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# Mural granulosa cell gene expression associated with oocyte developmental competence

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## Abstract

**Background:** Ovarian follicle development is a complex process. Paracrine interactions between somatic and germ cells are critical for normal follicular development and oocyte maturation. Studies have suggested that the health and function of the granulosa and cumulus cells may be reflective of the health status of the enclosed oocyte. The objective of the present study is to assess, using an *in vivo* immature rat model, gene expression profile in granulosa cells, which may be linked to the developmental competence of the oocyte. We hypothesized that expression of specific genes in granulosa cells may be correlated with the developmental competence of the oocyte.

**Methods:** Immature rats were injected with eCG and 24 h thereafter with anti-eCG antibody to induce follicular atresia or with pre-immune serum to stimulate follicle development. A high percentage (30-50%, normal developmental competence, NDC) of oocytes from eCG/pre-immune serum group developed to term after embryo transfer compared to those from eCG/anti-eCG (0%, poor developmental competence, PDC). Gene expression profiles of mural granulosa cells from the above oocyte-collected follicles were assessed by Affymetrix rat whole genome array.

**Results:** The result showed that twelve genes were up-regulated, while one gene was down-regulated more than 1.5 folds in the NDC group compared with those in the PDC group. Gene ontology classification showed that the up-regulated genes included lysyl oxidase (*Lox*) and nerve growth factor receptor associated protein 1 (*Ngfrap1*), which are important in the regulation of protein-lysine 6-oxidase activity, and in apoptosis induction, respectively. The down-regulated genes included glycoprotein-4-beta galactosyltransferase 2 (*Ggbt2*), which is involved in the regulation of extracellular matrix organization and biogenesis.

**Conclusions:** The data in the present study demonstrate a close association between specific gene expression in mural granulosa cells and the developmental competence of oocytes. This finding suggests that the most differentially expressed gene, lysyl oxidase, may be a candidate biomarker of oocyte health and useful for the selection of good quality oocytes for assisted reproduction.

## Introduction

Ovarian follicle development is a complex process. Paracrine interactions between somatic and germ cells are critical for normal follicular development [1]. Defects in meiotic maturation have been observed in mice lacking the granulosa cell oocyte junction protein connexin 37 [2], and somatic cells in ovaries are known to participate in regulating oocyte growth and development [3,4], meiosis [5], and global transcriptional activity [6,7]. On the other

hand, oocytes also promote granulosa cell proliferation and differentiation [1]. It has been shown that mouse oocytes promote granulosa cell proliferation in preantral and antral follicles *in vitro* [8] and that cumulus expansion and granulosa cell differentiation are dependent upon oocyte-derived factors [9,10]. In rodents, oocyte-secreted GDF-9 and BMP15 promote proliferation of granulosa cells from small antral follicles, and BMP15 inhibits FSH-stimulated progesterone production [11]. Evidence also indicates that while GDF9 suppresses expression of both *KitL-1* and *KitL-2* in granulosa cells from rat early antral follicles, *KitL-1* expression can be promoted by BMP15 *in vitro* [4]. In addition, we have recently shown that GDF-9

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from the oocyte promotes pre-antral follicles development by up-regulating granulosa cell FSH receptor mRNA expression and preventing granulosa cell apoptosis via activation of the phosphatidylinositol 3-kinase/Akt pathway [12]. Thus, while oocyte maturation is known to depend on secretory products of the granulosa and cumulus cells, proliferation, differentiation and apoptosis of these support cells is also under tight control of the oocyte, suggesting that the health and function of the granulosa and cumulus cells may be reflective of the health status of the enclosed oocyte.

The quality of the oocyte is largely dependent on its follicular environment, as shown in a number of animal and human studies [4,13]. During ovarian stimulation and ovulation induction, a cohort of heterogeneous follicles is recruited to develop and ovulate, irrespective of their differentiative state. This creates an asynchrony in the maturation process and heterogeneity in the quality of the oocytes recovered for assisted reproduction. The morphological appearance, which is widely used as the primary criterion for oocyte selection in the human fertility clinic, does not accurately predict the health of the oocyte [14]. In fact, only a small proportion of the oocyte population can develop to healthy embryos after fertilization and healthy fetuses after transfer.

Although multiple factors are at play in determining pregnancy outcome in assisted reproduction including age, sperm quality (male factor), fertilization capacity and number of embryos transferred, the effect of fertilization rate appears to be of less significance [15] and that intrinsic deficiencies of the oocyte and/or embryo account for greater than 50% of failed conceptions [16]. These findings suggest that the developmental competence of the oocytes is a major determinant in the establishment of successful pregnancy in assisted reproduction.

Two factors contributing to oocyte health are chromosomal constitution and gene expression patterns of the oocyte and the follicular micro-environment in which the oocyte grows and matures. It has been shown that eCG stimulates follicular development and oocyte maturation in immature rats [17]. After hCG treatment, superovulated oocytes in eCG-primed immature rats can be fertilized *in vitro* and developed to term after embryo transfer [18]. In addition, our model also indicates that eCG/hCG treatment resulted in decreased estradiol level at the time of oocyte collection, as also been reported in the bovine dominant preovulatory follicles [19]. This model is physiologically relevant since it is well established that high level of LH (e.g. LH surge) during preovulatory development is associated with marked decrease in follicular and circulatory estradiol levels and that insufficient gonadotropin support results in atresia of the subordinate follicles. In the latter context, withdrawal of gonadotropic support (e.g. anti-eCG

antibody treatment) in the present model induced granulosa cell apoptosis and follicular atresia [20-22]. Fertilization and developmental competence of oocytes from anti-eCG treated rats are dependent on the dilution of antibody used (Jiang *et al.*, unpublished data).

The objective of the present study is to assess, using an *in vivo* immature rat model, gene expression profile in granulosa cells, which may be linked to the developmental competence of the oocyte. We hypothesized that expression of specific genes in granulosa cells may be correlated with the developmental competence of the oocyte. These findings will facilitate future investigation on the identification of non-invasive biomarkers indicative of oocyte health status which would allow one to select only good-quality oocytes for *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) and to transfer fewer embryos for successful pregnancy.

## Materials and methods

### Materials

All reagents were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

### Animal care

Sprague-Dawley rats and New Zealand White Rabbits were purchased from Charles River Canada (Montreal, PQ, Canada). Rats were kept in polycarbonated cages with wood shavings on the floor at 21°C, 50% humidity and a light/dark cycle at 7:00 h/19:00 h. They were given bullet type commercial rat feed and tap water *ad libitum*. The studies were carried out in accordance to the Guide to Care and Use of Experimental Animals of the Canadian Council on Animal Care and approved by the Animal Care Committee of the Ottawa Health Research Institute.

### Production of anti-eCG antiserum

Three male rabbits (2.5 - 3.0 Kg body weight [BW]) were used to produce anti-eCG antisera as described previously [21]. Antibody titres were determined by ELISA. In the bioassay for the antiserum, immature female rats injected with 10 IU eCG were injected 24 h later with highest-titre antiserum or pre-immune serum (100  $\mu$ l of 1:5 to 1:200 dilution in PBS, *i.p.*). The ovaries were removed 24 h after treatment and weighed, the ability of various concentrations of the antiserum to prevent eCG-induced ovarian weight gain was assessed. The above dilution (1:5 to 1:200 dilution) of anti-eCG serum significantly decreased ovarian weight in eCG-primed rats.

### Animal treatment and collection of oocytes and mural granulosa cells

Eight immature rats were injected with eCG (10 IU; *s.c.*; G4877) and 24 h thereafter with either pre-immune

serum (control; to stimulate follicle development) or anti-eCG antibody (1:400, to induce follicular atresia). Twenty-four hours later, hCG (10 IU; i.p.; CG-5) was administered. Cumulus-oocyte complexes (COCs) and mural granulosa cells collected by follicle puncture 13 h after hCG were respectively subjected to *in vitro* fertilization or kept at  $-80^{\circ}\text{C}$  until the assessment of gene expression, as described hereafter.

#### ***In vitro* fertilization (IVF) and embryo transfer**

To assess the developmental competence of oocytes which were morphologically indistinguishable in both groups, COCs were inseminated *in vitro* and the fertilized oocytes were transferred into pseudo-pregnant rats as described previously [23]. Briefly, sperm suspensions ( $1 \times 10^6$  cells/ml) were pre-incubated in insemination media (400  $\mu\text{L}$  of IVF-30 supplemented with 30 mM NaCl) for 5 to 7 h at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  in air. COCs were then carefully transferred into the suspension drops and incubated for 12 h. The oocytes were transferred into 100  $\mu\text{L}$  of culture medium and freed from surrounding cumulus cells. The denuded oocytes were considered fertilized if they exhibited the presence of pronuclei with sperm tail(s) in the vitellus.

To assess the developmental competence *in vivo* of embryos fertilized *in vitro*, nine to ten embryos at the 1-cell stage were transferred to the oviducts of each pseudo-pregnant recipient at Day 1. Vaginal smear of recipients was examined on days 1 and 4 as well as days 12-14 after transfer to confirm successful induction of pseudo-pregnancy and signs of pregnancy, respectively. All recipients were sacrificed by day 24 of pregnancy regardless of delivering offspring, and their uterine horns were examined for implantation sites. The number of young was counted on the day of parturition.

#### **RNA isolation**

Total RNAs from mural granulosa cells collected from ovarian follicles were extracted using RNeasy Mini kit according to manufacturer's instructions and DNA contamination was removed by DNase I digestion (Qiagen Inc., Mississauga, ON, Canada). All total RNA specimens were quantified and checked for quality with a Bioanalyzer 2100 system (Agilent, Palo Alto, CA) before further manipulation.

#### **Affymetrix GeneChip hybridization and image acquisition**

A total of 4 NDC and 4 PDC samples were used, thus requiring a total of 8 GeneChips. The GeneChip hybridization and image acquisition were performed at the Ontario Genome Center. Briefly, two rounds of amplification were carried out to successfully generate sufficient labeled cRNA for microarray analysis from 100 ng of total RNA. For first round synthesis of double-

stranded cDNA, total RNA was reverse transcribed using the Two-Cycle cDNA Synthesis kit (Affymetrix) and oligo (dT) 24-T7 (5'-GGCCAGTGAATTGTAA-TACGACTCACTATAGGGAGGCGG-3') primer followed by amplification with the MEGAscript T7 kit (Ambion, Inc., Austin, TX). After cleanup of the cRNA with a GeneChip Sample Cleanup Module IVT Column (Affymetrix), a second-round double-stranded cDNA was produced using the IVT Labeling kit (Affymetrix). A 15  $\mu\text{g}$ -aliquot of labeled product was fragmented by heat and ion-mediated hydrolysis ( $94^{\circ}\text{C}$ , 35 minutes) in 24  $\mu\text{L}$   $\text{H}_2\text{O}$  and 6  $\mu\text{L}$  of 5 $\times$  fragmentation buffer (Affymetrix). The fragmented cRNA was made into hybridization cocktail and was hybridized (16 h,  $45^{\circ}\text{C}$ ) to an Affymetrix Rat 230.2 array. Washing and staining of the arrays with phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR) was completed in a Fluidics Station 450 (Affymetrix). The arrays were then scanned using a confocal laser GeneChip Scanner 3000 and GeneChip Operating Software (Affymetrix).

#### **Microarray data analysis**

Gene expression patterns were determined using Affymetrix Genechip Arrays Rat 230.2. Prior to any statistical analysis, raw data were normalized and compared using RMA (robust multichip average) method from the BioConductor package <http://www.bioconductor.org>, which uses a robust average of  $\log_2$ -transformed background-corrected perfect match probe signal intensities combined with a quantile normalization method [24,25]. The quality analysis of the slides was performed by checking the logarithmic scatter plots of probe set intensities in all the non-redundant pairs of replicated samples after the normalization procedure [26]. Normalized data were then filtered in three steps. First, probe sets called 'Absent' (A) over all conditions and replicates across the complete dataset were excluded. Second, a threshold as the 95<sup>th</sup> percentile of all the absent call signals of the entire dataset was set. All the remaining probe sets whose expression values were consistently below this value were removed in each sample [26]. To extract significant genes between two independent groups, the two-sample t statistic was used for filtered genes. In addition, multiple testing corrections were performed by computing adjusted p values using the Bonferroni and Sidak algorithm which provides experiment-wise (or Family-wise) type I control. Genes with a fold change of 1.2 (increase or decrease) relative to the poor oocyte developmental competence were subsequently used. Hierarchical clustering of samples and gene expression values based on similarities of expression levels was performed using the average linkage method and Euclidean distance measurements as implemented in the TIGR Multiexperiment Viewer (MeV) program

[27]. Gene Ontology (GO) analysis was performed with DAVID <http://david.abcc.ncifcrf.gov/>[28].

Reproducibility between experiments was assessed by calculating the pairwise concordance of presence calls, which was 92.1-97.9%, and by computing the pairwise Adjusted Coefficient of Determination of log-transformed signal intensities (average of 0.952). High correlation of array signals (low intra-experimental group variation) was observed between rat samples within the groups with oocytes showing normal and poor developmental competence (Data not shown).

#### Quantitative real-time PCR validation of microarray results

In order to validate the results of microarray, real time RT-PCR analysis was performed on all 8 samples. Briefly, 0.4  $\mu$ g of total RNAs extracted from mural granulosa cells of each rat ovarian follicles were reverse transcribed in a final volume of 40  $\mu$ l solution containing First-Strand Buffer, dNTPs, dithiothreitol (DTT), RevertAid Enzyme (Fermentas), and Random Decamer Primers (Ambion, Inc.). Ten representative genes whose expression levels were remarkably changed in microarray (see Table 1) were further validated, they are lysyl oxidase (*Lox*), glycoprotein-4-beta-galactosyltransferase 2 (*Ggbt2*; *UDP-Gal*), nerve growth factor receptor associated protein 1 (*Ngfrap1*), protein disulfide isomerase-associated 5 and 6 (*Pdia5* and *Pdia6*), myeloid ecotropic viral integration site 1 homolog (*Meis1*), CD83 antigen, lysozyme (*Lyz*), trinucleotide repeat containing 6 (*Tnrc6*), interleukin 13 receptor alpha 1 (*Il13ra1*). Real-time quantitative PCR analyses for those genes were performed using a LightCycler 2.0 System (Roche Diagnostic Corporation) and a QuantiTect SYBR Green PCR kit (Qiagen, Mississauga, ON, Canada). The thermal cycling conditions were comprised of an initial denaturation step at 95°C (15 min) and 40 cycles at 95°C (15 sec), 58°C (20 sec) and 72°C (30 sec). The primer sequence for each gene, their PCR product size, primer location on rat chromosome, and GeneBank access numbers were shown in Table 1. 18S ribosomal RNA was used as control. Target gene expression level was calculated by relative expression ratio (RER) of Normal Developmental Competence (NDC) to Poor Developmental Competence (PDC), all normalized by 18S as described previously [29]. Briefly, the Livak Method ( $2^{-\Delta\Delta C_t}$  method) was performed by the following formula: 1) Calculate crossing point change of NDC relative to housekeeping gene 18S,  $\Delta C_t$  (NDC) =  $C_t$  (target gene, NDC) -  $C_t$  (18S, NDC); 2) Calculate crossing point change of PDC relative to housekeeping gene 18S,  $\Delta C_t$  (PDC) =  $C_t$  (target gene PDC) -  $C_t$  (18S, PDC); 3) Calculate the difference of these changes between NDC and PDC group,  $\Delta\Delta C_t$  =  $\Delta C_t$ (NDC) -  $\Delta C_t$ (PDC); 4) finally

calculate  $RER = 2^{-\Delta\Delta C_t}$ . Fold changes by real-time qPCR in Table 1 were calculated by Mean of RER for NDC over PDC.

#### Statistical Analysis

Data in Table 2 and real-time PCR results (Fig. 1) were analyzed by student's t-test tests using Graph Pad Prism 3 software. Differences with  $P < 0.05$  were considered statistically significant.

#### Results

##### Production of oocytes with poor and normal developmental competence

Treatment of eCG-primed rats with low dose of anti-eCG antiserum (1:400 dilution) failed to significantly decrease paired ovarian weight ( $108.2 \pm 7.9$  mg versus  $93.3 \pm 4.7$  mg;  $P > 0.05$ ) and fertilization rates ( $93.5 \pm 2.7\%$  versus  $95.8 \pm 2.2\%$ ,  $P > 0.05$ ) when compared with those in eCG plus pre-immune serum-treated group (Table 2). However, anti-eCG antiserum injection resulted in the production of oocytes with poor developmental competence. No embryos in this group could develop to term after embryo transfer. In contrast, as high as 30%-50% of oocytes from eCG-primed rats developed to offspring ( $P < 0.05$ ). No significant differences in the number of implantation sites were observed between two groups (Table 2).

##### Microarray identification of differentially expressed genes

The global gene expression profiles in rat granulosa cell samples representing oocytes of poor and normal developmental competence were identified with microarray technique. Results in Fig. 2 (left panel) show that among the approximately 30,000 genes queried on Rat 230.2 array, there were more undetected genes than detected genes observed in all arrays. Mean expression intensities of detected genes were higher than those of undetected genes (Fig. 2, right panel). A  $\log_2$  signal intensity threshold of 98.3 was determined and only those genes with signal intensity smaller than 98.3 were filtered. 8985 genes were left for further analysis.

Of a total of about 30,000 probe sets, we observed that the expression of 701 genes (Table 3) were significantly different ( $P < 0.001$ ) between oocytes with poor developmental competence compared to normal one, 43 of which were altered  $> 1.2$ -fold, and 13 of which  $> 1.5$ -fold. Both up- or down-regulated genes are shown in Table 3. A Euclidean clustering of these differential genes is shown in Fig. 3. All four samples from poor oocyte developmental competence (PDC) group had similar gene expression patterns and were included in the same PDC cluster. On the other hand, all other four samples from normal oocyte developmental competence (NDC) group had similar gene expression patterns and were included in the same NDC

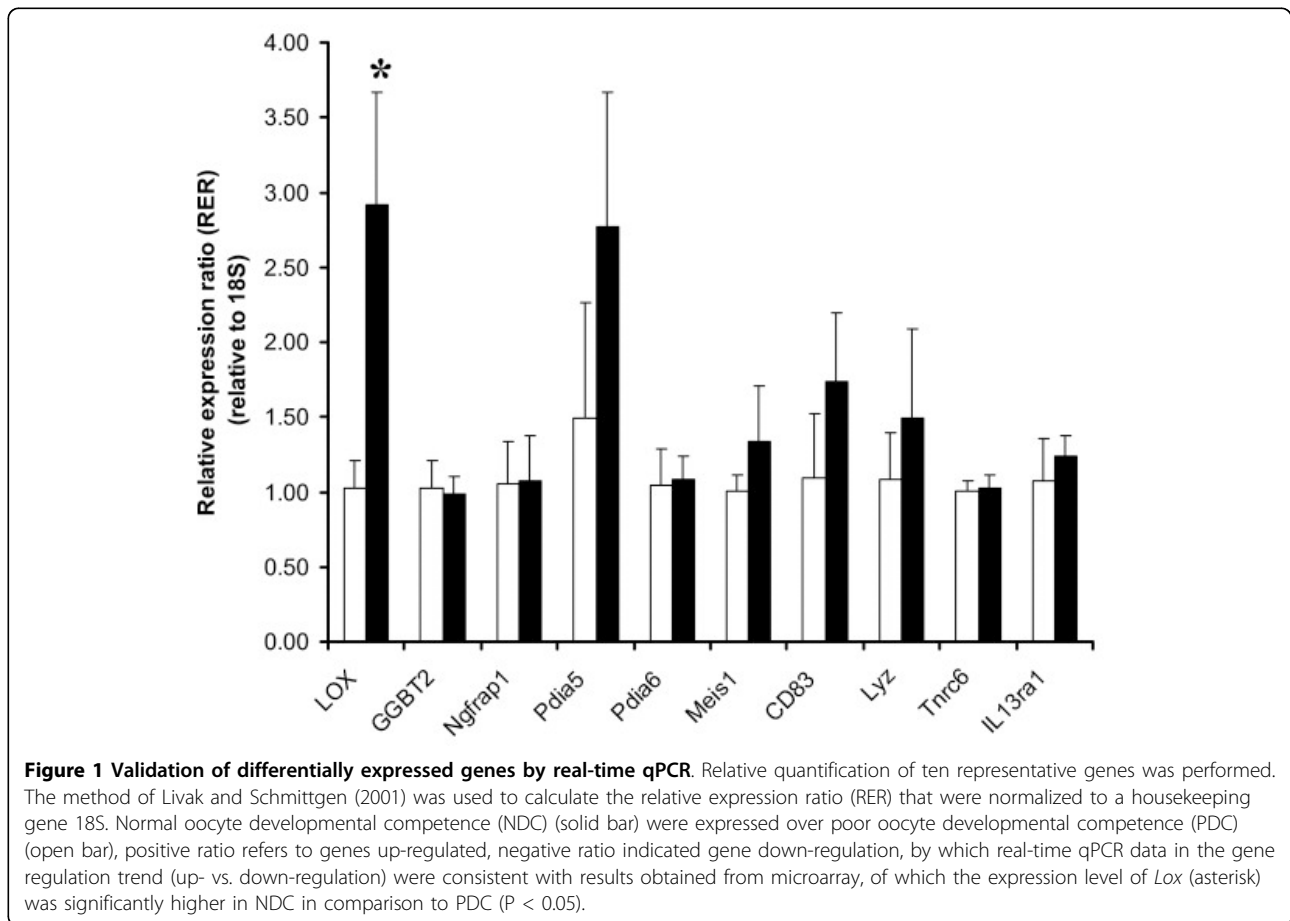
**Table 1 Summary on gene validation by RT-PCR in comparison with gene array results**

| Gene  | Primer sequence  | PCR product size (bp) | Location on rat chromosome | GenBank Access # | Fold changes (NDC/PDC) by |        |
|---|--|-----------------------|----------------------------|------------------|---------------------------|--------|
|   |  |                       |                            |                  | gene array                | RT-PCR |
| Lysyl oxidase<br>( <i>LOX</i> )   | RV:AGTCTCTGACA<br>TCCGCCCTA C<br>FW:ACCTGGTACCC<br>GATCCCTA        | 129                   | 18q11                      | NM_017061        | 2.8                       | 2.86   |
| Glycoprotein-4-<br>Beta-galactosyltrans-<br>ferase 2 ( <i>GGBT2</i> ;<br><i>UDP-Gal</i> )                 | FW:AGATAAAGATG<br>GGCGGCCGTTACT<br>RV:ACATGGTGTCT<br>CCAGCCTGATTGA | 186                   | 5q22                       | NM_053287        | -1.7                      | -1.04  |
| Nerve growth factor<br>receptor associated<br>protein 1 ( <i>Ngfrap1</i> ;<br><i>Bex3</i> ; <i>Nade</i> ) | FW:AATGATGGGTT<br>GGGTGGAGATGGA<br>RV:ACCGAAGTCAA<br>GGCATAAGGCAGA | 175                   | Xq35                       | NM_053401        | 1.6                       | 1.03   |
| Protein disulfide<br>isomerase-<br>associated 5 ( <i>Pdia5</i> )  | FW:ATATGACCGAG<br>CTGTGACGCTGAA<br>RV:ACATCTTTGGC<br>TCCAGGGTCTTCT | 185                   | 11q22                      | NM_001014125     | 1.8                       | 1.86   |
| Protein disulfide<br>isomerase-<br>associated 6 ( <i>Pdia6</i> )  | FW:ACCTTCTTCT<br>AGCGGTCACTGCT<br>RV:AGTGCACTTGC<br>TGCTTCTTCCAC   | 182                   | Chromo-<br>some 6          | NM_001004442     | 1.8                       | 1.04   |
| Myeloid ecotropic<br>viral integration site 1<br>homolog ( <i>Meis1</i> )                                 | FW:TAGCCACCAAT<br>ATCATGAGGGCGT<br>RV:TGAGTCCCGTA<br>TCTTGTGCCAACT | 99                    | 14q22                      | XM_223643        | 1.6                       | 1.33   |
| CD83 antigen  | FW:ATGTGCCTGAA<br>TACCACCTGGACA<br>RV:AGCCGCATGAA<br>ACATGAAGCTGAC | 193                   | 17p12                      | NM_001108410     | 1.7                       | 1.6    |
| Lysozyme ( <i>Lyz</i> )   | FW:TATGAACGCTG<br>TGAGTTCGCCAGA<br>RV:TGCTGAGCTAA<br>ACACACCCAGTCT | 95                    | 7q22                       | NM_012771        | 1.7                       | 1.37   |
| Trinucleotide repeat<br>containing 6 ( <i>Tnrc6</i> )   | FW:TGAAGTACCTC<br>CACGATTTGCGCA<br>RV:TGCTGTTCTGC<br>ACCTCTCCGTTAT | 176                   | 1q36                       | NM_001107549     | 1.7                       | 1.03   |
| Interleukin 13<br>receptor alpha 1<br>( <i>Il13ra1</i> )  | FW:AAGTGAGAAGC<br>CTAGCCCTTTGGT<br>RV:AGTTGGTGTCC<br>GGGCTTGATTCT  | 155                   | Xq12                       | NM_145789        | 1.4                       | 1.16   |
| 18S rRNA  | FW:CGCGGTTCTAT<br>TTTGTTGGT<br>RV:AGTCGGCATCG<br>TTTATGGTC         | 219                   | Chromo-<br>some 3          | M11188           | Housekeeping              |        |

**Table 2 In vitro fertilization and embryo transfer of oocytes from immature rats treated with eCG/anti-eCG/hCG**

| Experimental Group | Rat        | Paired Ovarian weight (mg) | No (%) of oocytes/fertilized | No (%) of pups/transferred embryos | No. of pups/implantation Sites |
|--------------------|------------|----------------------------|------------------------------|------------------------------------|--------------------------------|
| PDC                | A          | 104.0                      |                              |                                    |                                |
|                    | B          | 109.8                      |                              |                                    |                                |
|                    | C          | 87.3                       | 18/21(86)                    | 0/10(0)                            | 0/1                            |
|                    | D          | 131.6                      | 36/37(97)                    | 0/9(0)                             | 0/2                            |
|                    | Mean ± SEM | 108.2 ± 7.9                | (93.5 ± 2.7)                 | (0)                                | 0/4 ± 2                        |
| NDC                | E          | 98.7                       | 23/23(100)                   | 4/10(40)                           | 4/6                            |
|                    | F          | 104.9                      | 26/28(93)                    | 5/10(50)                           | 5/8                            |
|                    | G          | 79.9                       | 12/12(100)                   | 3/10(30)                           | 3/4                            |
|                    | H          | 89.5                       | 18/20(90)                    | 3/10(30)                           | 3/6                            |
|                    | Mean ± SEM | 93.3 ± 4.7                 | (95.8 ± 2.2)                 | (37.5 ± 4.2)                       | 4 ± 1/6 ± 1                    |

PDC: Oocytes with poor developmental competence; NDC: Oocytes with normal developmental competence.

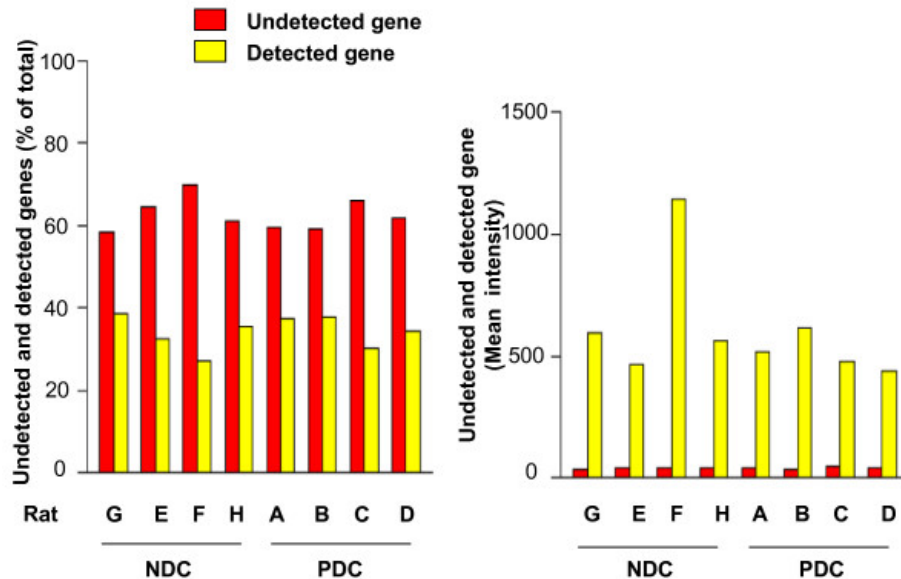


cluster. The gene expression patterns were very different between PDC and NDC clusters.

**Gene ontology analysis**

Gene ontology analysis showed that up-regulated genes in oocytes with normal developmental competence were

linked to transcription regulation, protein phosphorylation and signal transduction, microtubule cytoskeleton organization and movement (Table 3). The genes participating in transcriptional regulation included nucleosome assembly protein 1-like 1, *Necdin*, *Meis 1* and TAF9 RNA polymerase II and a transcribed locus



**Figure 2 Percentages (left panel) and mean gene expression intensities (right panel) of detected and undetected genes in 8 gene arrays.** The number of undetected genes was higher than that of detected genes in all arrays (left panel). However, the mean gene expression intensities of detected genes were much higher than those of undetected genes in all arrays (right panel).

homologous to polymerase I-transcript release factor (*PTRF*), while those involved in the control of protein phosphorylation and signal transduction were *Lox*, *Pdia5* and *Pdia6*, golgi autoantigen and cell division cycle 2-like 5. The genes having a role in microtubule cytoskeleton organization and movement include CD83 antigen, *Tnrc6*, *Goliath*, vesicle-associated membrane protein 8 (Table 3).

Twelve genes were up-regulated, and one gene down-regulated, more than 1.5 folds in NDC group than those in PDC group. Gene ontology classification showed that the up-regulated genes included *Lox* and *Ngfrap1*. *Lox* is important in the regulation of copper ion binding [30]. *Ngfrap1* plays an important role in apoptosis induction [31]. The down-regulated gene is *Ggbt2* known to be involved in the regulation of extracellular matrix organization and biogenesis [32].

#### Identification of signaling pathways contributing to the normal oocyte developmental competence

To determine the signaling pathways of up-regulated genes associated with normal oocyte developmental competence, all genes with more than 1.2-fold change were subjected to the pathway analysis by Pathwayexplorer <https://pathwayexplorer.genome.tugraz.at/>. Although no directly related pathways were found, a potential signaling pathway of the highest-regulated gene, *Lox*, could be envisaged since oocyte-derived factors such as GDF-9 increases gene expression of *Lox* which induces differentiation of mural granulosa cells [33].

#### Quantitative real-time PCR validation of microarray data

Ten representative genes, the expression levels of which were remarkably changed in microarray (Table 3), were selected for further validation by RT-PCR analyses. Of ten genes selected, *Lox*, *Pdia5*, and CD83 antigen mRNA abundance of mural granulosa cells in normal oocyte developmental competence group were higher (fold changes > 1.6) than that in poor oocyte developmental competence group, consistently in both gene microarray and quantitative RT-PCR analyses. The fold change from microarray and that from RT-PCR exhibit excellent concordance, with Pearson correlation equal to 0.94 ( $p < 0.0001$ ). However, only *Lox* was statistically significantly different between the two groups (fold changes > 2.8,  $P < 0.05$ , Fig. 1 and Table 1). Our data suggested that the profile of *Lox* gene in mural granulosa cells could be a likely candidate for a potential biomarker for follicular maturity and oocyte quality.

#### Discussion

In the present study, using whole genome gene expression profiling of mural granulosa cells, we have demonstrated that mural granulosa cells isolated from follicles containing oocytes with normal developmental competence are distinct from those with oocytes exhibiting poor developmental competence. The dissimilarity between these two groups was clearly shown through unsupervised hierarchical clustering of these samples and was substantiated using binary tree prediction as well as expression data from independent arrays. The

**Table 3 Expression and their biological functions of genes in mural granulosa cells of follicles containing oocyte with normal developmental competence compared to those with poor developmental competence, as determined by Gene Ontology Analysis**

| Probe position at array                          | Fold changes* | Gene   | Biological functions  |
|--|---------------|--|---|
| <b>Transcription regulation genes</b>            |               |  |   |
| 1367847  | 1.8           | Nuclear protein 1  | Unknown   |
| 1384308  | 1.6           | Meis1 (myeloid ecotropic viral integration site 1 homolog)                     | Regulation of transcription, DNA dependent  |
| 1371947  | 1.6           | Necdin   | Unknown   |
| 1371822  | 1.5           | RNA polymerase III (DNA directed) polypeptide D                                | Regulation of progression through cell cycle  |
| 1375414  | 1.5           | TAF9 RNA polymerase II [TATA box binding protein (TBP)-associated factor]      | Negative regulation of transcription from RNA polymerase II promoter  |
| 1390116  | 1.4           | Transcribed locus: similar to polymerase I-transcript release factor (PTRF)    | Unknown   |
| 1374780  | 1.3           | Transcribed locus  | Unknown   |
| 1372093  | 1.3           | Max interacting protein 1  | Unknown   |
| 1373978  | 1.3           | Nuclear cap binding protein subunit 1 (80 kDa)                                 | RNA splicing and Mrna cleavage  |
| 1385486  | 1.3           | Transcribed locus  | Unknown   |
| 1380827  | 1.3           | Similar to C1orf25   | tRNA processing   |
| 1370826  | 1.3           | Nucleosome assembly protein 1-like 1   | DNA replication, nucleosome assembly and positive regulation of cell proliferation                                  |
| 1376597  | 1.3           | Ninc finger, CCHC domain containing 10   | Unknown   |
| 1388067a   | -1.3          | Glucocorticoid modulatory element binding protein 2                            | Regulation of transcription, transcription from RNA polymerase II promoter  |
| <b>Post-translation regulation genes</b>         |               |  |   |
| 1368171  | 2.8           | Lysyl oxidase  | Protein modification, copper ion binding oxidoreductase activity, cancer metastasis, granulosa cell differentiation |
| 1374828  | 1.8           | Protein disulfide isomerase-associated 5                                       | Electron transport, protein folding and response to stress  |
| 1370859  | 1.5           | Protein disulfide isomerase associated 6                                       | Electron transport, protein folding and electron transport  |
| 1398895  | 1.4           | Golgi autoantigen, golgin subfamily a,7  | Protein amino acid palmitoylation   |
| 1392149  | 1.3           | Transcribed locus  | Unknown   |
| 1368653a   | 1.3           | Parkinson disease (autosomal recessive, early onset) 7                         | Protein folding, cell proliferation and adult locomotory behavior   |
| 1387258a   | 1.3           | Protein-L-isoaspartate (D-aspartate) O-methyltransferase 1                     | Protein methylation, S-adenosylhomocysteine metabolism and protein modification                                     |
| 1386164  | 1.3           | Cell division cycle 2-like 5 (cholinesterase-related cell division controller) | Protein phosphorylation, regulation of mitosis and positive regulation of cell proliferation                        |
| 1398343  | 1.2           | DNAJ (Hsp40) homolog, subfamily A, member 4                                    | Protein folding   |
| 1383475  | -1.3          | Protein phosphatase 1A, magnesium dependent, alpha isoform                     | Protein dephosphorylation, positive regulation of IκB kinase/NFκB cascade   |
| <b>Microtubule cytoskeleton regulation genes</b> |               |  |   |
| 1370154  | 1.7           | Lysozyme   | Antimicrobial activity in human follicular fluid, ovulation   |
| 1390529  | 1.7           | CD83 antigen   | Defense response, humoral immune response and signal transduction   |
| 1375664  | 1.7           | Trinucleotide repeat containing 6  | Microtubule-based movement  |
| 1369948  | 1.6           | Nerve growth factor receptor associated protein 1                              | Induction of apoptosis, increase in PCO ovaries   |
| 1374321  | 1.4           | Similar to RIKEN cDNA 2700081O15   | Unknown   |
| 1388711  | 1.4           | Interleukin 13 receptor, alpha 1   | Cell surface receptor linked signal transduction  |
| 1372330  | 1.4           | Goliath  | Apoptosis and proteolysis   |
| 1372682  | 1.3           | Similar to RIKEN cDNA 2810432L12   | Unknown   |



**Table 3: Expression and their biological functions of genes in mural granulosa cells of follicles containing oocyte with normal developmental competence compared to those with poor developmental competence, as determined by Gene Ontology Analysis (Continued)**

|          |      |   |   |
|----------|------|---|---|
| 1372093  | 1.3  | Max interacting protein 1   | Unknown   |
| 1386952a | 1.3  | Dynein, cytoplasmic, intermediate chain 2                         | Microtubule-based movement  |
| 1380577  | 1.3  | ATP-binding cassette, sub-family G (WHITE), member 2              | Drug transport  |
| 1369970  | 1.3  | Vesicle-associated membrane protein 8                             | Protein complex assembly and vesicle-mediated transport   |
| 1367716  | 1.2  | T-cell immunomodulatory protein                                   | Unknown   |
| 1373090  | 1.2  | Signal sequence receptor, alpha                                   | Cotranslational protein targeting to membrane, positive regulation of cell proliferation  |
| 1376874a | 1.2  | Adaptor-related protein complex AP-4, beta 1                      | Intracellular protein transport, vesicle-mediated transport   |
| 1383206  | 1.2  | Component of oligomeric golgi complex 3                           | Intracellular protein transport   |
| 1369549  | -1.3 | Killer cell lectin-like receptor subfamily K, member 1            | Unknown   |
| 1371073  | -1.7 | UDP-Gal: betaGlcNAc beta 1,4-galactosyltransferase, ploypeptide 1 | Promote apoptosis, N-acetylglucosaminylglycopeptide beta-1,4-galactosyltransferase activity, carbohydrate metabolism, development of secondary sexual characteristics, extracellular matrix organization and biogenesis, galactose metabolism, integral to membrane, lactose synthase activity, oligosaccharide biosynthesis, transferase activity, |

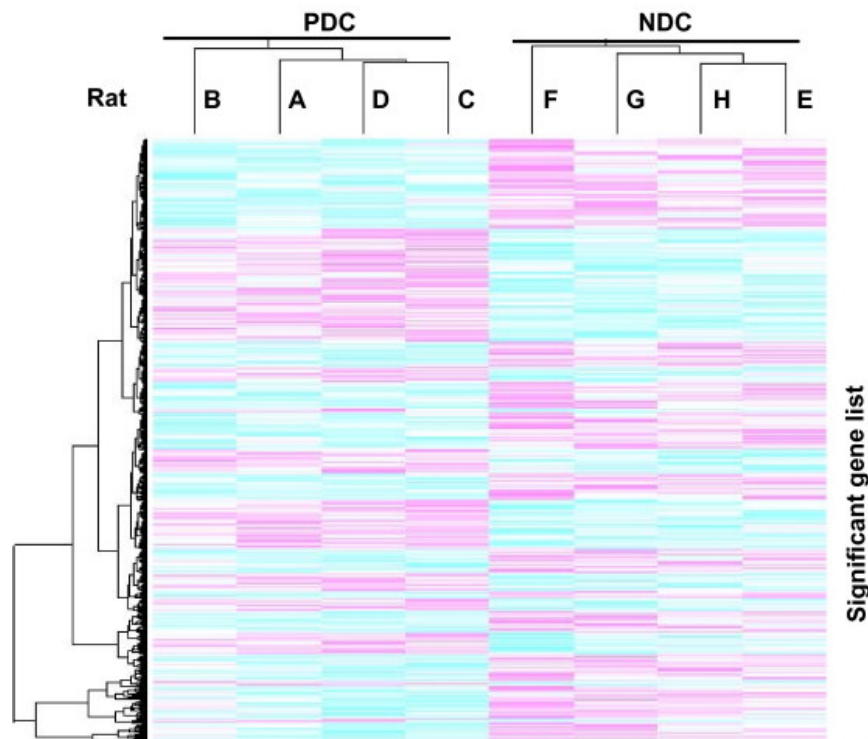
\*Fold changes represent difference of gene expression in granulosa cells from follicles containing oocytes with normal developmental competence compared with that with poor developmental competence. "-": down-regulation; others: up-regulation

identification of two unique branches containing normal and poor oocyte developmental competence is consistent with the distinct developmental outcome after embryo transfer. Meanwhile, our comparison of gene expression profiles between different samples within the same group showed that there was a high "within-group" similarity, demonstrating the quality of our gene expression experiment. Differentially expressed genes in these two groups might be further tested as potential biomarkers of oocyte quality, in particular the highest changed gene encoding lysyl oxidase that plays an important role in the regulation of differentiation of mural granulosa cells.

The assessment of differential gene expression between two groups, in conjunction with gene ontology analysis, showed that differences in genes were associated with regulation of transcription and DNA replication and cell cycle progression, protein folding, phosphorylation and signaling pathways, microtubule cytoskeleton organization and movement, and receptor signaling and apoptosis. Of principal importance was the gene "*Lox*" which, with the largest difference in expression, has been shown to be involved in the regulation of mural granulosa cell differentiation. *Lox* was expressed 2.8-fold higher in mural granulosa cells in follicles producing normal oocyte than poor oocyte developmental competence. This enzyme oxidizes peptidyl lysine to peptidyl aldehyde residues within collagen and elastin, initiating formation of the covalent cross-

linkages that insolubilize these extracellular proteins [34]. This enzyme is also present and active within rat vascular smooth muscular cell nuclei, exhibits its catalytic activity on histone H1 [35,36], suggesting that it may regulate chromatin remodeling involved in the regulation of transcription [37]. It has been shown that *Lox* is expressed in cultured bovine granulosa cells and involved in the maintenance of cell differentiation [30]. The activity of this enzyme is increased in rabbit ovarian follicles after hCG-induced ovulation and its mRNA expression is up-regulated at the time of ovulation in perch ovary [38,39]. However, rat granulosa cell *Lox* transcripts were significantly suppressed 48 h after eCG injection compared with untreated controls and were further reduced during hCG-induced luteinization [38]. Furthermore, FSH dose-dependently inhibited *Lox* mRNA and enzyme activity in cultured rat granulosa cells [33].

In the present study, *Lox* mRNA abundance was 2.8-fold higher in mural granulosa cells isolated from follicles containing oocytes which exhibit normal developmental competence when compared with poor ones. This result was validated by real-time PCR. It has been demonstrated that TGFβ1 and GDF9 increase *Lox* mRNA expression in human lung fibroblasts [40] and rat granulosa cells [33], respectively. Since the actions of TGFβ superfamily members are mediated via the Smad2/Smad3 pathways [33], these findings raise the interesting possibility that the GDF9-induced preantral



**Figure 3 Unsupervised hierarchical clustering analysis of 701 differentially expressed probe sets in all arrays.** To identify the relationships between samples, a 1 - correlation metric with centroid linkage was applied to those probe sets. A dendrogram containing two distinct arms was identified. All four samples from poor oocyte developmental competence (PDC) group had similar gene expression patterns and were included in the same PDC cluster. On the other hand, all other four samples from normal oocyte developmental competence (NDC) group had similar gene expression patterns and were included in the same NDC cluster. The gene expression patterns were very different between PDC and NDC clusters.

follicular growth in vitro [12] involves increased mural granulosa cell *Lox* mRNA expression. Whether this indeed is the case awaits further investigation.

In addition to *Lox*, *Pdia5* is also up-regulated at less extent in the normal oocyte developmental competence group. Although *Pdia5* plays an important role in the regulation of electron transport, protein folding and stress response [41], posttranslational protein modification and is essential for normal cell function [42], the differences between the two experimental groups are not statistically significant as determined by real-time PCR. The physiological significance of this observation remains unclear.

### Conclusions

The present studies demonstrate a close association between the expression of *Lox* in mural granulosa cells and the developmental competence of oocytes. These findings suggest that the most differentially expressed gene, lysyl oxidase, may be a potential biomarker for oocyte health in assisted reproduction. Further studies are required to confirm this notion.

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#### Authors' contributions

JYJ designed the experiment, conducted animal studies (including development and injection of eCG-antibody, IVF/embryo transfer and collection of cells), extracted RNA and prepared manuscript. HX analyzed gene array data and was assisted by XX. MC performed real-time RT-PCR. MAS assisted in experimental design. BKT involved in designing this study and developing the manuscript. All authors have read and approved the final manuscript.

#### Competing interests

The authors declare that they have no competing interests.

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#### References

1. Eppig JJ: Oocyte control of ovarian follicular development and function in mammals. *Reproduction* 2001, **122**:829-838.
2. Carabatsos MJ, Sellitto C, Goodenough DA, Albertini DF: Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Dev Biol* 2000, **226**:167-179.
3. Brower PT, Schultz RM: Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth. *Dev Biol* 1982, **90**:144-153.
4. Thomas FH, Vanderhyden BC: Oocyte-granulosa cell interactions during mouse follicular development: regulation of kit ligand expression and its role in oocyte growth. *Reprod Biol Endocrinol* 2006, **4**:19.
5. Chesnel F, Wigglesworth K, Eppig JJ: Acquisition of meiotic competence by denuded mouse oocytes: participation of somatic-cell product(s) and cAMP. *Dev Biol* 1994, **161**:285-295.
6. de la Fuente R, Eppig JJ: Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling. *Dev Biol* 2001, **229**:224-236.
7. Matzuk MM, Burns KH, Viveiros MM, Eppig JJ: Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science* 2002, **296**:2178-2180.
8. Vanderhyden BC, Telfer EE, Eppig JJ: Mouse oocytes promote proliferation of granulosa cells from preantral and antral follicles in vitro. *Biol Reprod* 1992, **46**:1196-204.
9. Vanderhyden BC, Caron PJ, Buccione R, Eppig JJ: Developmental pattern of the secretion of cumulus expansion-enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation. *Dev Biol* 1990, **140**:307-17.
10. Buccione R, Vanderhyden BC, Caron PJ, Eppig JJ: FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Dev Biol* 1990, **138**:16-25.
11. Otsuka F, Yamamoto S, Erickson GF, Shimasaki S: Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. *J Biol Chem* 2001, **276**:11387-92.
12. Orisaka M, Orisaka S, Jiang JY, Craig J, Wang Y, Kotsuji F, et al: Growth differentiation factor 9 is antiapoptotic during follicular development from preantral to early antral stage. *Mol Endocrinol* 2006, **20**:2456-2468.
13. Hunter MG, Brankin V, Quinn RL, Ferguson EM, Edwards SA, Ashworth CJ: Oocyte-somatic cell-endocrine interactions in pigs. *Domest Anim Endocrinol* 2005, **29**:371-384.
14. McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, et al: Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum Reprod* 2004, **19**:2869-2874.
15. Rhodes TL, McCoy TP, Higdon HL III, Boone WR: Factors affecting assisted reproductive technology (ART) pregnancy rates: a multivariate analysis. *J Assist Reprod Genet* 2005, **22**:335-346.
16. Racowsky C: High rates of embryonic loss, yet high incidence of multiple births in human ART: is this paradoxical? *Theriogenology* 2002, **57**:87-96.
17. Jiang JY, Umezu M, Sato E: Improvement of follicular development rather than gonadotrophin secretion by thyroxine treatment in infertile immature hypothyroid rdw rats. *J Reprod Fertil* 2000, **119**:193-199.
18. Jiang JY, Miyoshi K, Umezu M, Sato E: Superovulation of immature hypothyroid rdw rats by thyroxine therapy and the development of eggs after in vitro fertilization. *J Reprod Fertil* 1999, **116**:19-24.
19. Mihm M, Baker PJ, Ireland JL, Smith GW, Coussens PM, Evans AC, et al: Molecular evidence that growth of dominant follicles involves a reduction in follicle-stimulating hormone dependence and an increase in luteinizing hormone dependence in cattle. *Biol Reprod* 2006, **74**:1051-1059.
20. Boone DL, Carnegie JA, Rippstein PU, Tsang BK: Induction of apoptosis in equine chorionic gonadotropin (eCG)-primed rat ovaries by anti-eCG antibody. *Biol Reprod* 1997, **57**:420-427.
21. Kim JM, Boone DL, Auyeung A, Tsang BK: Granulosa cell apoptosis induced at the penultimate stage of follicular development is associated with increased levels of Fas and Fas ligand in the rat ovary. *Biol Reprod* 1998, **58**:1170-6.
22. Wang H, Jiang JY, Zhu C, Peng C, Tsang BK: Role and regulation of nodal/activin receptor-like kinase 7 signaling pathway in the control of ovarian follicular atresia. *Mol Endocrinol* 2006, **20**:2469-2482.
23. Jiang JY, Tsang BK: Optimal conditions for successful in vitro fertilization and subsequent embryonic development in Sprague-Dawley rats. *Biol Reprod* 2004, **71**:1974-1979.
24. Bolstad BM, Irizarry RA, Astrand M, Speed TP: A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003, **19**:185-193.
25. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP: Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 2003, **31**:e15.
26. Pelizzola M, Pavelka N, Foti M, Ricciardi-Castagnoli P: AMDA: an R package for the automated microarray data analysis. *BMC Bioinformatics* 2006, **7**:335.
27. Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, et al: TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 2003, **34**:374-378.
28. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, et al: DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 2003, **4**:3.
29. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001, **25**:402-408.
30. Kendall NR, Marsters P, Scaramuzzi RJ, Campbell BK: Expression of lysyl oxidase and effect of copper chloride and ammonium tetrathiomolybdate on bovine ovarian follicle granulosa cells cultured in serum-free media. *Reproduction* 2003, **125**:657-665.
31. Nocentini G, Giunchi L, Ronchetti S, Krausz LT, Bartoli A, Moraca R, et al: A new member of the tumor necrosis factor/nerve growth factor receptor family inhibits T cell receptor-induced apoptosis. *Proc Natl Acad Sci USA* 1997, **94**:6216-6221.
32. Asano M, Furukawa K, Kido M, Matsumoto S, Umehashi Y, Kochibe N, et al: Growth retardation and early death of beta-1,4-galactosyltransferase knockout mice with augmented proliferation and abnormal differentiation of epithelial cells. *EMBO J* 1997, **16**:1850-1857.
33. Harlow CR, Rae M, Davidson L, Trackman PC, Hillier SG: Lysyl oxidase gene expression and enzyme activity in the rat ovary: regulation by follicle-stimulating hormone, androgen, and transforming growth factor-beta superfamily members in vitro. *Endocrinology* 2003, **144**:154-162.
34. Li W, Nellaippan K, Strassmaier T, Graham L, Thomas KM, Kagan HM: Localization and activity of lysyl oxidase within nuclei of fibrogenic cells. *Proc Natl Acad Sci USA* 1997, **94**:12817-12822.
35. Kagan HM, Li W: Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J Cell Biochem* 2003, **88**:660-672.
36. Mello ML, Contente S, Vidal BC, Planding W, Schenck U: Modulation of ras transformation affecting chromatin supraorganization as assessed by image analysis. *Exp Cell Res* 1995, **220**:374-382.
37. Grattarola M, Borghi C, Emionite L, Lulli P, Chessa L, Vergani L: Modifications of nuclear architecture and chromatin organization in ataxia telangiectasia cells are coupled to changes of gene transcription. *J Cell Biochem* 2006, **99**:1148-1164.
38. Slee RB, Hillier SG, Lague P, Harlow CR, Miele G, Clinton M: Differentiation-dependent expression of connective tissue growth factor and lysyl

oxidase messenger ribonucleic acids in rat granulosa cells. *Endocrinology* 2001, **142**:1082-1089.

39. Langenau DM, Goetz FW, Roberts SB: **The upregulation of messenger ribonucleic acids during 17alpha, 20beta-dihydroxy-4-pregnen-3-one-induced ovulation in the perch ovary.** *J Mol Endocrinol* 1999, **23**:137-152.
40. Roy R, Polgar P, Wang Y, Goldstein RH, Taylor L, Kagan HM: **Regulation of lysyl oxidase and cyclooxygenase expression in human lung fibroblasts: interactions among TGF-beta, IL-1 beta, and prostaglandin E.** *J Cell Biochem* 1996, **62**:411-417.
41. Turano C, Coppari S, Altieri F, Ferraro A: **Proteins of the PDI family: unpredicted non-ER locations and functions.** *J Cell Physiol* 2002, **193**:154-163.
42. Knizetova P, Vancova I, Kocakova P, Slovak M, Proost P, Kopacek J: **New member of the protein disulfide isomerase (PDI) family identified in *Amblyomma variegatum* tick.** *Insect Biochem Mol Biol* 2006, **36**:943-953.

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