cells into specific hormone-producing cells. Differentiation of hormone-producing cells occurs under the control of various transcription factors, the expression levels of which are spatiotemporally regulated. Among these, Prophet of PIT-1 (Prop1) is specifically expressed in the adenohypophysis and is responsible for the differentiation of hormone-producing cells [1], as well as roles involving progenitor cell migration [2], normal cell differentiation and tumor susceptibility [3], and dorsal-ventral patterning [4]. Notably, a relationship between Prop1 and pituitary progenitor cells has been postulated [5–7]. Yoshida et al. (2009) demonstrated that Prop1 emerges in SOX2-positive pituitary stem/progenitor cells, which then continue to express SOX2 [7]. Thereafter, extensive characterizations of Prop1-positive cells were subsequently reported [8–14].

Prop1 specifically emerges in SOX2-positive cells of the adenohypophysis as early as oral ectoderm invagination on rat embryonic day (E) 11.5, and after 2 days, all cells in the pituitary primordium of Rathke’s pouch remain Prop1-positive [7]. Thereafter, the proportion of Prop1-positive cells promptly decreases, followed by differentiation into committed cells [7]. These changes are controlled at the transcriptional level during pituitary development [8], indicating the presence of unique mechanisms regulating Prop1 expression. Although Ward et al. (2007) attempted to elucidate the mechanisms underlying tissue-specific Prop1 expression using comparative genomics [15], little is currently known about the regulatory factors and mechanisms involved, with the exception of recombination-signal-binding protein for immunoglobulin kappa J region (RBP-J), which is a primary mediator of Notch signaling [16]. Recently, we demonstrated that 21 transcription factors putatively active in the embryonic pituitary gland are potentially involved in Prop1 regulation [17]. Nevertheless, the mechanisms controlling tissue-specific expression of Prop1 remain unclear.

Here, we focused on the epigenetic regulation of Prop1 by studying CpG methylation, which is a potent epigenetic regulatory mechanism for nearly half of all tissue-specific genes [18, 19], and examined DNA methylation involvement in regulating Prop1 expression. Our results revealed differences in CpG-methylation levels in four regions at the Prop1 locus as determined by comparison of pituitary and liver DNA on E13.5. These regions did not exhibit marked changes in their methylation profiles in subsequent developmental
periods, indicating that epigenetic regulation of Prop1 expression by DNA methylation is likely limited to early pituitary primordium development.

**Materials and Methods**

**Animals**

Wistar-Imamichi rats were housed individually in a temperature-controlled room under a 12 h light-dark cycle. All animal experiments were performed following approval from the Institutional Animal Experiment Committee of Meiji University and in accordance with the Institutional Regulations of Animal Experiments and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

**Immunohistochemistry**

Immunohistochemical staining for PROP1 was performed as described previously [7]. Briefly, the pituitary glands of Wistar-Imamichi rats on E13.5, E14.5, postnatal day (P) 4, and P30 were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), followed by immersion in 30% trehalose (Wako, Osaka, Japan) in 20 mM HEPES for tissue cryoprotection. Cryosections of 6 µm thick were treated with guinea pig IgG against rat PROP1 (2 ng/µl; produced in our laboratory), followed by Cy3-conjugated AffiniPure donkey anti-guinea pig IgG (1:500 dilution; Jackson ImmunoResearch, West Grove, PA, USA). The sections were enclosed in VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Immunofluorescence was observed using fluorescence microscopy with a BZ-8000 microscope (Keyence, Tokyo, Japan).

**Reverse transcription polymerase chain reaction (RT-PCR)**

Rat total RNAs were prepared from whole pituitary glands on E13.5 (n = 15), from the anterior and intermediate/posterior lobes on P4 (n = 4) and P30 (n = 6), and from the liver on E13.5 (n = 5), P4 (n = 3), and P30 (n = 3) using ISOGEN (Nippon Gene, Tokyo, Japan). After DNase I treatment, cDNAs were synthesized with PrimeScript reverse transcriptase (Takara Bio, Otsu, Japan) and amplified in a mixture (10 µl) containing a specific primer set for each gene (6 pmol each) and 0.6 U Taq DNA polymerase (TOYOBO, Tokyo, Japan), with reaction conditions of 94°C for 1 min, followed by cycles of 94°C for 15 sec, 62°C for 15 sec, and 72°C for 45 sec. Nucleotide sequences of the primers were as follows: rat Prop1, 5′-TCCTGACATCTGGGTTCGAG-3′ and 5′-ACATCTGGGTTCGAG-3′ of rat TATA-box-binding protein (Tbp), 5′-GATCAAACCCAGAATTGTTCTTC-3′ and 5′-ATGTGGTCTTCCTGAATCCC-3′. The resulting PCR products were analyzed on 3% agarose gels.

**Bioinformatics analysis**

The presence of CpG islands within a region spanning from −4,000 b to + 6,700 b relative to the transcription start site (+ 1 b) of rat Prop1 (accession no. NM_153627) was analyzed with EMBOS Cpgplot (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/).

**Isolation of genomic DNA and sodium bisulfite sequencing**

Fresh pituitary glands and livers were removed in 20 mM HEPES buffer under a stereomicroscope (Leica S8AP0, Leica Microsystems, Newcastle, UK). Genomic DNA was extracted using a Nucleospin Tissue XS (MACHEREY-NAGEL, Düren, Germany) from pooled embryonic (sex unknown) and postnatal male tissues as follows: pituitary gland on E13.5 (n = 10), E14.5 (n = 8), P4 (n = 5), and P30 (n = 2); and liver on E13.5 (n = 2), P4 (n = 5), and P30 (n = 2), respectively. Bisulfite conversion of genomic DNA was performed using a MethylEasy Xceed kit (Human Genetic Signatures Pty, North Ryde, Australia) according to manufacturer instructions. Following bisulfite conversion, genomic DNA was amplified by PCR using the BIOTAQ DNA polymerase (Bioline Reagents, London, UK) with primer sets specific for bisulfite-converted genomic DNA sequences (Table 1). PCR conditions were as follows: 94°C for 10 min, 12
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Variations in the PROPI1-positive cell population during pituitary development

Immunohistochemical staining for PROPI1 was performed to examine the proportion of PROPI1-positive cells during pituitary organogenesis [7]. Figure 1 shows immunostaining of the pituitary glands for PROPI1 on E13.5 (composed of almost all stem/progenitor cells), E14.5 (initiating commitment), P4 (prior to the postnatal growth wave of the anterior lobe), and P30 (after the postnatal growth wave of the anterior lobe). Almost all cells were PROPI1-positive, with the exception of cells in the rostral tip (RT) (pars tuberalis primordium) on E13.5 (Fig. 1A). The population of PROPI1-positive cells decreased slightly by E14.5 (Fig. 1B), and the number of PROPI1-positive cells in the postnatal pituitary gland was markedly decreased, with the remaining cells localizing among cells of the marginal cell layer (MCL) or scattered in the parenchyma of the anterior pituitary on P4 (Fig. 1C). The population of PROPI1-positive cells disappeared from the MCL and markedly decreased in the parenchyma by P30 (Fig. 1D). The populations of PROPI1-positive cells in each lobe (anterior vs. intermediate/posterior) was quantified using postnatal immunostaining images (Fig. 1E), revealing their absence from the intermediate and posterior lobes on P30. On E13.5 and E14.5, PROPI1-positive cells comprised ~83.1% and ~72.6% of the population (Fig. 1E), respectively. However, the number of these cells decreased to 21.4% on P4 and 12.3% on P30 in the anterior lobe, whereas their proportion in the intermediate/posterior lobes remained at < 5% on both postnatal days (Fig. 1E). Furthermore, RT-PCR indicated PROPI1 expression in Rathke’s pouch, the anterior lobe, and the intermediate/posterior lobe, but not in the liver (Fig. 1F).

CpG site distribution at the rat Prop1 locus

Prop1 consists of three exons and two introns (Fig. 2). The positions of CpG sites, the ratio of observed to expected CpG sites, and the GC content in rat Prop1 were analyzed using EMBOSS Cgpplot software. The results showed relatively low GC content and CpG frequency (Fig. 2A and B, respectively). Along with evolutionarily conserved regions [15] and the binding site of the RBP-J transcription factor in the first intron [16], we selected seven regions that were relatively enriched with CpG sites, except for two sites located at isolated positions exhibiting low GC content and in unsuitable regions for primer synthesis (Fig. 2).

DNA methylation at the rat Prop1 locus

The results of our analysis of genomic DNA methylation in samples from the pituitary gland and liver are shown in Fig. 3. The regional methylation levels in pituitary primordium DNA on E13.5 were 59.4%, 88.3%, 85.7%, 35.1%, 25.0%, 50.7%, and 88.9% for Region-1 to Region-7, respectively, and the liver DNA on E13.5 showed methylation levels of 90.6%, 76.2%, 78.6%, 53.6%, 58.3%, 80.8%, and 85.2% for Region-1 to Region-7, respectively. Comparisons of the pituitary and liver DNA on E13.5 revealed that Prop1 Region-2, -3, and Region-7 exhibited relatively high methylation levels in both tissues. By contrast, Region-1, -4, -5, and Region-6 exhibited 20% to 30% lower DNA methylation levels in the pituitary primordium. Notably, unmethylated clones were obtained from Region-4, -5, and Region-6 in pituitary DNA on E13.5. Additionally, five CpG sites in Region-1, two CpG sites in Region-2, two CpG sites in Region-5, and five CpG sites in Region-6 (Fig. 2, boxed) exhibited methylation levels lower by 32% to 58% in the pituitary gland. The DNA samples from the pituitary gland and liver on E14.5, P4, and P30 all showed similar methylation levels with those obtained from the same organ on E13.5.

Separate DNA samples were obtained from the anterior and intermediate/posterior lobes on P4 and P30, and their methylation levels were analyzed. The DNA samples from these two areas did not show any marked differences in methylation patterns, and Region-1 through Region-7 did not show increases in methylation from E13.5 to E14.5 (data not shown). However, the methylation of liver DNA ranged from 52.9% to 96.3% and differed by <20% from E13.5 to P30. These results indicated that some CpG sites in the region from ~2805 b to +1801 b of rat Prop1 were differentially methylated on E13.5, but did not show significant changes after E14.5.

We previously showed that SOX2 is consistently found in PROPI1-positive cells [7], and we hypothesized that SOX2 might participate in the regulation of Prop1 expression. We subsequently tested the potential involvement of many pituitary transcription factors in controlling Prop1 expression and identified SOX2 and 18 other factors (FOXJ1, HES1, HEY1, HEY2, KLF6, MSH1, MSH2, PAX6, PIT1, PITX1, PITX2, Pou5f2, RUNX1, SOX8, SOX11, TEAD2, YBX2, and ZFP36L1) as putative Prop1 regulators [17]. An overlay of the CpG sites from this study and the consensus binding sequences of the putative regulators are shown in Fig. 4.

Discussion

Prop1 plays crucial roles in pituitary organogenesis and is present specifically within pituitary stem/progenitor cells during the embryonic and postnatal periods. PROPI1 exclusively emerges in SOX2-positive cells of the invaginating oral ectoderm on rat E11.5 and becomes expressed in all cells within the subsequent 2 days [7]. Thereafter, Prop1 expression promptly decreases and becomes limited to the stem/progenitor cell niches scattered in the parenchyma of the postnatal anterior lobe [12, 20]. Clarification of the regulatory mechanisms governing Prop1 expression will be indispensable for understanding pituitary organogenesis. A previous comparative genomic analysis [15] and promoter assays examining several transcription factors [17] did not provide solid conclusions. Given these circumstances, this study characterized rat Prop1 CpG methylation by comparing genomic DNA from the developing pituitary gland with genomic DNA from the developing liver, with our results indicating possible
involvement of DNA methylation in regulating rat Prop1 during the early period of development on E13.5.

Although differences in methylation levels were observed between the liver and pituitary gland in Region-1, -4, -5, and Region-6 on E13.5, considerable CpG methylation (25–60%) was observed in these regions in the pituitary primordium. Cells in the RT (pars tuberalis primordium) do not leave the pituitary primordium and never express Prop1; however, the RT comprises ~20% to ~30% of pituitary primordium cells (Fig. 1). Given that the Prop1-promoter region is methylated in the RT, the DNA methylation levels in Prop1-expressing cells on E13.5 and E14.5 would likely be lower than those in cells of the whole pituitary primordium, including the RT. While the proportion of PROP1-positive cells remarkably decreased together with a decline in Prop1 expression during pituitary

Fig. 1. Immunohistochemistry and RT-PCR analysis of the rat pituitary gland during development. Immunohistochemical staining was performed on rat pituitary tissues obtained on E13.5 (A, composed of almost all stem/progenitor cells), E14.5 (B, initiating commitment), P4 (C, prior to the postnatal growth wave of the anterior lobe), and P30 (D, after the postnatal growth wave of the anterior lobe), and PROP1 and nuclei were visualized with Cy3 (red) and DAPI (blue), respectively. Dotted lines indicate the outline of Rathke’s pouch (RP) (A and B) and the anterior (AL), intermediated (IL), and posterior (PL) lobes (C and D). The marginal cell layer (MCL) of the anterior lobe, which is a known stem/progenitor niche, is facing the cleft. Scale bars indicate 100 μm. RT, rostral tip. The populations of PROP1-positive cells were counted, and their proportions are indicated as percentages of the total DAPI-stained cells in (E), where n indicates the number of tissue samples analyzed in each case. RT-PCR analysis of Prop1 in the rat pituitary gland and liver during development (F) was performed using RNAs prepared from tissues on E13.5, P4, and P30. RT-PCR products of Prop1 (42 cycles) and Tbp (34 cycles) were analyzed on 3% agarose gels.
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Development (Fig. 1), methylation status did not differ markedly, indicating that DNA methylation did not change during subsequent development. We previously showed that PROP1-positive cells in the intermediate lobe rapidly decreased prior to those in the anterior lobe [7]. However, the methylation levels between the anterior and intermediate/posterior lobes on P4 and P30 were similar. Although the involvement of DNA methylation in the early stage of pituitary development may be definitive, it does not appear responsible for the decrease in Prop1 expression during pituitary development after E13.5. Similarly, expression of the Oct4 and Nanog genes that are crucial for maintaining pluripotency is downregulated following induction of differentiation without an obvious increase in DNA methylation within their promoter regions [21]. Certain other regulatory mechanisms, such as spatiotemporal variation in transcription factor expression or repressive histone modification, may also be involved.

The proximal region of the transcription start site is important for recognition and binding by numerous factors for which the methylation of CpG sites can be crucial. The third CpG site in Region-4 is located at the putative transcription start site (−1 b to +1 b) and exhibited moderate methylation in DNA from Rathke’s pouch (45%) as compared with DNA from the liver (58%) on E13.5. Similarly, lower methylation was observed at CpG sites on both sides of the putative transcription start site in the pituitary DNA compared to the liver DNA at E13.5. Additionally, during the embryonic period, all CpG sites in region four were relatively hypomethylated, and several clones without any methylated CpG sites were isolated, suggesting a possible role for this region in regulating Prop1 expression.

Our data showed that DNA methylation occurs in the Prop1 5′ region during the early period of pituitary development, but do not explain the overall regulation of Prop1 expression. RBP-J is a primary mediator of Notch signaling, which is responsible for embryonic

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**Fig. 2.** G/C content, CpG islands, and exons near the 5′ region of the rat Prop1 gene. CpG site frequency as a ratio of observed to expected (A) is mapped across the 5′ region of Prop1, and the average GC content in this region is shown (B). The structure of rat Prop1 (C) is shown with open and closed boxes indicating the untranslated region and the coding regions, respectively. Enlarged region of the rat Prop1 gene (D), with shaded boxes indicating regions that are evolutionarily conserved among several mammals. Vertical lines in (C) and (D) indicate the positions of CpG sites, and the numbers indicate nucleotide positions, with the putative transcription start site indicated at +1 b. Boxed nucleotide numbers in (D) indicate sites that are hypomethylated in pituitary DNA as compared with liver DNA on E13.5, where the difference in methylation is > 30%.
Fig. 3. Analysis of CpG methylation in the \textit{Prop1} region during pituitary development. DNA methylation status of each PCR clone was determined by sodium bisulfite sequencing of genomic DNA samples prepared from rat pituitary glands on E13.5, E14.5, P4, and P30 and rat livers on E13.5, P4, and P30. Anterior and intermediate/posterior lobes were separately collected from postnatal pituitary glands on P4 and P30. The open and closed circles on the grids indicate unmethylated and methylated CpG sites, respectively, and numbers indicate the percentage of the CpG sites methylated in each region. Boxed CpG sites are hypomethylated in E13.5 pituitary glands, DNA samples as compared with E13.5 liver DNA samples, where the difference is $>30\%$. 
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development. Thus far, RBP-J was the only transcription factor reported to regulate Prop1, the expression of which was decreased in a conditional knockout of Rbp-j, with a RBP-J-response element identified in the first intron of the mouse gene [16]. The same response-element sequence (TTTCCCACG) is conserved at +846 b to +854 b of the first intron of the first intron of rat Prop1 in Region-5. The CpG site at the 3’ end of the RBP-J binding sequence showed a lower methylation level in the pituitary primordium (17%) than in the liver (34%) at E13.5, but the significance of the difference is uncertain at present. Additionally, an overlay of the CpG site and the binding sites of putative Prop1 regulators [7] demonstrated that the binding sites of SOX2, FOXJ1, HES1/HES2/HEY1, PITX1, and PITX2 are located close to the hypomethylated CpG sites and within 20 bases of Region-1, -5, and Region-6 in the pituitary gland on E13.5. This correlation between transcription factor binding sites and CpG methylation sites requires further investigation.

In summary, this study revealed that CpG sites in cells of the pituitary primordium were hypomethylated in four regions (Region-1, -4, -5, and Region-6) as compared with the same regions in DNA from the liver. Furthermore, no marked increases in methylation were observed in the anterior or intermediate/posterior lobes during later stages of development. Our results indicated that epigenetic regulation of Prop1 by DNA methylation might occur to some extent during the early period of pituitary organogenesis along with concerted modulation by pituitary transcription factors.

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