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The DNA methylation profile of oocytes in mice with hyperinsulinaemia and hyperandrogenism as detected by single-cell level whole genome bisulphite sequencing (SC-WGBS) technology

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Abstract. Polycystic ovary syndrome (PCOS), a familial aggregation disease that causes anovulation in women, has well-recognised characteristics, two of which are hyperinsulinaemia and hyperandrogenaemia. To determine whether the DNA methylation status is altered in oocytes by high insulin and androgen levels, we generated a mouse model with hyperinsulinaemia and hyperandrogenaemia by injection of insulin and human chorionic gonadotrophin and investigated DNA methylation changes through single-cell level whole genome bisulphite sequencing. Our results showed that hyperinsulinaemia and hyperandrogenaemia had no significant effects on the global DNA methylation profile and different functional regions of genes, but did alter methylation status of some genes, which were significantly enriched in 17 gene ontology (GO) terms ($P < 0.05$) by GO analysis. Among differently methylated genes, some were related to the occurrence of PCOS. Based on our results, we suggest that hyperinsulinaemia and hyperandrogenaemia may cause changes in some DNA methylation loci in oocytes.

Additional keywords: assisted reproduction, fertility, hyperandrogenaemia, insulin, mouse model, PCOS.

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Introduction

Hyperinsulinaemia and hyperandrogenism are well-recognised characteristics of polycystic ovary syndrome (PCOS). Both of them are always caused by unhealthy lifestyle habits along with the symptoms of obesity, irregular menstrual cycles and even increased risk of Type 2 diabetes mellitus or cardiovascular diseases (Wild *et al.* 2000; Carmina 2009).

DNA methylation is an important epigenetic marker that is critical for embryo development and has its specificity in relation to species and tissues. It is deemed as stable and a potential marker for diagnostic purposes. At present, the DNA methylation status of adipose tissue (Kokosar *et al.* 2016), granulosa cells (Xu *et al.* 2016) and ovaries (Zhang *et al.* 2014; Yu *et al.* 2015) has been examined in humans with PCOS. Although it has been confirmed that DNA methylation changes with PCOS in some reproductive tissues, it is still not known whether the DNA methylation status is changed by hyperinsulinaemia and hyperandrogenism or by some other stimulus.

We have previously generated a mouse model with hyperinsulinaemia and hyperandrogenism by injection of insulin and human chorionic gonadotrophin (hCG) and found that hyperinsulinaemia and hyperandrogenism evidently affected oocyte quality and developmental competence (Ou *et al.* 2012). In the present study we used this mouse model to investigate whether hyperinsulinaemia and hyperandrogenism could influence DNA methylation status in oocytes. We chose a new technique named single-cell level whole genome bisulphite sequencing (SC-WGBS) to examine the global DNA profile. Global DNA methylation status was compared between normal oocytes and the oocytes from model mice.

Materials and methods

Hyperinsulinaemia and hyperandrogenism mouse model

Female ICR mice at 30 or 34 days of age were purchased from SPF Biotechnology Co., Ltd. The feeding conditions were in accordance with the stipulations promulgated by the Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences. The mice were randomly divided into three groups: (1) subcutaneous (s.c.) injection of saline (control), (2) s.c. injection of insulin (insulin) and (3) s.c. injection of insulin and hCG (insulin–hCG). The injection dose of insulin was 0.05 IU twice a day for 16 days, then the dose was increased gradually to 0.8 IU for 6 days (Ou *et al.* 2012). The injection dose of hCG was 0.075 IU twice a day (Lima *et al.* 2006).

Serum detection and oocyte collection

The model mice were given an i.p. injection with 8 IU pregnant mare serum gonadotrophin (PMSG; Ningbo). Intraperitoneal injection with 8 IU hCG (Ningbo) was performed 48 h later, then 14 h after hCG blood was collected by removing the eyeball and MII oocytes were recovered and cumulus cells were removed by 1 mg mL^{-1} hyaluronidase (Sigma) treatment. The zona pellucida was removed by treatment with Tyrode's solution (Sigma). The blood was stored overnight at 4°C and was centrifuged at 4000*g* for 10 min at 37°C. Then serum was used to detect insulin and testosterone levels, which were conducted at the biochemical laboratory (Kemei Co.). If the insulin and testosterone levels were significantly higher than the control, then the oocytes were prepared for SC-WGBS.

SC-WGBS analysis

SC-WGBS was performed by Annoroad Gene Tech. Co., Ltd. A total of 30 oocytes were collected from five mice in each group. The samples were kept in 1.5 μL 20 μg mL^{-1} proteinase K (Sigma) at 37°C for 1 h. Bisulphite conversion was performed on cell lysates using an EZ DNA Methylation-Gold Kit (Zymo). CT Conversion Reagent (65 μL) was added into the sample and incubated at 98°C for 10 min then 64°C for 3 h and stored at 4°C; the following steps were consistent with the kit's instructions. λDNA was added in this process to evaluate the conversion efficiency. Finally, DNA was eluted from the column in 10 mM Tris-Cl. Biotin-labelled oligo 1 was added together with dNTPs and reaction buffer and the mix was incubated at 65°C for 3 min, then cooled down to 4°C for primer annealing. Klenow exo– (50 U; NEB) was used for structuring the first strand and following steps were consistent with Smallwood *et al.* (2014). We used 0.8× Agencourt Ampure XP beads and M-280 Streptavidin Dynabeads (Life Technologies) for DNA purification. Klenow exo– (100 U; Enzymatics) was used for structuring the second strand, then the purified DNA was used for polymerase chain reaction (PCR) amplification, which included enzyme activation (95°C for 2 min), amplification (12–13 cycles of 94°C for 80 s, 65°C for 30 s and 72°C for 30 s) and final cooling at 4°C. After PCR amplification, 0.8× Agencourt Ampure XP beads were again used for purification. An Agilent Bioanalyzer and StepOnePlus Real-Time PCR System was used for library quantification and 125-bp paired-end sequencing was executed by a HiSeq2500 platform.

Statistical analyses

The original data was converted to raw data by CASAVA (Illumina), then filtered to get clean reads (see Table S1 available as Supplementary Material to this paper). Both clean reads and the mouse genome were carried on C-to-T (Forward) and G-to-A (Reverse) conversion. The converted reads were mapped to the converted genome by Bismark (Krueger and Andrews 2011; ver. 0.9.0; bismark -p -n -1 -2 -un –bowtie2 -path_to_bowtie -bam -samtools_path -o). Potential methylation site statistics was performed in accordance with the methods of Guo *et al.* (2013). The methylation status of the genome was divided by sliding a 2-kb window and then used for cluster analysis by pair-wise Pearson's correlation. Cluster analysis was also carried out on the methylation status of each locus between different groups. Data was analysed by R software (http://www.r-project.org; accessed 1 September 2009). Gene ontology (GO) function analysis was performed by DAVID software (http://david.abcc.ncifcrf.gov/; accessed 1 September 2017).

Results

Establishment of hyperinsulinaemia and hyperandrogenism mouse model

For the reason that hyperinsulinaemia and hyperandrogenaemia are two important diagnostic criteria for PCOS, we generated a mouse model with these two parameters by injection with insulin and hCG. Through different treatments, the mice were divided into three groups: control mice (control), hyperinsulinaemia mice (insulin) and hyperinsulinaemia–hyperandrogenism mice (insulin–hCG). This mouse model was previously established by our laboratory in 2012 (Ou *et al.* 2012). We detected insulin and testosterone levels in our treated mice and the results were consistent with our previous report.

The overview of SC-WGBS

To investigate the DNA methylation profile in oocytes, we adopted a single-cell level whole genome bisulphite sequencing (SC-WGBS) approach. More than 160×10^6 clean reads were obtained in each group (Table S1) and the clean reads were transformed and compared with the genome. The mapped rates were 51.42, 49.19 and 49.22% in the control, insulin-treated and insulin–hCG-treated mice respectively (Table S2).

CpG island analysis

The methylation status of CpG islands (CGIs) is important for gene transcription, especially in mammals. We used the mapped reads to find the CpG islands of the whole genome (Fig S1 available as Supplementary Material to this paper). The CpG islands statistical analysis showed the relationship between coverage depth and ratios in Fig. 1 (Table S3). The overall methylation level in each group is shown in Fig. 2 and pair-wise Pearson's correlation is shown in Fig. 3 (Table S4). The methylation levels of different functional regions of genes are shown in Fig. 4. There was no significant difference in global methylation and methylation distribution of different gene functional regions between the three groups.

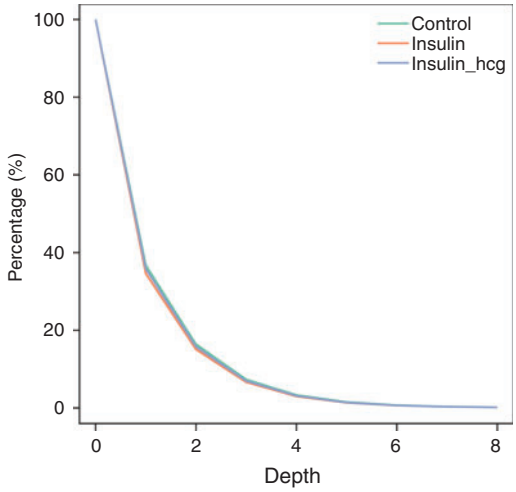

Fig. 1. The cumulative distribution of CpG sites: *x*-axis represents the effective sequencing depth, *y*-axis represents the proportion of accumulated C bases.

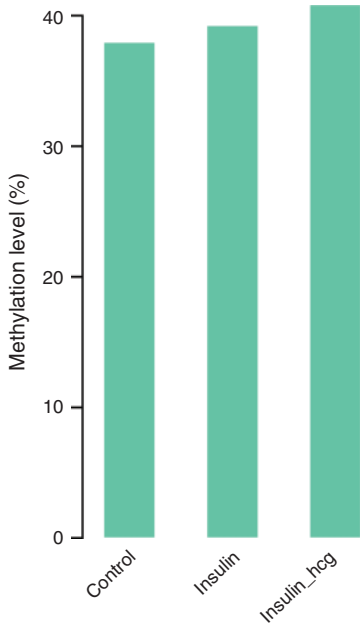

Fig. 2. Whole genome methylation level distribution: *x*-axis represents different groups, *y*-axis represents methylation level.

Differentially methylated regions (DMRs) and cluster analysis

Differentially methylated regions (DMRs) were found between different groups. There were 53 DMRs between the control and insulin groups, 44 DMRs between the control and insulin–hCG groups and 52 DMRs between the insulin and insulin–hCG groups (Table S5; Figs S2–S4). Cluster analyses between different groups were carried out and are shown by their heat maps (Fig. 5). It helped us confirm the DMRs with significant differences.

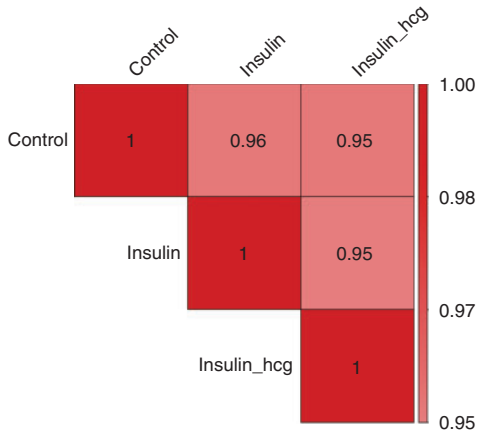

Fig. 3. Pair-wise Pearson's correlation analysis. The higher the correlation, the darker the colour.

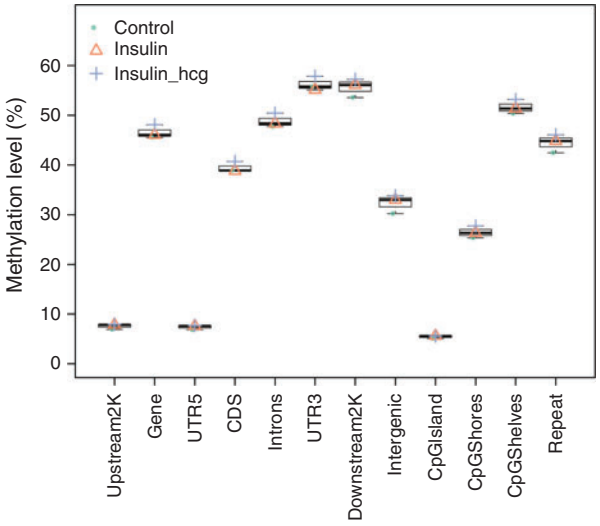

Fig. 4. The methylation distribution of different gene functional regions.

Differential genes and GO analysis

After we found DMRs, we confirmed the corresponding gene names in Table 1. There were 17 GO terms that were significantly enriched ($P < 0.05$; Fig. 6; Tables 2, S6).

Discussion

PCOS is a disease that always shows familial aggregation. Numerous studies have been conducted by using ovary, blood or other tissues from patients, trying to find the genetic sites associated with PCOS by high-throughput sequencing technology and differentially expressed genes have been confirmed. These approaches are direct, but it is nevertheless hard to distinguish the causes of PCOS since, in addition to genetic inheritance, epigenetic inheritance and hormone changes are always affected by environmental stress. In order to determine whether environment stress affects the DNA methylation of the

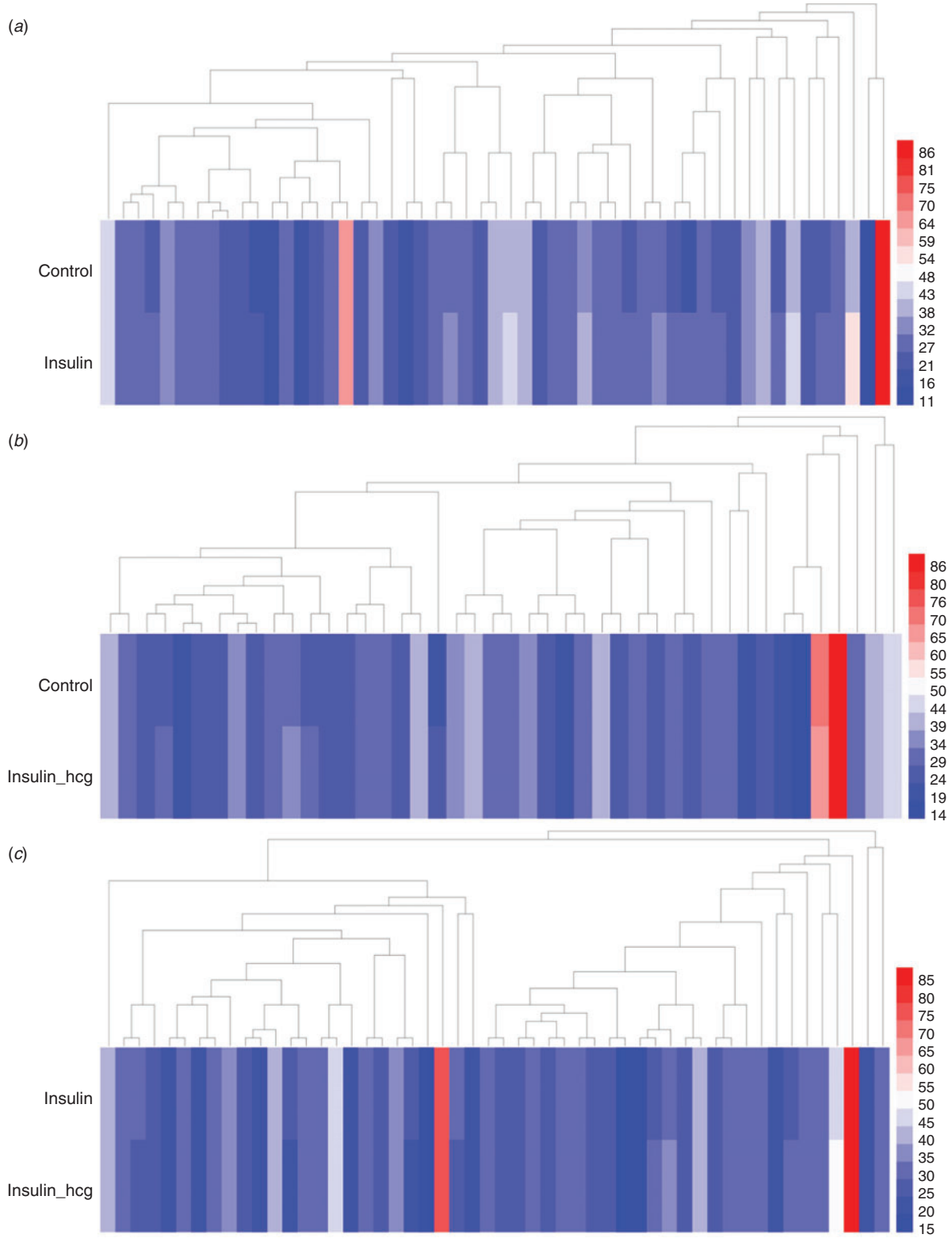

Fig. 5. Heat map by cluster analysis between different groups. Red represents increased expression, blue represents decreased expression.

Table 1. Differently methylated genes

3110070M22Rik, *A830018 L16Rik*, *Adra1b*, *Atp1b3*, *Atp7b*, *Cenpv*, *Ctu1*, *Flg2*, *Gm7120*, *Hace1*, *Hdac5*, *Hsdl1*, *Ncor2*, *Nim1k*, *Papln*, *Pigm*, *Prdm16*, *Ptprn2*, *Slc4a5*, *Tmem267*, *Topors*, *Vwf*. Note: These names are official names (Provided by MGI), which can be researched via NCBI

Comparison groups		Gene names
Control	Insulin	*Gm7120*, *Ptprn2*, *Cenpv*, *Hsdl1*, *Ctu1*, *Prdm16*, *Topors*
Control	Insulin–hCG	*Tmem267*, *3110070M22Rik*, *Ptprn2*, *Cenpv*, *Hsdl1*, *Ctu1*, *Prdm16*, *Topors*
Insulin	Insulin–hCG	*A830018 L16Rik*, *Pigm*, *Flg2*, *Prdm16*, *Ncor2*, *Slc4a5*, *Vwf*, *Atp7b*, *Atp1b3*, *Hace1*, *Adra1b*, *Hdac5*, *Papln*, *Nim1k*

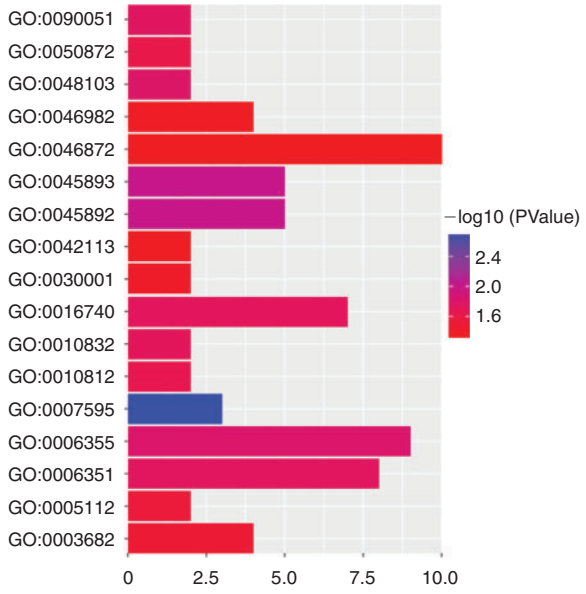

Fig. 6. GO analysis of differently methylated genes.

patients, given the premise that the genetic background is consistent, we chose to use the model animals. We generated hyperinsulinaemia and hyperandrogenaemia mouse models by injection of insulin and hCG to find the relationship between environmental factors and DNA methylation in oocytes of PCOS-like mice.

Because environmental factors and experimental individuals were important for epigenetic experiments, the mice used for the experiments were specific pathogen-free (SPF) animals, whose feeding conditions were strictly kept. The weight of these mice differed by 2 g when they were bought. During the 22-day treatment, we tried to avoid the influence of environmental factors and experimental individuals as carefully as possible to ensure a consistent outcome. The oocytes and serum from a single mouse were collected at the same time. When the hormone was significantly different between different groups, then the oocytes were used for SC-WGBS. A total of 30 oocytes

Table 2. GO enrichment analysis of differently methylated genes

GO accession	Description	*P*-value
GO:0007595	Lactation	0.002134
GO:0045893	Positive regulation of transcription, DNA-templated	0.009990
GO:0045892	Negative regulation of transcription, DNA-templated	0.010170
GO:0006355	Regulation of transcription, DNA-templated	0.015290
GO:0048103	Somatic stem cell division	0.016308
GO:0090051	Negative regulation of cell migration involved in sprouting angiogenesis	0.017777
GO:0006351	Transcription, DNA-templated	0.018063
GO:0016740	Transferase activity	0.018917
GO:0010832	Negative regulation of myotube differentiation	0.019245
GO:0010812	Negative regulation of cell-substrate adhesion	0.022174
GO:0050872	White fat cell differentiation	0.023635
GO:0005112	Notch binding	0.029404
GO:0003682	Chromatin binding	0.031223
GO:0030001	Metal ion transport	0.036693
GO:0046982	Protein heterodimerisation activity	0.040005
GO:0042113	B-cell activation	0.042442
GO:0046872	Metal ion binding	0.047530

from five mice in each group were used for SC-WGBS to make the data as reliable as possible.

We chose oocytes at MII stage, the last phase before fertilisation. Since it is difficult to obtain a large number of oocytes for analysis, we thus chose a new technique named single-cell level whole genome bisulphite sequencing to get the global DNA methylation status. Our results showed that the hyperinsulinaemia and hyperandrogenaemia did not cause changes in the global DNA methylation profile and different functional regions of genes in oocytes, but some differently methylated genes were found and were enriched in 17 GO terms.

The pathogenesis of PCOS always shows familial clustering. The data from 1332 monozygotic twins and 1873 dizygotic twins proved that genetic factors have a significant influence on PCOS (Vink *et al.* 2006); however, the causal genetic factors were not identified. With the development of sequencing technology, genome-wide association studies (GWAS) was used to determine the loci for PCOS risk (Chen *et al.* 2011; Shi *et al.* 2012; Lee *et al.* 2015). For these loci, some studies have been carried out for genetic screening and found several PCOS candidate genes including *LHCGR*, *FSHR*, *INSR* and *DENND1A* (Gammoh *et al.* 2015; McAllister *et al.* 2015).

The differently methylated genes that we found were correlated with *LHCGR*, *FSHR*, *INSR* and *DENND1A* in humans, as revealed by STRING (https://string-db.org/cgi/input.pl; accessed 1 September 2017; Szklarczyk *et al.* 2015, 2017; Fig. 7). We also used the gene expression profiles differentially expressed in PCOS patients between non-hyperinsulinaemia and hyperinsulinaemia granulosa cells to

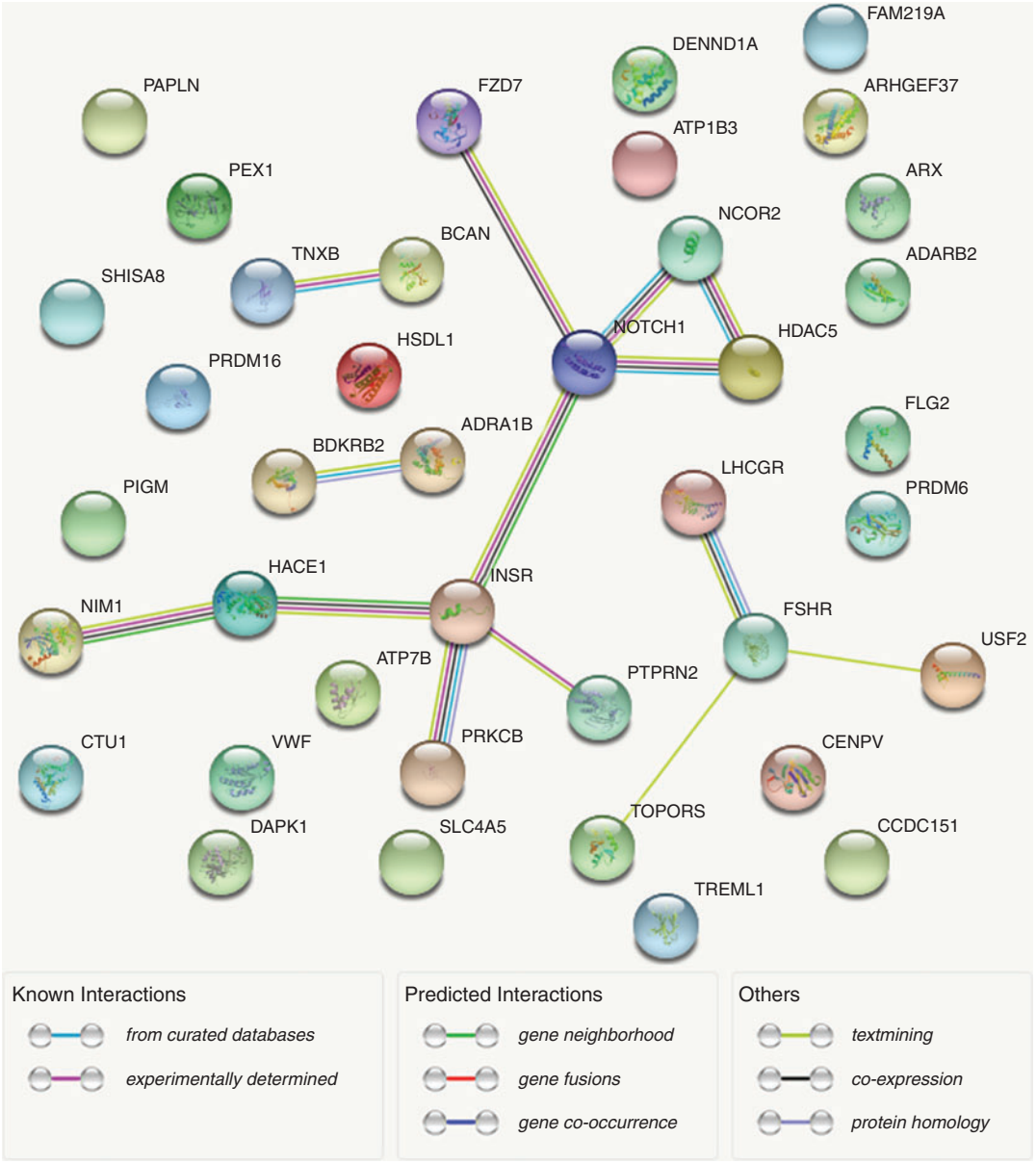

Fig. 7. The relationships between differently methylated genes and four differential genes in PCOS patients by STRING. The four differential genes in PCOS patients are *LHCGR*, *FSHR*, *INSR* and *DENND1A*.

calculate the correlation coefficient between each different methylation gene and the four differential genes in PCOS patients (Kaur *et al.* 2012; Fig. 8). It showed that most of them (*Adarb2*, *Adra1b*, *Arhgef37*, *Arx*, *Atp1b3*, *Bdkrb2*, *Ccdc151*, *Cenpv*, *Dapk1*, *Fam219a*, *Flg2*, *Fzd7*, *Hace1*, *Hdac5*, *Hsdl1*, *Ncor2*, *Papln*, *Pex1*, *Pigm*, *Prdm16*, *Prdm6*, *Slc4a5*, *Topors*, *Treml1*, *Usf2*) were highly correlated with one of the four important genes.

In conclusion, hyperinsulinaemia and hyperandrogenaemia do not have a significant effect on the global DNA methylation status and different functional regions of genes in oocytes, but they do affect methylation of some genes. These genes were associated with four PCOS-related genes, *LHCGR*, *FSHR*, *INSR* and *DENND1A* in humans. We hypothesise that environment stress alters some DNA methylation loci in oocytes.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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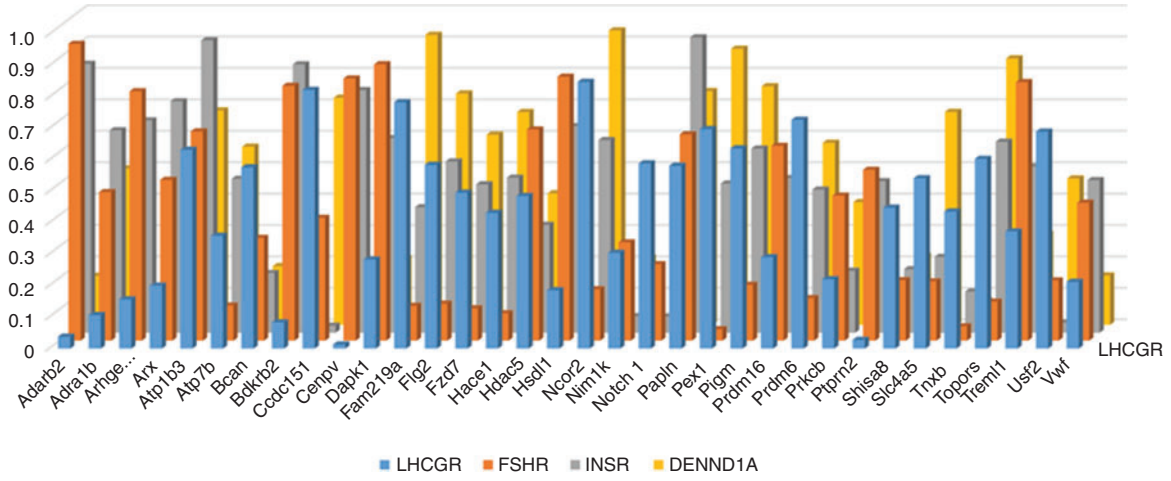

Fig. 8. Correlation coefficient between each differently methylated gene and the four differential genes in PCOS patients.

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