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DNA methylation contributes to the tissue-specific expression of the rPL-Iv gene

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ABSTRACT

To understand the tissue-specific expression of the rat placental lactogen-I variant (rPL-Iv) gene, we investigated the methylation pattern of the 5′-flanking region of this gene in various rat tissues. We report that the 5′-flanking region of the rPL-Iv gene was hypomethylated in placenta that expressed the gene and hypermethylated in those tissues that did not express the gene. Moreover, the intron region of the rPL-Iv gene was hypomethylated in the placenta, but hypermethylated in the liver, kidney and pituitary. Although there are 5 CpG sites and the density of CpG dinucleotide is lower within 2 kb of the rPL-Iv 5′-flanking region, the methylated promoter reporter gene produced strong repression in the transcriptional activity of the gene. In addition, the 5′-flanking and intron regions of the rPL-Iv gene were hypomethylated on day 12 of gestation, and the methylation pattern in the placenta remained unchanged from mid-pregnancy until term. The entire genomic region of the rPL-Iv gene might be hypermethylated in tissues other than the placenta, within which its methylated status repress expression of the placenta-specific rPL-Iv gene. Interestingly, the methylation status of the intron region of the rPL-Iv in proliferating Rcho-1 cells was changed to the unmethylated status on day 8 and 12 of differentiation of Rcho-1 cells. These results demonstrate that demethylation in the rPL-Iv upstream region was induced at an early stage of placental development, and once the 5′-flanking region of the rPL-Iv had been demethylated, its status on the rPL-Iv genomic region was continued during pregnancy. Taken together, these results suggest that DNA methylation is responsible for the silencing of tissue-specific genes in non-expressing cells, while defined combinations of trophoblast factors dictate the expression of unmethylated rPL-Iv gene in placenta trophoblast cells.

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1. Introduction

The placenta is one of the major endocrine organs in the body and produces various secretory proteins that function during pregnancy. More than 20 genes of the placental lactogen (PL) and prolactin (PRL)-family are known, which are organized into a single locus of the genome [1–3]. Their family members are expressed in placental trophoblast cells, including primary and secondary trophoblast giant cells (TGCs) and spongiotrophoblasts, whose signal regulates the functioning of TGCs [4,5]. The TGCs induce blastocyst attachment into uterus and are a major component of the placenta with regulatory functions in the development of both the fetal and maternal compartments of the placenta.

Many studies have reported that each gene of the PL and PRL-family shows a characteristic stage and tissue-specific expression pattern in TGCs [1,3]. This family of genes is expressed not only in specific types of TGCs but also in the ectoplacental cone, spongiotrophoblast cells and glycogen trophoblast cells in placenta [3]. The rat placental lactogen-I variant (rPL-Iv) is expressed during pregnancy in the placenta, and its production developmentally begins at mid-pregnancy and continues throughout the remainder of the pregnancy [6]. However, the rPL-I gene is expressed at maximum levels by day 13 of gestation, and disappears on day 16 in the placenta [7]. The rPL-II gene expression begins after day 13 of gestation and continues until near term [8]. The temporal and cell-specific transcriptional activation are common characteristics in the expression mode of the placental PRL-family genes, suggesting that the gene transcription is tightly regulated in the placenta during

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pregnancy. Differential expression or availability of transcription factors is likely to be involved in developmental regulation of its expression. It has been reported that the hand1, Stra13 and Gcm1 transcription factors override FGF signaling to promoter terminal differentiation of trophoblast cells [9]. The transcription factors AP-1, Fos, and Jun have been implicated in transcriptional control of the placental-specific genes with differentiation of the TGCs [10]. GATA-2 is highly expressed in differentiated trophoblast Rcho-1 cells and increased levels of GATA-2 result in greater transcriptional activity of the r*PL-I* promoter in Rcho-1 cells [11,12].

The rPL-Iv and rPL-I are highly homologous and the similarity of the nucleotide sequence of cDNA is more than 90% [6,7]. Moreover, the upstream sequence-500 bp in the 5′-flanking region of the both genes is very similar. Comparison of the 5′-flanking region of both genes suggests that expression of the genes may be regulated in a similar manner. Previous studies from our lab have well demonstrated the expression of rPL-I in the placenta, in which the 5′-flanking region of the rPL-I gene is hypomethylated. This region was shown to be hypermethylated in various tissues that do not express the gene [13]. Moreover, the dynamic changes of DNA methylation regulate the expression of bovine placental lactogen and bovine prolactin-related protein-1 genes in bovine placenta trophoblasts [14].

The negative regulation of the tissue-specific gene is associated with DNA methylation which mainly occurs at the fifth position of cytosine within CpG dinucleotides and plays a fundamental role in inhibiting gene expression [15,16]. Recent studies have established that epigenetic regulation is an important mechanism in the expression control for the tissue-specific gene. Many studies have focused on the coordinated set of epigenetic silencing and DNA methylation on the activation of humorous placental genes [17–19]. The placenta strongly expresses DNMT-1, which is the most abundant DNA methyltransferase and considered to be the key maintenance methyltransferase [20]. The analyses of the methylation status of global genomic DNAs in tissues including the placenta showed that there are placenta-specific methylated or unmethylated loci in genomic DNA, and that the formation of a DNA methylation pattern contributes to placental development [21].

Given the placental-specific expression of the rPL-Iv gene the precise mechanism of the tissue-specific regulation of the rPL-Iv expression is not known. An aim of the present study was to clarify the link between placenta-specific gene expression and CpGs methylation at 5′-flanking region of the *rPL-Iv gene.* In the present study, we show that the 5′-flanking region of rPL-Iv gene is hypomethylated in the placenta in which the demethylation of the genomic region at the early stage occurred, and the methylation status of the region inversely correlates with the gene expression among tissues.

2. Materials and methods

2.1. Animal preparation

Adult Wistar-Imamichi rats were purchased from the Imamichi Institute for Animal Reproduction (Ibaraki, Japan). Day 0 of pregnancy was designated when sperm was observed in virginal smears on the next morning of housing with a male rat. Pregnant rats were sacrificed by decapitation for sample dissection. The junctional layers of placenta at days 12, and 20 were dissected, frozen in liquid nitrogen and stored at −80 °C until RNA isolation and genomic DNA extraction. RNA and DNAs in the junctional layer of the placenta tissue was used in all experiments.

2.2. Cell culture

The Rcho-1 trophoblast cell line was derived from a rat choriocacinoma and is capable of differentiating along the trophoblast giant cell lineage [22]. These cells were cultured in NCTC-135 culture medium supplemented with 20% heat-inactivated FBS, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 95% air−5% CO_2 at 37 °C according to the method described previously. The cells were routinely maintained in subconfluent conditions and the culture media was changed at 2-day intervals. The cells were induced to differentiate by growing them to near confluence in FBS-supplemented culture medium and then replacing the FBS with 10% horse serum.

2.3. Analysis of the rPL-Iv expression by RT-PCR

Total RNA was obtained from cells and tissues with TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. We obtained the first-strand cDNA with oligo (deoxythymidine) primers and Superscript II reverse transcriptase (Life Technologies, Inc.). RT-PCR was performed using the following primers: rPL-Iv-sense, 5′-ATGCAGCTGACTTTGACTCTT-3′; rPL-Iv-antisense, 5′-TCAAAAGGTGGACACTCCA-3′. Amplification of beta-actin was performed with the following primers: sense primer, 5′-GACAACGGCTCCGGCATGTGCAAAG-3′; antisense primer, 5′-TTCACGGTTGGCCTTAGGGTTCAG-3′. The PCR product was confirmed to be derived from the rPL-Iv mRNA by nucleotide sequencing (data not shown).

2.4. Southern blotting

Genomic DNA from the dissected tissues was extracted as previously described [12]. Genomic DNAs were digested with EcoRV and a methyl-sensitive enzyme, *Hha*I, which digests only unmethylated GCGC sites. The resulting fragments were size-fractionated on a 0.8% agarose gel, transferred to nylon filters, and hybridized with [α-^{32}P] deoxy-CTP (Amersham Pharmacia Biotech, NJ)-labeled probe (Fig. 2A) corresponding to the rPL-Iv 5′-flanking region. The signals were visualized by a Fujix BAS-2000 analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

2.5. Sodium bisulfite genomic sequencing

Sodium bisulfite sequencing was carried out as previously described [13]. Genomic DNAs digested with NheI were denatured by adding 0.3 M NaOH and incubating for 15 min at 37 °C. After the incubation, sodium metabisulfite and hydroquinone (Nacalai, Osaka, Japan) were added to final concentrations of 2.0 M and 0.5 mM, respectively, and the mixture was incubated at 55 °C for 10 h. The bisulfite reaction was terminated by adding 0.3 M NaOH, and modified DNAs were purified with Wizard DNA Clean-Up system (Promega, Madison, WI). The solution was neutralized by adding NH_4OAc, and the DNA was ethanol precipitated, dried, and resuspended in water. After the bisulfite reaction, the genome was amplified by PCR with various sets of primers to cover the whole sequence of the 5′-flanking region, and each amplified fragment was sequenced to determine the methylated cytosine sites. The amplified PCR products were cloned into pGEM-T easy vector (Promega) and sequenced.

2.6. Reporter gene constructs preparation

The 5′-flanking region of the rPL-Iv gene from rat genomic DNA was cloned into pGL vector (Promega, WI) to construct a reporter gene plasmid to study the promoter activity. To generate the −2025 Luc and −202 Luc constructs, the genomic fragments from −2025 or −202 to +1 (the translation start site was designated +1) were cloned into pGL3-Basic vector. PCR was performed using Ex tag polymerase (Takara) with specific sense primers and a common antisense primer (5′-TCTCTAAGTAGATCCACGACAGTG-3′) to generate genome fragments of luciferase reporter gene. The resultant vectors were designated according to the positions of the fragments as −202 Luc and −2025 Luc which were generated by using each specific sense primer of the following sequences respectively: 5′-GAAGAGCATCTGTGTTCTTTCCAGC-3′ (−2025 to 1) and 5′-ACTGTGCTAAATG-TAAAGAGGATG-3′ (−202 to 1).

2.7. In vitro methylation of plasmid DNA

Reporter gene constructs of the rPL-Iv promoter were methylated in vitro with 3 U *Sss*I methylase (New England Biolabs, Inc., Beverly, MA) for each microgram of DNA in the presence of 160 μM S-adenosylmethionine at 37 C° for 3 h. We confirmed by *Hha*I restriction enzyme digestion followed by electrophoresis and bisulfite sequencing that methylation at all CpG sites in the promoter region were completed (data not shown).

2.8. Luciferase assays

Luciferase assays were carried out according to the method described elsewhere [12] with a slight modification. Rcho-I cells were transfected with 2 μg of reporter luciferase construct and 0.1 μg of control plasmid expressing Renilla luciferase with Lipofectamine (Gibco BRL). The activities of both luciferases were determined by means of a Dual-Luciferase Reporter System (Promega). Each construct was transfected into Rcho-1 cells on day 0 (undifferentiated state) or day 6 of differentiation. Cultures were assayed for luciferase expression 48 h later. All luciferase assay experiments were performed twice independently in triplicate. All results are shown as the mean ± SD.

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