the skeletal muscle of adult mice, where its mRNA expression was very low (Fig. 1*F*). DNA methylation levels of the alternative *Gpam* promoter were low in the skeletal muscle and liver, and GPAT1-variant expression levels were similar between the skeletal muscle and liver (Fig. 1*G*). These observations, taken together, suggest that DNA methylation of the *Gpam* promoter, containing SREs, and its mRNA expression are inversely correlated in the skeletal muscle.

DNA methylation of the *Gpam* promoter in primary culture of neonatal mouse hepatocytes. We next examined DNA methylation of the *Gpam* promoter in primary culture of neonatal mouse hepatocytes. In this study, the gene expression of albumin, a marker of mature hepatocytes, increased during the course of culture, suggesting the maturation of primary hepatocytes (Fig. 2*A*). The DNA methylation levels of the *Gpam* promoter was high at 0 h but gradually decreased at 48 and 96 h, and GPAT1 mRNA levels were low at 0 h and increased at 48 and 96 h (Fig. 2*B*). By contrast, DNA methylation levels of IAP remained high at both 0 and 96 h (Fig. 2*B*), suggesting that decreased methylation is not likely due to total demethylation of the whole genome. These observations also suggest an inverse correlation between DNA methylation of the *Gpam* promoter and its mRNA expression in primary culture of neonatal mouse hepatocytes.

Reporter activity from the *Gpam* promoter with in vitro DNA methylation. In an in vitro reporter assay, we examined whether DNA methylation of the *Gpam* promoter affects the SREBP-1c–induced transcriptional activity. Cotransfection of the *Gpam* promoter-Luc without DNA methylation and SREBP-1c expression plasmid in HEK293 cells caused increased reporter activity from the *Gpam* promoter (Fig. 2*C*). By contrast, upon methylation of the *Gpam* promoter, Luc activity was not increased above the basal level even in the presence of the SREBP-1c expression plasmid (Fig. 2*C*).

Adenovirus-mediated SREBP-1c overexpression and ChIP analysis of the *Gpam* promoter in neonatal and adult primary hepatocytes. To address whether DNA methylation of the *Gpam* promoter is critical for SREBP-1c–dependent GPAT1 expression, we overexpressed SREBP-1c in neonatal (high DNA methylation) and adult (low DNA methylation levels in the *Gpam* promoter) primary hepatocytes through the adenoviral technique. We observed similarly high expression of nuclear form of SREBP-1c protein in neonatal and adult primary hepatocytes (Fig. 2*D*). In this experiment, we observed less expression of GPAT1 with lower recruitment of SREBP-1 to the *Gpam* promoter in the neonatal primary hepatocytes relative to adult primary hepatocytes (Fig. 2*D*). The data indicated that GPAT1 expression is low even in the presence of considerable SREBP-1c expression, suggesting that DNA methylation of the *Gpam* promoter is critical for SREBP-1c–dependent GPAT1 expression.

ChIP analysis of the *Gpam* promoter in neonatal and adult liver. Dnmt1, -3a, and -3b mRNA expression was detected in the neonatal and adult livers, although the

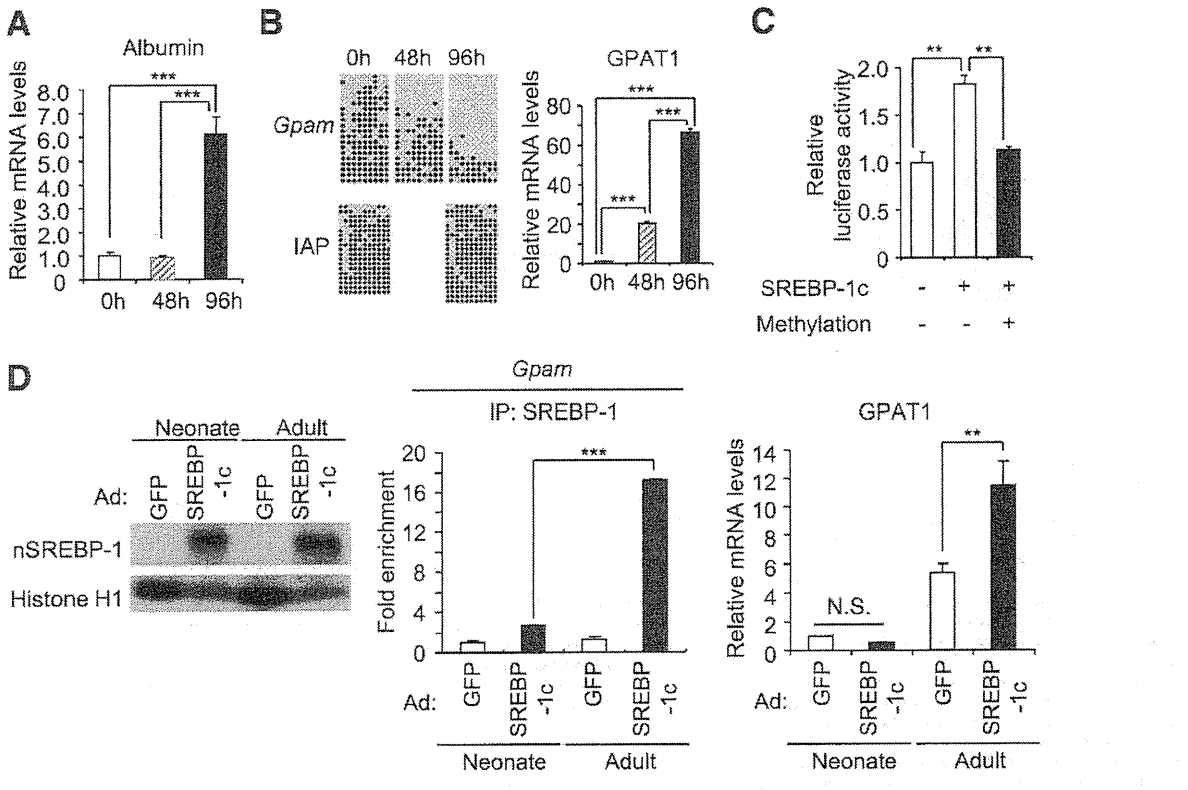

FIG. 2. In vitro analysis of DNA methylation and SREBP-1 recruitment of the *Gpam* promoter. mRNA expression levels of albumin (*A*) and DNA methylation and mRNA expression levels (*B*) of the *Gpam* in neonatal primary hepatocytes, cultured at 0, 48, and 96 h. The *upper panels* are the results of the *Gpam* promoter and lower panels are those of IAP. ***$P < 0.001$. *C*: In vitro methylation reporter assay. Relative luciferase activities are shown. Values without DNA methylation and in the absence of SREBP-1c expression vector are set at 1. **$P < 0.01$; $n = 4$. *D*: *Left*: Western blot analysis of primary hepatocytes from day 0 (neonate) and 10 weeks (adult) of age, overexpressing SREBP-1c and control GFP. *Middle*: ChIP analysis; recruitment of SREBP-1c to the *Gpam* promoter in those cells. *Right*: Quantitative real-time PCR analysis of GPAT1 mRNA. Values for the neonatal hepatocytes with GFP were set at 1. **$P < 0.01$, ***$P < 0.001$; $n = 3$.

hepatic Dnmt1 mRNA levels were lower in the adult liver than in the neonatal liver (Fig. 3*A*). Therefore, we performed ChIP analysis to compare the recruitment of SREBP-1 and Dnmts to the *Gpam* promoter between the neonatal and adult livers. In this study, SREBP-1 was not recruited to the *Gpam* promoter in the neonatal liver (day 0 after birth), although it was clearly recruited in the adult liver (Fig. 3*B*). Importantly, Dnmt3b was strongly recruited to the *Gpam* promoter in the neonatal liver, but not in the adult liver (Fig. 3*B*). Dnmt3a was also recruited, although weakly, to the *Gpam* promoter in the neonatal liver, but not in the adult liver (Fig. 3*B*). We also found that levels of histone H3 lysine-4-trimethylation (H3K4me3) and lysine-9-acetylation (H3K9Ac), two transcriptionally active histone codes, are increased and those of the repressive histone H3 lysine-9-di-methylation (H3K9me2) are decreased at the *Gpam* promoter in the adult liver relative to the neonatal liver (Fig. 3*B*). The recruitment of Dnmt3a and Dnmt3b to the *Scd1* promoter did not differ significantly between the neonatal and adult livers (Fig. 3*C*). In this study, we observed that the recruitment of SREBP-1 and level of H3K9Ac are increased and that of H3K9me2 is decreased at the *Scd1* promoter in the adult liver relative to the neonatal liver (Fig. 3*C*).

DNA methylation of the *Gpam* promoter and GPAT-mediated TG/DG synthesis in primary hepatocytes with Dnmt3b overexpression. With adenoviral transduction of Dnmt3b, which was strongly recruited to the *Gpam* promoter

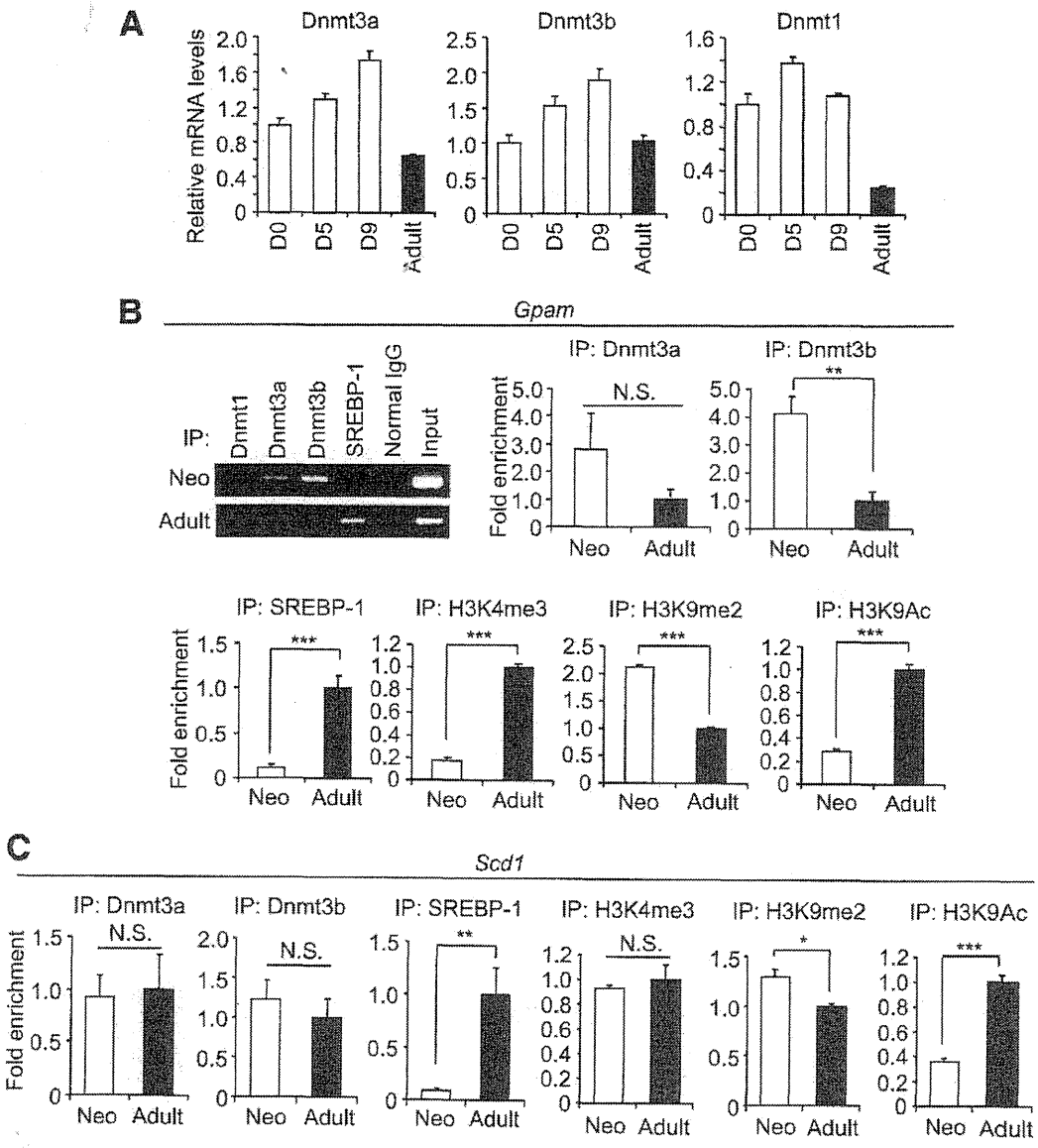

FIG. 3. Recruitment of SREBP-1c and Dnmts to the *Gpam* and *Scd1* promoters containing SREs, with changes in histone modification. *A*: Relative Dnmts mRNA levels in the neonatal (days after birth: 0, 5, and 9) and adult livers (10 weeks of age) examined by quantitative real-time PCR. Values for day 0 were set at 1. $n = 3$ to 4. *B*: ChIP analysis. Neonatal (day 0) and adult (10 weeks) livers were used for ChIP analysis with the indicated antibodies. Amplified PCR primers are as shown in Fig. 1*B*. The *top left panel* is a representative gel electrophoresis photo. Input is a PCR product from the aliquot of liver lysate before immunoprecipitation (IP). The graphs demonstrate quantitative PCR analysis. The graph of Dnmt1 is not shown because signals from the neonatal and adult livers are lower than the negative control IgG signals. *C*: ChIP analysis in the *Scd1* promoter in the samples used in *B*. Values of adult sample are set at 1. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, $n = 3$ to 4.

in the neonatal liver (Fig. 3*B*), we next examined DNA methylation of the *Gpam* promoter, its mRNA expression, and TG/DG synthesis in primary hepatocytes, which were obtained from adult mice with low DNA methylation levels of the *Gpam* promoter. In this study, we confirmed Dnmt3b overexpression causes significant Dnmt3b protein expression (Fig. 4*A*). We found increased DNA methylation of the *Gpam* promoter (Fig. 4*B*), but not *Scd1* and *Fasn* promoters (Fig. 4*C*), in Dnmt3b-expressing hepatocytes relative to control GFP-expressing hepatocytes. The GPAT1 mRNA levels were markedly lower in hepatocytes with Dnmt3b overexpression than those with control GFP (Fig. 4*D*). In the *Gpam* promoter (Fig. 4*E*), but not the *Scd1* promoter (Fig. 4*F*), SREBP-1 was less recruited in Dnmt3b-expressing hepatocytes than in control GFP-expressing hepatocytes. In addition, TG/DG synthesis and TG contents were lower in Dnmt3b-expressing hepatocytes than in GFP-expressing hepatocytes (Fig. 4*G*). These data suggest that increased DNA methylation suppresses SREBP-1 recruitment in the *Gpam* promoter, decreased GPAT1 expression, and TG/DG synthesis.

DNA methylation of the *Gpam* promoter in the neonatal offspring liver of the HF/HS diet–fed dams. We next examined whether the observed DNA methylation of the *Gpam* promoter is affected by environmental factors in vivo. A high-fat or high-calorie diet fed to female animals during gestation and lactation has been reported to increase lipogenic gene expression and TG levels in the liver of offspring (27). In this study, we fed a high-calorie, lipogenic HF/HS diet to dams and examined DNA methylation of the *Gpam* promoter in the offspring. In pups of the HF/HS diet–fed dams, DNA methylation levels of the *Gpam* promoter were lower, and GPAT1 mRNA levels and SREBP-1 recruitment, but not SREBP-1c mRNA levels, were higher than those in the pups of standard diet–fed dams (Fig. 5*A–D*). In this study, hepatic TG levels increased in the pups of the HF/HS diet–fed dams relative to those of the standard diet–fed dams (Fig. 5*E*).

DISCUSSION

The DNA methylation status has been considered to be relatively stable except during embryogenesis and carcinogenesis. However, recent studies showed that DNA methylation can be modulated in normal tissues even after birth (28). In this study, we show that expression of *Gpam*, which encodes a rate-limiting enzyme for TG biosynthesis, increases in the liver during weaning in response to the physiologic demand of TG biosynthesis. This may be related to the dynamic change in the DNA methylation status of the *Gpam* promoter during liver maturation. In the neonatal liver, the *Gpam* promoter region, containing three SREs, shows high DNA methylation levels with low *Gpam* expression, whereas in the adult liver, it shows low DNA methylation levels with high *Gpam* expression. Importantly, SREBP-1c is recruited to the promoter in the adult liver but not in the neonatal liver. In this study, in vitro analysis revealed that DNA methylation of the *Gpam* promoter can suppress the

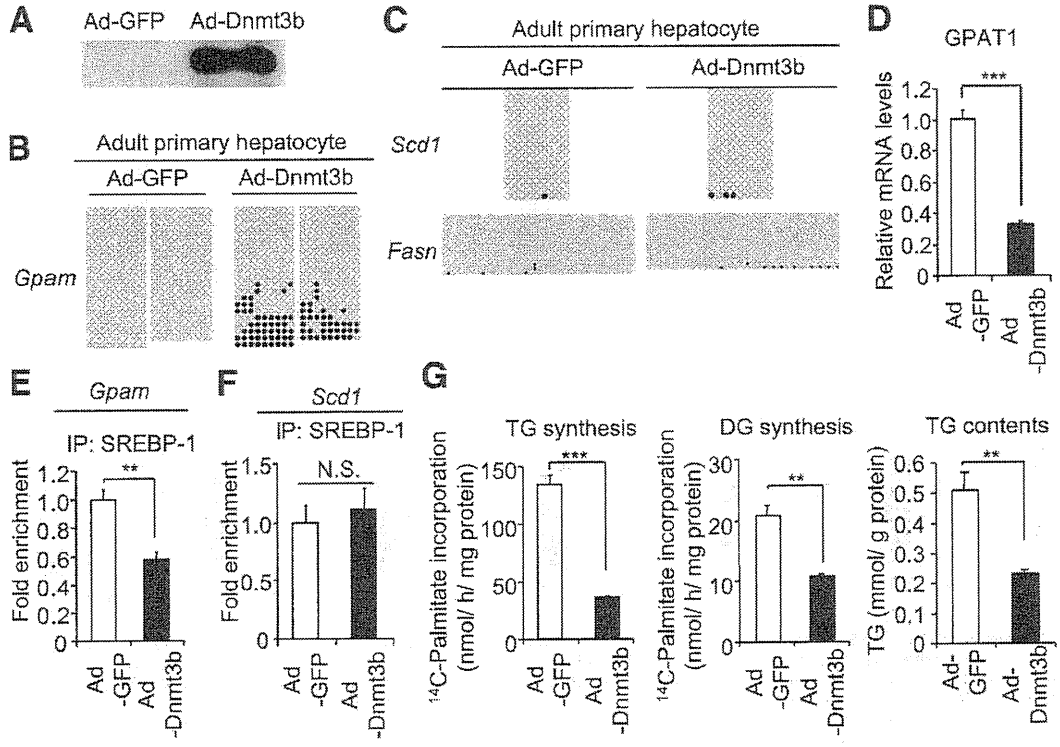

FIG. 4. DNA methylation level of the *Gpam* promoter and GPAT-mediated TG/DG synthesis in the adult primary hepatocytes overexpressing Dnmt3b. *A*: Protein expression levels of Dnmt3b in adult primary hepatocytes overexpressing Dnmt3b and control GFP. Endogenous Dnmt3b protein expression is known to be high in early embryo and embryonic stem cells, but very low in adult tissues (33) and was not detected in this experiment using adult primary hepatocyte samples. *B*: DNA methylation level of the *Gpam* promoter in adult primary hepatocytes overexpressing Dnmt3b and control GFP. Results of two independent dishes from each group are shown. *C*: DNA methylation level of the *Scd1* and *Fasn* promoters. *D*: Expression of GPAT1 mRNA levels and recruitment of SREBP-1 to the *Gpam* (*E*) and *Scd1* (*F*) promoters. Values of GFP cells are set at 1. *G*: TG/DG synthesis levels and TG contents. $^{**}P < 0.01$, $^{***}P < 0.001$. $n = 3$.

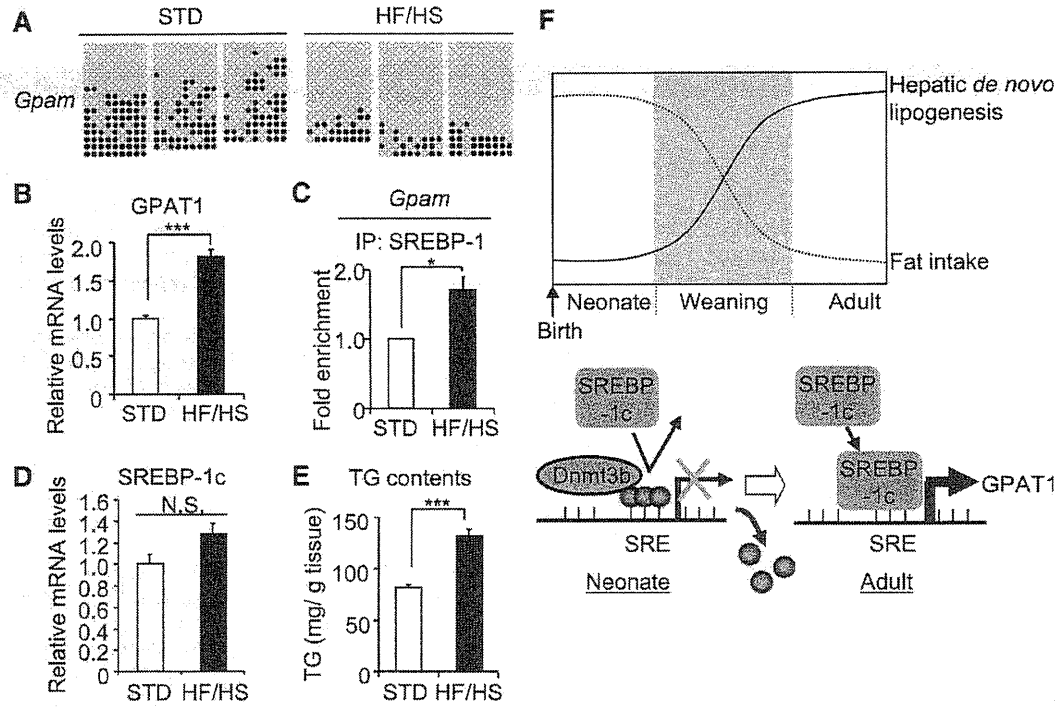

FIG. 5. DNA methylation of the *Gpam* promoter and its mRNA expression in pups of dams fed an HF/HS diet during gestation and lactation. *A*: DNA methylation of the *Gpam* promoter in pups (day 5) of the HF/HS or standard (STD) diet–fed dams. Representative bisulfite sequencing data from three animals in each group. Relative GPAT1 mRNA levels (*B*), recruitment of SREBP-1 to the *Gpam* promoter (*C*), and relative SREBP-1c mRNA levels (*D*) in pups of the HF/HS and STD diet–fed dams. *E*: Liver TG levels of neonatal liver (day 5). Open bar, STD-fed dams; filled bar, HF/HS diet-fed dams. *$P < 0.05$, ***$P < 0.001$; $n = 7$ (open bars), $n = 4$ (filled bars). *F*: Schematic model of the DNA methylation-mediated regulation of *Gpam* expression in the neonatal and adult livers. The graph shows the time course of the dietary fat content and rate of hepatic de novo lipogenesis before and after weaning. In the neonatal liver, the *Gpam* promoter may be highly methylated at least in part by Dnmt3b, when SREBP-1c cannot make access to the promoter. In the adult liver, the *Gpam* promoter is less methylated, with SREBP-1c being recruited to the promoter, thereby activating GPAT1 mRNA expression. Environmental factors, such as nutritional state, may change the DNA methylation status. Closed circle denotes methyl group.

SREBP-1c–mediated transcriptional activation. Less SREBP-1 recruitment in the *Gpam* promoter in Dnmt3b-overexpressing hepatocytes suggests that DNA methylation of the *Gpam* promoter inhibited SREBP-1c recruitment. These observations, taken together, suggest the role of DNA methylation in the suppressed *Gpam* expression in the neonatal liver even in the presence of SREBP-1c. This above discussion is consistent with a previous observation that the extent of hepatic de novo lipogenesis in the neonatal liver is lower than that in the adult liver (1). Consistent with the decreased DNA methylation, transcriptionally active H3K4me3 and H3K9Ac are increased, whereas repressive H3K9me2 is decreased at the *Gpam* promoter in the adult liver. It is conceivable that in the neonatal liver, DNA methylation of the *Gpam* promoter plays roles in the formation and/or maintenance of transcriptionally repressive chromatin conformation, thereby inhibiting the recruitment of SREBP-1c. In contrast, in the adult liver, decreased DNA methylation and increased active histone modifications may lead to the recruitment of SREBP-1c to the *Gpam* promoter.

The mechanism underlying the altered DNA methylation of the genome has not been thoroughly investigated. In this study, we found that Dnmt3b, which is implicated in de novo DNA methylation, is strongly recruited to the *Gpam* promoter in the neonatal liver but not in the adult liver. Dnmt3b overexpression markedly increases DNA methylation levels of the *Gpam* promoter in primary adult hepatocytes. Dnmt3b plays important roles in embryogenesis (10). It has been recently reported to be involved in colon cancer (29) and hormonal gene regulation in renal tubular cells (30). Therefore, it is likely that the decreased recruitment of Dnmt3b plays a role in the decreased DNA methylation of the *Gpam* promoter, and additional mechanism(s) for DNA demethylation may be involved in the process.

The H3K4 methylation level is reported to be reciprocal with the DNA methylation level, and H3K4me2/3 and DNA methylation occur mutually exclusively (11,31). In the region of H3K4me2/3, the DNA methylation levels are generally low. Alternatively, Dnmt3 may preferentially bind to genomic DNA without H3K4 methylation. In this study, the H3K4me3 level in the *Gpam* promoter was low in the neonatal liver and high in adult liver, whereas the H3K4me3 level in the *Scd1* promoter was similar in neonatal and adult livers. Thus, it is possible that histone modification is involved in the regulation of specificity of DNA methylation in *Gpam* and *Scd1* promoters. In addition, because GPAT1 contributes to the transfer of fatty acid to glycerol, whereas SCD1 and FAS are involved in fatty acid biosynthesis (3), the differential positioning of GPAT1, SCD1, and FAS in the TG biosynthesis pathway may explain the different mechanisms of their gene expression. Further studies are required to elucidate how *Scd1* and *Fasn* transcription is regulated in the neonatal liver.

In this study, we also found that GPAT1 mRNA levels are much lower in the adult skeletal muscle than those in the adult liver, although SREBP-1c is highly expressed in

both tissues (19). This is consistent with a previous report that the GPAT1 enzymatic activity in the skeletal muscle is much lower than that in the liver (32). The low expression of *Gpam* in the adult skeletal muscle may be because the high DNA methylation of the *Gpam* promoter suppresses the SREBP-1c–mediated transcriptional activation. Therefore, it is conceivable that DNA methylation of the *Gpam* promoter is involved in the SREBP-1c–dependent tissue-specific regulation of *Gpam* expression. In contrast, the expression of the GPAT1-variant transcript from the alternative promoter without SRE in the neonatal liver is roughly comparable to that in the adult liver. This is consistent with our observation that the alternative promoter is unmethylated in both the neonatal and adult livers, thereby suggesting the role for DNA methylation in the promoter-specific regulation of *Gpam* in the liver.

The change in hepatic gene expression before and after weaning could be regulated in response to nutritional demand. Indeed, we demonstrated that the lipogenic HF/HS diet fed to female mice during pregnancy and lactation results in decreased DNA methylation of the *Gpam* promoter in the liver of offspring, suggesting that the DNA methylation status can be modulated, at least in part, by the nutritional factors. Thus, the change in DNA methylation of the *Gpam* promoter is likely to be affected by the fetal and neonatal environments, such as nutrition, and/or be a programmed process of liver maturation.

Whether DNA methylation would affect hepatic de novo lipogenesis later in life is an important issue to be addressed. Previous reports showed that maternal overnutrition in animals contributes to the development of nonalcoholic fatty liver disease in adult offspring (27), suggesting that hepatic lipid metabolism is nutritionally affected early in life. In this study, maternal overnutrition caused decreased *Gpam* promoter methylation with increased GPAT1 expression and TG level in the liver of the offspring; however, the possibility cannot be excluded that increased lipid flux from dam, as well as increased de novo TG biosynthesis, might have affected the liver TG content of offspring. Further studies are required to understand whether DNA methylation can affect hepatic de novo lipogenesis and susceptibility to fatty liver-related diseases in later life.

In conclusion, this study is the first demonstration of reciprocal change in DNA methylation of the *Gpam* promoter and its mRNA expression in the mouse liver before and after weaning (Fig. 5*F*). Our data suggest that in the neonatal liver, DNA methylation of the *Gpam* promoter containing SREs, which is likely to be induced by Dnmt3b, inhibits the recruitment of SREBP-1c, whereas in the adult liver, the decreased DNA methylation may result in active chromatin conformational change, thereby allowing the recruitment of SREBP-1c. This is the first detailed analysis of the DNA methylation-dependent regulation of TG biosynthesis gene in the liver, thereby leading to the better understanding of the molecular mechanism underlying the epigenetic regulation of metabolic genes and thus metabolic diseases.

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T.E., Y.K., and Y.O. designed the research. T.E., Y.K., M.Tak., X.Y., S.K., E.T., M.Tan., T.Y., and S.M. performed research. T.S. and M.O. analyzed data. O.E. and M.O. contributed new reagents and analytic tools. Y.K. and Y.O. wrote the manuscript. All authors contributed to the discussion. Y.O. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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ORIGINAL

Standardized centile curves and reference intervals of serum insulin-like growth factor-I (IGF-I) levels in a normal Japanese population using the LMS method*

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Abstract. Measurements of insulin-like growth factor-I (IGF-I) are useful not only for diagnosis and management of patients with growth hormone (GH)-related disorders but also for assessing nutritional status. We reported population-based references of serum IGF-I in 1996. However, they did not properly reflect data in the transition period from puberty to maturity. The aim of the present study was to re-establish a set of normative data for IGF-I for the Japanese population. The study included 1,685 healthy Japanese subjects (845 males, 840 females) from 0 to 83 years old. Subjects suffering from diseases that could affect IGF-I levels were excluded. Obese or extremely thin adult subjects were also excluded. IGF-I concentrations were determined by commercially available immunoradiometric assays. The reference intervals were calculated using the LMS method. Median IGF-I levels reached 310 ng/mL in males at the age of 14 years and 349 ng/mL in females at the age of 13 years, falling to 124 ng/mL and 103 ng/mL, respectively, by the age of 70 years. The mean pretreatment IGF-1 SD scores in patients with severe GH deficiency (GHD) obtained from the database of the Foundation for Growth Science and from clinical studies for adult GHD were -2.1±1.6 and -4.9±2.5, respectively. The present study established age- and gender-specific normative IGF-I data for the Japanese population and showed the utility of these references for screening patients with severe GHD.

Key words: Insulin-like growth factor-I, Reference intervals, LMS method, Japanese population

SERUM LEVELS of insulin-like growth factor-I (IGF-I) reflect endogenous growth hormone (GH) secretion. Measurements of IGF-I are particularly useful for diagnosis and management of patients with GH-related disorders such as GH deficiency (GHD) and excess GH [1, 2]. IGF-I is also dependent on nutritional status [2]. Recent studies have suggested that IGF-I levels represent a prognostic factor for systemic diseases including cardiovascular disease [3, 4], cancer [5], osteoporosis [6], and dementia [7]. Serum IGF-I levels are known to vary with age, gender, pubertal stage, physiological condition, and ethnicity [8-10]. Therefore, it is necessary to establish normative IGF-I data specifically for the Japanese population.

We previously reported population-based reference ranges of serum IGF-I for Japanese based on the data from 586 children and 724 healthy adults, in 1996 [11, 12]. These references were widely used and acceptable in clinical practice. However, as the references of children and those of adults were established independently, they did not properly reflect data in the transition period,

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*This article is dedicated to the late Prof. Kenji Fujieda who passed away in March, 2010.

when serum IGF-I levels rapidly decline. Children with all degrees of mild to severe GHD are considered candidates for GH replacement, whereas in adulthood, only patients with severe GHD are currently indicated for GH therapy. Therefore, re-evaluation of the diagnosis at the transition period, when the major pediatric goal of treatment, i.e. the adult height, has been reached, is vital. Measurement of serum IGF-I concentration is recommended for screening for patients with severe GHD [13, 14]. In Japan, GH therapy for adults with severe GHD was approved by the Ministry of Health, Labor and Welfare in 2006. From these points of view, there is an urgent need to establish normative IGF-I references that can be utilized throughout one's life.

In this cross-sectional study, we re-analyzed serum IGF-I levels of a previously defined cohort of children [12] and measured serum IGF-I levels in an additional 1,120 healthy adult subjects [15] in order to establish normative data for the Japanese population from birth to senescence.

Subjects and Methods

A total of 586 children (293 boys and 293 girls) of the defined cohort were re-analyzed using the raw data described previously [12]. We considered that these data were applicable for the present study, because nutritional status of Japanese children did not change so much between 1996 and 2011 judging from the national nutrition survey [16], and auxological standards of Japanese children (i.e. height, weight and body mass index) are based on the national survey in 2000. A total of 1,120 participants over 18 years old were recruited by Sogo-Rinsho-Yakuri Institute [15]. We excluded subjects from medical histories who had pituitary disorders, thyroid disease, gigantism or acromegaly, short stature, diabetes mellitus, liver disease (liver cirrhosis in particular), renal disease, and severe cardiac disease, those who were pregnant or lactating and others who had suffered from an acute illness within 4 weeks. Moreover, those whose serum creatinine was elevated to more than 1.8 mg/dL, whose fasting blood glucose concentration was more than 116 mg/dL, or whose ALT level was 3-fold that of the upper normal values were also excluded. And obese or extremely thin subjects with a body mass index above 30 kg/m^2 or below 17 kg/m^2, respectively, were also excluded from this study; cut-off values were based on the WHO definition of obesity and mild thinness. No subjects were using any drug that could affect IGF-I levels such as oral contraceptives or hormone replacement therapy. The subjects visited the hospital after an overnight fast and their medical history was taken. Height, weight and waist circumference were measured and blood was withdrawn from the ante-cubital vein. Fasting plasma glucose, serum ALT, total cholesterol and creatinine were measured by routine methods. Blood samples for the determination of IGF-I were centrifuged at room temperature for 10 minutes at 3,000 rpm. The sera obtained were stored on ice and placed into freezers at -20 °C on the same day. They were studied in the laboratory of the Mitsubishi Chemical Medience Co., Tokyo, Japan.

Serum IGF-I concentrations were determined by commercially available immunoradiometric assay (IRMA) kit (IGF-I assay "Daiichi"; TFB, Inc., Japan) after dissociation of IGF-I from IGF-binding proteins by non-extraction methods [17]. One hundred randomly selected serum samples were also measured by another commercially available IRMA kit (Somatomedin-C-II "Siemens"; Mitsubishi Chemical Medience Co., Japan [18, 19]). Both assays have been calibrated against the WHO International Reference Reagent 87/518. The intra- and inter-assay coefficients of variation were less than 5% and 8%, respectively, at analyte levels of 400 and 50 ng/mL.

To evaluate the efficacy of IGF-I in screening for patients with severe GHD, we obtained anonymized pretreatment IGF-I data of subjects with severe GHD from the database of the Foundation for Growth Science, Japan [20] and from clinical studies of GH therapy for adult GHD [21-24]. In total, 747 children (523 boys and 224 girls) aged two months to 18 years and 67 adults (34 males and 33 females) aged 18 to 24 years with childhood-onset severe GHD diagnosed according to the criteria by the Hypothalamo-Pituitary Dysfunction Study Group of the Japanese Ministry of Health, Labor and Welfare [25] with recombinant human GH measurements were available for this purpose.

The Ethical Committee of the National Hospital Organization Kyoto Medical Center and of the Sogo-Rinsho-Yakuri Institute approved the clinical study [15]. Written informed consent was obtained from all of the newly recruited participants.

Statistical Analyses

Construction of the centile curves was performed with the LMS Chart Maker Pro version 2.3 software

program (The Institute of Child Health, London), which fit smooth centile curves to the reference data of serum IGF-I using the LMS method. This method summarizes percentiles at each age based on the power of the age-specific Box-Cox power transformations that are used to normalize data [26]. The final curves of the percentiles were produced by three curves representing skewness (L curve), the median (M curve) and coefficient of variation (S curve). The L, M, S values were smoothed for each age and gender using cubic spline curves [27]. Two subjects whose serum creatinine was elevated to more than 1.8 mg/dL or whose ALT level was 3-fold that of the upper normal values were excluded. We excluded 11 children and 8 adults as extreme outliers from the analysis by the LMS method since their IGF-I values were above or below the median ± 2.81 standard deviation (SD); therefore, the final analysis set consisted of a total of 1,685 cases (845 males and 840 females).

Results

Table 1 shows the numbers and gender of subjects in each age group. Since the serum IGF-I concentration did not show a normal distribution, peaked at puberty and declined with age after puberty in both males and females, we adopted the LMS method to analyze these data. The calculated median ± 2SDs of serum IGF-1 and L, M, S values by age for males and females are shown in Table 2a and b, respectively. Scatter plots and smoothing curves of +2SD, +SD, median, -SD and -2SD of serum IGF-I for males and females are depicted in Fig. 1a and b, respectively. There was a gender difference in IGF-I levels: females had higher values than males during childhood and adolescence, and males had higher values than females after 35- years of age.

The two commercially available IRMAs showed very similar values. The coefficient of correlation of the two assays was as high as 0.9864: the conversion equation by the Deming correlation method is expressed as (Daiichi) = 18.03 + 0.822 (Siemens), as shown in Fig. 2.

The SD score (Z-score) of serum IGF-I measurement (y value) was calculated from the L, M and S curves, using values appropriate for the age and gender, by the following equation: $Z= [(y/M)^{L} -1] / (L \times S)$, and if L=0, Z=ln (y/M)/S. With this equation, we calculated SD scores (SDS) of pretreatment IGF-1 levels in children with severe GHD whose data were obtained from the Foundation for Growth Science, Japan and from clinical studies of GH therapy for adult GHD patients [20-24]. The average pretreatment IGF-1 SDS in children with severe GHD was -2.1±1.6 (males, -1.7±1.0: females, -3.0±2.2) and that in childhood-onset adult GHD was -4.9±2.5 (males, -3.3±1.3: females, -6.6±2.4). The percentages of patients showing less than -2 SD were 45.5% (males, 37.1%: females, 65.2%) and 94.0% (males, 88.2%: females, 100%) in childhood and adult patients, respectively.

Discussion

In the present study, we established the age- and gender-specific ranges for serum IGF-1 levels measured by IRMA from birth to senescence in a sample of 1,685 healthy Japanese subjects. The median IGF-1 levels increased slowly in prepubertal children from 70-150 ng/mL with a further steep increase during puberty to approximately 350 ng/mL. Peaks of serum IGF-I levels were observed at 13 years of age in

Table 1 Age and gender distribution of the study population

Age (years)	Male	Female
0-0.99	45	33
1-1.99	12	13
2-2.99	19	11
3-3.99	18	19
4-4.99	14	17
5-5.99	12	10
6-6.99	42	48
7-7.99	10	16
8-8.99	10	24
9-9.99	10	13
10-10.99	12	17
11-11.99	14	9
12-12.99	25	12
13-13.99	18	14
14-14.99	11	11
15-15.99	14	7
16-16.99	2	4
17-17.99	3	6
18-19.99	25	24
20-24.99	51	53
25-29.99	50	51
30-34.99	50	50
35-39.99	50	53
40-44.99	50	49
45-49.99	50	50
50-54.99	52	49
55-59.99	50	51
60-64.99	51	50
65-69.99	51	51
70-	24	25
Total	845	840

Table 2a Serum IGF-I reference ranges, and L, M, and S parameters for IGF-I for males age 0 to 77 years

Age(years)	L	M	S	-2SD	-1SD	Median	+1SD	+2SD
0	0.658	67	0.524	11	35	67	105	149
1	0.647	69	0.498	14	38	69	106	148
2	0.635	74	0.469	18	42	74	111	154
3	0.622	82	0.432	24	50	82	120	164
4	0.607	93	0.390	32	60	93	132	176
5	0.592	108	0.349	44	73	108	148	193
6	0.577	124	0.324	55	86	124	166	215
7	0.561	142	0.325	63	99	142	192	247
8	0.545	165	0.335	72	114	165	225	292
9	0.527	195	0.342	84	134	195	267	350
10	0.509	233	0.348	99	159	233	321	423
11	0.493	272	0.355	113	184	272	377	499
12	0.481	301	0.359	125	203	301	419	557
13	0.473	315	0.353	133	214	315	436	579
14	0.468	315	0.342	138	217	315	433	570
15	0.465	310	0.331	141	217	310	422	552
16	0.464	307	0.326	142	216	307	416	543
17	0.464	306	0.324	142	216	306	414	540
18	0.462	301	0.317	142	214	301	405	526
19	0.461	292	0.306	143	210	292	389	501
20	0.458	280	0.293	142	204	280	368	470
21	0.456	265	0.280	139	197	265	345	436
22	0.454	251	0.269	135	188	251	323	405
23	0.452	237	0.260	131	180	237	304	379
24	0.449	226	0.252	128	173	226	287	356
25	0.447	216	0.245	125	167	216	273	337
26	0.561	212	0.248	119	163	212	268	329
27	0.556	208	0.248	116	159	208	262	322
28	0.550	203	0.249	114	155	203	256	315
29	0.544	199	0.249	111	152	199	251	309
30	0.537	195	0.249	109	149	195	246	303
31	0.529	191	0.250	107	146	191	241	297
32	0.520	187	0.250	105	143	187	237	292
33	0.511	184	0.250	103	141	184	233	287
34	0.500	181	0.251	102	138	181	229	283
35	0.488	178	0.251	100	136	178	226	279
36	0.476	175	0.251	99	134	175	222	275
37	0.462	173	0.252	97	132	173	219	272
38	0.448	171	0.252	96	131	171	217	269
39	0.432	168	0.252	95	129	168	214	266
40	0.417	166	0.252	94	127	166	212	263
41	0.401	165	0.253	94	126	165	209	261
42	0.384	163	0.253	93	125	163	207	259
43	0.368	161	0.253	92	124	161	206	257
44	0.352	160	0.253	92	123	160	204	255
45	0.337	159	0.253	91	122	159	202	253
46	0.324	157	0.254	90	120	157	199	250
47	0.311	156	0.254	90	120	156	199	250
48	0.300	154	0.254	89	118	154	197	248
49	0.291	153	0.255	88	117	153	196	246
50	0.283	152	0.255	87	116	152	194	245
51	0.276	151	0.256	87	115	151	193	243
52	0.272	149	0.257	86	114	149	192	242
53	0.269	148	0.258	85	114	148	190	240
54	0.267	147	0.259	84	113	147	189	239
55	0.268	146	0.260	84	112	146	188	238
56	0.270	145	0.261	83	111	145	187	237
57	0.274	144	0.263	82	110	144	186	236
58	0.281	143	0.264	81	109	143	185	235
59	0.289	142	0.266	80	108	142	184	233
60	0.300	141	0.268	79	107	141	182	232
61	0.312	140	0.270	77	105	140	181	230
62	0.327	138	0.272	76	104	138	180	228
63	0.344	137	0.274	75	103	137	178	226
64	0.363	135	0.276	73	101	135	176	224
65	0.383	134	0.278	72	100	134	174	221
66	0.405	132	0.280	70	98	132	172	219
67	0.428	130	0.282	68	96	130	170	216
68	0.453	128	0.284	66	95	128	168	213
69	0.479	126	0.287	65	93	126	165	209
70	0.506	124	0.289	63	91	124	162	206
71	0.533	122	0.291	61	89	122	160	202
72	0.562	119	0.294	58	87	119	157	198
73	0.591	117	0.296	56	84	117	153	194
74	0.620	114	0.299	54	82	114	150	190
75	0.650	112	0.301	52	80	112	147	185
76	0.680	109	0.303	50	78	109	144	181
77	0.710	106	0.306	48	75	106	140	177

Values were calculated by the LMS method with lms Chart Maker.

Table 2b Serum IGF-I reference ranges, and L, M, and S parameters for IGF-I for females age 0 to 77 years

Age(years)	L	M	S	-2SD	-1SD	Median	+1SD	+2SD
0	0.563	69	0.510	15	38	69	107	154
1	0.490	85	0.480	23	49	85	130	186
2	0.419	99	0.451	32	60	99	150	213
3	0.350	108	0.422	40	69	108	161	227
4	0.283	116	0.395	48	77	116	169	238
5	0.220	126	0.372	56	86	126	181	252
6	0.162	147	0.355	69	102	147	207	287
7	0.109	183	0.346	89	129	183	257	357
8	0.065	224	0.342	111	159	224	314	438
9	0.028	264	0.339	133	188	264	370	517
10	0.000	302	0.333	155	217	302	422	588
11	-0.021	333	0.323	175	241	333	460	638
12	-0.036	348	0.312	188	255	348	476	654
13	-0.045	349	0.301	193	259	349	473	643
14	-0.050	344	0.294	193	257	344	463	625
15	-0.052	341	0.290	192	256	341	456	614
16	-0.052	340	0.289	192	255	340	455	611
17	-0.054	335	0.286	191	252	335	447	599
18	-0.057	326	0.279	188	247	326	431	574
19	-0.060	311	0.271	182	238	311	408	539
20	-0.062	293	0.261	175	226	293	381	499
21	-0.063	275	0.252	168	214	275	355	459
22	-0.062	259	0.243	161	204	259	331	425
23	-0.060	247	0.235	155	195	247	312	397
24	-0.056	237	0.228	151	189	237	297	375
25	-0.051	228	0.222	147	183	228	286	358
26	0.078	223	0.209	146	180	223	274	336
27	0.099	217	0.210	141	176	217	267	328
28	0.120	212	0.211	137	171	212	261	320
29	0.140	206	0.212	133	166	206	254	312
30	0.158	201	0.213	129	162	201	248	304
31	0.176	196	0.214	126	158	196	242	297
32	0.192	192	0.215	122	154	192	237	290
33	0.206	187	0.217	119	150	187	231	283
34	0.219	183	0.218	115	146	183	226	277
35	0.231	178	0.220	112	142	178	221	271
36	0.241	174	0.221	109	139	174	216	265
37	0.250	170	0.223	106	135	170	211	260
38	0.257	166	0.225	103	132	166	207	254
39	0.263	163	0.227	100	129	163	203	250
40	0.268	159	0.229	98	126	159	199	245
41	0.271	156	0.231	95	123	156	195	240
42	0.273	153	0.233	93	120	153	191	236
43	0.273	150	0.235	90	117	150	188	233
44	0.272	147	0.237	88	115	147	185	229
45	0.269	145	0.239	87	113	145	182	226
46	0.266	142	0.241	85	111	142	180	224
47	0.261	140	0.243	83	109	140	177	221
48	0.255	138	0.244	82	108	138	176	219
49	0.249	137	0.246	81	106	137	174	218
50	0.241	135	0.248	80	105	135	172	216
51	0.233	134	0.250	79	104	134	171	215
52	0.225	133	0.251	78	102	133	169	213
53	0.216	131	0.253	77	101	131	168	212
54	0.208	130	0.254	76	100	130	167	211
55	0.200	129	0.256	75	99	129	165	210
56	0.193	128	0.257	74	98	128	164	208
57	0.186	126	0.259	73	97	126	162	207
58	0.180	125	0.260	72	95	125	161	205
59	0.176	123	0.262	71	94	123	159	203
60	0.172	121	0.263	70	93	121	157	201
61	0.170	120	0.264	69	91	120	155	198
62	0.168	118	0.266	68	90	118	153	196
63	0.167	116	0.267	66	88	116	151	194
64	0.168	114	0.269	65	87	114	149	191
65	0.169	112	0.270	64	85	112	146	188
66	0.170	110	0.272	62	84	110	144	186
67	0.173	109	0.273	61	82	109	142	183
68	0.177	107	0.275	60	80	107	139	180
69	0.181	105	0.276	59	79	105	137	177
70	0.185	103	0.278	57	77	103	135	175
71	0.189	101	0.279	56	76	101	133	172
72	0.194	100	0.281	55	75	100	131	170
73	0.199	98	0.282	54	73	98	129	167
74	0.203	96	0.284	53	72	96	127	165
75	0.208	95	0.286	52	71	95	125	163
76	0.212	93	0.287	50	69	93	123	160
77	0.217	92	0.289	49	68	92	121	158

Values were calculated by the LMS method with lms Chart Maker.

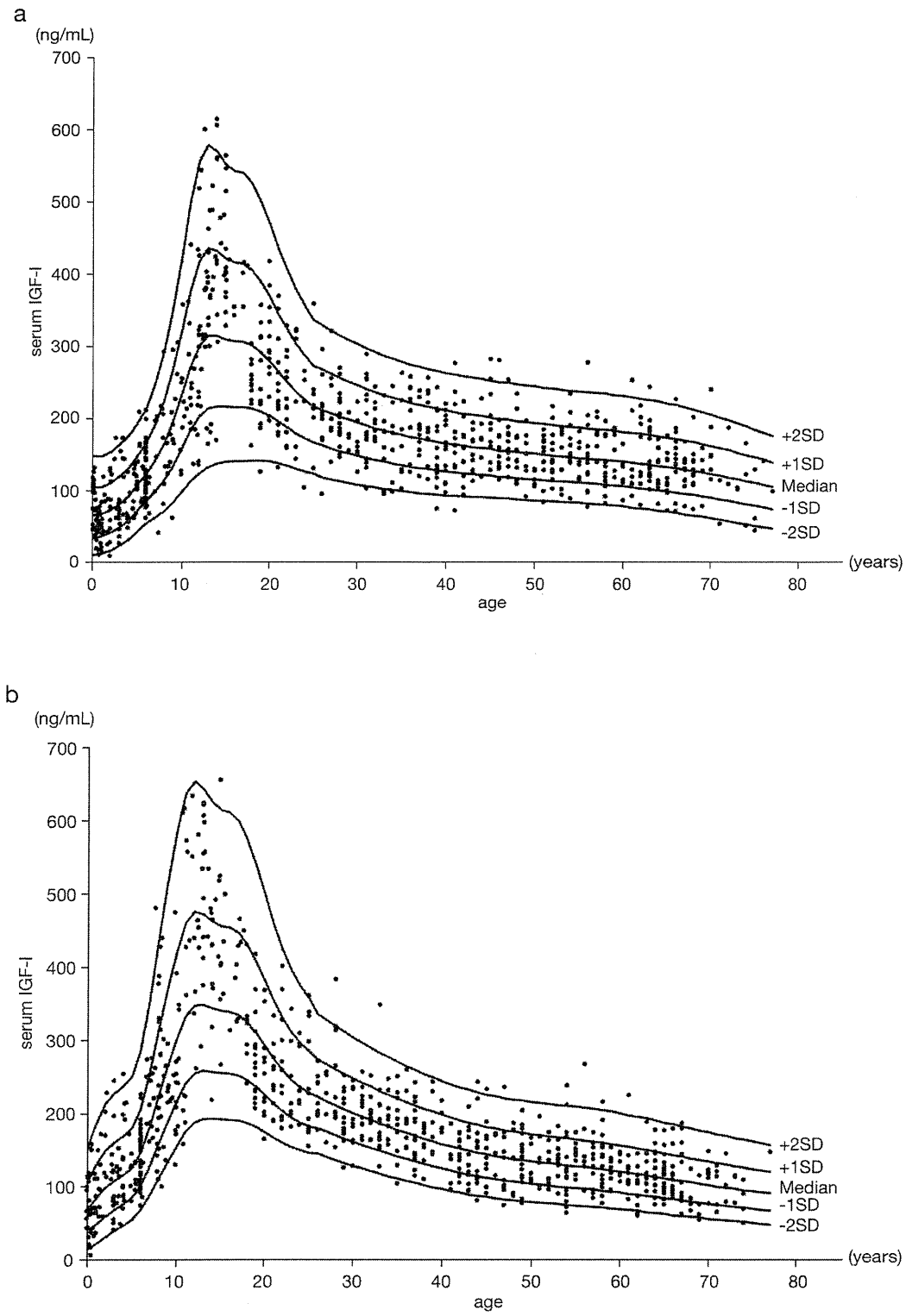

Fig. 1 Scatter plots and smoothing curves of +2SD, +SD, Median, -SD, -2SD of serum IGF-I values in Japanese male (a) and female (b) subjects

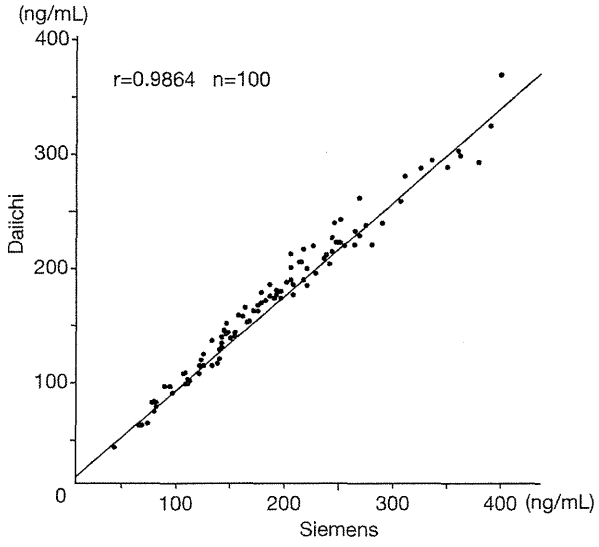

Fig. 2 Correlation between IGF-I values determined by IRMA IGF-I "Daiichi" kit and somatomedin-C-II "Siemens" kit using linear regression analysis
(Daiichi)=18.03+0.822 (Siemens)

girls and 14 years of age in boys, compatible with the results of previous studies [9, 10, 28-32]. These gender differences were in line with the observation that girls experience peak height velocity earlier than boys. After puberty, observed serum IGF-I levels decreased rapidly, reaching a plateau in early adulthood. A subsequent continuous fall in circulating IGF-I levels was apparent throughout adulthood to a mean of 100 ng/mL at the age of 70 years. The decline was steeper in women, who had higher levels initially, but had lower levels than men by the age of 35 years. These gender differences have also been reported in other studies [33, 34], and might result from the diminished estrogen levels with aging in women [34], as estrogens play a major and positive role in regulating the GH/IGF-I axis in both genders [35]. Accordingly, we believe that new Japanese references have been successfully produced and can be adequately used from birth to senescence.

The newly established reference values were constructed by the LMS method with all subjects together, which we believe is one of the most widely applicable approaches for age-related reference intervals [36]. There were three reasons for our adopting the LMS method to construct the reference charts, although other investigators have used other statistical methods such as the polynominal equation model [29] and quantile regression model [33]. The first reason was that with the LMS method, we could easily produce convincing centile curves even when the skewness of data changed with age. In the present study, indeed, the serum IGF-I measurements were not normally distributed, and the skewness of the distribution varied with age. The reference values in the charts constructed by the polynomial equation model are offset from the conditional mean dependent on the global SD of the initially transformed variables because that model assumes that the reference distribution follows a normal distribution without age-variation in skewness. From this point of view, we thought that the LMS method was superior to the polynomial equation model in the present study. The second reason was that the resulting L, M and S curves contained the information needed to draw any kind of centile curve, and to convert measurements into an exact SDS. With reference curves constructed by a quantile regression model, we cannot calculate the exact SDS with any measurement. When we utilize these references to analyze many measurements, SDS is very useful for both clinical management and research purposes. The third reason was that in the LMS method maximum penalized likelihood was used to estimate the age-related curves for each of L, M, and S by natural cubic splines, which indicated that subjective grouping step was removed and the curve fitting across age was controlled directly by the values of equivalent degrees of freedom for three smoothing parameters [36]. Actually, adopting the LMS method with all subjects together which included adequate number of subjects for smoothing centile curves, we could represent the rapid decline from puberty to early adulthood smoothly in the newly established centile curves, compared with those established in the previous studies [10, 28-31], although numbers of subjects around 16 years of age were very small. On the other hand, centile curves constructed by the polynominal equation model or quantile regression model would be undersmoothed, and a bump or a dip would be observed, which reflected the sampling error with small numbers of subjects around 16 years of age. Based on these factors, we decided to adopt the LMS method to generate percentile curves of serum IGF-I levels.

We also found the utility of IGF-I in screening for severe GHD patients, especially in the transition period aged 18 to 24 years. The mean pretreatment IGF-I SDS in patients with severe GHD obtained from the clinical studies for childhood-onset adult GHD was -4.9±2.5 (male, -3.3±1.3: female, -6.6±2.4), and its sensitivity in detecting patients less than -2 SD was 94.0% (male,

88.2%: female, 100%). Moreover, serum IGF-I levels of all subjects except one male were less than 200 ng/mL, which is the cut-off level for screening severe GHD patients recommended in the Japanese guideline for the management of GH-treated adolescents in transition to adult care [14]. These results let us conclude that newly established references for serum IGF-I levels were useful for screening severe GHD patients in the transition period. On the other hand, the utility of IGF-I for severe GHD in childhood was limited, as the percentage of these patients showing less than -2 SDS was 45.5% (male, 37.1%: female, 65.2%). These results may be explained by the fact that IGF-I appears to be regulated by multiple factors and a variable percentage of severe GHD patients have completely normal results [37]. However, the mean SDS of serum IGF-I levels in children with severe GHD was -2.1±1.6 (boy, -1.7±1.0: girl, -3.0±2.2), which was low enough to consider GHD as a differential diagnosis. The combination of serum IGF-I level with auxological information would provide ample information for diagnosis. Taken together, we considered that these references are useful for screening for patients with severe GHD even in childhood.

In conclusion, the present study established age- and gender-specific normative data of IGF-I for the Japanese population and showed the utility of this reference for screening patients with severe GHD before GH replacement therapy. These reference intervals of serum IGF-I levels could be widely used in clinical practice and would assist in calculating patients' SDS.

Acknowledgments

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Conflict of Interest

SY received a research grant from Novo Nordisk Pharma Ltd. KT is employed by JCR Pharmaceuticals Co. Ltd. Others have nothing to declare.

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Successful pregnancy in a patient suffering from recurrent mid-trimester miscarriage with C9 deficiency after receiving cervical cerclage followed by clindamycin and progesterone: A case report

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Abstract

Complement component 9 (C9) deficiency is relatively common, especially in Japan. Here we present the case of a 27-year-old Japanese woman whose obstetric history involved three mid-trimester miscarriages (at 22 weeks', 18 weeks' and 21 weeks' gestation) and one early spontaneous miscarriage. Her fifth pregnancy was successfully managed by cervical cerclage at 13 weeks' gestation, followed by clindamycin administration (600 mg/day for 7 days) and progesterone injections (250 mg/week). She gave birth to a healthy 3326-g male infant at 40 weeks and 1 day gestation after natural onset of labor. After delivery, the serum complement components were analyzed. C9 protein and activity were undetectable in the patient's serum. We suggest that an immunologic disorder such as C9 deficiency should be considered as a potential complication of undiagnosed recurrent miscarriages.

Key words: complement component 9, mid-trimester, miscarriage, pregnancy.

Introduction

The complement system is the effector arm of non-specific humoral immunity, providing first-line defense against infectious agents, including bacteria, viruses, fungi, yeasts and parasites. The incidence of complement component 9 (C9) deficiency in Japan has been reported to be 0.036–0.150% in the general population.[1–3] There are a few reports of C9-deficient patients suffering from intractable infections,[4,5] but most experience good health;[6] however, several studies have demonstrated slow and weak bactericidal activity in C9-deficient serum in vivo.[7,8] Little data exists regarding the effect of C9 deficiency on pregnancy outcome. Here we present the case of a C9-deficient patient who had a successful pregnancy after three consecutive mid-trimester miscarriages and one early miscarriage.

Case Report

The 27-year-old Japanese woman (gravida 4, para 0) had an unremarkable medical history and family history. Although she had no past history of frequent

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infection during the non-pregnant period, she had a poor obstetric history, as described below.

- *First pregnancy: miscarriage at 22 weeks' gestation.* The first pregnancy, which occurred when the patient was 23 years old, ended in a miscarriage at 22 weeks' gestation. Although she felt no abdominal symptoms, such as uterine contractions, the cervix was dilated and labor progressed; finally, membrane rupture occurred at 22 weeks and 1 day gestation.
- *Second pregnancy: miscarriage at 18 weeks' gestation.* The second pregnancy, which occurred when the patient was 24 years old, ended in a miscarriage at 18 weeks' gestation. Again, no abdominal symptoms accompanied cervical dilation, which was noticed at 16 weeks' gestation; membrane rupture occurred despite tocolytic treatment.
- *Third pregnancy: early miscarriage.*
- *Fourth pregnancy: miscarriage at 21 weeks' gestation.* Before the fourth pregnancy, antiphospholipid syndrome was considered, but antiphospholipid antibody titers were negative. This pregnancy occurred when she was 26 years old and ended in a miscarriage at 21 weeks' gestation. The pregnancy was managed in our hospital, where the patient underwent cervical cerclage at 13 weeks' gestation after a diagnosis of cervical insufficiency. Prophylactic tocolysis by continuous ritodrine infusion was initiated after surgery; however, membrane rupture occurred at 21 weeks 0 days' gestation and signs of infection (elevated white blood cell [WBC] count and C-reactive protein [CRP]) were observed. Values of WBC, CRP and fibrinogen were 7300/mm^3, <0.2 mg/dL and 390 mg/dL, respectively, at 13 weeks' gestation (i.e. at Shirodkar's operation); 7340/mm^3, <0.2 mg/dL and 304 mg/dL, respectively, at 20 weeks 6 days' gestation; and 11 780/mm^3, 2.9 mg/dL and 518 mg/dL, respectively, at 21 weeks 0 days' gestation. Normal ranges for these parameters are 3500–9100/mm^3 for WBC, <0.3 mg/dL for CRP and 217–339 mg/dL for fibrinogen.

The fifth pregnancy occurred after the patient conceived naturally. She visited our hospital for care at 5 weeks' gestation. Cervical cerclage was performed at 13 weeks' gestation and prophylactic tocolysis therapy was initiated. Because infection was one of the causes of miscarriage in the previous pregnancy, she received oral clindamycin (600 mg/day) for one week.

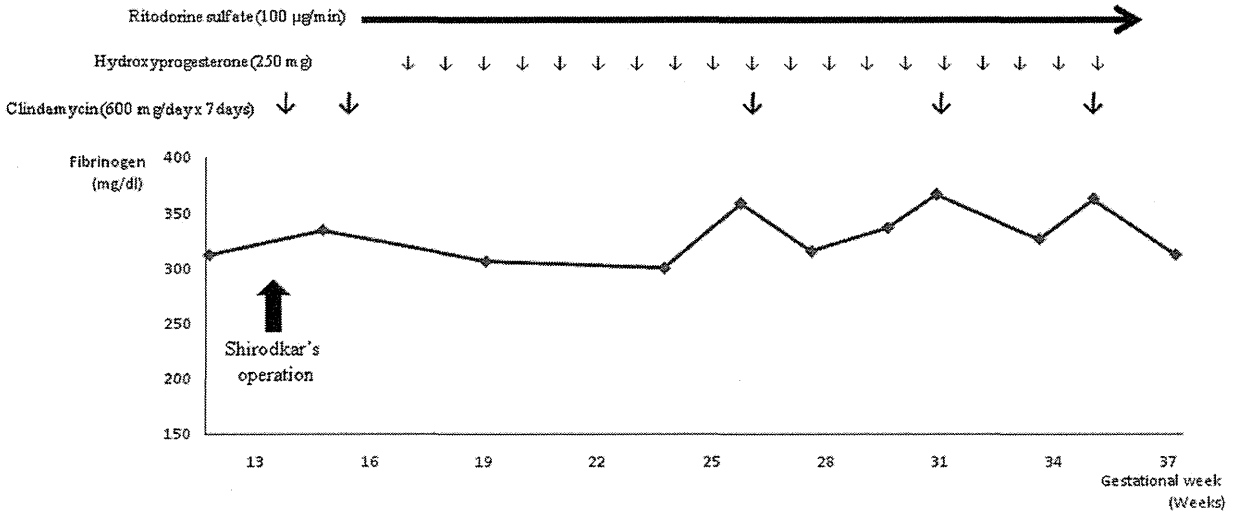
To determine whether the previous three mid-trimester miscarriages were due to immunological abnormalities induced by pregnancy or surgical suture of the cervix, we performed blood tests for autoimmune disease, such as antiphospholipid syndrome and systemic lupus erythematosus, at 14 weeks' gestation. Complement hemolytic activity (CH50) was 15.3 U/mL (normal range: 30–40 U/mL), but complement component 3 (C3) was 90 U/mL (normal range: 86–160 U/mL) and complement component 4 (C4) was 24 U/mL (normal range, 17–45 U/mL). The results of other tests for systemic lupus erythematosus and antiphospholipid syndrome were normal. Rather than consumption of complement, deficiency appeared to be the cause of the abnormal complement result; however, commercial laboratory tests were not available for components other than C3 and C4. Clindamycin treatment was initiated given that elevated fibrinogen levels had been detected before the elevation of WBC and CRP in a previous miscarriage and because the decreased CH50 value suggested an immunological defect. Hydroxyprogesterone caproate (Proge Depot; Mochida Pharmaceutical, Tokyo, Japan) intramuscular injection (250 mg/week) was initiated at 16 weeks' gestation and continued to 36 weeks' gestation, based on evidence that it improved outcomes after previous premature delivery.[9,10] The clinical course and values for fibrinogen, WBC and CRP are shown in Figure 1.

Cervical sutures were removed at 36 weeks' gestation. The patient gave birth to a healthy male infant (3326 g) at 40 weeks and 1 day's gestation after the natural onset of labor. The 1- and 5-minute Apgar scores were both 9, and no structural abnormalities in the baby were observed. After delivery, component deficiency was determined with blood sampled at 14 weeks' gestation (Table 1). C9 activity and concentration were markedly decreased compared with the normal control, confirming that the decreased CH50 value was due to C9 deficiency.

Discussion

A mid-trimester miscarriage is a very painful experience for women who hope to have a baby. In the present case, we detected C9 deficiency as a possible cause of recurrent mid-trimester miscarriage after delivery of a healthy infant.

Causes of mid-trimester abortion include anatomical abnormalities, cervical incompetence, genital tract infection, multiple pregnancy, auto-immune factors, vaginal bleeding and fetal abnormalities.[11] Although causes cannot be confirmed in many cases, cervical insufficiency is often diagnosed in women who



Gestational weeks at blood sampling	11w6d	14w6d	19w1d	23w6d	25w6d	27w5d	29w5d	31w0d	33w5d	35w1d	37w2d
WBC (/mm³)	7960	7960	6660	6810	6520	7330	7340	6620	6870	6960	6850
CRP (mg/dl)	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Fibrinogen (mg/dl)	312	334	306	300	358	315	334	366	326	362	312

* Ritodorine sulfate was administered by continuous drip infusion (→)
** Hydroxyprogesterone was administered by intramuscular injection once a week (↓)
*** Clindamycin was orally administered for 7 days (↓)
**** Normal range: WBC, 3500-9100 (/mm³); CRP, below 0.3 (mg/dl); Fibrinogen, 217-339 (mg/dl)

Figure 1 The patient's clinical course and levels of fibrinogen, white blood cells and C-reactive protein throughout the pregnancy.

Table 1 Complement hemolytic activity (CH50) and complement component C3–C9

Hemolytic activity	CH50 (U/mL)	C3 (U/mL)	C5 (U/mL)	C6 (U/mL)	C7 (U/mL)	C8 (U/mL)	C9 (U/mL)	C9 protein concentration (mg/dL)
Normal human serum (NHS)	44.75	2500	2800	3000	4000	3850	3550	60
(NHS%)	100%	100%	100%	100%	100%	100%	100%	100%
The present patient	14.40	2100	2790	2230	3050	3000	0	0
(NHS%)	32%	84%	100%	74%	76%	78%	0%	0%

CH50 was measured using the Meyer method (normal range: 30–50 U/mL); C3–C9 were measured using the microplate method (detection limit: <5 U/mL); C9 concentration was measured using the single radial immunodiffusion method (detection limit: <10 mg/dL). Blood used in the diagnosis was sampled at 14 weeks' gestation.

experience painless uterine cervical dilation. The diagnosis of cervical insufficiency is based on an obstetric history of recurrent second-trimester or early third-trimester miscarriage following painless cervical dilation, prolapse, or rupture of the membranes and expulsion of a live fetus despite minimal uterine activity. Prophylactic cervical cerclage is an option for women with cervical insufficiency.[12] In the present case, cervical cerclage was performed in the fourth and fifth pregnancies because of two consecutive mid-trimester miscarriages without abdominal symptoms.

Infection is one of the most serious complications after cervical cerclage; it is important to prevent cervical infection and chorioamnionitis to achieve a good pregnancy outcome.[13] In an in vitro study using the serum of a patient with C9 deficiency, slow and weak bactericidal activity against *Escherichia coli* was reported.[7] Although in vitro bactericidal potential has

not been tested in patients with C9 deficiency in previous studies or in the patient of this study, slow and causal bactericidal potential might be associated with the recurrent miscarriages in the present case.

It is not clear why our patient had a successful outcome in her fifth pregnancy; however, there were two important aspects of her prenatal care besides the surgical procedure for cervical insufficiency. The first is the choice of antibiotic and duration of its use. Prophylactic clindamycin administration in the second trimester has been reported to improve the outcomes of patients who have suffered previous preterm births,[14] although it is not clear whether single or repeated administration is better. Another report showed that continuous low-dose antibiotic treatment and cervical cerclage improved the outcomes of patients who suffered from recurrent second trimester miscarriages.[15] In the present case, we administered oral clindamycin (600 mg/day) for 7 days, beginning the day of the operation. Clindamycin was also administered when the patient's fibrinogen levels exceeded the upper limit of normal, because an elevated fibrinogen titer had preceded the elevation of WBC and CRP in the miscarriage of the fourth pregnancy; further, an immunological defect was suspected because of the decreased CH50 titer. Fibrinogen levels were normalized by the administration of clindamycin; however, the clinical significance of fluctuating fibrinogen levels in the present case was unclear. We speculate that the increase in fibrinogen levels reflected early infection or inflammation and the decrease in fibrinogen levels was a result of clindamycin treatment. Fibrinogen is known to be involved in inflammation. Treiber reported a decrease in plasma fibrinogen levels after eradication of *Helicobacter pylori* infection in patients with ischemic heart disease.[16] This finding suggested that treatments directed to *H. pylori* reduced fibrinogen levels in patients with negative CRP. Although causal bacteria were not detected in any of the pregnancies in the present case, clindamycin treatment may have controlled early infections before the WBC and CRP levels increased. We speculate that the normalization of fibrinogen levels can be attributed to the anti-inflammatory and anti-infectious responses induced by clindamycin.

The second was the role of progesterone. Several authors have reported that progesterone improved outcomes of patients at increased risk of preterm birth.[9,10] Progesterone treatment was also reported to induce a shift in cytokine bias by inhibiting proinflammatory and increasing anti-inflammatory cytokine production.[17] In the present case, we think that progesterone treatment may have improved immune function reduced by C9 deficiency.

An immunological disorder such as C9 deficiency should therefore be considered as a potential cause of undiagnosed recurrent miscarriage. More cases will be needed to support an association between mid-trimester miscarriage and C9 deficiency.

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Disclosure

None declared.

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