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ORIGINAL RESEARCH

Demethylation of *LHR* in dehydroepiandrosterone-induced mouse model of polycystic ovary syndrome

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ABSTRACT: The cause of polycystic ovary syndrome (PCOS), a complex endocrine disorder, is unknown, but its familial aggregation implies underlying genetic influences. Hyperandrogenemia is regarded as a major endocrine character of the PCOS. In this study, we employed bisulfite sequencing and bisulfite restriction analysis to investigate the DNA methylation status of *LHR*, *AR*, *FSHR* and *H19* in dehydroepiandrosterone (DHEA)-induced mouse PCOS model. The result showed that methylation of *LHR* was lost in ovary from induced PCOS mouse. However, *AR*, *FSHR* and *H19* had similar methylation pattern in DHEA-treated group and control groups. These data provide evidence for close linkage between DNA demethylation of *LHR* and PCOS.

Key words: DNA demethylation / *LHR* / polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women. The estimated prevalence of PCOS in reproductive age women is about 5–7% (Carmina and Lobo, 1999) and it is characterized by hyperandrogenism and chronic anovulation. It has long been recognized that more than one gene contributes to the heterogeneous disease which can result in hypersecretion of circulating luteinizing hormone (LH) but displays lower or equivalent follicle-stimulating hormone (FSH) levels (Franks *et al*., 1997; Urbanek *et al*., 1999; Abbott *et al*., 2002). Although the negative feedback of LH secretion mediated by either estradiol or progesterone in women with PCOS is impaired, the mechanism for LH hypersecretion is not well understood (Eagleson *et al*., 2000; Abbott *et al*., 2002). In PCOS, elevated LH pulse amplitude and increased LH pulse frequency cause a 2- to 3-fold elevation in circulating LH versus FSH levels (Waldstreicher *et al*., 1988; Dumesic *et al*., 2007). The LH and FSH signals are mediated by the LH receptor and FSH receptor, respectively. LHR mRNA is overexpressed in thecal and granulosa cells from PCOS and may be prematurely expressed in granulosa cells from PCOS (Jakimiuk *et al*., 2001). Demethylation of the promoter CpG sites is necessary for maximal stimulation of *LHR*, whereas deoxyribonucleic acid (DNA) methylation levels do not affect the histone code of this gene promoter (Zhang *et al*., 2005). Although the lower FSH levels are unlikely to be the primary cause for abnormality in PCOS, androgen treatment significantly increases granulosa cell FSHR mRNA abundance in primates including humans (Weil *et al*., 1999; Franks *et al*., 2000). Site-specific methylation of the promoter is a major factor in the repression of *FSHR* gene expression (Griswold and Kim, 2001).

Hyperandrogenemia is the major endocrine characteristic of PCOS (Legro *et al*., 1998a, b). The abnormal receptor-mediated androgen activity leads to changes in the control of follicle development and maturation (Franks, 1995). The androgen receptor (*AR*) gene which is the only X-linked candidate gene for PCOS contains a polymorphic trinucleotide (CAG) repeat sequence in its first exon. The frequency distributions of CAG repeat alleles and *AR*'s differential methylation patterns have been correlated with the disease process leading to PCOS manifestation (Hickey *et al*., 2002). Only two HpaII methylation pattern sites have been assessed so far by PCR-based assays after digestion of DNA with methylation-sensitive HpaII (Vottero *et al*., 1999; Hickey *et al*., 2002, 2006) and it is important to investigate the methylation pattern of *AR*'s entire sites near the polymorphic CAG repeats. Those sites may be involved in regulating *AR* expression in the active X chromosome.

Although it has been difficult to evaluate the etiology and development of PCOS and to obtain ovarian tissue from patients, the mouse model using dehydroepiandrosterone (DHEA) for induction of PCOS has allowed investigations into various aspects of the pathology (Luchetti *et al*., 2004; Elia *et al*., 2006; Sander *et al*., 2006). It has been confirmed that the DHEA–PCOS mouse model exhibits the salient features of human PCOS (Lee *et al*., 1991; Anderson *et al*., 1992). Although the cause of PCOS is unknown, its familial occurrence implies underlying genetic components (Legro *et al*., 1998a, b; Azziz and Kashar-Miller, 2000; Kahsar-Miller *et al*., 2001). In addition, epigenetic modifications by environmental determinants of PCOS may alter the clinical presentation (Hickey *et al*., 2006). Epigenetic modifications are the heritable changes that occur without change in DNA sequence (Wolffe and Matzke, 1999). DNA methylation is thought to be a particularly important epigenetic modification mechanism in mammals. Gene silencing is a major biological consequence of DNA methylation, which plays a role in the etiology of human disease (Bird and Wolffe, 1999; Colot and Rossignol, 1999).

To date, no studies of DNA methylation have been conducted in PCOS genes except for the androgen receptor gene. In the present study, we used bisulfite sequencing and bisulfite restriction approaches to analyze the DNA methylation of several genes related to PCOS susceptibility by using a DHEA-induced mouse model.

Materials and Methods

Animals and experimental protocols

All procedures described here were reviewed and approved by the ethical committee of the Institute of Zoology, Chinese Academy of Sciences. The hyperandrogenized environment of PCOS was reproduced in mice by injection of DHEA (Luchetti *et al*., 2004; Elia *et al*., 2006; Sander *et al*., 2006). Briefly, thirty female prepuberal (25-day-old) mice of the BALB/c strain were daily injected (sc) with DHEA (IL,USA) [6 mg/100 g body weight, dissolved in 0.01 ml 95% ethanol (Aragno *et al*., 1997, 2002), and mixed with 0.09 ml sesame oil] for 20 consecutive days (DHEA group). The control group consisted of thirty mice injected with 0.09 ml sesame oil and 0.01 ml 95% ethanol daily for 20 consecutive days (oil group) and thirty untreated mice (blank group). All of these mice were raised and housed under controlled temperature (22°C) and illumination (14 h light:10 h dark; lights on at 05:00 h) and allowed free access to Purina rat chow and water. After 20 days of treatment, animals were anaesthetized with ether and killed by decapitation. Ovaries were collected and stored at −20°C before use.

Genomic DNA isolation and bisulfite treatment

In each group, all of the 60 ovaries from 30 mice were used in the DNA extraction. One extraction included 20 ovaries. So there were three DNA samples for each group. Two separate bisulfite modification treatments were performed in one DNA sample. Isolation and bisulfite treatment of genomic DNA was carried out as previously described (Liang *et al*., 2008). Briefly, genomic DNA from ovaries was isolated using Wizard SV Genomic DNA Purification System (Promega, USA), followed by using the Methylamp DNA Modification Kit (Epigentek, USA) according to the manufacture's instructions. Bisulfite chemically converts cytosine residues in single-stranded DNA to uracil, whereas leaving 5-methylcytosine unconverted. Cloning the PCR product and sequencing individual clones can detect methylated cytosines (Warnecke *et al*., 1998).

PCR amplification

The preferential amplification of methylated or unmethylated sequences from a mixed population of starting molecules is a potential problem in bisulfite genomic sequencing (Warnecke *et al*., 1998). Therefore, to fully characterize the methylation profile, two individual nested or semi-nested PCR was performed using 2 μl bisulfite-converted DNA in the first round PCR of 25 μl reaction system and 6 μl of the first round PCR products as templates in the second round PCR of 50 μl reaction system. All reactions contained 0.4 mM primers, 0.2 mM dNTPs, 50 mM KCl, 10 mM Tris–HCl, 1.5 mM $MgCl_2$ and 1.25 units of rTaq hotstart polymerase (TaKaRa, Japan). PCR was performed with a Peltier Thermal Cycler-100 (MJ Research) using the following programs. First round: 1 time at 94°C for 6 min, 35 times at 94°C for 1 min, annealing temperature (AT) for 2 min, 72°C for 3 min and 1 time at 72°C for 5 min; second round: 1 time at 94°C for 5 min, 30 times at 94°C for 40 s, AT for 45 s, 72°C for 50 s, 1 time at 72°C for 5 min. For *LHR*, we examined 16 CpG sites in a 219-bp fragment including a CpG island (Fig. 1). The first 173 bp promoter domain which is GC-rich with several SP-1 binding domains was within the fragment examined (El-Hefnawy *et al*., 1996; Nikula *et al*., 2001). The primers used for *LHR* were 5′ TTTAGGTTAAGGAGAATAGGGATAGG 3′ (Out/Inside forward), 5′ CAACATTACCAACACCAACAACTAT 3′ (Inside reverse) and 5′ CTACTTCAACACCAACATTACCAAC 3′ (Outside reverse). For *AR*, we examined 12 CpG sites in a 287-bp fragment including a polymorphic CAG repeat sequence. The primers used for *AR* were 5′ GAGTTTGTTTTGGGATTGGGTTTAGG 3′ (Outside forward), 5′ AAGAGGGGTTTTTAAAGGTTATAGTG 3′ (Inside forward), 5′ CTCTAATTCTCCCAACAAACTAACTC 3′ (Inside reverse) and 5′ CCCCTCTAATACCCTCTCAACCTCC 3′ (Outside reverse). For *FSHR* and *H19*, the primers specific to bisulfite-converted DNA were previously described (Davis *et al*., 2000; Griswold and Kim, 2001; Lucifero *et al*., 2002).

DNA sequencing and restriction analysis of PCR products

Three independent amplifications were performed in one bisulfite converted DNA sample. The PCR products from one DNA sample (six independent amplifications) were pooled together for gel-purifying using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). The purified fragments were subcloned into T vector (TaKaRa, Japan). The six or seven positive clones confirmed by PCR from one DNA sample were sequenced using an automated sequencer (ABI PRISM-77). Twenty clones represented all animals (30 mice) in each group. The remaining portion of the purified fragments were digested with appropriate restriction enzymes TaqI (New England Biolabs) (for *LHR*, *AR* and *H19*) and BasAI (New England Biolabs) (for *FSHR*), respectively. The digested fragments were electrophoresed on 3.0% agarose gels.

Statistical analysis

The methylation rates in each group were expressed as mean ± standard error from bisulfite sequencing. Data were analyzed by One Way Anova, and the difference between group was determined by the Tamhane's T2 Test. $P < 0.05$ was considered statistically significant.

Results

DNA methylation pattern of *LHR*, *FSHR*, *AR* and *H19* were examined in DHEA, oil and blank groups. The bisulfite sequencing and bisulfite restriction analysis results are summarized in Figs 2 and 3, respectively. The data showed that DNA methylation of the *LHR* promoter was

Figure 1 Nucleotide sequences for a 237 bp fragment of mouse *LHR* promoter and 5′ region (−174/+63, upper strands) and its bisulfite converted version (lower strands). The binding domains for Sp1 (GGCGGG) and AP-2 (CCCCGGGC) are shown in bold. Primer sequences are underlined. The CpG dinucleotides are boxed.

lost in the DHEA group compared with the oil and blank groups. The differences between DHEA group and oil and blank group were significant ($P < 0.01$). Compared with the oil and blank group, the majority of PCR products from the DHEA group were undigested (Fig. 3). Unexpectedly, most CpG sites of the *LHR* promoter in the oil group were hypermethylated, whereas it was semi-methylated in the blank group (Fig. 2). However, statistical analysis showed that the difference between the oil and blank group was non-significant ($P > 0.05$). The PCR products from the oil groups were digested (Fig. 3). This indicated that the effects of oil may be site-specific.

Although the results of bisulfite sequencing analysis showed that the number of clones in which all CpG sites of *FSHR* were unmethylated in the DHEA group was higher than that in the oil or blank groups, the difference between each group was non-significant ($P > 0.05$). The 319 bp region of the mouse *FSHR* core promoter encompassed six CpG sites. Three of these sites are found in the major regulatory elements including the consensus E box element (the third CpG site), the Inr region (the fourth CpG site) and the E2F element (the sixth CpG site) (Griswold and Kim, 2001). Methylation level of CpG sites in the three major regulatory elements were plotted (Fig. 4) using the bisulfite sequencing analysis (Fig. 2). As shown in Fig. 4, the three CpG sites within the three major regulatory elements displayed lower methylation levels in DHEA group than in the oil group, but the difference was non-significant ($P > 0.05$). Furthermore, bisulfite restriction analysis did not clearly reveal the slight demethylation of the third CpG site (Fig. 3).

AR showed a similar methylation pattern of heterogeneity in all three groups (Figs 2 and 3) ($P > 0.05$). The polymorphic CAG repeat was located between the fourth and the fifth CpG site. The upstream CpG sites of the polymorphic CAG repeat were hypermethylated, whereas the downstream CpG sites were hypomethylated. The paternally imprinted *H19* also had the same methylation pattern in three groups (Figs 2 and 3). No significant difference was found among DHEA, oil and blank groups in bisulfite sequencing ($P > 0.05$) and bisulfite restriction analysis.

Discussion

PCOS is a common disorder of unknown etiology, but it is known that genetic and environmental factors play a role in the origin and development of this disorder (Franks *et al.*, 1997; Kahsar-Miller and Azziz, 1998; Escobar-Morreale *et al.*, 2005). Environmental determinants of PCOS may alter the clinical presentation via epigenetic modifications (Hickey *et al.*, 2006). DNA methylation, a particularly important epigenetic modification mechanism, regulates gene expression without altering the gene code. Epigenetic differences in DNA methylation and/or histone modification are thought to be involved in the etiology of complex diseases (Fraga *et al.*, 2005). In the present study, the PCOS mouse model created by DHEA induction was used to examine the methylation status of *LHR*, *FSHR*, *AR* and *H19*. Methylation of *LHR* was lost in the DHEA–PCOS group. However, there were no significant changes in methylation of *FSHR*, *AR* and *H19*.

Although the heritability of PCOS is very complex, analysis of linkage and association between one (or several) candidate gene(s) with the PCOS susceptibility may guide further studies (Urbanek *et al.*, 1999). Loss of DNA methylation of *LHR* may be a candidate molecular diagnostic marker. The LHR plays a pivotal role in the reproductive process; *LHR* knockout arrests post-natal sexual development and results in compromised ovulation and infertility (Lei *et al.*, 2001; Zhang *et al.*, 2001; Rao and Lei, 2002). Epigenetic silencing and activation of the *LHR* gene is achieved through coordinated regulation at both the histone and DNA levels (Zhang *et al.*, 2005).

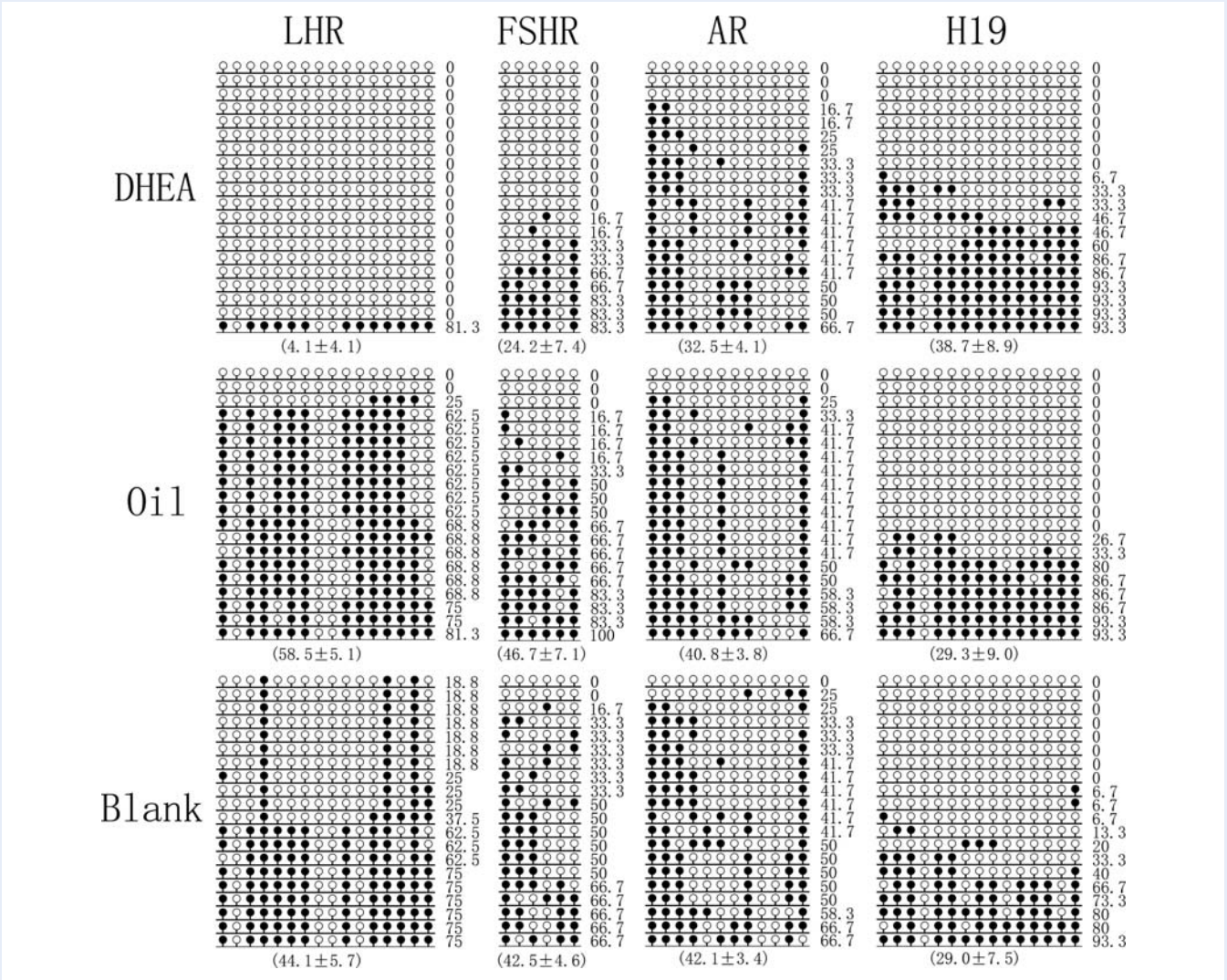

Figure 2 Methylation status of *LHR*, *FSHR*, *AR* and *H19* in DHEA-treated, oil and blank groups. The methylation profiles were obtained by bisulfite sequencing. Each line represents an individual clone allele, with open circles for unmethylated and filled circles for methylated CpG site. Numbers on the right of each line represent the percentage of methylated CpG sites relative to the whole CpG sites examined in each line (line methylation level). The mean methylation level of each group (mean ± standard error) was indicated in parentheses below each group. $P < 0.05$ was considered statistically significant.

Demethylation of the promoter CpG sites is necessary for maximal stimulation of *LHR* expression, although DNA methylation levels did not affect the histone code of the *LHR* gene promoter (Zhang *et al.*, 2005). Although the histone code was not investigated in the present study, full demethylation of the promoter CpG sites indicates that the *LHR* gene may be highly expressed. Study of methylation in DNA from the whole ovary may not be able to detect a difference at a cell-type level. Presumably premature overexpression of *LHR* in some cells of the follicles affects ovulation. It is well-known that DHEA can be converted to androstenedione, testosterone and dihydrotestosterone, and be aromatized to estrogens (Longcope, 1996). In this mouse model of PCOS, demethylation of *LHR* may be potentially due to estrogens or other derivatives. So it is necessary to further investigate how DHEA are related, directly or indirectly, to demethylation of *LHR*.

Unexpectedly, the oil affects DNA methylation status of *LHR* promoter though the difference was non-significant and probably site-specific. This is consistent with a previous study in honeybees which showed that nutritional inputs differentially altered the DNA methylation status (Kucharski *et al.*, 2008). It supports indications that DNA methylation is susceptible to environmental changes; environmental changes such as dietary factors may affect the susceptibility of PCOS via DNA methylation of *LHR*. In addition, stress hormones are potentially elevated for hypothalamic–pituitary–adrenal stress response that may be induced by injection daily with treatments during pubertal development. Stress hormones and its receptors have a possible role in the epigenetic programming of stress response (Meaney *et al.*, 2007; Oberlander *et al.*, 2008). So aberrant methylation of *LHR* in oil-only treated animals may be induced by oil and/or stress hormones. But it is not clear how stress hormones affect the methylation of *LHR*.

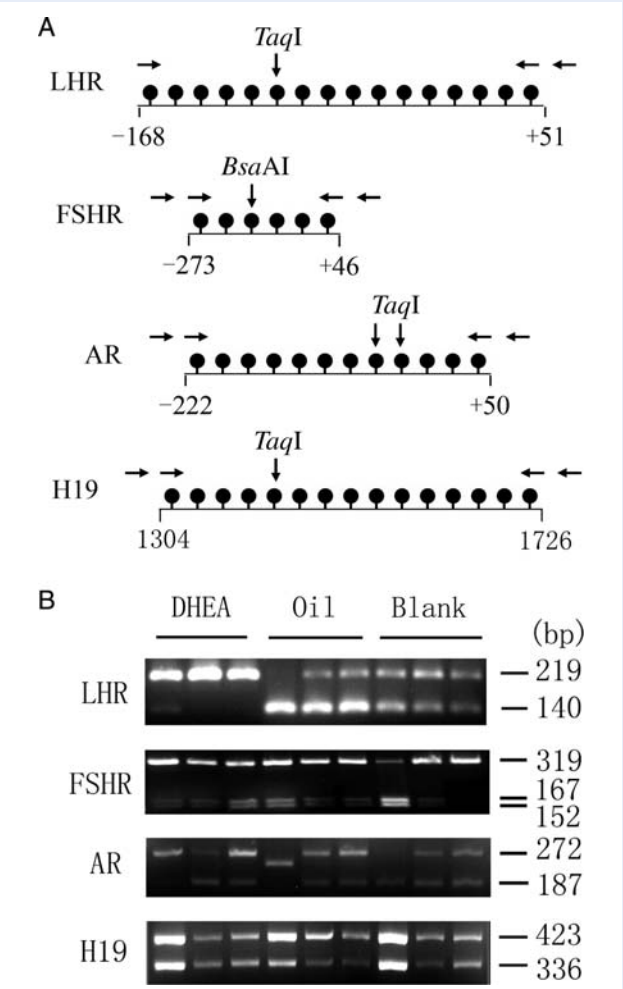

Figure 3 Overall methylation profiles of *LHR*, *FSHR*, *AR* and *H19* as revealed by bisulfite restriction analysis. (**A**) Horizontal arrows represent the primers, and vertical arrows indicate the unique bisulfite PCR restriction enzyme sites. For *LHR*, *FSHR* and *AR*, numbers indicate base positions with respect to the translation initiation site. For *H19*, numbers refer to GenBank accession no. U19619. (**B**) The same bisulfite-treated DNA sample used for sequencing was digested with the restriction enzymes which cut DNA only if the site was methylated at the positions indicated in (A). Sizes of digested fragments are indicated on the right. One DNA sample represents 20 ovaries.

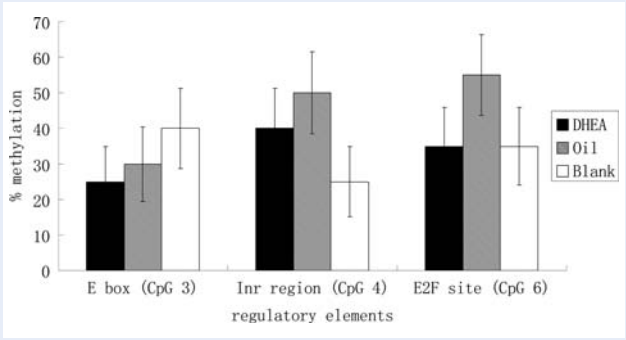

Figure 4 Methylation of *FSHR* major regulatory elements in DHEA-treated, oil and blank groups. The bisulfite sequencing (Fig. 2) was used to plot the number of methylated CpGs percentage of the total number of CpG sites. The results were presented as mean ± standard error.

However, demethylation of *FHSR* promoter regions was not observed in the PCOS mouse model. The FSH signal is mediated by FSHR whose expression is highly restricted to granulosa cells in the ovary (Griswold and Kim, 2001). Site-specific methylation of the promoter, involving direct interference with the binding of transcription factors, prevents *FSHR* gene transcription (Griswold and Kim, 2001). Therefore, other CpG sites' methylation may have limited function. We have analyzed the data more thoroughly to identify the specific CpG sites that are most susceptible to alteration by the treatment of DHEA, although it is uncertain which of these may be critical. The three major regulatory regions have a same methylation level in each group (Fig. 4). This indicates that *FSHR* may have equivalent levels in PCOS.

We further investigated the methylation status of sequences upstream of the highly polymorphic (CAG)n repeat region in the *AR* gene. There was no evident difference between the PCOS and control groups. This is different from another study in which a different method was employed (Vottero *et al.*, 1999; Hickey *et al.*, 2002, 2006). In this report (Vottero *et al.*, 1999; Hickey *et al.*, 2002, 2006), only one or two CpG sites were examined by the PCR-based assays after digestion of DNA with methylation-sensitive HpaII. The two studies are not contradictory because the methylation pattern showed heterogeneity. The polymorphic CAG repeat was located between the fourth and the fifth CpG site in the present study. The upstream CpG sites of the polymorphic CAG repeat were hypermethylated, whereas the downstream CpG sites were hypomethylated. The different methylation patterns were not affected by the DHEA. The upstream and downstream sequences of the polymorphic CAG repeat may have different roles in regulating the AR expression. The frequency distributions of CAG repeat alleles may be more closely correlated with the development of PCOS than methylation patterns. Although the region is only highly polymorphic in humans and has no functional relevance in mice because it is on the mouse X chromosome, it is necessary to investigate the methylation pattern of entire CpG sites upstream of the CAG repeat region in human because of heterogeneity. Furthermore, demethylation of *H19* imprinting control region was not observed in the PCOS mouse model. This demonstrates that methylation imprints may be steadily maintained in the occurrence of PCOS, and this also serves as a control.

In summary, in the present study, we have showed that a high level of DHEA induces demethylation of *LHR* in an artificial PCOS mouse model. However, mouse model may be different from human PCOS. A recent study refutes the concept that prenatal androgen exposure leads to PCOS (Kuijper *et al.*, 2009). Thus, DHEA-induced *LHR* demethylation may be applicable only to this mouse model, rather than the human situation. Further studies in human PCOS are needed to assess whether *LHR* demethylation in part plays a role in the occurrence of PCOS. This may also be valuable for assessing the use of DHEA that is available as an over-the-counter supplement in many countries.

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